The biological effects of constitutively active mutants of the common β subunit of the human IL-3, IL-5 and GM-CSF receptors

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ABSTRACT

To date several activating mutations have been discovered in the common signal-transducing subunit (hβc) of the receptors for human GM-CSF, IL-3 and IL-5, which enable it to signal in the absence of ligand. Two of these mutations, V449E and I374N, result in single amino acid substitutions in the transmembrane and extracellular domains of hβc, respectively. A third, FIΔ, results in a 37 amino acid duplication in the extracellular domain. The aim of this project was to study the biological effects and leukaemic potential of these mutants using murine models.

Firstly, the effects of these mutants on the growth and differentiation of primary haemopoietic cells was studied. To achieve this, all three mutants were expressed in primary haemopoietic cells derived from murine foetal liver. When cultured without growth factors all three mutants conferred factor-independent survival and proliferation on these cells. However, the cell types induced differed between transmembrane and extracellular hβc mutants. Hence whilst the extracellular mutants, FIΔ and I374N, conferred factor independence on cells of the neutrophil and monocyte lineages only, V449E could do so on all five myeloid lineages as well as erythroid cells. Moreover, immature cells (blast cells and myelocytes) containing the V449E mutant displayed an extended lifespan in culture and in some cases lead to the development of factor-independent cell lines, one of which was tumorigenic in a proportion of injected mice.

During the course of these studies, two growth factor dependent cell lines were isolated, termed FDB1 and FDB2. These lines showed the unique properties of growth in murine IL-3 and growth arrest and terminal granulocyte-macrophage differentiation in response to murine GM-CSF. When the above factor-independent hβc mutants were expressed in these cell lines, the transmembrane mutant, V449E, induced continuous growth of these lines. However, the extracellular mutants, FIΔ and I374N, lead to granulocyte-macrophage differentiation in a manner analogous to murine GM-CSF. Hence in addition to their cell-type specific signalling, transmembrane and extracellular hβc mutants deliver a qualitatively different signal to multipotential cell lines.

Finally, in order to study the effects of hβc mutants *in vivo*, all three mutants were expressed in the haemopoietic system of mice using the technique of bone marrow reconstitution. The extracellular hβc mutants FIΔ and I374N induced a chronic myeloproliferative disorder marked by elevated levels of neutrophils, red blood cells and platelets in the blood. In contrast, the transmembrane mutant, V449E induced an acute disorder of blast cell accumulation in the blood, reminiscent of leukemias such as AML. Hence whilst activated hβc mutants are capable of inducing haemopoietic disorders, the type of disorder induced depends on the location of the activating mutation.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any other university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published by any other person, except where due reference has been made in the text. I give consent for this thesis to be made available for loan and photocopying.

Signed,

Matthew McCormack

6 November 1998.

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PUBLICATIONS

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D'Andrea, R.J., Harrison-Findik, D., Butcher, C.M., Finnie, J., Blumbergs, P., Bartley, P., **McCormack, M.P.**, Jones, K., Rowland, R., Gonda, T.J., and Vadas, M.A. (1998). Dysregulated hematopoiesis and a progressive neurological disorder induced by expression of an activated form of the human common beta chain in transgenic mice. J. Clin. Invest. (in press).

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ABBREVIATIONS

A	adenine
A_X	optical density at X nm
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
(α-^{32}P)dATP	alpha-labelled 2'-deoxyadenosine-5'-triphosphate
bp	base pair(s)
BFU-E	erythroid burst-forming unit
BSA	bovine serum albumin
C	cytosine
cDMEM	complete Dulbecco's modified Eagle's medium
cDNA	deoxyribonucleic acid complementary to ribonucleic acid
CFU-E	erythroid colony-forming unit
Ci	curie
cIMDM	complete Iscove's modified Dulbecco's medium
CIP	calf intestinal phosphatase
CLL	chronic lymphocytic leukaemia
CML	chronic myeloid leukaemia
CRD	cytokine receptor domain
CRM	cytokine receptor module
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deocycytosine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTPs	dATP, dCTP, dGTP, dTTP
DTE	dithioerythritol
DTT	dithiothreitol
dTTP	2'-deoxythymidine-5'-triphosphate
E.coli	*Escherischia coli*

EDTA	ethylenediaminetetraacetic acid
Epo	erythropoietin
FACS	fluorescence-activated cell sorting
FCS	bovine foetal calf serum
FITC	fluorescein isothiocyanate
F-MuLV	Friend murine leukaemia virus
5-FU	5-fluorouracil
G	guanine
G-CSF	granulocyte colony-stimulating factor
GM	GM-CSF eg. hGMR
GM-CSF	granulocyte-macrophage colony-stimulating factor
gp	glycoprotein
Gy	Grays
h	human
HEPES	N'-[2-hydroxyethyl]piperazine-N'-2-ethanesulphonic acid
Ig	immunoglobulin
IL-	interleukin
IMDM	Iscove's modified Dulbecco's medium
JCML	juvenile chronic myelogenous leukaemia
kb	kilobase pair(s)
LB	Luria broth
m	murine
M	mole(s) per litre
M-CSF	macrophage colony-stimulating factor
MPLV	myeloproliferative leukaemia virus
p	protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIPES	Piperazine-N,N'-bis[2-ethanesulphonic acid]
PV	polycythaemia vera
R	receptor eg. hIL-3R
RBC	red blood cells (erythrocytes)

RNase	ribonuclease
SCF	stem cell factor
SDS	sodium dodecyl sulphate
SFFV	Friend spleen focus-forming virus
SSC	saline sodium citrate
T	thymidine
TAE	tris/acetic acid/EDTA
Taq	*Thermus aquaticus*
TE	tris/EDTA
Tpo	thrombopoietin
Tris	tris[hydroxymethyl]amino methane
U	units
UV	ultra-violet
WBC	white blood cells (leukocytes)
% (v/v)	percentage volume per volume
% (w/v)	percentage weight per volume

The haemopoietic system is one which is remarkable both for its magnitude and the complexity of its regulation. It involves the controlled development of a small number of pluripotent haemopoietic stem cells to generate functional end cells of the eight lineages of which it is comprised. Due to the short lifespan of most of these end cells, it is a system in a constant state of flux. As such, almost one billion (10^{12}) blood cells per day are turned over in an adult (Ogawa, 1993). This development, which occurs chiefly in the bone marrow, must be balanced with self-renewal so as not to deplete the small pool of haemopoietic stem cells, which are mostly quiescent and remain in the bone marrow throughout life. Such enormity demands stringent control, which is achieved by the influence of polypeptide growth factors, which may be either soluble or membrane-bound. These growth factors exert control at four levels; those of cell survival, proliferation, differentiation, and mature cell activation. With regard to survival and proliferation both stimulatory and inhibitory growth factors have been described.

1.1 Haemopoietic growth factors in normal haemopoiesis - a stochastic or instructive process?

A key question in understanding the role of growth factors in haemopoiesis concerns whether this process is instructive or stochastic. Debate over this point has gone on for more than 30 years since Till et al. (1964) first proposed a stochastic model of haemopoiesis, and is yet to reach a clear conclusion. The models seek to explain the two fundamental choices faced by a haemopoietic stem cell. Firstly, there is the decision whether to divide or to differentiate, and secondly, having committed to differentiate, there is the choice of lineage specification. The stochastic or permissive model proposes that the decision of a stem cell to self-renew or to differentiate is a random process, as is the lineage specification of the differentiating stem cell. The probability of self-renewal is slightly higher than 0.5, ensuring that stem cell depletion does not occur (Schofield et al., 1980; Nakahata et al., 1982). The model proposes that the role of growth factors in this process is merely to provide survival signals, hence inasmuch as the receptor for a growth factor is expressed in a haemopoietic lineage, its cognate growth factor will drive proliferation and differentiation of that lineage. On

the other hand, the instructive, or deterministic model proposes that growth factors play important roles in determining whether a stem cell divides or differentiates, and in the lineage specification of differentiating multipotential cells. I will briefly review each model, as well as more recent models combining elements of each.

1.1.1 Evidence for instructive models

The majority of evidence suggesting that lineage determination is an instructive process has come from *in vitro* studies performed using transformed cell lines. Several cell lines exhibit the properties of proliferation in one growth factor and differentiation in another, implying that these growth factors can influence differentiation directly. The murine myeloid cell line 32D has the properties of continuous proliferation in murine IL-3 and differentiation in response to murine G-CSF (Rodel and Link, 1996), whilst a variant of the murine IL-3-dependent cell line FDC-P1 exhibits partial differentiation in response to murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (Matsuguchi et al., 1997). The human cell line UT-7 has the properties of growth and spontaneous granulocytic differentiation in the presence of IL-3 or GM-CSF, and partial erythroid differentiation in response to erythropoietin (Hermine et al., 1992).

Moreover, the ectopic expression of several growth factor receptors in various murine haemopoietic cell lines leads to differentiation in response to their cognate ligands. These include the receptors for Macrophage colony stimulating factor (M-CSF) (Rohrschneider and Metcalf, 1989), G-CSF (Fukunaga et al., 1993), GM-CSF (Smith et al., 1997), Thrombopoietin (Tpo) (Porteu et al., 1996), and Erythropoietin (Epo) (Liboi et al., 1993). These studies indicate that growth factors can affect the cellular decision whether to divide or differentiate in an instructive manner and that growth factor receptors transduce differentiation "signals".

It is also now clear that growth factor concentration can influence the differentiation commitment in a phenomenon known as "differentiation downgrading". Using paired progenitors (two daughter cells derived from a single parent), Metcalf (1980) showed that low concentrations of GM-CSF promote granulocyte-macrophage progenitors to form macrophage colonies where higher concentrations promote granulocyte formation from sister progenitors. Moreover, the

combination of cytokines present appears to affect lineage commitment similarly. Using replated blast cell colonies, Metcalf (1991) showed that when combined with stem cell factor (SCF), GM-CSF and IL-3 increase the proportion of granulocyte progenitors in blast colonies and IL-3 also increases the proportion of eosinophil progenitors, compared to blast colonies formed in the presence of SCF alone. This was taken as evidence that haemopoietic growth factors have the ability to induce selective lineage commitment in the progeny of multipotential progenitors. However, as GM-CSF and IL-3 also increased the numbers of secondary colonies in these experiments, they do not exclude the possibility that the progenitors committed to alternative lineages die in the absence of favourable growth factor conditions, as a stochastic model would predict.

1.1.2 Evidence for stochastic models

Till et al. (1964) first proposed a stochastic model of stem cell proliferation based on correlations between the numbers of secondary spleen colony forming units (CFU-S) in individual murine CFU-S and a random computer simulation. In this study the number of "undifferentiated" CFU-S in individual murine CFU-S appeared to follow a random distribution pattern. Similar observations have been made *in vitro* with regard to murine blast cell and mixed myeloid/erythroid colonies by Humphries et al. (1981) and Nakahata et al. (1982) respectively. Using paired progenitors, Suda et al. (1984) showed that the types and numbers of lineages formed by such cells was often disparate, consistent with a stochastic model of differentiation commitment. However, Novak and Stewart (1991) re-interpreted much of these data as being consistent with deterministic models provided a sufficiently complex control mechanism is in place.

Further evidence for the stochastic model has come from recent studies looking at ectopically expressed growth factor receptors in transgenic mouse models. Nishijima et al. (1995) found that when the human GM-CSF receptor (GMR) is expressed in transgenic mice, bone marrow cells derived from these mice form all types of colonies in response to GM-CSF, without the granulocyte-macrophage restriction usually found on stimulation with this factor. Similarly, ectopic expression of the receptors for lineage-specific growth factors such as IL-5 (Takagi et al., 1995) and Epo (Dubart et al., 1994) in murine haemopoietic cells leads to multilineage colony formation without

lineage restriction. Moreover expression of activated EpoR or CSF-1R mutants in murine pluripotent progenitors does not alter their developmental potential (Pharr et al., 1993; Pharr et al., 1994). These studies suggest that the lineage specific differentiation normally induced by these factors is a function of the location of the expression of their cellular receptors, causing them to act at the level of the committed progenitor. Moreover, they imply that haemopoietic cells contain the signalling machinery which can be utilised by a variety of growth factor receptors, if present.

Fairbairn et al. (1993) showed that suppression of apoptosis is sufficient to induce the synchronous granulocyte, macrophage and erythroid differentiation of an IL-3-dependent multipotential haemopoietic cell line in the absence of growth factors. These data demonstrate that differentiation can occur without exogenous growth factors, providing apoptosis is suppressed, suggesting that haemopoietic precursors contain an intrinsic differentiation program and that the role of growth factors in differentiation is enabling rather than inductive.

1.1.3 Evidence for Hybrid Models

In light of the apparent differences between studies using haemopoietic growth factors in transformed lines and primary cells above, models have recently been described which combine aspects of both inductive and stochastic models.

Based on the fact that autocrine GM-CSF can induce the differentiation of murine IL-3-dependent multipotent haemopoietic cell lines, Just et al. (1991) proposed a hybrid model of haemopoietic differentiation. According to the model, haemopoietic growth factor receptors signal both proliferation and differentiation, but in different proportions. In this system, then, GM-CSF signals more differentiation and less proliferation than IL-3. The nature of the differentiation is then dependent on the intrinsic program characteristic of the responding cell type. In other words, growth factors play an inductive role in the first differentiation decision, that of whether to divide or differentiate, but a permissive role in the second decision, that of lineage specification.

Consistent with this model are the findings that receptors which induce differentiation of haemopoietic cell lines tend to invoke similar differentiation programs. For instance, the signalling through ectopically expressed hybrid IL-

3Rα/IL-3Rβ or GMRα/IL-3Rβ dimeric receptors in the pro-B cell line Ba/F3 leads to partial erythroid differentiation, as does signalling through ectopically expressed Epo receptors (EpoRs) (Pless et al., 1997). The authors explain this phenomenon by suggesting that any cytokine receptor which fits the structural requirement of homodimerisation upon activation causes erythroid differentiation in this line. This suggests that the Epo-induced erythroid differentiation of this line is a result of an endogenous erythroid differentiation program in these cells, and is not specified by EpoR signalling in particular. Indeed, there is no single instance of two growth factor receptors overexpressed in the same cell line invoking disparate differentiation programs, and neither has this been conclusively shown in primary cells. Future studies should aim to discern whether such instances exist.

1.2 IL-3, IL-5 and GM-CSF in normal haemopoiesis

1.2.1 IL-3

IL-3 was first described as an activity produced by the myelomonocytic murine leukaemia line WEHI-3B, from which it was eventually cloned (Fung et al., 1984). The primary source of IL-3 is activated T cells and basophils, from which it is thought to play a role in inflammation and immunity (Wodnar-Filipowicz et al., 1989; Mosmann et al., 1986); however it is also produced by epidermal cells (Luger et al., 1985), astrocytes (Frei et al., 1986) and endothelial cells (Nilsen et al., 1998), hence IL-3 may also play a role in basal haemopoiesis.

IL-3 has the broadest effects of any known colony stimulating factor. *In vitro* it stimulates the formation of murine neutrophil, macrophage, eosinophil, basophil, megakaryocyte, mixed myeloid/erythroid and blast cell colonies (Rennick et al., 1985; Metcalf et al., 1987; Hapel et al., 1985). It is the only regulator known to stimulate mouse mast cell colony formation in isolation, however this is strongly augmented by the additional presence of SCF (Tsuji et al., 1991). It also effects development of the megakaryocyte and erythroid lineages, however terminal maturation requires the presence of thrombopoietin (Tpo) and Erythropoietin (Epo) respectively (Wu et al., 1995; Williams et al., 1984; Emerson et al., 1985). As well as its proliferative effects it supports the functional activation of neutrophils, basophils and monocytes (Weiser et al., 1987; Lopez et al., 1990; Carlson et al., 1993).

When injected into mice IL-3 causes dose-related increases in peripheral blood neutrophils, eosinophils and monocytes, splenomegaly and increases in neutrophils, eosinophils, nucleated erythroid cells, megakaryocytes and mast cells in the spleen (Metcalf et al., 1987a; Metcalf et al., 1986a). The *in vivo* effects of IL-3 have also been studied using a more powerful system, that of bone marrow reconstitution of irradiated mice with cells expressing IL-3 from a retroviral promoter (Chang et al., 1989). Using this system extremely high levels of IL-3 were achieved in the sera of transplanted animals, leading to a lethal but non-neoplastic myeloproliferative disorder characterised by elevated levels of neutrophils, monocytes and eosinophils in the blood and extensive neutrophil infiltration of the spleen, lung, liver and muscle. Hence IL-3 can effect production of all 5 myeloid lineages and early erythroid cells *in vivo*, but is not in itself transforming.

Surprisingly given the broad effects of IL-3 on haemopoiesis *in vitro* and *in vivo*, IL-3-deficient mice show no defect in basal haemopoiesis (Lantz et al., 1998). Accordingly, several inbred mouse strains which are haematologically normal show near-complete defects in response to IL-3 due to a splicing branch-point deletion in the IL-3Rα gene causing altered mRNA splicing and very low expression (Leslie et al., 1996; Ichihara et al., 1995; Muthuswamy and Muller, 1995; Morris et al., 1990). However, IL-3 deficient mice show defective mast cell production and immunity in response to parasite infection, indicating that IL-3 plays a role in host defence against infection (Lantz et al., 1998). Accordingly, IL-3 is not detectable in normal serum or tissues, hence it may play a role only as an emergency haemopoietic regulator in states that involve T lymphocyte activation (Metcalf et al., 1987a).

1.2.2 GM-CSF

Murine GM-CSF was first cloned in 1984 from a partial amino acid sequence derived from protein purified from mouse lung conditioned medium (Gough et al., 1984). It is produced by several cells of the immune system in response to inflammatory stimuli, including T (Chan et al., 1986) and B lymphocytes (Pluznik et al., 1989), macrophages (Thorens et al., 1987) and mast cells (Wodnar-Filipowicz et al., 1989), suggesting a key role as an inflammatory mediator. However GM-CSF is also produced by several cell types of non-haemopoietic origin including fibroblasts

(Zucali et al., 1986; Munker et al., 1986) and endothelial cells (Munker et al., 1986; Bagby, Jr. et al., 1986) in response to cytokine stimulation, suggesting that it may play a role in bone marrow haemopoiesis.

Indeed, GM-CSF is a potent, multilineage haemopoietic growth factor *in vitro* inducing, in order of increasing concentration, macrophage, neutrophil, granulocyte-macrophage, eosinophil, megakaryocyte and immature (CFU-blast) colonies from murine bone marrow (Koike et al., 1987; Metcalf et al., 1986; Metcalf et al., 1986). Blast cell colonies induced by GM-CSF are a subpopulation of those induced by IL-3, suggesting that this factor is not active as early in haemopoietic development as IL-3 (Koike et al., 1987). It has also been found to stimulate proliferation of human erythroid burst-forming units (BFU-E) but not their terminal maturation (Donahue et al., 1985; Metcalf et al., 1986). GM-CSF is also active on cells of non-haemopoietic origin, stimulating proliferation and migration of human endothelial cells (Bussolino et al., 1989) and proliferation of several non-haemopoietic tumour cell lines as well as normal marrow fibroblast precursors (Dedhar et al., 1988).

As well as its proliferation and maturation effects, GM-CSF stimulates activation of the effector functions of a number of mature haemopoietic cell types, including neutrophils (Lopez et al., 1986b; Weisbart et al., 1985), macrophages (Cannistra et al., 1988), eosinophils (Silberstein et al., 1986; Lopez et al., 1986b) and basophils (Lopez et al., 1990), underscoring its potential role in inflammation and immunity.

The biological activity of GM-CSF has also been studied *in vivo* in several systems. Transgenic mice overexpressing GM-CSF ubiquitously develop blindness due to macrophage infiltration of the eyes, and tissue damage due to macrophage infiltrates in the skeletal muscle and peritoneum of the mice (Lang et al., 1987). Unexpectedly, these mice showed no hyperplasia of the neutrophil lineage, however the pattern of transgene expression suggests that the gene was only active in macrophages. In contrast are bone marrow reconstituted mice whose haemopoietic cells express GM-CSF from a retrovirus (Johnson et al., 1989). These mice show much higher serum levels of GM-CSF than the transgenic mice. Accordingly, the mice succumbed to a lethal non-neoplastic myeloproliferative syndrome with elevated levels of neutrophils, monocytes and eosinophils in the peripheral blood, neutrophil and macrophage infiltration of the spleen, lung, liver, and peritoneal cavity and neutrophil, macrophage

and eosinophil infiltration of heart and skeletal muscle. These data indicate that GM-CSF causes production of neutrophils, monocytes and eosinophils *in vivo*. Whilst overexpression of GM-CSF did not in itself lead to neoplastic transformation, it is known that GM-CSF can play a role in leukaemic progression (see below).

To assess the requirement of GM-CSF for haemopoiesis, GM-CSF knockout mice have been developed (Stanley et al., 1994; Dranoff et al., 1994). Surprisingly, given the *in vitro* and *in vivo* activities of the factor, the haemopoietic system of these mice was completely normal in terms of numbers progenitors and circulating leucocytes. The only discernible defect in these mice was alveolar proteinosis and surfactant accumulation in the lungs due to defective activation of alveolar macrophages, leading to a susceptibility to lung infection. These data suggest that GM-CSF has a critical role in the activation of certain types of tissue macrophages, however its role in blood cell production, if any, can be compensated for by other factors.

1.2.3 IL-5

IL-5 was first cloned as a B cell growth factor (BCGFII) (Kinashi et al., 1986). However it was found to be identical to the previously identified factors eosinophil differentiation factor (EDF) and T cell replacing factor (TRF) (Takatsu et al., 1987; Campbell et al., 1987). Like GM-CSF and IL-3, its primary source is activated T cells (Swain et al., 1988). Treatment of human or mouse bone marrow cultures with IL-5 leads to the growth of eosinophil colonies specifically (Yokota et al., 1987; Campbell et al., 1987). Whilst GM-CSF and IL-3 also stimulate eosinophil development, IL-5 is unique in its specificity for this lineage. Unlike GM-CSF or IL-3, IL-5 exerts its actions at the level of the eosinophil progenitor. leading to maturation of these progenitors without expansion in number (Clutterbuck and Sanderson, 1990). In addition to stimulating their production, IL-5 activates both murine and human eosinophils in a number of assays (Lopez et al., 1988; Lopez et al., 1986a).

As well as its activities on eosinophils, IL-5 has effects on lymphoid development, stimulating proliferation and the final steps of maturation of B cells in mice but not humans (reviewed in Sanderson et al. (1988)) and inducing T cell activity (Takatsu et al., 1987).

Transgenic mice carrying the IL-5 gene under control of the CD2 locus control region develop a profound eosinophilia with modest increase in blood neutrophil, lymphocyte and monocyte numbers (Dent et al., 1990). Hence IL-5 is relatively specific for the eosinophil lineage *in vivo*. However, in a separate study of mice expressing IL-5 under control of the metallothionein promoter, the CD-5^+ (B-1) B cell subfraction was also elevated, confirming the actions of IL-5 on these cells *in vivo* (Tominaga et al., 1991). These biological functions were confirmed by the generation of IL-$5^{-/-}$ mice (Kopf et al., 1996). These mice showed reduced levels of $CD5^+$ B cells in young mice which recovered with age. Basal eosinophil levels were 2- to 3-fold reduced in these mice, and the mice were unable to mount an eosinophilic response to parasite infection. The lack of eosinophilic response is unlikely to be due to the abnormal haemopoietic system of these mice, since treatment of normal mice with an anti-IL-5 antibody inhibits eosinophil production in response to parasitic infection similarly (Coffman et al., 1989). A requirement for IL-5 for emergency eosinophilopoiesis is consistent with previous reports detecting IL-5 in the sera of mice undergoing experimental eosinophilia due to parasitic infection (Strath and Sanderson, 1986). Hence IL-5 has non-redundant roles in both basal and emergency eosinophilopoiesis and in B cell development *in vivo*.

1.3 The cytokine receptor superfamily

1.3.1 Subunit composition

The cytokine receptor family is a large growth factor receptor family defined by structural characteristics. This family is divided into subfamilies based on oligomeric structure and shared components (Watowich et al., 1996). Subfamily I cytokine receptors bind their cognate cytokine as homodimers. This includes the receptors for Epo, Tpo, G-CSF, Growth Hormone (GH) and Prolactin (Prl). Subfamily IIa receptors consist of two subunits; α subunits specific for each cytokine whose role is primarily ligand binding, but which may also play a role in signal transduction, and a shared β subunit (hβc) which is responsible for increasing the affinity of ligand binding and for signal transduction. This subfamily includes the receptors for IL-3, IL-5 and GM-CSF. Subfamily IIb is also characterised by receptors sharing a common signalling subunit, gp130. This includes the receptors for IL-6, IL-11, ciliary neurotrophic factor (CNTF),

leukaemia inhibitory factor (LIF) and oncostatin M (OSM). In the case of the IL-6 and LIF receptors the receptor consists of a ligand-binding subunit and gp130, the other receptors also have LIFR as a shared component. Subfamily III are receptors sharing the IL-2Rγ subunit. This subfamily, each of which is composed of two or three subunits, includes the receptors for IL-2, -4, -7, -9, -13, and -15.

1.3.2 Protein structure

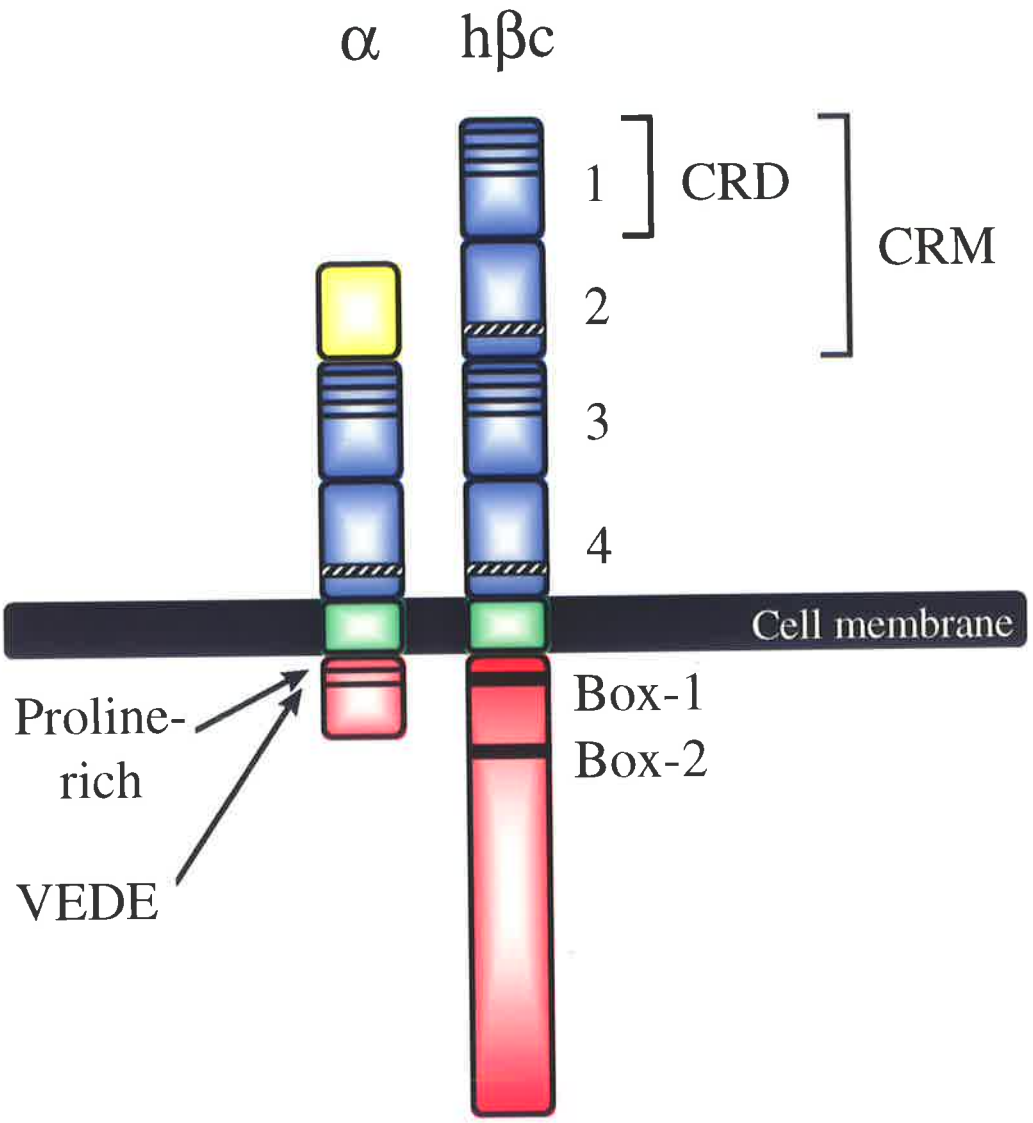
Members of the cytokine receptor superfamily contain either one or two copies of a conserved domain of approximately 200 amino acids in their extracellular regions, termed the cytokine receptor module (CRM). This motif is comprised of two globular subdomains each of which are related to the fibronectin type III domain. These subdomains have been termed the cytokine receptor domains (CRDs). Each forms a 7-stranded β-barrel structure which was first predicted by Bazan (1990) and has since been confirmed by the solving of the crystal structure of the human Growth Hormone (GH) and prolactin (Prl) receptors (Somers et al., 1994; De Vos et al., 1992). Each β strand is named in order from the N-terminus, with those in the N-terminal CRD designated A-F and those in the C-terminal subdomain termed A′-F′. Within each CRM are several features conserved throughout the cytokine receptor superfamily (Figure 1.1). In the membrane-distal (N-terminal) subdomain are 4 conserved cysteine residues in the pattern of $CX_{9-10}CXWX_{26-32}CX_{10-15}C$, where X represents any amino acid, which are involved in forming intra- and inter-molecular disulfide bonds to stabilise either monomeric or dimeric structures. The membrane proximal (C-terminal) subdomain contains a pattern of alternating hydrophobic residues (consensus YXVXVRVR) and the most highly conserved WSXWS motif which is the hallmark of the cytokine receptor superfamily. The role of this motif has been an area of intense investigation which has shown an involvement in protein folding, receptor expression, ligand binding and receptor internalisation in various cytokine receptors, with the most consistent defects being in protein folding (Miyazaki et al., 1991; Hilton et al., 1996; Quelle et al., 1992; Yoshimura et al., 1992; Baumgartner et al., 1994).

The cytoplasmic domains of the cytokine receptors do not contain any known enzymatic domains such as kinase domains. However, in the membrane proximal portion of this domain are two regions conserved throughout several signal

Figure 1.1. Structure of the receptors for human IL-3, IL-5 and GM-CSF

The 'α' subunit is representative of the IL-3, IL-5 or GM-CSF α subunit. CRDs are shown in blue, transmembrane domains in green, intracellular regions in red and the approximately 100 amino acid N-terminal domain of the α subunits unrelated to the CRM is depicted in yellow. Conserved features are shown as follows; extracellular conserved cysteine residues are depicted as solid lines, striped boxes represent the WSXWS motif. In the intracellular regions Box-1 and Box-2 of the hβc subunit and the proline-rich and VEDE motifs of the α subunits are depicted as solid lines as indicated. Cytokine receptor domains 1-4 are indicated.

CRD- cytokine receptor domain. CRM- cytokine receptor module.


α
hβc
1
CRD
CRM
2
3
4
Cell membrane
Box-1
Box-2
Proline-
rich
VEDE

transducing members of this superfamily, termed the box 1 and box 2 motifs (Murakami et al., 1991). These motifs are involved in the binding of members of the Janus (JAK) family of protein tyrosine kinases (Ihle, 1995).

1.3.3 Activation

It is now well established that activation of growth factor receptors is dependent on oligomerisation of signal-transducing receptor components. This is true of both receptor tyrosine kinases (RTK) family and cytokine receptors (reviewed in Lemmon and Schlessinger, (1995), Heldin, (1994)). The importance of dimerisation for activation is parallelled in several aspects of signal transduction through these receptors, in that several kinases and transcription factors activated by cytokine receptors function as dimers.

Like members of the RTK family, several cytokine receptors function as homodimers in which the cognate growth factor binds two receptor molecules at separate sites, leading to dimerisation. These include the receptors for Epo, Growth Hormone, Prolactin (Prl), Tpo and Granulocyte Colony Stimulating Factor (Watowich et al., 1992; Fukunaga et al., 1990; Sarica et al., 1995; D'Andrea et al., 1994; Cunningham et al., 1991; Chang et al., 1998). Moreover, the signal transducing components of several multimeric cytokine receptors form homodimers upon ligand binding. For example, IL-6 binding leads to homodimerisation of the gp130 signal-transducing component of its receptor (Murakami et al., 1993). The stoichiometry of this receptor complex has been solved using analytical ultracentrifugation, and is a hexamer consisting of two copies each of IL-6, IL-6α, and gp130 proteins (Ward et al., 1994).

Further evidence for this concept has come from studies using activated mutants of cytokine receptors. A spontaneously arising mutation in the EpoR, R129C, converts an asparagine residue in the extracellular domain to cysteine, leading to activation (Yoshimura et al., 1990b). Based on an alignment with the growth hormone receptor, Arg^{129} is predicted to reside in the interface formed between wild-type EpoR proteins in the presence of ligand (Watowich et al., 1994). The activating mutation leads to the formation of a disulfide bond between the introduced cysteine residues in the absence of ligand, causing homodimerisation (Watowich et al., 1992). Several other residues

placed at the same site which do not homodimerise are not activating implying that disulfide-linked homodimerisation is essential for the activated phenotype. Moreover, introduction of cysteine residues at other sites predicted to lie in the EpoR dimer interface leads to activation (Watowich et al., 1994). Based on these studies, Alexander et al. (1995) introduced cysteine residues into regions of the Tpo receptor (c-Mpl) predicted to lie at the receptor interface. Similarly to the case of the EpoR, these mutants were constitutively active and formed disulfide-linked dimers in the absence of ligand. These results imply that dimerisation is an important step in activation of cytokine receptors, and may be sufficient to do so.

1.4 The Receptors for IL-3, IL-5 and GM-CSF

IL-3, IL-5 and GM-CSF are pleiotrophic growth factors which exhibit a number of functional similarities. As outlined above, IL-3 and GM-CSF stimulate the formation of similar colony types from murine bone marrow. Moreover, both growth factors activate mature neutrophils, mast cells and eosinophils. Their activities on eosinophil colony formation and activation are also shared with IL-5. Moreover these cytokines cross-compete for binding to human cells (Lopez et al., 1989; Lopez et al., 1991). These functional redundancies and cross-competition were explained, at least in part, by the finding that the receptors for these factors share a common signal-transducing subunit. On the basis of their shared signalling component and similarity in receptor composition these receptors constitute the IIa subfamily of the cytokine receptors superfamily.

1.4.1 Structure

The receptors for IL-3, IL-5 and GM-CSF are comprised of two membrane spanning subunits (Figure 1.1). Firstly, there are specific α subunits which bind each growth factor with low affinity but cannot deliver a proliferative signal (Tavernier et al., 1991; Murata et al., 1992; Kitamura et al., 1991; Gearing et al., 1989). Rather, these α subunits complex with a common signal transducing β subunit (hβc), which does not on its own detectably bind any of these factors, to form a high affinity signalling receptor complex (Tavernier et al., 1991; Kitamura et al., 1991; Hayashida et al., 1990). In mice this situation is complicated by the presence of a second, IL-3 specific

β subunit, $m\beta_{IL-3}$, in addition to a murine homologue of hβc, mβc, which is shared between all three receptors (Hara and Miyajima, 1992; Gorman et al., 1990). These two subunits have arisen by chromosomal duplication late in evolution such that even relatively closely related species such as rats do not have a second β subunit (Gorman et al., 1992; Appel et al., 1995). As such, the two subunits are highly homologous, sharing 91% amino acid sequence identity with most changes being homologous (Hara and Miyajima, 1992). Targeted inactivation of each subunit suggests that they deliver similar signals upon IL-3 stimulation (Nishinakamura et al., 1995).

All three α subunits as well as hβc are members of the cytokine receptor superfamily. The α subunits each contain one CRM in the extracellular region whereas hβc contains two. The four CRDs in hβc have been termed domain 1-4 in order from the N-terminus. In addition to the CRM, the extracellular domains of the α subunits contain an approximately 100 amino acid N-terminal domain unrelated to the CRM. Likewise the intracellular domain of hβc is larger than those of the α subunits (432 amino acids compared to 53, 55 and 54 for human IL-3Rα, IL-5Rα and GMRα respectively). It contains two regions of similarity with other cytokine receptors, termed box 1 and 2. In the cytoplasmic domains of the α subunits are two motifs conserved between all three; a proline rich sequence and a VEDE motif (Figure 1.1).

1.4.2 Functional Activation

As discussed above, receptor oligomerisation is an important step in cytokine receptor activation. Accordingly, chimeric receptors consisting of the extracellular and transmembrane domains of hIL-2Rβ, hIL-4R, hEpoR and mEpoR fused to the cytoplasmic domain of $m\beta_{IL-3}$ are functionally active in the presence of hIL-2, hIL-4, hEpo and mEpo respectively (Sakamaki et al., 1993; Chiba et al., 1993). Hence forced dimerisation of the cytoplasmic domain of $m\beta_{IL-3}$ appears sufficient to induce signal transduction. However, hβc exists as a homodimer in the absence of ligand, implying that physical proximity is not sufficient for activation (Muto et al., 1996). Moreover, the human GM-CSF receptor exists as a preformed complex of hGMRα and hβc in the absence of GM-CSF. This was first predicted by Ronco et al. (1994) who showed that a mutant hGMRα subunit which was unable to bind GM-CSF with low affinity was still capable of forming a high-affinity receptor when expressed with hβc. This

has since been confirmed by two lines of evidence. Firstly, Murray et al. (1996) showed that a soluble hGMRα was retained on the surface of cells expressing hβc, and subsequently Woodcock et al. (1997) co-immunoprecipitated the preformed complex. The intrinsic interaction of these subunits is weak, is not covalently linked, and is stabilised over 1000-fold by the presence of GM-CSF. In the case of the IL-3 and IL-5 receptors no such preformed complex has been detected. Recently it has been shown that truncated forms of GMRα behave as dominant negatives, suggesting the presence of 2 GMRα subunits in the active complex (Lia et al., 1996). Hence the likely stoichiometry of the active human GMR is GM-CSF$_2$:α_2:β_2.

Ligand binding induces covalent-linked heterodimerisation of both IL-3 and GM-CSF receptors and phosphorylation of the β subunit but not the α subunits (Woodcock et al., 1997; Stomski et al., 1996). In the case of the hIL-3 receptor, disulfide linkage is essential for receptor activation as measured by tyrosine phosphorylation but not for ligand binding (Stomski et al., 1996).

1.4.3 Signal transduction

Signal transduction through the IL-3, IL-5 and GM-CSF receptors requires the cytoplasmic domains of both α and β subunits (Barry et al., 1997; Takaki et al., 1994; Sakamaki et al., 1992; Polotskaya et al., 1993; Muto et al., 1995). Based on the fact that hβc has a large cytoplasmic domain compared to the α subunits and is tyrosine phosphorylated on receptor activation, this subunit is thought to be responsible for the majority of signalling through these receptor complexes. Accordingly, chimeric receptors consisting of the cytoplasmic portion of hβc connected to the extracellular domains of GMRα and IL-5Rα deliver ligand-dependent survival and proliferation signals to murine haemopoietic cell lines, implying that the hβc cytoplasmic domain is sufficient for these functions (Kouro et al., 1996; Takaki et al., 1994; Eder et al., 1994).

As mentioned previously, the cytoplasmic domains of the cytokine receptor superfamily contain no intrinsic enzymatic domains such as kinases. However, ligand stimulation leads to rapid tyrosine phosphorylation of several substrates, including the receptors themselves. This is achieved through the recruitment of cytoplasmic tyrosine

kinases. Tyrosine kinases which have been implicated in IL-3/IL-5/GM-CSF signalling include Lyn, Fps/Fes, Tec, Syk, Btk, JAK1, Tyk2 and JAK2 (Tsubokawa et al., 1997; Torigoe et al., 1992; Silvennoinen et al., 1993; Nagata and Todokoro, 1996; Mano et al., 1995; Li et al., 1995; Hanazono et al., 1993). Other than JAK2, the role of these kinases in signal transduction is at present ill-defined. In contrast, however, JAK2 is critical for signal transduction by these growth factors. Progenitors from $JAK2^{-/-}$ mice are unable to respond to a number of cytokines, including IL-3 (Parganas et al., 1998). However, introduction of JAK2 into these progenitors by retroviral transduction restores their ability to respond to IL-3. These results imply that JAK2 is essential for IL-3 signalling in mice. Further evidence for this has come from *in vitro* studies. Expression of a dominant negative form of JAK2 completely blocks hGMR-mediated proliferation of the murine pro-B cell line BAF-B03 (Watanabe et al., 1996). Moreover, deletion of the Box-1 motif which JAK2 binds via its N-terminal region results in loss of JAK2 activation, hβc phosphorylation and growth signal transduction through hβc (Itoh et al., 1996; Zhao et al., 1995; Quelle et al., 1994). These results indicate that JAK2 is required for phosphorylation of hβc and is crucial for signal transduction through hβc.

Members of the Janus kinase family (JAKs) associate with cytokine receptors either constitutively or following ligand binding (Ihle, 1995). Their activation in thought to be brought about by dimerisation caused by the oligomerisation of their bound receptors. In support of this, a chimeric protein consisting of the extracellular and transmembrane domains of the epidermal growth factor (EGF) receptor fused to the JAK2 kinase domain induced EGF-dependant activation of the kinase domain, implying that dimerisation is sufficient for activation of JAK2 (Nakamura et al., 1996). Among the substrates of JAKs are cytokine receptors themselves, as well as members of the signal transducers and activators of transcription (STAT) family of transcription factors (Ihle, 1995). STATs are a family of latent cytoplasmic transcription factors which upon cytokine stimulation become phosphorylated, dimerise and translocate to the nucleus where they bind to specific DNA sequences and activate transcription. IL-3/IL-5/GM-CSF signalling in mice induces activation of two STAT5 homologues, STAT5a and STAT5b, which are 96% identical at the amino

acid level, and to a lesser extent STAT1 and STAT3 (Nagata and Todokoro, 1996; Mui et al., 1995).

STAT activation is generally thought to occur through binding to phosphotyrosine motifs on activated cytokine receptors followed by phosphorylation by JAKs. However, a hGMR with all cytoplasmic tyrosines mutated is still able to activate STAT5, implying that in this case JAK2 activates STAT5 directly (Okuda et al., 1997). Accordingly, whilst STAT5 interacts with the tyrosine phosphorylated IL-3 receptor through its *src* homology (SH)2 domain, STAT5 activation can also occur through a direct interaction with the JH2 domain of members of the JAK family (Barahmand-Pour et al., 1998; Fujitani et al., 1997; Chin et al., 1996). STAT5 is required for maximal IL-3-dependent proliferation and for the induction of several genes including *cis*, *osm*, and *pim-1* (Mui et al., 1996). Whilst the role of STAT5 proteins in differentiative signalling through hβc has not been shown, they have been implicated in Epo-induced erythroid differentiation, and may function similarly in signalling through hβc (Wakao et al., 1997; Iwatsuki et al., 1997). However, mice lacking STAT5 proteins have no defects in erythroid development, unlike $Epo^{-/-}$ mice, suggesting a redundant role of these proteins in erythroid development (Wu et al., 1995; Teglund et al., 1998). In contrast, there are quantitative defects in colony formation induced by IL-3, IL-5 or GM-CSF in mice lacking both STAT5a and STAT5b proteins, suggesting a role for these proteins in IL-3/IL-5/GM-CSF signalling *in vivo* (Teglund et al., 1998).

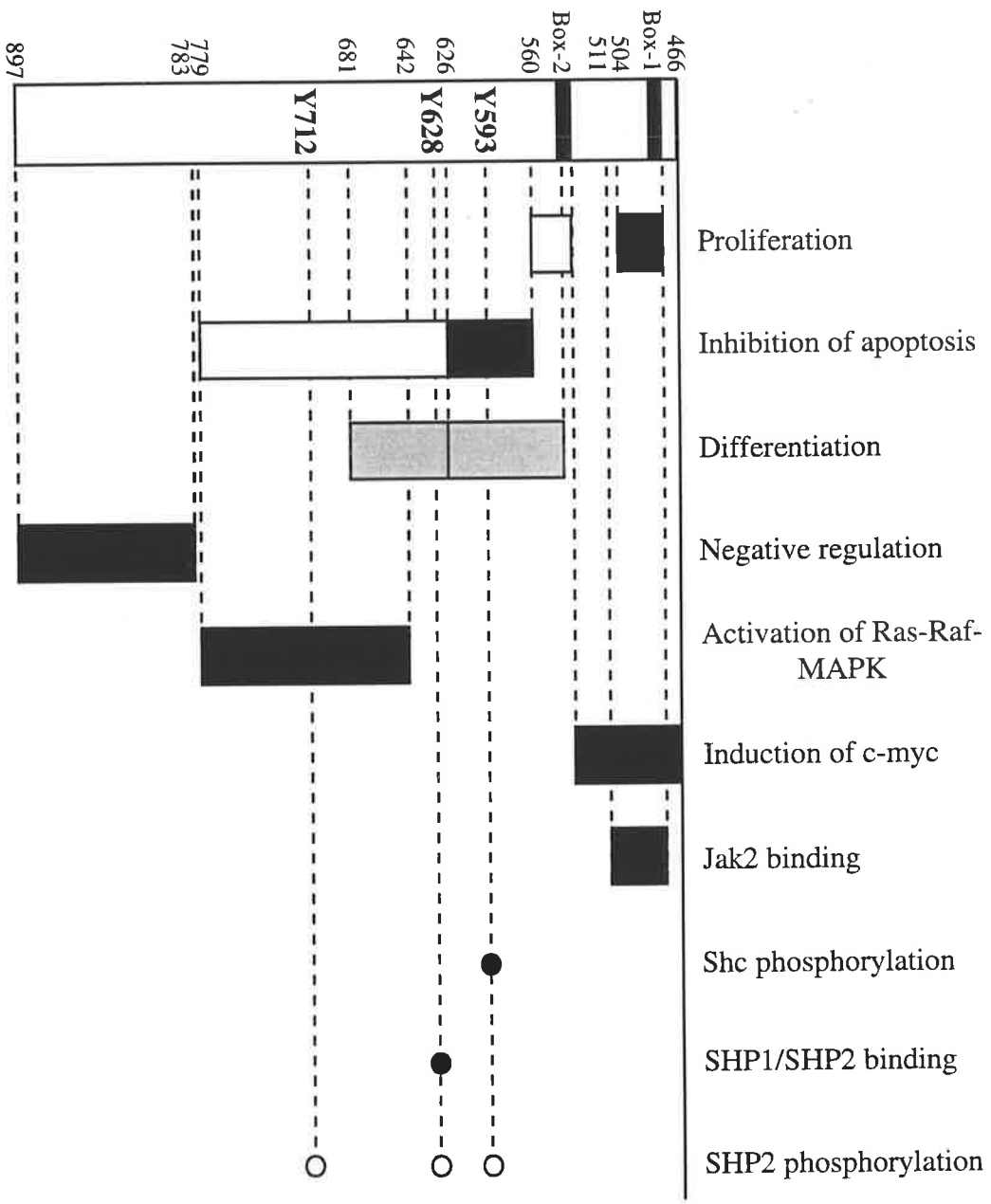
The minimal region of hβc cytoplasmic domain required to achieve cellular proliferation is a membrane proximal domain of 31 amino acids (amino acids 473-504), containing the box 1 motif, which is a similar region to that sufficient for JAK2 binding. However, a more distal 25 amino acid region containing Box 2 (amino acids 535-560) is required for maximal growth stimulation (Figure 1.2) (Sakamaki et al., 1992).

Whilst the hβc cytoplasmic region up to amino acid 560 will efficiently mediate cell proliferation, Kinoshita et al. (1995) showed that this proliferation was transient and that long-term proliferation requires supression of apoptosis, the minimal essential region for which lies between amino acids 560 and 779. Expression of Ras was sufficient to rescue the defect in suppression of apoptosis of a hGMR lacking this

Figure 1.2. Cytoplasmic domains of hβc critical for functions of the hGM-CSF receptor in various murine haemopoietic cell lines

Shown are the cytoplasmic regions of hβc required for proliferation (Sakamaki et al., 1992), inhibition of apoptosis (Smith et al., 1997; Kinoshita et al., 1995; Inhorn et al., 1995), differentiation (Smith et al., 1997; Matsuguchi et al., 1998), negative regulation (Smith et al., 1997), activation of the Ras-Raf-MAPK pathway (Sato et al., 1993b), induction of c-myc (Sato et al., 1993b), JAK2 binding (Zhao et al., 1995; Quelle et al., 1994), Shc phosphorylation (Okuda et al., 1997; Itoh et al., 1996; Durstin et al., 1996), binding of SHP1/SHP2 (Bone et al., 1997) and SHP2 phosphorylation (Okuda et al., 1997). It should be noted that most of the data shown are from C'-terminal truncation or internal deletion studies hence the shown functions may require more N'-terminal regions also. For example, JAK2 binding is thought to be required for all of the outcomes listed.

The entire hβc intracellular domain is shown. Amino acids are numbered from the first amino acid of the hβc leader sequence. Box-1 and Box-2 are shown as black boxes in the hβc cytoplasmic domain. Tyrosine residues with known functions are shown. Dark shading indicates regions of the hβc cytoplasmic domain required for a particular outcome, whilst white shading shows regions which enhance a response, or which are required for some outcomes but not others. Where there is direct controversy grey shading is shown. Circles indicate specific tyrosine residues implicated in a response.


897
779
783
681
642
626
560
Box-2
504
511
Box-1
466
Y712
Y628
Y593
Proliferation
Inhibition of apoptosis
Differentiation
Negative regulation
Activation of Ras-Raf-MAPK
Induction of c-myc
Jak2 binding
Shc phosphorylation
SHP1/SHP2 binding
SHP2 phosphorylation

region, suggesting that the defect may be due to an inability to activate Ras. However, the region required was later refined by Smith et al. (1997), who showed that truncations to amino acid 626 allowed long-term proliferation of CTLL-2 cells, and hence postulated that the essential region for suppression of apoptosis lies between amino acids 560 and 626 (Figure 1.2).

As well as its proliferative and anti-apoptotic effects, the hGMR mediates differentiation signalling in myeloid leukaemic cell lines. The regions required for this were examined by Smith et al. (1997). It was found that whilst a regions between amino acids 541 and 626 were required for differentiation of the murine myeloid leukaemic cell line M1, a further 157 amino acids (amino acids 626 - 793) were required for differentiation of a different murine leukaemic cell line, WEHI-3B D+. A region within this membrane-distal domain, encompassing amino acids 626 - 681, was found to be required for differentiation of a variant of the FDC-P1 cell line, WT19 (Matsuguchi et al., 1998). Hence the regions required for differentiation appear to vary between different cell lines (Figure 1.2).

Ligand-induced phosphorylation of hβc allows binding of several proteins containing SH2 domains. Amongst these are the STAT5 proteins as well as SHP1, SHP2 (Bone et al., 1997; Yi et al., 1993) and Shc, the phosphorylation of which leads to activation of the Ras-Raf-MAP kinase pathway (Pratt et al., 1996). The membrane-proximal 60 amino acids of hβc are sufficient for induction of *c-myc*, and *pim-1* but not for activation of the Ras-Raf-1-MAP kinase cascade, p70 S6 kinase, induction of *c-fos* and *c-jun*, these actions require a more distal region of 137 amino acids (amino acids 642-779) (Kinoshita et al., 1995) (Figure 1.2). Intriguingly, Pratt et al. (1996) showed that Shc binds to tyrosine 593. Since Shc activation is thought to be responsible for activation of the Ras-Raf pathway, this seems incongruous with the previous finding that a more distal region is required. However, a hβc protein with Tyr^{593} mutated can still mediate partial Shc phosphorylation and activate the Ras-Raf pathway, hence other regions of hβc are also important to achieve this (Durstin et al., 1996).

Signal transduction through the human IL-3/IL-5/GM-CSF receptors requires the cytoplasmic domain of the α subunits in addition to that of hβc subunits. (Weiss et al., 1993; Takaki et al., 1994). The critical region of hGMRα required for mitogenesis and

JAK2 activation is within the membrane-proximal 29 amino acids, which contains the conserved proline-rich region and VEDE motif (Figure 1.1) (Weiss et al., 1993; Doyle and Gasson, 1998). Mutation of the proline-rich region abolishes signalling through the hIL-5R, whilst deletion of the VEDE motif does so through the hGMR, confirming the importance of these conserved regions (Doyle and Gasson, 1998; Kouro et al., 1996). However, the ability of chimeric hGMRα/hβc proteins to signal proliferation and activate JAK kinases suggests that the cytoplasmic domain of hβc is sufficient to transduce proliferative signals and that the role of the cytoplasmic domains of the α subunits may be simply to stabilise the active receptor complexes (Eder et al., 1994).

1.4.4 Negative Regulation

Signal transduction through the IL-3/IL-5/GM-CSF receptors is balanced by negative regulation, which occurs through two distinct mechanisms. The first of these is feedback inhibition. STAT5 activation leads to induction of a several members of the suppressor of cytokine signalling (SOCS) family, including SOCS-1, -2, -3 and CIS (Yoshimura et al., 1995; Starr et al., 1997). Whilst SOCS-1 interacts with the JAK2 kinase domain and inhibits JAK2 activity, CIS binds to the phosphorylated IL-3 receptor and is a negative modulator of STAT5 activation and IL-3-induced signal transduction (Yoshimura et al., 1995; Matsumoto et al., 1997; Endo et al., 1997). Similarly, haemopoietic cell phosphatase (HCP), also known as SHP1, binds to tyrosine 628 of hβc and inhibits IL-3-induced mitogenesis (Figure 1.2) (Yi et al., 1993; Bone et al., 1997). In this case the likely mode of inhibition is through the direct binding of SHP1 to JAK2 leading to its dephosphorylation and the termination of proliferative signals (Jiao et al., 1996).

Secondly, ligand binding and subsequent phosphorylation of the IL-3 receptor leads to internalisation and proteolytic cleavage of the receptor complex (Mui et al., 1992). This is thought to be the general method for long-term suppression of cytokine-mediated signalling.

Deletion of the C-terminal 114 amino acids of hβc leads to an approximately 10-fold hyper-responsive form of the hGMR, implying that this region comprises a

negative regulatory domain, the nature of which is at present unknown (Figure 1.2) (Smith et al., 1997).

1.5 Haemopoietic Growth Factors in Neoplastic Haemopoiesis

Normal haemopoietic cells are absolutely dependent on the presence of exogenous growth factors for survival and proliferation. However, cells from haemopoietic disorders such as leukaemia frequently display reduced or absent growth factor requirements. Abrogation of cellular growth factor requirement is one mechanism whereby neoplastic cells escape normal regulatory constraints. This abrogation can occur by several mechanisms, including autocrine growth factor production, paracrine growth stimulation, activation of growth factor receptors and constitutive activation of signal transduction pathways normally utilised by growth factor receptors.

1.5.1 Autocrine growth factor production

A minority of blast cells from most samples of acute myeloid leukaemia (AML) can be cultured *in vitro* either in liquid suspension cultures or in colony form in semi-solid medium. The clonogenic cells are generally a small proportion of the sample, and display a more immature surface antigen phenotype than non-clonogenic leukaemic cells (Griffin and Lowenberg, 1986). Blast cells from AML have been shown to respond to exogenous growth factors such as GM-CSF, IL-3 and G-CSF (Griffin et al., 1986; Vellenga et al., 1987; Kelleher et al., 1987; Hoang et al., 1986; Delwel et al., 1987; Delwel et al., 1988), however such cells often exhibit autonomous growth. Reilly et al. (1989) divided AML blasts into four categories based on behaviour in semi-solid methylcellulose culture. Group 1 blasts fail to grow in this culture system, group 2 blasts require an exogenous growth factor stimulus for growth, group 3 blasts show a degree of autonomous growth which is cell density dependent and can be supplemented by the addition of growth factors, whilst group 4 blasts exhibit totally autonomous growth. In separate studies 18/25 and 14/14 cases of AML were shown to exhibit some degree of autonomous growth (Reilly et al., 1989; Murohashi et al., 1989).

It is now clear that in most cases the autonomous growth of AML blasts is due to autocrine production of GM-CSF (Young and Griffin, 1986; Kaufman et al., 1988;

Young et al., 1987). However, AML blasts frequently produce other factors such as IL-1, IL-6, and G-CSF, M-CSF and TNFα (van der Schoot et al., 1989; Cheng et al., 1988). In most cases the growth of cells expressing GM-CSF can be blocked by neutralising antibody against this growth factor. However, in some cases blast cells which express GM-CSF transcripts are not blocked by anti-GM-CSF antibodies, but are blocked by antisense GM-CSF oligonucleotides (Rogers et al., 1994). This is taken as evidence for internal autocrine loops operating in these cases without growth factor secretion. A similar mechanism was observed in *in vitro* studies of FDC-P1 cells expressing GM-CSF in an autocrine manner (Lang et al., 1985). In addition to AML, autocrine GM-CSF production has been demonstrated in juvenile chronic myelogenous leukaemia (JCML) and acute lymphoblastic leukaemia (ALL) (Gualtieri et al., 1989; Freedman et al., 1993).

That growth factor independence is a step in oncogenesis is also supported by several lines of evidence from *in vitro* studies. Firstly, the murine myelomonocytic leukaemia cell line WEHI-3B synthesises murine interleukin-3 constitutively due to retroviral insertion of a retrovirus-like intracisternal A particle (IAP) near the gene (Ymer et al., 1985). A similar IAP insertion has lead to overexpression of the *Hox-B8* (previously *Hox-2.4*) gene in these cells, expression of which immortalises mouse myelomonocytic progenitors without conferring factor-independence (Kongsuwan et al., 1989; Perkins and Cory, 1993; Blatt et al., 1988). Accordingly, retroviral expression of both *Hox-B8* and IL-3 in murine bone marrow cells yields leukaemic cell lines resembling WEHI-3B (Perkins et al., 1990). Hence autocrine IL-3 synthesis plus cellular immortalisation is sufficient to elicit myeloid leukaemia in this system, whereas neither does so alone. Similar observations have been made regarding autocrine GM-CSF synthesis and immortalisation using the cellular proto-oncogene *c-myb* (Gonda et al., 1989).

Moreover, autocrine expression of GM-CSF or IL-3 confers factor-independence and tumorigenicity on the otherwise GM-CSF- or IL-3-dependent, non-tumorigenic murine myelomonocytic cell line, FDC-P1 (Lang et al., 1985; Hapel et al., 1986). It has also been shown that autocrine IL-5 expression in a murine myeloid cell line K-5 confers tumorigenicity on this line. Hence whilst IL-3, IL-5 and GM-CSF can

contribute to oncogenicity in murine cell lines via autocrine mechanisms, only GM-CSF has been demonstrated to contribute to human leukaemia by such a mechanism.

1.5.2 Paracrine growth factor production

In addition to autocrine growth factor production, abrogation of cellular growth factor dependence can eventuate by paracrine growth factor production. Blast cells from some AML samples produce IL-1, a factor which has been shown to induce expression of GM-CSF and other cytokines from bone marrow stroma and leukaemic blasts themselves (Cozzolino et al., 1989; Sakai et al., 1987; Griffin et al., 1987; Delwel et al., 1989). In several cases antibodies to IL-1 suppress the growth of leukaemic blasts due to the inhibition of autocrine GM-CSF production by these blasts (Rodriguez-Cimadevilla et al., 1990; Bradbury et al., 1990). Hence autocrine IL-1 production by AML blasts is thought to contribute to leukaemogenesis by upregulating GM-CSF production by bone marrow stroma and the by inducing autocrine production by the blasts themselves. This may explain the lack of GM-CSF gene rearrangement found in most cases of AML (Falcinelli et al., 1991).

1.6 Activated Growth Factor Receptors in Neoplastic Haemopoiesis

Another method whereby haemopoietic cells can overcome the requirement for exogenous growth factors is by mutation the receptors for such growth factors leading to constitutively active forms which can signal in the absence of growth factors. This has been demonstrated for both tyrosine kinase receptors and cytokine receptors.

1.6.1 Tyrosine Kinase Receptors

The first examples of activated growth factor receptors were provided by transforming retroviruses which had transduced altered forms of cellular tyrosine kinase receptors. For example, the McDonaugh strain of feline sarcoma virus responsible for fibrosarcomas in cats has transduced an altered form of the macrophage colony stimulating factor (M-CSF) receptor, *v-fms*. Unlike its cellular homologue, *c-fms*, *v-fms* exhibits constitutive tyrosine kinase activity and leads to factor-independence and tumorigenicity of factor-dependent myeloid cell lines (Wheeler et al., 1986; Wheeler et al., 1987). Similarly, the *v-ErbB* gene transduced by the avian erythroblastosis virus

and the *v-kit* gene transduced by the feline sarcoma virus are activated forms of the cellular genes encoding the receptors for Epidermal Growth Factor and Steel Factor, respectively (Graf and Beug, 1983; Downward et al., 1984). These studies indicate that activated forms of cellular tyrosine kinase receptors can contribute to oncogenesis. Moreover, activated forms of the human receptors for Steel Factor and Fibroblast Growth Factor have been found in human mast cell leukaemia and achondroplastic dwarfism respectively, indicating that activated tyrosine kinase receptors can play a role in human disorders (Webster and Donoghue, 1996; Furitsu et al., 1993).

1.6.2 Cytokine Receptors

Whilst not as well studied as the activated forms of cellular tyrosine kinases, it has recently become apparent that the activated forms of several cellular cytokine receptors can contribute to oncogenesis. Indeed, this might be expected, as tyrosine kinase receptors and cytokine receptors stimulate many of the same signal transduction pathways and often deliver similar signals to the same cell types, for example, macrophage development and activation by both c-fms and the GM-CSFR. To date activating mutations have been found in the receptors for thrombopoietin, erythropoietin and the common β subunit of the IL-3, IL-5 and GM-CSF receptors (hβc).

1.6.2.1 The TpoR

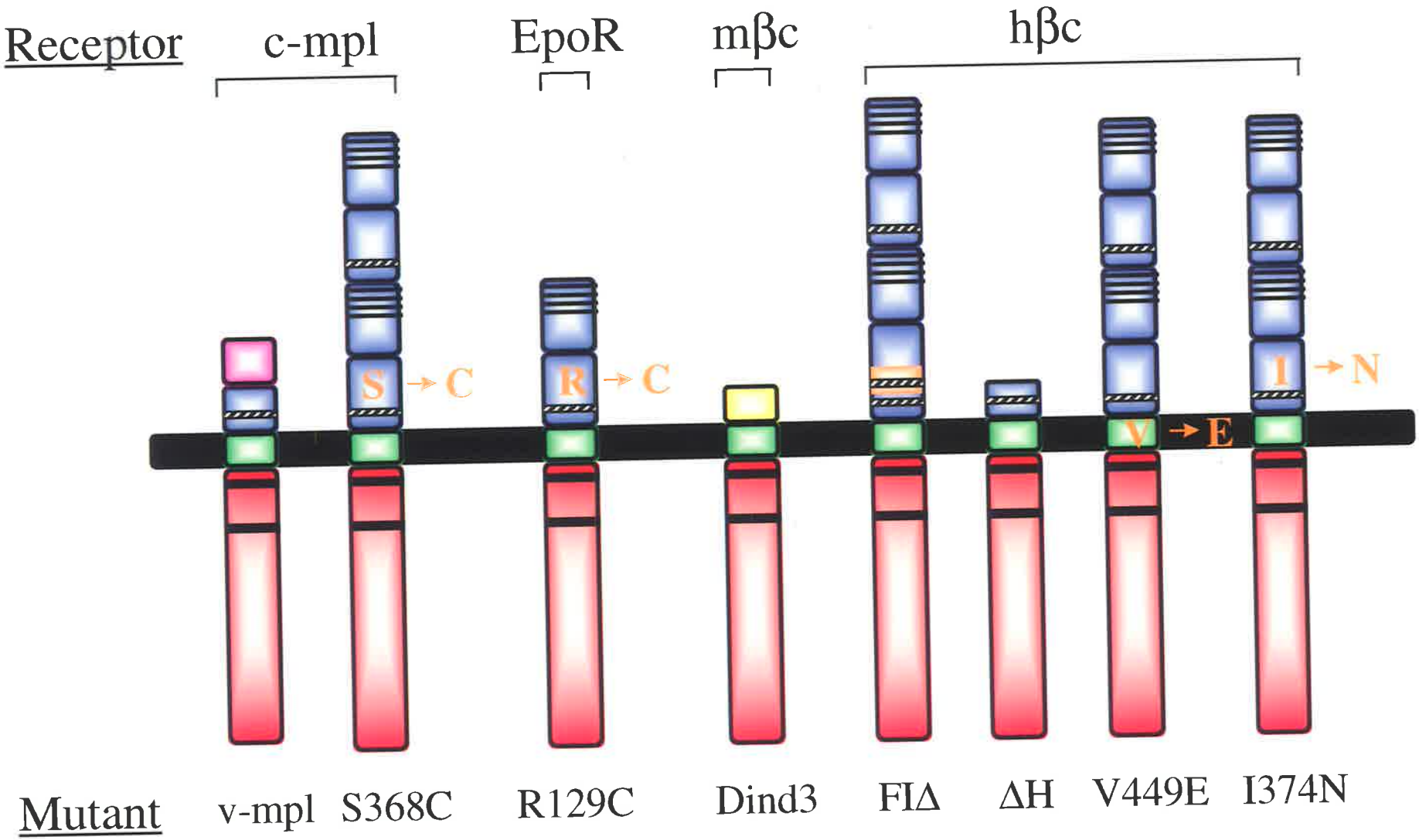
The human thrombopoietin receptor gene, *c-mpl* was first cloned as a homologue of the *v-mpl* gene transduced by the myeloproliferative leukaemia virus (MPLV), a replication-defective retrovirus which in conjunction with the replication-competent Friend murine leukaemia virus (F-MuLV) causes an acute multilineage haemopoietic disorder in mice (Wendling et al., 1986; Vigon et al., 1992). The *v-mpl* gene is a truncated form composed of the extracellular 43 amino acids of *c-mpl* and fused at the N-terminus to two parts of the F-MuLV *env* gene, with a 3 amino acid deletion at the C-terminus (Figure 1.3) (Souyri et al., 1990). Infection of murine bone marrow with helper-free MPLV leads to factor-independence and immortalisation of a variety of haemopoietic lineages (Souyri et al., 1990). A region of the *v-mpl* cytoplasmic domain

Figure 1.3. Location of activating mutations in members of the cytokine receptor superfamily

CRDs are shown in blue, transmembrane regions in green and intracellular regions in red. The MPLV *env* glycoprotein is shown in pink, and the unique extracellular sequence transduced by the Dind3 mutant is shown in yellow. Mutated residues are shown in orange, as is the duplicated region of the FIΔ mutant. Conserved features of the cytokine receptor superfamily are as for Figure 1.1.

Note: the wild-type EpoR has one CRM whereas the other receptor subunits shown have two.

Data are from (Yoshimura et al., 1990; Souyri et al., 1990; Jenkins et al., 1996; Hannemann et al., 1995; D'Andrea et al., 1996; D'Andrea et al., 1994; Alexander et al., 1995).


Receptor
c-mpl
EpoR
mβc
hβc
S → C
R → C
I → N
V → E
Mutant
v-mpl
S368C
R129C
Dind3
FIΔ
ΔH
V449E
I374N

is required for MPLV pathogenicity, and *env-mpl* is sufficient to reconstitute myeloproliferative disorders when delivered in a distinct retroviral vector, indicating that the *env-mpl* fusion gene is a determinant of MPLV pathogenesis (Benit et al., 1993; Benit et al., 1994). Moreover, expression of the *env-mpl* fusion protein confers factor-independence on human and murine factor-dependent cell lines (Benit et al., 1994). Hence *v-mpl* appears to encode a constitutively active form of the cellular thrombopoietin receptor *c-mpl*. Whilst the mechanism of activation is unknown, a variant of the env-mpl fusion protein, del3-mpl, which is also constitutively active, forms disulfide-linked homodimers constitutively (Courtois et al., 1995). By analogy, *env-mpl* may also homodimerise leading to activation. Moreover, the env proteins encoded by retroviruses generally oligomerise (Yang et al., 1990; Earl et al., 1990), hence it is possible that the env protein sequences of the env-mpl fusion increase oligomerisation of mpl.

Furthermore, constitutively active point mutants of c-mpl have been isolated which homodimerise in the absence of ligand (Alexander et al., 1995). Insertion of cysteine residues in the region of c-mpl homologous to the dimer interface region of the active GHR lead to constitutive disulfide-linked dimerisation and activation. When expressed in Ba/F3 cells these mutants cause factor-independence and tumorigenicity. These results imply that dimerisation of c-mpl leads to activation.

1.6.2.2 The EpoR

The first indication that activation of the EpoR can contribute to oncogenesis came from studies using the Friend spleen focus-forming virus (SFFV), a defective murine leukaemia virus which causes an acute erythroleukaemia in mice and erythroblastosis in murine bone marrow cultures (Hankins and Troxler, 1980). The envelope glycoprotein, gp55, which is required for the induction of erythroblast proliferation *in vitro* and for pathogenesis of the virus *in vivo*, binds to the murine EpoR and triggers growth activation in the absence of Epo (Li et al., 1990). It is interesting that similar viral envelope glycoproteins are implicated in the activation of members of the cytokine receptor superfamily in MPLV and SFFV. Binding of gp55 to the EpoR does not affect ligand binding, hence gp55 is unlikely to be acting as an Epo mimetic (Yoshimura et al., 1990a). Moreover, the env protein of SFFV has been shown to

dimerise (Li et al., 1990; Bestwick et al., 1985). Hence the likely mechanism of SFFV-induced Epo-independence is by constitutive dimerisation of the EpoR.

A second example of ligand-independent activation of the EpoR was provided by the *in vitro* generation of a constitutively active form of this receptor. When murine Ba/F3 cells expressing the EpoR and growing in an Epo-dependent manner were selected for growth in decreasing concentrations of Epo, two classes of mutant were obtained (Yoshimura et al., 1990b). The first of these, R129C, is a constitutively active point mutant in the extracellular domain converting the Arginine^{129} residue to Cysteine (Figure 1.3). Like the c-Mpl mutations described above, the Arginine^{129} residue corresponds to the dimer interface region of the GHR. Similarly to the case with c-Mpl, other insertion of cysteine at other residues in this interface region, E^{132} or E^{133} also lead to constitutive activity (Watowich et al., 1994). All three constitutively active mutants formed disulfide linked homodimers in the absence of ligand. Substitutions of Serine, Glutamic Acid or Proline at the R^{129} position do not lead to homodimerisation and are not activating, implying that the activity of this mutant is due to the disulfide-linked homodimerisation caused by the presence of a cysteine residue at this position (Watowich et al., 1992). R129C confers both factor independent proliferation and tumorigenicity on Ba/F3 cells (Yoshimura et al., 1990b). Moreover, a Friend SFFV complex in which R129C is inserted in place of the gp55 envelope glycoprotein causes erythroleukaemia in mice, unlike a virus expressing the wild-type EpoR, indicating that a constitutively active EpoR can contribute to oncogenesis *in vivo* (Longmore and Lodish, 1991).

A second type of EpoR mutant was isolated by Yoshimura et al. (1990b) in which the C-terminal 42 amino acids are deleted and replaced with alanine and leucine. This mutation rendered the EpoR 10-fold hypersensitive to Epo, but did not lead to factor-independence or tumorigenicity of Ba/F3 cells. Hence the C-terminus of the EpoR appears to function as a negative regulatory domain. An explanation for this came from the finding that SHP1 binds to this region and leads to inactivation of JAK2 and termination of proliferative signals (Yi et al., 1995; Klingmuller et al., 1995). Similarly truncated forms of the EpoR have been found in patients with Primary Familial and Congenital Polycythemias and Sporadic Primary Polycythemia (Sokol et al., 1994; Sokol et al., 1995; Kralovics et al., 1997; de la Chapelle et al., 1993a; de la

Chapelle et al., 1993b). In families with Primary Familial Polycythemia the EpoR mutations are linked to the polycythemia phenotype, suggesting that the mutations are at least partly responsible for the erythroproliferative disorder.

1.6.2.3 The human IL-3/IL-5/GM-CSF receptor common β subunit (hβc)

There are now several examples of constitutively active mutants of hβc which deliver proliferative signals in the absence of growth factors to the murine IL-3/GM-CSF-dependent myelomonocytic cell line, FDC-P1. In all cases tested such mutants have conferred tumorigenicity on this cell line, suggesting the possibility that hβc mutants may play a role in oncogenesis.

The first example of an activated hβc mutant was a spontaneously arising 37 amino acid duplication in the membrane-proximal CRD, encompassing several conserved regions such as the WSXWS motif and the adjacent basic region, YXVXVRVR (Figure 1.3) (D'Andrea et al., 1994). Subsequently, Jenkins et al. (1995) combined random mutagenesis of the central third of hβc with retroviral expression screening in FDC-P1 cells to identify a point mutant, I374N, which converts an isoleucine residue in the extracellular membrane-proximal CRD to asparagine (Figure 1.3). Interestingly, the Ile^{374} residue is encompassed by the FIΔ duplication, suggesting that this region is important in hβc activation. Using molecular modelling techniques Jenkins et al. (1995) suggested that Ile^{374}, which lies in the C′ strand of the membrane proximal CRM, participates in van der Waals interactions with the conserved Trp^{358} and Leu^{356} residues in the B′ strand, forming part of a hydrophobic core. On this basis it was predicted that disruption of these interactions by insertion of a hydrophilic residue, such as isoleucine, causes a conformational change leading to activation of hβc. Accordingly, substitution of Trp^{358} or Leu^{359} with isoleucine leads to constitutive activation of hβc. Moreover, insertion of several different residues at these positions as well as position 374 showed that hydrophilic substitutions result in the greatest degree of activation, with hydrophobic substitutions having lesser or no effect. These results suggest that disruption of interactions between I^{374} and Leu^{356} or Trp^{358} in the hydrophobic core of domain 4 of hβc leads to a conformational change resulting in constitutive activity.

The functional importance of domain 4 is also evidenced by the fact that extracellular truncation mutants of hβc which retain domain 4 or the membrane-proximal 45 amino acids of domain 4, termed hβcΔQP and hβcΔH respectively, are constitutively active in FDC-P1 cells (Figure 1.3) (D'Andrea et al., 1996). However, a further truncation mutant of hβc which retains only 7 amino acids of the extracellular region is not activating, implying that sequences within the deleted 38 amino acid region of domain 4 are required for the constitutively active phenotype. The region retained by hβcΔH is analogous to that of the v-mpl protein (above), implying that these two mutant receptors may function similarly. Moreover, a constitutively active mutant of the murine βc has been described in which the entire extracellular domain is replaced by a unique 34 amino acid sequence (Figure 1.3) (Hannemann et al., 1995). Hence extracellular truncation may be a general mechanism of cytokine receptor activation. Interestingly, an extracellular truncation mutant of hβc which retains domains 3 and 4 is not constitutively active (D'Andrea et al., 1996). From these results it was hypothesised that the N-terminal region of hβc, and in particular domain 3, masks regions in domain 4 which are involved in activation, possibly by dimerisation with hβc itself or another signalling component. These regions can then be unmasked either by ligand binding or by extracellular truncation of the receptor. Based on their similar behaviour in various murine cell lines (see below) it is possible that the extracellular point mutants described above also act by unmasking regions of hβc involved in protein-protein interaction.

As well as the I374N mutant, the random screen performed by Jenkins et al. (1995) identified a second point mutant, V449E, which converts a transmembrane valine residue to glutamic acid (Figure 1.3). Interestingly, when placed in the murine IL-3 dependent pro-B cell line BAF-B03, the transmembrane mutant, V449E confers factor independence on this cell line whereas the extracellular mutants I374N and FIΔ do not. Moreover, none of these three mutants confer factor independence on the murine IL-2 dependent cell line CTLL-2. These results suggest that the transmembrane and extracellular hβc mutants act by different mechanisms, and that they require cell type-specific molecules for function. In this respect extracellular hβc mutants appear to behave similarly, as evidenced by the fact that the extracellular truncation mutants described above are also unable to confer factor-independence on BAF-B03 cells.

The V449E mutant bears striking resemblance to a previously identified mutant of the rat *neu* proto-oncogene which also converts a valine residue in the transmembrane domain to glutamic acid. This activated form of the *neu* proto-oncogene was isolated repeatedly from chemically induced rat neuroblastomas and glioblastomas in a NIH-3T3 focus-forming assay (Bargmann et al., 1986b; Bargmann et al., 1986b). An identical mutation was found to occur spontaneously in NIH 3T3 cells bearing the *neu* proto-oncogene, which conferred tumorigenicity on this line (Hung et al., 1989). Hence this mutation is not particular to the chemical mutagenesis used to generate the neuro/glioblastomas from which it was derived in rats. The neu gene product encodes a receptor tyrosine kinase which is 50% identical to the EGF-R (Bargmann et al., 1986b). Its ligand has now been cloned, and dubbed heregulin or neu differentiation factor (Peles et al., 1992; Wen et al., 1992; Holmes et al., 1992). The V664E mutant of neu homodimerises in the absence of ligand leading to constitutive activation of the tyrosine kinase activity (Yarden, 1990; Weiner et al., 1989; Peles et al., 1991; Bargmann and Weinberg, 1988). By analogy, it is likely that V449E also homodimerises leading to constitutive activity.

Several groups have studied the conformation of the transmembrane region of inactive and active forms of neu. Using conformational energy analysis, Brandt-Rauf et al. (1989; 1990) predicted that the transmembrane region of inactive neu is an α-helix with a sharp bend at residues Val^{664} and Gly^{665}. In contrast, the conformation of the active mutant is all α-helical, implying that the presence of the Glu residue at position 664 leads to a conformational change resulting in activation. An alternative hypothesis was proposed by Sternberg and Gullick (1989) who used stereochemical modelling to predict that the area around residue 664 is α-helical, and that in the oncogenic neu protein the carboxylate groups of the Glu^{664} residues form symmetrical hydrogen bonds with the carbonyl oxygen of the Ala^{661} residue of a separate neu protein, thereby stabilising a dimeric interaction. Interestingly, hβc also has an alanine residue 3 positions N-terminal to Val^{449}, further suggesting that V449E is functioning similarly to the activated neu protein. Using NMR analysis of synthetic peptides representing part of the neu transmembrane region, Gullick et al. (1992) confirmed that the region is α-helical and showed that there was no detectable conformational

change between proto-oncogenic and oncogenic forms of neu, arguing against the model proposed by Brandt-Rauf et al.

1.7 Tyrosine Kinase Oncogenes in Neoplastic Haemopoiesis

Another method whereby haemopoietic cells can abrogate growth factor dependence is by the direct activation of mitogenic signal transduction pathways by the activation of tyrosine kinases. This was first exemplified by a family of viral tyrosine kinase oncogenes including v-*src* and v-*abl*, which lead to factor-independence of haemopoietic cells through a non-autocrine mechanism (Overell et al., 1987; Cook et al., 1985). That such oncogenes can be responsible for leukaemogenesis is evidenced by the fact that greater than 90% of patients with chronic myelogenous leukaemia (CML) express a fused transcript of the breakpoint cluster region (*bcr*) and c-*abl* genes, resulting in an active form of c-*abl* similar in structure to v-*abl* (reviewed in Verfaillie et al. (1998)).

Recently the JAK-STAT pathways utilised by cytokine receptors have been implicated in leukaemogenesis. The first evidence for their involvement was the finding that JAK2 and JAK3 proteins are constitutively active in cell lines transformed by the Human T-cell leukaemia virus (HTLV-)1 which causes adult T cell leukaemia (Xu et al., 1995). Subsequently, constitutively active JAK and/or STAT proteins were found in CML, acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML), chronic lymphocytic leukaemia (CLL) and T-cell lymphoma (Zhang et al., 1996; Gouilleux-Gruart et al., 1996; Frank et al., 1997; Chai et al., 1997). Moreover, constitutively activated JAK proteins have been shown to play a causative role in leukaemia. A point mutant of the *Drosophila melanogaster* JAK kinase *hopscotch* leads to a leukaemia-like syndrome in fruit flies (Luo et al., 1995). Furthermore, chromosomal translocations leading to fusion of the *TEL* gene to *JAK2* have been found in human CML and ALL (Peeters et al., 1997; Lacronique et al., 1997). These fusion genes demonstrate constitutive tyrosine kinase activity in murine cell lines and cause fatal myelo- and lympho-proliferative disorders in mice (Schwaller et al., 1998). By analogy with other TEL-tyrosine kinase fusion proteins, it is proposed that the constitutive kinase activity of the TEL-JAK2 fusion proteins is brought about by constitutive homodimerisation of JAK2 mediated by the TEL

pointed (PNT) domain (Schwaller et al., 1998). Since the constitutively active hβc mutants described above activate JAK2 in murine cell lines, the finding that constitutively active forms of JAK2 are involved in human leukaemia further imply that these mutants have the potential to be involved in human leukaemia (Jenkins et al., 1998).

1.8 PROJECT AIMS AND APPROACH

The aims of this project are to determine the biological effects and leukaemic potential of three recently-described mutants of the hβc protein, V449E, I374N and FIΔ. As described above, based on their ability to function in the murine IL-3 dependent pro-B cell line BAF-B03 it is proposed that the extracellular mutants, FIΔ and I374N, and the transmembrane mutant, V449E, act by different mechanisms. Moreover, unlike the wild-type hGMR, none of these mutants confer factor-independence on the murine IL-2 dependent T cell line, CTLL-2, implying that both transmembrane and extracellular mutants require cell-type specific molecules for function. Given the apparent cell-type specificity of these mutants, it is of interest to determine their ability to function, if any, in primary haemopoietic cells, and to determine the cell types which are permissive to factor-independent signalling.

As abrogation of GM-CSF dependence of haemopoietic cells is a well-defined step in leukaemic oncogenesis (see section 1.5.1), and all three hβc mutants confer factor-independence and tumorigenicity on the murine GM-CSF-dependent myelomonocytic cell line FDC-P1 (Jenkins et al., 1995), it is possible that they may play a role in leukaemogenesis. In order to gain insight into the leukaemogenic potential of these mutants, their effects on the growth and differentiation of primary murine haemopoietic cells will be tested both *in vitro* and *in vivo*. Firstly they will be expressed *in vitro* in primary murine haemopoietic cells using the technique of retroviral transduction. Retroviral transduction is a simple and efficient technique whereby the effects of transduced proteins can be tested in haemopoietic cells (Miller, 1992). The reasons for using murine haemopoietic cells for these studies are threefold. Firstly, they are inexpensive and easy to isolate. Secondly, techniques of retroviral transduction are more developed in murine systems than is the case with human cells. Thirdly, using murine cells for the initial *in vitro* studies allows correlation with later *in vivo* work, which must necessarily be carried out in an animal model system, the simplest and most defined of which is murine. Following transfer of constitutively-active hβc mutants to primary haemopoietic cells, cells expressing constitutively active hβc mutants will be assessed with regard to the magnitude of factor-independent proliferation as well as the cell types induced by each mutant. Furthermore, studies in primary cells will allow an assessment of effects of these

mutants on haemopoietic cell differentiation. This aspect of cytokine receptor signal transduction has not yet been studied with regard to these mutants, as the transformed cell lines used in previous studies are not capable of differentiation, unlike primary haemopoietic cells.

Secondly, the potential constitutively active hβc mutants to contribute to neoplastic haemopoiesis will be tested *in vivo* in mice. Studies *in vivo* will provide a more realistic setting in which to assess the potential of these mutants to contribute to neoplastic haemopoiesis. The bone marrow reconstitution technique which will be employed allows the expression of proteins of interest in the entire haemopoietic system of mice (Williams et al., 1984). This technique has been used previously to demonstrate the oncogenic potential of number of genes, and the biological effects of overexpression of a number of growth factors including GM-CSF and IL-3 (Johnson et al., 1989; Chang et al., 1989). Moreover, bone marrow reconstitution is simple and rapid to perform, which will allow all three hβc mutants to be studied *in vivo* in parallel.

2.1 Chemicals, reagents and consumables

Standard chemicals were of analytical grade where possible, obtained from Ajax Chemicals (Auburn, NSW, Australia), BDH Laboratory Supplies (Poole, UK) and Sigma Chemical Company (St Louis, MO, USA). The source of other chemicals and reagents is as follows;

DMSO, glycerol, hydrogen peroxide, phenol: BDH Laboratory Supplies, Poole, UK.

SDS: Bio-Rad Laboratories, Hercules, CA, USA.

Glycogen: Boehringer Mannheim, Mannheim, Germany.

Bacto-Agar, bacto-tryptone, bacto-yeast extract: Difco Laboratories, Detroit, MI, USA.

dNTPs: Pharmacia Biotech, Uppsala, Sweden.

Agarose, ampicillin, bromophenol blue, DTT, EDTA, ethidium bromide, Ficoll, Hepes, herring sperm DNA, mineral oil, xylene cyanol: Sigma Chemical Company, St Louis MO, USA.

2.2 Solutions and buffers

Bacterial transformation solution	60mM calcium chloride, 15% glycerol, 10 mM PIPES, pH 7.
Buffer saturated phenol	Phenol containing 0.1% (w/v) 8-hydroxyquinoline mixed 1:1 with 50 mM Tris-HCl, pH 8.0.
Buffer saturated phenol-chloroform	TE-saturated phenol mixed with 1:1 with chloroform.
FACS fixative	1% (v/v) formaldehyde, 2% (w/v) glucose, 0.2% (v/v) azide in PBS.
10 × Ficoll loading buffer	0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 25% (w/v) Ficoll.

Genomic DNA extraction buffer	0.1 M sodium chloride, 0.05 M Tris pH 8.0, 1 mM EDTA.
2 × HEBES	0.25 M sodium chloride, 1.5 mM disodium hydrogen orthophosphate pH 7.0, 50 mM HEPES pH 7.1.
10% neutral buffered formalin	0.35% (w/v) sodium di-hydrogen orthophosphate, 0.65% (w/v) di-sodium hydrogen orthophosphate, 10% (v/v) formaldehyde.
PBS	130 mM sodium chloride, 10 mM di-sodium hydrogen orthophosphate, 10 mM sodium di-hydrogen orthophosphate, pH 7.2.
RBC lysis buffer	1 part 20 mM Tris pH 7.65 added to 9 parts 0.83% (w/v) ammonium chloride, used immediately.
1 × SSC	150 mM sodium chloride, 15 mM tri-sodium citrate, pH 7.4.
10 × Super Duper buffer	330 mM Tris acetate pH 7.8, 625 mM potassium acetate, 100 mM magnesium acetate, 40 mM spermidine, 5 mM DTE.
1 × TAE buffer	40 mM Tris pH 8.2, 20 mM sodium acetate, 1 mM EDTA.

2.3 Restriction endonucleases

The restriction endonucleases used in this study were obtained from New England Biolabs (Beverley, MA, USA), Pharmacia Biotech (Uppsala, Sweden) and Amersham (Buckinghamshire, England).

2.4 Antibodies

The murine anti-hβc monoclonal antibody 4F3 (Stomski et al., 1996) was kindly provided by Dr. Qiyu Sun (Hanson Centre for Cancer Research, Adelaide, South Australia).

The FITC-conjugated anti-mouse Ly5.1 and Ly5.2 monoclonal antibodies A20.1 and ALI-4A2 respectively were generously provided by Dr. Ivan Bertoncello (Peter MacCallum Cancer Institute, Melbourne, Victoria, Australia).

The rat anti-mouse Mac-1 (Springer et al., 1979), Gr-1 (Holmes et al., 1986), Thy-1.2 (Ledbetter and Herzenberg, 1979), F4/80 (Austyn and Gordon, 1981), B220 (Coffman and Weissman, 1981) and Sca-1 (Spangrude et al., 1989) monoclonal antibodies are those described previously, and were generously provided by Dr. Ivan Bertoncello (Peter MacCallum Cancer Institute, Melbourne, Victoria, Australia), Dr. Chung Li (Queensland Institute of Medical Research, Brisbane, Queensland, Australia) and Dr. Andrew Hapel (John Curtin School of Medical Research, Canberra, ACT, Australia).

The rat anti-mouse c-kit antibody ACK-2 (Okada et al., 1991) was a kind gift of Dr. Tony Cambereri (Hanson Centre for Cancer Research, Adelaide, South Australia)

The rat anti-mouse Ter-119 antibody (Ogawa et al., 1991) was purchased from PharMingen, San Diego, CA, USA.

FITC-conjugated anti-mouse CD4 and CD8 antibodies were generously provided by Dr. Richard D'Andrea (Hanson Centre for Cancer Research, Adelaide, South Australia)

The M2 anti-FLAG monoclonal antibody was purchased from Kodak (New Haven, CT, USA).

To detect non-conjugated primary mouse antibodies, FITC-conjugated $F(ab')_2$ sheep anti-mouse IgG (Silenus, Hawthorn, Victoria, Australia) antibody was used. Alternatively, to achieve more sensitive staining, biotin-labelled $F(ab')_2$ anti-mouse IgG antibody (Vector Lab Inc., Burlingame, CA, USA) followed by phycoerythrin-conjugated streptavidin (Caltag Laboratories, San Francisco, CA, USA) were used where indicated. To detect non conjugated rat primary antibodies, FITC-conjugated $F(ab')_2$ mouse anti-rat antibody (Silenus) was used.

All antibodies were used at previously determined optimal concentrations.

2.5 Growth factors

Recombinant murine GM-CSF (mGM-CSF) was obtained and used as crude yeast supernatant kindly supplied by Dr. Tracy Wilson, Walter and Eliza Hall Institute, Melbourne.

Recombinant murine IL-3 (mIL-3) was produced from a baculovirus vector kindly supplied by Dr. Andrew Hapel, John Curtin School of Medical Research, Canberra.

Recombinant human IL-6 (hIL-6) was kindly provided by Dr. Paul Simmons (Hanson Centre for Cancer Research, Adelaide, South Australia)

Recombinant human erythropoietin (hEpo) was purchased from Janssen Cilag, Lane Cove, NSW, Australia.

Recombinant murine Stem Cell Factor (mSCF) supplied by Immunex Corporation (Seattle, Washington) was kindly provided by Dr. Leonie Ashman, Hanson Centre for Cancer Research, Adelaide, South Australia.

2.6 Radiochemicals

(α-^{32}P)dATP (5 mCi/ml) was obtained from Bresatec, Adelaide, South Australia.

2.7 Bacterial culture media

2.7.1 Liquid media

Luria Broth (LB)	1% (w/v) bacto-tryptone, 1% (w/v) bacto yeast extract, in milli-Q water, pH adjusted to 7.5 with sodium hydroxide and solution sterilised by autoclaving.

2.7.2 Solid media

L-agar	LB containing 1% (w/v) bacto-agar (Difco Laboratories, Detroit, MI, USA).
L-Amp	L-agar cintaining 100 µg/ml ampicillin

2.8 Tissue culture media

2.8.1 Liquid media

Dulbecco's modified Eagle's medium (DMEM) was supplied by the Media Production Unit, Institute of Medical and Veterinary Science, Adelaide, South Australia, and was supplemented with 0.2% (v/v) sodium bicarbonate, 2 mM L-glutamine, 12 μg/ml penicillin, 16 μg/ml gentamicin, 2.5 μg/ml fungizione (Trace Scientific, Clayton, Victoria, Australia). To make complete Dulbecco's medium (cDMEM) DMEM was supplemented with 10% FCS (Commonwealth Serum Laboratories, Parkville, Victoria, Australia).

Iscove's modified Dulbecco's medium (IMDM) powder was purchased from Trace Scientific. To make 2 × IMDM, 500 g of this powder was dissolved in 500 ml sterile water along with 3.024 g sodium bicarbonate and the pH checked to be within 7.2 - 7.4. To make complete Iscove's medium (cIMDM) 2 × IMDM was diluted 1:1 in sterile water and supplemented with 12 μg/ml penicillin, 16 μg/ml gentamicin, 2.5 μg/ml fungizone and 15% FCS.

2.8.2 Semi-solid media

Methyl-cellulose medium consisted of IMDM containing 1.4% α-methyl-cellulose (Gibco BRL, Gaithersburg, MD, USA), 25% FCS and 1% BSA.

Methyl-cellulose medium used in erythroid colony-forming unit (CFU-E) assays was purchased from Stem Cell Technologies, Vancouver, Canada and consisted of 0.9% methyl-cellulose in Alpha modified Eagle's medium, 30% FCS, 1% BSA, 10^{-4} M 2-Mercaptoethanol and 2 mM L-glutamine.

2.9 Molecular weight standards

EcoRI-digested bacteriophage SPP1 DNA markers (high range) were purchased from Bresatec (Adelaide, South Australia) and used at a final concentration of 50 ng/μl in 1 × Ficoll loading buffer, with 5 μl loaded typically. Fragment sizes are 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48 and 0.36 kb.

2.10 Vectors and cDNAs

The pRufNeo retroviral vector (Figure 2.1A) is a version modified from that described by Rayner and Gonda (Rayner and Gonda, 1994) by the removal of the *Nco*I restriction site from the multiple cloning region.

The pRufPuro retroviral vector (Figure 2.1B) has been described previously (Jenkins et al., 1995).

The hβc cDNA (Hayashida et al., 1990) used here was that described by Barry et al. (1994).

The FIΔ (D'Andrea et al., 1994), V449E and I374N (Jenkins et al., 1995) hβc mutants have been described previously.

2.11 Molecular biology techniques

2.11.1 Cloning

2.11.1.1 Bacteria

The DH10B strain of *E.coli* (F^- *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ80d*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *end*A1*ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, λ^-, *rps*L, *nup*G) was used. This strain was cultured in LB, whilst clones harbouring pRufNeo pRufPuro plasmids were maintained in LB containing 100 μg/ml Ampicillin.

2.11.1.2 Long-term storage of bacteria

E.coli was stored as glycerol stocks prepared by mixing 500 μl fresh overnight culture with 500 μl glycerol, vortexing thoroughly and placing at -70°C.

2.11.1.3 Preparation of plasmid DNA

2.11.1.3.1 Mini scale preparations (1-2 μg)

Either a single fresh colony of *E.coli* or a drop of glycerol stock was isolated on a sterile toothpick and used to inoculate 2 ml LB containing 100 μl Ampicillin and incubated overnight with shaking. Mini-preps were performed on 1.5 ml of this culture using the Wizard^R Plus Mini-preps DNA Purification System (Promega Corporation, Madison, WI, USA) using the equipment, solutions and protocols supplied by the manufacturer.

Figure 2.1. The pRufNeo and pRufPuro retroviral expression vectors

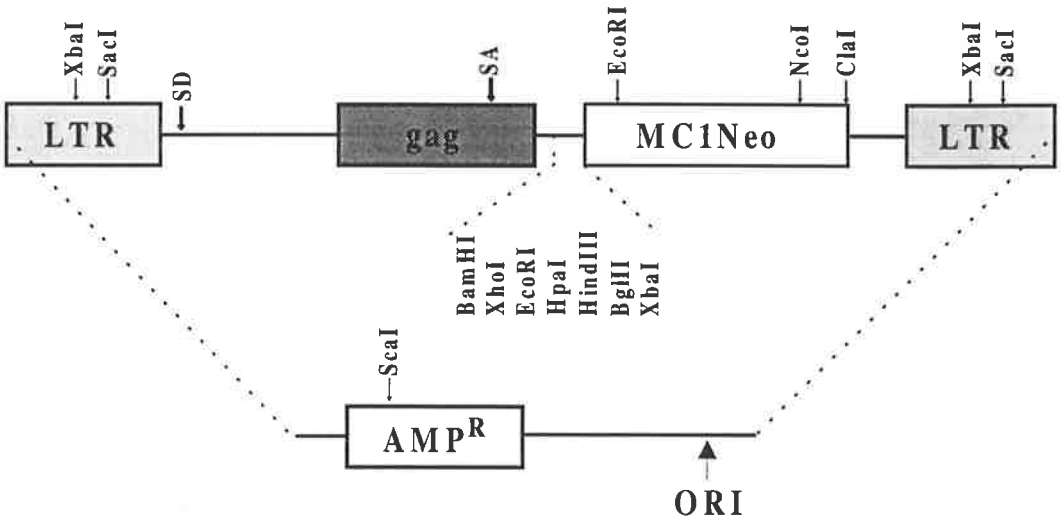
Structure of the pRufNeo (Rayner and Gonda, 1994) (**A**) and pRufPuro (Jenkins et al., 1995) (**B**) retroviral expression vectors, showing important restriction endonuclease digestion sites, the origin of replication (ORI) and the splice donor (SD) and splice acceptor (SA) sites used to generate subgenomic RNA. The provirus units (spanned by the LTRs) are 3248 bp and 3224 bp for RufNeo and RufPuro respectively.

LTR: long terminal repeat, Amp^R: ampicillin resistance gene, MC1Neo: Neomycin resistance gene under control of the MC1 promoter, SVPuro: puromycin resistance gene under control of the SV40 early promoter.

A

pRufNeo

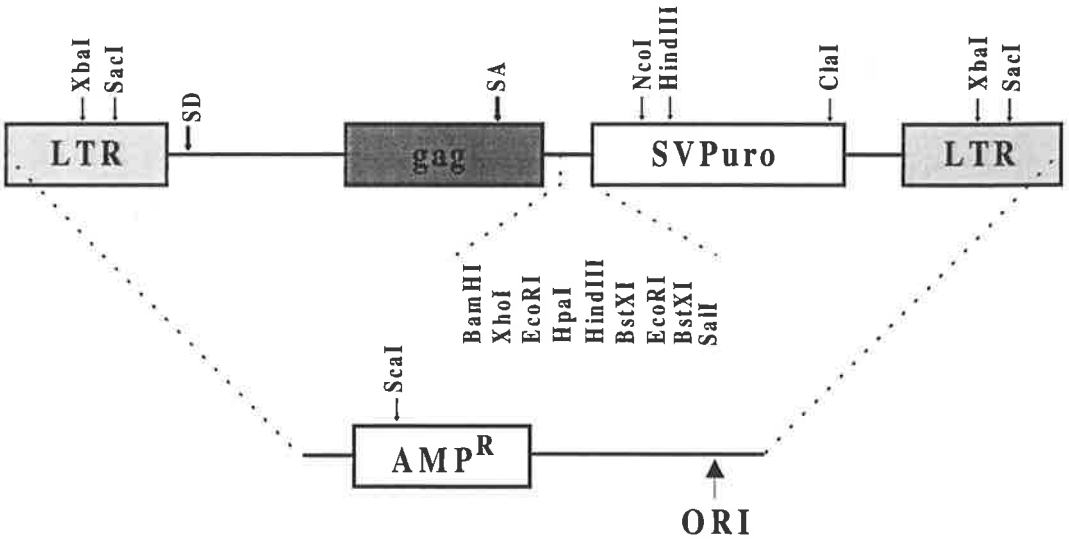
(5650 bp)



B

pRufPuro

(5626 bp)



2.11.1.3.2 Midi scale preparations (50-100 µg)

100 ml of LB containing 100µg/ml Ampicillin was inoculated as above and incubated overnight with shaking. Midi-preps were performed on the entire cultures using the Plasmid Midi Kit (QIAGEN, Chatsworth, CA, USA) using the equipment, solutions and protocols supplied by the manufacturer. DNA yield and purity was quantified by measuring A_{260}/A_{280} using a DU^R-64 Spectrophotometer (Beckman Instruments, Fullerton, CA, USA).

2.11.1.4 Ethanol precipitation of DNA

Plasmid vectors and genomic DNA were ethanol precipitated at -20°C for at least 20 min in the presence of 0.3 M sodium acetate pH 5.5 or 0.4 M sodium chloride and 70% (v/v) ethanol. When ≤ 0.1 µg of DNA was present 1 µl of 20 mg/ml glycogen was used as carrier. DNA was pelleted by centrifugation at 13,000 rpm for 10 min at 4°C, washed in 70% ethanol and resuspended in milli-Q water.

2.11.1.5 Restriction endonuclease digestion

DNA was digested using at least 10 units of the appropriate restriction enzyme per 1 µg DNA to be digested, in a volume so as to make the restriction enzyme stock not more than 10% of the final reaction volume. Diagnostic digestions were performed in 1 × Super Duper buffer, whilst digestions to yield cloning vectors and inserts were carried out using the Boehringer-Mannheim incubation buffer set for restriction enzymes (Boehringer-Mannheim, Mannheim, Germany).

2.11.1.6 Agarose gel electrophoresis

Analytical DNA electrophoresis was performed in agarose gels made of 0.8 - 2% (w/v) agarose in 1 × TAE buffer, which was submerged in 1 × TAE buffer. To visualise DNA gels were stained in 10 µg/ml ethidium bromide for 5 min, rinsed with water and exposed to UV light. DNA was photographed using Polaroid positive land film, type 667 (Polaroid, Hertfordshire, England).

2.11.1.7 Preparation of cloning vectors and inserts

Restriction endonuclease digested plasmid DNA was separated by agarose gel electrophoresis and visualised as described above and the required fragment excised from the gel using a scalpel. DNA was recovered and purified using the BRESA-CLEAN™ DNA extraction kit (Bresatec). The yield of DNA was approximated using agarose gel electrophoresis by comparison with molecular weight standards.

Cloning vectors were treated with CIP (Boehringer-Mannheim) according to the manufacturers instructions and recovered by ethanol precipitation.

2.11.1.8 Ligation

Ligation reaction mixes consisted of 50 ng vector DNA, sufficient insert DNA to give a 4-fold molar excess, 1 × T4 DNA ligase buffer and 0.1 units T4 DNA ligase (New England Biolabs, Beverley, MA, USA). Ligation reactions were carried out overnight at room temperature.

2.11.1.9 Preparation of competent *Escherichia coli* (*E.coli*)

A single colony of *E.coli* cells was used to inoculate 50 ml LB, which was incubated overnight with shaking. The next day, 4mls of this culture was used to inoculate 400 ml LB in a sterile 2 litre flask. This was incubated with shaking and the OD_{590} of the culture determined periodically using a DU^R-64 Spectrophotometer (Beckman). When the culture had reached an OD_{590} of 0.37 - 0.4 it was aliquoted into pre-chilled, sterile 50 ml polypropylene tubes (Corning) and left on ice 5 to 10 min. The culture was then centrifuged for 7 min at 3,000 rpm at 4°C in a Beckman GPR benchtop centrifuge. The pellets were resuspended in 10 ml ice cold bacterial transformation solution. The cells were centrifuged for 5 min at 3,000 rpm at 4°C, and the pellets were resuspended in 10ml ice-cold bacterial transformation solution and left on ice for 30 min. The cells were then centrifuged for 5 min at 3,000 rpm at 4°C and the pellets resuspended in 2 ml ice-cold bacterial transformation solution. The cells were dispensed into pre-chilled, sterile 1.5 ml polypropylene types in 250 µl aliquots, which were stored at 70°C until required.

2.11.1.10 Transformation of competent *E.coli*

One half of a ligation reaction as described above was aliquoted into a 1.5 ml test tube (Eppendorf) and placed on ice. An aliquot of competent *E.coli* was taken from -70°C storage and allowed to thaw on ice. 100 µl competent cells were dispensed into the test tubes containing the ligation mix, vortexed thoroughly and incubated on ice for 10 min. The cells were then heat shocked at 42°C for 2 min to aid internalisation of plasmid DNA. The cells were dispensed into 2 ml LB and incubated for one hour with shaking to allow expression of antibiotic resistance genes. This mixture was centrifuged at 3,000 rpm for 10 min at 4°C in a Beckman GPR benchtop centrifuge and the pellets resuspended by vortexing, plated on L-amp plates and incubated at 37°C overnight.

2.11.2 Construction of retroviral expression plasmids

The pRufNeo-hβc plasmid used is that described by D'Andrea et al. (D'Andrea et al., 1994). The pRufNeo plasmids containing the FIΔ, V449E and I374N mutants have been described previously (Jenkins et al., 1995).

The pRufPuro-hβc and pRufPuro-FIΔ plasmids were obtained from Richard D'Andrea, Hanson Centre for Cancer Research, Adelaide, South Australia, whilst the pRufPuro-V449E and pRufPuro-I374N plasmids were obtained from Brendan Jenkins, Hanson Centre for Cancer Research. These plasmids all contained hβc cDNAs inserted between the *Eco*RI sites of the pRufPuro polylinker.

To construct pRufPuro vectors expressing FLAG-tagged (FLAG)hβc, (FLAG)FIΔ, (FLAG)V449E and (FLAG)I374N cDNAs, pRufNeo constructs containing (FLAG)hβc and (FLAG)FIΔ (D'Andrea et al., 1996) were obtained from Dr. Richard D'Andrea, Hanson Centre for Cancer Research, Adelaide, South Australia. An approximately 650 bp AccI restriction enzyme fragment of pRufNeo-V449E and pRufNeo-I374N containing the 5' 550 bp of the hβc cDNA and the adjacent 58 bp of RufNeo was excised and replaced with that derived from of pRufNeo-(FLAG)hβc, which incorporated the FLAG tag (amino acid sequence DYKDDDDK) but did not incorporate the sites of the V449E or I374N mutations. The FLAG tag is located between hβc residues A24 and E25 (numbered from the first amino acid of the signal peptide). To optimise cleavage of the signal peptide, residues 18-24 of hβc have been

altered from ERSLAGA to VLSGAQA in the FLAG-tagged proteins. The (FLAG)hβc, (FLAG)FIΔ, (FLAG)V449E and (FLAG)I374N cDNAs were excised from pRufNeo using *Bam*HI and *Eco*RI and inserted into the corresponding sites in the RufPuro plasmid. The presence of the FLAG tag and the FIΔ, V449E and I374N mutations was confirmed in these constructs by sequencing as described in Section 2.11.5. To sequence the FLAG epitope tag sequence a primer directed against the pRufPuro *gag* sequence, RCF1 (Rayner and Gonda, 1994) was used. To sequence the hβc mutations the hβc#2 primer described in Section 2.11.4 was used.

2.11.3 Southern blot analysis of genomic DNA

2.11.3.1 Genomic DNA preparation

The procedure used was that described by Hughes et al. (1979). Briefly, 10^7 cells were collected by centrifugation for 5 min at 1,200 rpm in a Beckman GPR benchtop centrifuge, resuspended in 10 ml PBS and centrifuged again. The cell pellet was resuspended in 1 ml genomic DNA extraction buffer to which was added SDS to 0.5% (w/v), RNase A to 100 µg/ml and proteinase K to 500 µg/ml. This mixture was incubated for 1 hour at 37°C and SDS was added to a final concentration of 1% (w/v). DNA was extracted once with TE-saturated phenol/chloroform and again with chloroform and ethanol precipitated. The resultant DNA pellet was dissolved in 0.4 ml Milli-Q water overnight at room temperature and the yield and purity quantified by measuring A_{260}/A_{280} using a DU^R-64 Spectrophotometer (Beckman).

2.11.3.2 Southern blot analysis

Genomic DNA was digested with BamHI or KpnI restriction endonucleases and 10 µg digested DNA was separated on a 0.8% agarose gel. The DNA was then transferred to a Hybond^{TM}-N nylon membrane (Amersham) and immobilised by UV cross-linking at 0.12 Joules/cm^2 using a UV Stratalinker 800 (Stratagene, La Jolla, CA, USA).

Membranes were prehybridised in a solution of 5 × SSC, 50% (v/v) formamide, 5 × Denhardt's reagent, 0.1% (v/v) SDS, 10 mM Hepes, 1 mM EDTA, 2 mM sodium pyrophosphate and 100 µg/ml herring sperm DNA in roller bottles (Hybaid, Middlesex, UK) at 42°C for at least 6 hours with rotation in a Hybaid oven. (α-

^{32}P)dATP labelled random probes were made from agarose gel-purified cDNAs using the Megaprime kit (Amersham) according to the manufacturers instructions. Unincorporated nucleotides were removed by centrifuging through a Bio-Rad spin column. The probe was heat-denatured by incubation at 100°C for 5 min and added to the prehybridisation mix and the membranes were hybridised overnight at 42°C.

Following hybridisation membranes were washed twice in 2 × SSC at room temperature for 10 min and once in 0.1 × SSC, 0.1% (w/v) SDS at 65°C for 30 min. Membranes were rinsed in 2 × SSC, wrapped in clingwrap and exposed to a Phosphorimage screen (Molecular Dynamics, Sunnyvale, CA, USA) overnight. Phosphorimage screens were analysed with a Phosphorimager SP (Molecular Dynamics).

To strip membranes for reprobing, boiling 0.1 × SSC, 0.1% (w/v) SDS was added and the membrane incubated at room temperature for 1 hr with rocking. This procedure was then repeated and the membranes were rinsed in 2 × SSC prior to prehybridisation and hybridisation as above.

2.11.4 Polymerase chain reaction (PCR) from genomic DNA

Polymerase chain reaction (PCR) reactions were performed directly from whole cell lysates essentially as described by Cassel et al. (1993). Briefly, 5×10^3 cells were washed in PBS and resuspended in 0.4 ml milli-Q water. Samples were heated at 100°C for 10 min, 0.4 ng proteinase K was added and the samples were incubated at 37°C for 1 hour. Samples were incubated at 100°C for 10 min and 10 µl of this mix was used in a PCR reaction.

The primers used for amplification of hβc were hβc#2: 5'-TGGAGCCCAGAGGTTTGCAGGGACT-3' (hβc nucleotides 713-737) and hβc#3: 5'-GGGCCCATTGAAGTCAAAGCTGGAA-3' (versus hβc nucleotides 1804-1780), spanning a 1091 bp fragment.

The GAPDH primers used in control reactions were 5'GAP: 5'-ACCACAGTCCATGCCATCAC-3' and 3'GAP: 5'-TCCACCACCCTGTTGCTGTA-3', spanning an approximately 400 bp fragment.

Reactions were carried out in a volume of 50 µl in the presence of 0.2 mM dNTPs, 1.5 mM magnesium chloride, 1 × *Taq* DNA polymerase buffer and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). Reactions were denatured at 95°C for 5 min then cycled for 30 cycles of 95°C for 1 min, 68°C for 1 min and 72°C for 1 min in a DNA Thermal Cycler (Perkin-Elmer). PCR products were analysed by agarose gel electrophoresis as described in section 2.11.1.6.

2.11.5 Sequencing of DNA

DNA sequencing was performed using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit with Ampli*Taq* DNA Polymerase, FS (Perkin-Elmer). Briefly, 100-500 ng plasmid DNA was sequenced with 100 ng primer and 4 µl Terminator Ready Reaction Mix in a 20 µl volume. Cycle sequencing was performed using a DNA Thermal Cycler (Perkin-Elmer) for 25 cycles of 96°C for 30 sec, 50°C for 15 sec and 60°C for 4 min. The DNA was then precipitated using 2 µl sodium acetate and 50 µl 95% ethanol for 10 min on ice. Samples were then centrifuged at 12,000 rpm for 10 min at 4°C the resultant pellets were washed with 70% ethanol and dried under vacuum. Sequence data was obtained by running the reaction products on an ABI Prism 377 DNA Sequencer. Sequence data was obtained by the Institute for Medical and Veterinary Science (Adelaide, South Australia) sequencing facility.

2.12 Tissue culture

All cells used in this study were maintained at 37°C in a mixture of 5% CO_2 in air in a humidified tissue culture incubator (Forma Scientific, Marietta, Ohio, USA).

2.12.1 Cell lines

The Ψ2 (Mann et al., 1983) and BOSC 23 (Pear et al., 1993) ecotropic retroviral packaging cell lines were maintained in cDMEM. Ψ2 cells producing RufNeo retroviruses were maintained in cDMEM containing 200 µg/ml geneticin (G418; Gibco BRL).

The murine IL-3/GM-CSF-dependent myelomonocytic cell line, FDC-P1 (Dexter et al., 1980) was maintained in cDMEM containing 50 U/ml murine GM-CSF. FDC-P1

cells containing RufPuro retroviruses were maintained in growth medium containing 1 μg/ml puromycin (Calbiochem, San Diego, CA, USA).

The murine myeloid cell lines FDB1 and FDB2 (described in Chapter 4) were maintained in cIMDM containing 1,000 U/ml murine IL-3. FDB1/2 cells containing RufPuro retroviruses were maintained in growth medium containing 1 μg/ml puromycin. For differentiation assays cells were placed in cIMDM containing 500 U/ml mIL-3 and 500 U/ml mGM-CSF where indicated.

2.12.2 Preparation of primary haemopoietic cells

2.12.2.1 Foetal liver

Murine foetal liver cells were obtained from day 14 pregnant CBA mice as described by Johnson and Metcalf (1978). Briefly, foetuses were isolated from pregnant mice, placed in a petri dish containing ice-cold DMEM + 2% FCS, and the livers removed with the aid of a dissecting microscope (Olympus Australia, Richmond, South Australia, model SZ-11) into a sterile polypropylene tube containing 5 ml cold DMEM. The livers were homogenised by gentle pipetting with a 5 ml pipette and the tissue debris was allowed to settle. The cells were decanted, centrifuged at 1,200 rpm for 5 min at 4°C in a pre-cooled Beckman GPR benchtop centrifuge, resuspended in 1 ml cold cIMDM and used immediately.

2.12.2.2 Bone Marrow

Bone marrow cells were isolated as described by Metcalf (1984). Briefly, femurs were dissected out of a freshly sacrificed mouse, the ends removed using a scalpel and the bone marrow extracted by flushing through 2 ml ice-cold DMEM + 2% FCS with a 21 gauge needle. The cells were dispersed by gentle pipetting and the tissue debris was allowed to settle. The cells were decanted, centrifuged at 1,200 rpm for 5 min at 4°C in a pre-cooled Beckman GPR benchtop centrifuge, resuspended in 1 ml cold cIMDM and used immediately.

2.12.2.3 Spleen

Spleens were removed from freshly sacrificed mice and weighed in a sterile 1.5 ml test tube. The cells were removed by mashing with forceps in 4 mls cold DMEM +

2% FCS in a petri dish and the residual cell debris was discarded. The cells were dispersed by gentle pipetting, centrifuged at 1,200 rpm for 5 min at 4°C in a pre-cooled Beckman GPR benchtop centrifuge, resuspended in 1 ml cold cIMDM and used immediately.

2.12.2.4 Peripheral Blood

To isolate peripheral blood, mice were generally anaesthetised in a mixture of 2% (v/v) Flouthane (Zeneca, Cheshire, UK) and 10% (v/v) nitrous oxide in air using a Boyle anaesthetic apparatus (British Oxygen Company, London, UK). Peripheral blood samples of approximately 150 µl were obtained by puncture of the retro-orbital plexus using a 0.2 mM heparinised capillary tube and immediately placed into 4 ml tubes pre-treated with EDTA as an anticoagulant (Vacuette, Greiner Labortechnik, Gloucestershire, UK).

2.12.2.5 White Blood Cells

To lyse erythrocytes 1 ml ice-cold fresh RBC lysis buffer was added per 100 µl peripheral blood the cells placed on ice for 10 min. The white blood cells were collected by centrifugation at 1,200 rpm for 5 min at 4°C in a pre-cooled Beckman GPR benchtop centrifuge, washed in 10 ml ice-cold DMEM 2% FCS and recentrifuged. The cells were resuspended in ice-cold DMEM 2% FCS and the cells used immediately.

2.12.3 Long-term storage of murine cells

Cells (10^6 - 2×10^7) were collected by centrifugation at 1,200 rpm for 5 min at 4°C and resuspended in 0.8 ml cold freezing medium, which consisted of 10% (v/v) DMSO in FCS. Cells were transferred to pre-chilled 1 ml freezing vials (Corning) and placed in polystyrene racks which were sealed and stored at -80°C overnight. The next day the vials were transferred to liquid nitrogen tanks for long-term storage.

Cells were thawed rapidly by placing vials in a 37°C waterbath. The cell suspension was transferred to a 15 ml sterile polypropylene tub (Corning) and 10 ml cDMEM was added dropwise whilst mixing. The cells were centrifuged at 1,200 rpm for 5 min at room temperature and resuspended in growth medium.

2.12.4 Removal of fibroblasts by protease treatment

To remove fibroblasts the medium was aspirated and the cells rinsed in PBS. To 25 cm^2 tissue culture flasks 0.3 ml of a solution of 0.05% (w/v) trypsin (Difco) and 0.5 mM EDTA in PBS was added. The cells were incubated at 37 °C for 3 min and removed by agitation. 2.7 ml cDMEM was added and the cells were dispersed by gentle pipetting.

2.12.5 Stable transfection of Ψ2 cells

5×10^5 Ψ2 cells were seeded into a 60 mM dish containing 4 ml cDMEM and incubated overnight. The next day, air was bubbled through 250 µl 2 × HEBES whilst 250 µl of 0.25 M calcium chloride solution containing 10 µg plasmid DNA was added dropwise. As controls, mock-transfections were performed containing no plasmid DNA. The mixture was allowed to stand for 15 min at which time a fine precipitate was visible. The mixture was then added dropwise to the dishes containing Ψ2 cells and mixed by gently swirling the dishes. After overnight incubation, the medium was removed, 1 ml DMEM + 15% (v/v) glycerol was added and the dishes were left for 4 min with gentle rocking. The medium was then removed and the cells washed with 5 ml DMEM. The DMEM was removed and replaced with cDMEM and the cells were incubated overnight. The next day the cells were removed and half the cells placed in 75 cm^2 tissue culture flasks selected in cDMEM containing 400 µg/ml G418 to select for transfected cells. Once all mock-transfected Ψ2 cells had died the resultant stably transfected pools were maintained in cDMEM containing 200 µg/ml G418.

2.12.6 Infection of haemopoietic cells

2.12.6.1 Foetal liver cells

Ψ2 retrovirus packaging cells were transfected with the pRufNeo retroviral vector (Rayner and Gonda, 1994) containing wild-type or mutant hβc cDNAs. Transfected Ψ2 cells were selected in cDMEM containing 400 µg/ml G418 and pooled. The resultant pools were selected for cell surface expression of hβc by flow cytometry as described in section 2.12.11.2, and used to infect murine foetal liver cells obtained from 14 day pregnant CBA mice. To achieve this, the Ψ2 cells were first irradiated

with 30 Gy from a Cs^{137} source in an IBL Blood Product Irradiator at the Blood Transfusion Unit of the Royal Adelaide Hospital. 3×10^6 irradiated Ψ2 cells were co-cultivated with 3×10^6 foetal liver cells for 48 hours in 75 cm^2 flasks in cIMDM containing 500 U/ml mIL-3, 500 U/ml mGM-CSF and 2 U/ml hEpo. The foetal liver cells were harvested, washed three times in DMEM, resuspended in 1 ml cIMDM and placed in liquid culture or semi-solid methyl-cellulose medium. Where indicated 500 U/ml IL-3, 500 U/ml GM-CSF, 2 U/ml hEpo and 1 mg/ml G418 were added.

2.12.6.2 5-FU treated bone marrow

3.3×10^6 Ψ2 cells stably transfected with pRufNeo or stably transfected with pRufNeo containing wild-type and mutant hβc subunits and sorted for hβc expression as described above were irradiated as above and co-cultivated with 5-FU treated bone marrow derived from 3.3 femurs in 75 cm^2 tissue culture flasks in 17 ml of cIMDM containing 500 U/ml mIL-3, 100 ng/ml mSCF and 10 ng/ml hIL-6. As a control, 3×10^5 Ψ2 cells were co-cultivated with the 5-FU treated bone marrow cells derived from 0.3 femurs in 1.5 ml the above medium in a 6-well tissue culture dish. After 2 days, the non-adherent cells were removed, collected by centrifugation and replaced in identical fresh medium. After a further day of culture the non-adherent cells were removed, washed once in 10 ml PBS and resuspended in PBS. Cells equivalent to 0.3 femurs were used to assess the infection frequency *in vitro* in methylcellulose culture, whilst those equivalent to 1 femur were used to reconstitute recipient mice.

2.12.6.3 Infection of FDB1 and FDB2 cells with RufPuro-hβc constructs

10^6 Ψ2 cells stably infected with pRufPuro constructs containing wild-type and mutant hβc cDNAs were irradiated as described in Section 2.12.6.1 and co-cultivated with 10^6 FDB1 or FDB2 cells in cIMDM containing 500 U/ml mIL-3. After 2 days the non-adherent (FDB1/2) cells were decanted, collected by centrifugation and the infected cells selected by culture in the above medium plus 1 μg/ml puromycin.

2.12.6.4 Infection of murine myeloid cell lines with RufPuro-(FLAG)hβc constructs

2×10^6 BOSC 23 cells were seeded in a 60 mM petri dish in 4 ml cDMEM. The next morning the medium was removed and replaced with cDMEM containing 25 μM chloroquine. The cells were then transfected using the calcium phosphate procedure as described for Ψ2 cells in section 2.12.5, except that for each dish transfected 500 μl of 0.25 M calcium chloride containing 10 μg plasmid DNA was added to 500 μl 2 × HEBES to make 1 ml of precipitate. After 7 hours, the medium was removed and replaced with cDMEM and the cells were incubated overnight. The next day, 2×10^5 target cells (FDC-P1 or FDB1/2) were added in the appropriate maintenance medium plus 4 μg/ml polybrene. After 48 hours the target cells were removed, collected by centrifugation and the infected cells selected by culture in growth medium plus 1 μg/ml puromycin.

2.12.7 Liquid culture of foetal liver cells

Foetal liver cells were cultured in cIMDM. Where indicated 500 U/ml mGM-CSF, 500 U/ml mIL-3, 2 U/ml hEpo and 1 mg/ml G418 was added. To perform differential cell counts, these cultures were agitated by gentle pipetting and counts performed as described in Section 2.13.8.

For total cell counts non-adherent cells were decanted and adherent cells were washed in DMEM and incubated in DMEM containing 0.4% Lidocaine (Sigma) for 3-5 min. Cells were then agitated and FCS was added to a final concentration of 10%. The cells were washed twice in DMEM containing 10% FCS, combined with non-adherent cells and viable cell counts performed as described in Section 2.12.13.

2.12.8 Colony assays

2.12.8.1 CFU-E

To assay CFU-E, 5×10^4 bone marrow cells or 5×10^5 spleen cells were plated in 1 ml methyl-cellulose medium. Where indicated 2 U/ml hEpo, 50 ng/ml mSCF and 1 mg/ml G418 were added. After 2 days incubation, 1 ml of a solution of 1 mg/ml 3'-3'-diaminobenzidine (ICN Biomedicals, Seven Hills, NSW, Australia) and 0.015% (v/v) hydrogen peroxide in PBS was carefully layered on top of the methylcellulose. This solution rapidly diffused into the methyl-cellulose medium and within 5 min at room

temperature haemoglobinised cells had developed a dark brown stain. Colonies of ≥ 8 haemoglobinised cells were scored as CFU-E with the aid of an inverted microscope (Olympus model CK-2).

2.12.8.2 Other colony types

10^4 5-FU treated bone marrow cells, 2×10^4 foetal liver cells, 5×10^4 bone marrow cells and 5×10^5 spleen cells were cultured in 1 ml methyl-cellulose medium. Where indicated 500 U/ml mIL-3, 10 ng/ml hIL-6, 500 U/ml mGM-CSF, 50 ng/ml mSCF, 2 U/ml hEpo and 1 mg/ml G418 were added. Colonies containing greater than 50 cells were scored at the seventh day of culture with the aid of a dissecting microscope (Olympus model SZ-11) using indirect lighting. To assess colony types individual colonies were removed at days 7-11 of culture with a micropipette, spread on glass slides using compressed air until dry as described by Metcalf (1984), stained with Wright-Giemsa and the colony types determined microscopically using a light microscope (Olympus model BH-2).

2.12.9 Cytocentrifugation

Approximately 10^5 cells were placed in 100 - 200 µl cDMEM or cIMDM and cytocentrifuged at 500 rpm for 5 min onto glass microscope slides (Knittel Glaser, Germany) in a Cytospin cytocentrifuge (Shandon, Cheshire, UK). Microscope slides were removed and allowed to air dry for 5 min before staining. Alternatively, slides were preserved by desiccation at 4°C for staining at a later date.

2.12.10 Cytochemical Staining

All cytochemical staining was performed on cytocentrifuged and air dried cells. The following stains were performed in this study;

2.12.10.1 Wright Giemsa

Wright-Giemsa staining was performed using a Shandon Varistain 12 staining machine in the Diagnostic Services Laboratory of the Institute for Medical and Veterinary Science, Adelaide, South Australia. Cells were fixed in methanol for 2 min then stained with Jenner's Stain for 2 min and Giemsa stain for 6 min before being

washed in a buffer of pH 7 and air dried. The cells were mounted in DePX mounting medium (Gurr, BDH Laboratory Supplies).

2.12.10.2 Myeloperoxidase

The myeloperoxidase staining technique employed was similar to that used by Hu et al. (1993). Briefly, cells were fixed in a mixture of 1.25% (v/v) gluteraldehyde and 1% (v/v) formamide in PBS for 1 min. The slides were rinsed in PBS then immersed in a freshly made solution of 0.5 mg/ml 3'-3'-diaminobenzidine (ICN Biomedicals) and 0.3% (v/v) hydrogen peroxide in 0.05 M Tris buffer, pH 7.6 for 1 min. The slides were then rinsed 3 times in PBS and immersed in a solution of 5 mg/ml copper sulphate in 0.05 M Tris buffer, pH 7.6 for 1 min. Cells were counterstained with haematoxylin.

2.12.10.3 Haematoxylin

Slides were immersed in Harris haematoxylin (Labaids, Narrabeen, Victoria, Australia) for 30 seconds then rinsed in tap water and "blued" in running tap water for 5 min.

2.12.11 Flow cytometry

2.12.11.1 Cell surface antigen analysis

10^6 - 10^7 cells were collected by centrifugation and resuspended in 50 µl of DMEM 2% FCS. The cells were incubated with the primary antibody on ice for 20 min. Where the primary antibody was FITC-conjugated, the cells were washed in 2 ml DMEM 2% FCS, resuspended in 300 µl FACS fixative and stored at 4°C in the dark. Where the primary antibody was not conjugated, cells were washed in 2 ml DMEM, 2% FCS, collected by centrifugation and stained with FITC-conjugated anti-isotype antibodies for 20 min on ice. Where indicated, to enhance staining, following primary antibody staining the cells were washed, incubated with a biotin-conjugated anti-mouse immunoglobulin antibody for 20 min on ice, washed and incubated with phycoerythrin-labelled streptavidin for 20 min on ice. After washing and centrifugation the cells were resuspended in 300 µl FACS fix and stored in the dark at 4°C.

Fluorescence was measured using an Epics Profile II analyser (Coulter Electronics, Hileah, FL, USA).

2.12.11.2 Cell sorting

To sort cells expressing various receptor subunits cells were stained as above except that they were resuspended in 300 µl maintenance medium in the final step. Cells expressing the desired antigens were collected using a FACStar^{PLUS} flow cytometer (Becton-Dickinson, San Jose, CA, USA). Cells were washed in 10 ml cDMEM, resuspended in maintenance medium and cultured as in Section 2.12.1.

2.12.12 Cell proliferation assay

Cells were collected by centrifugation and washed twice in DMEM. 5×10^3 cells were cultured in triplicate in 96-well microtiter plates in cDMEM with or without 50 U/ml mGM-CSF for 72 hours. Cell proliferation was measured with a formazan reduction assay using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega). Absorbance was measured on a Bio-Rad Model 3550 Microplate Reader and Bio-Rad Laboratories Microplate Manager® v.2.2 software for Macintosh.

2.12.13 Viable cell counts

Cell suspensions were mixed 1:1 with 0.2% (w/v) Trypan Blue in PBS. The number of cells excluding trypan blue was scored using a haemocytometer (Weber Scientific, Middlesex, UK) and light microscope (Olympus model BH-2).

2.12.14 Cell viability assays

To assay cell viability, viable cell counts were performed as above and the proportion of cells excluding trypan blue (viable cells) was determined from at least one hundred cells scored.

2.13 Studies using mice

2.13.1 Strains used

8 week old CBA mice and day 14 pregnant CBA mice were obtained from the Animal Resource Centre, Perth, Western Australia.

C57BL/6J mice were obtained from the Animal Facility, WAITE Institute, Adelaide, South Australia.

B6.SJL mice were obtained from the Animal Resource Centre, Perth, Western Australia.

2.13.2 Housing and conditions

CBA mice were housed in the Basement Animal House of the Veterinary Services division of the Institute for Medical and Veterinary Science, Adelaide, South Australia.

C57BL/6J and B6.SJL mice were housed in sterile conditions in a quarantine barrier at the Adelaide University Medical School Animal House.

When necessary mice were sacrificed by cervical dislocation.

2.13.3 Tumorigenicity assay

To assess the tumorigenicity of cell lines, cells were collected by centrifugation and resuspended in PBS at a concentration of 2.5×10^7 cells/ml. 8 week old male CBA mice were generally anaesthetised as described in section 2.12.2.4 and 200 µl of the cell suspension was injected subcutaneously at the nape of the neck. Mice were monitored twice weekly and upon tumour formation the mice were sacrificed. When required the tumours were recovered by dissection, homogenised, cytocentrifuged, Wright-Giemsa stained and the cellular morphology examined microscopically.

2.13.4 Bone Marrow Reconstitution

8 week old male C57BL/6J mice (donor mice) were injected intraperitoneally with 150 mg/kg 5-fluorouracil (5-FU, Faulding, David Bull Laboratories, Victoria, Australia). 4 days post-injection the mice were sacrificed and bone marrow harvested. The resultant 5-FU treated bone marrow cells were infected with RufNeo constructs as described in section 2.12.6.2. After the 3 day infection period, 8 week old female B6.SJL mice (recipient mice) were lethally irradiated with two doses of 5 Grays each, 4 hours apart, from a Caesium^{137} source in an IBL Blood Product Irradiator at the Blood Transfusion Unit of the Royal Adelaide Hospital. The irradiated recipients were

injected intravenously with the infected 5-FU treated cells derived from one donor mouse femur, in a volume of 0.2 ml PBS. Tail vein injections were performed by Hayley Ramshaw, Division of Human Immunology, The Hanson Centre for Cancer Research, Adelaide, South Australia. Mice were monitored weekly and their peripheral blood analysed monthly. At the first sign of illness mice were sacrificed and their haemopoietic tissues analysed.

2.13.5 Quantification of haemopoietic reconstitution

In order to quantify the haemopoietic reconstitution of bone marrow reconstituted mice, the donor and recipient mice used were of congenic strains each carrying antigenically distinct forms of the leukocyte common antigen, Ly5, also known as CD45 (Morse, 1992). Donor C57BL/6 mice carry the *Ly5*^{a} allele of specificity Ly5.1 (also known as the *ptprc*^{b} allele of specificity CD45.2). Recipient mice of the strain B6.SJL carry the *Ly5*^{b} allele of specificity Ly5.2 (also known as the *ptprc*^{a} allele of specificity CD45.1).

Bone marrow reconstituted mice were bled at 4-8 weeks post-reconstitution and white blood cells prepared as described in section 2.12.2.5. White blood cells equivalent to 20 µl peripheral blood were stained with FITC-conjugated anti-Ly5.1 and anti-Ly5.2 monoclonal antibodies and analysed by flow cytometry as described in section 2.12.11.1. To achieve homogeneous staining, lymphocytes, which comprise approximately 80% of normal murine leukocytes, were gated by scatter profile and the relative proportions of donor and recipient cells determined. White blood cells from unreconstituted C57BL/6J and B6.SJL mice were used as controls.

2.13.6 Peripheral blood analysis

To quantify peripheral blood white blood cells, erythrocytes, platelets, haemoglobin and packed cell volume, 40 µl peripheral blood was diluted 1:5 in PBS, analysed using a Coulter blood analyser (Coulter Electronics) and the results multiplied to allow for dilution.

2.13.7 Histological analysis of organs

Mice were dissected and the whole or part organs fixed in 10% neutral buffered formalin for at least 16 hours. These specimens were paraffin embedded, sectioned at 5 μm and stained with Haematoxylin/Eosin by the Division of Histopathology, IMVS, Adelaide, South Australia.

2.13.8 Differential cell counts

White blood cells, bone marrow cells, spleen cells and foetal liver cells were cytocentrifuged, Wright-Giemsa stained and mounted using DePX mounting medium. Differential cell counts were performed using an inverted microscope.

2.13.9 Immunohistochemistry

Cells were fixed in a solution of 47.5% (v/v) acetone, 47.5% (v/v) methanol and 5% (v/v) formaldehyde for 90 seconds, washed in PBS and stained using the Mouse To Mouse Alkaline Phosphatase kit (ScyTek Laboratories, Logan, Utah, USA) using the equipment and protocol supplied by the manufacturer, using the mouse anti-hβc monoclonal antibody 4F3 (Woodcock et al., 1994) as primary antibody. Antibody staining was visualised with an fast red stain detecting an alkaline phosphatase antibody conjugate, with cells expressing hβc staining red. Cells were counterstained with haematoxylin as described in Section 2.12.10.3.

CHAPTER 3. Function of activated hβc mutants in primary murine haemopoietic cells

3.1 Introduction

Several activating mutations in hβc have recently been identified via their ability to deliver a proliferative signal to the murine IL-3- or GM-CSF-dependent myelomonocytic cell line FDC-P1 in the absence of growth factors. As detailed in Chapter 1, one interesting property of these mutants is their apparent cell-type specificity. That is, whilst the transmembrane mutant, V449E, can deliver a constitutive proliferative signal to the murine pro-B cell line, BAF-B03, the extracellular mutants, FIΔ and I374N, cannot (Jenkins et al., 1995). This result implies that the extracellular mutants require other, perhaps myeloid-specific molecules for function, which are not required by V449E. Moreover, none of these three activated hβc mutants could deliver proliferative signals to the murine IL-2 dependent T cell line, CTLL-2, whilst a wild-type hGM-CSF receptor could do so (Jenkins et al., 1995). This implies that the transmembrane mutant, V449E, also requires cell-type specific molecules for function, which are absent in CTLL-2 cells but present in FDC-P1 and BAF-B03 cells.

In light of the apparent differences in signalling between the transmembrane and extracellular hβc mutants, it is of interest to determine their function in primary haemopoietic cells. The aims of this work are twofold; firstly, to determine the primary haemopoietic cell types, if any, to which these mutants can deliver proliferative signals. The identification of permissive cell types will provide important information as to the leukaemic potential of these mutants in a more "realistic" setting than the already transformed cell lines employed thus far. Secondly, it is hoped to determine the effects of the hβc mutants on cellular differentiation, an issue which was not addressed by the previous studies which were performed in differentiation-arrested cell lines.

Here I report a functional study of the FIΔ, I374N and V449E mutants in primary murine haemopoietic cells derived from foetal liver, using the technique of retroviral transduction. This technique has been previously shown to be a rapid and efficient method for gene transfer into primary haemopoietic cells both *in vitro* and *in vivo* (for

review see Miller (1992)). The retrovirus used, RufNeo, has been shown to give high titres and efficient infection of murine haemopoietic cells using the technique of co-cultivation of ecotropic virus producers with target cells (Rayner and Gonda, 1994).

3.2 Results

3.2.1 Generation of factor-independent foetal liver cells

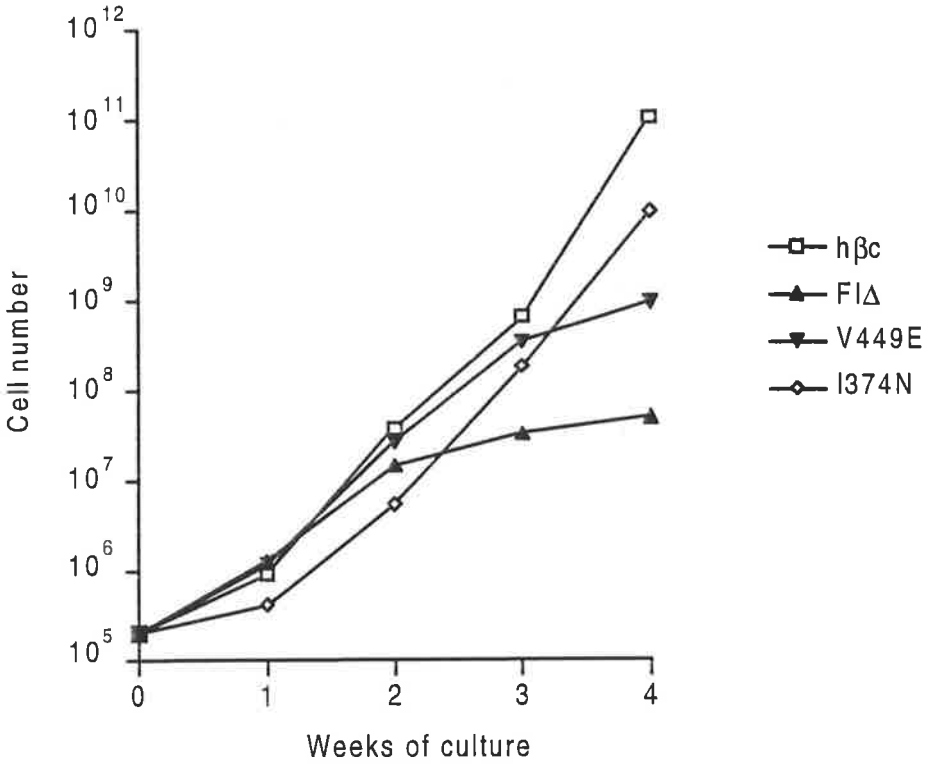
To investigate the effect of expression of activated hβc mutants on haemopoietic cell growth and differentiation, three separate constitutively active mutant hβc subunits were expressed in murine foetal liver cells using the RufNeo retrovirus (Rayner and Gonda, 1994). As described in Section 2.12.6.1, foetal liver cells were co-cultivated with Ψ2 ecotropic retrovirus packaging cells producing RufNeo containing either a wild type or mutant hβc cDNA (as a control, parallel co-cultivations were carried out with untransfected Ψ2 cells). These co-cultivations were performed in the presence of saturating concentrations of IL-3, GM-CSF and Epo to maximally stimulate survival and growth of a broad range of myeloid and erythroid progenitor cells. When subsequently placed in liquid culture without exogenous growth factors, foetal liver cells infected with a retroviral construct containing wild type hβc rapidly died (Figure 3.1), as did mock-infected controls (data not shown). When cells infected with viruses encoding any of the three mutant hβc subunits were placed in culture without exogenous growth factors, some cell death was observed (Figure 3.1). This was presumably due to some cells not being infected, or not expressing sufficient levels of mutant hβc subunits to allow factor-independent proliferation. However, a subset of each of these populations was able to proliferate in the absence of growth factors. The factor-independent response was greatest from cells infected with the V449E vector, but was less in all cases than that obtained with added GM-CSF, IL-3 and Epo.

Examination *in situ* of the factor-dependent and -independent cells revealed that whilst cells grown in the presence of growth factors contained a mixture of adherent and non-adherent cells of various morphologies (as expected) (Figure 3.2A), factor-independent cells containing the transmembrane mutant, V449E, were almost exclusively small, non-adherent cells (Figure 3.2B). In contrast, cultures infected with either extracellular hβc mutant (FIΔ and I374N) showed an initial small flourish of non-adherent cell growth; however these cells died after approximately 11 days,

Figure 3.1. Time-course of proliferation of infected foetal liver cells in liquid culture.

Murine foetal liver cells were infected with RufNeo retroviral constructs containing the indicated hβc subunits as described in Section 2.12.6.1. The cells were then washed and 2×10^5 cells cultured with (**A**) and without (**B**) mIL-3, mGM-CSF and hEpo. Viable cell counts were performed at weekly intervals.

A


Cell number
10^{12}
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
0
1
2
3
4
Weeks of culture
hβc
FIΔ
V449E
I374N

B

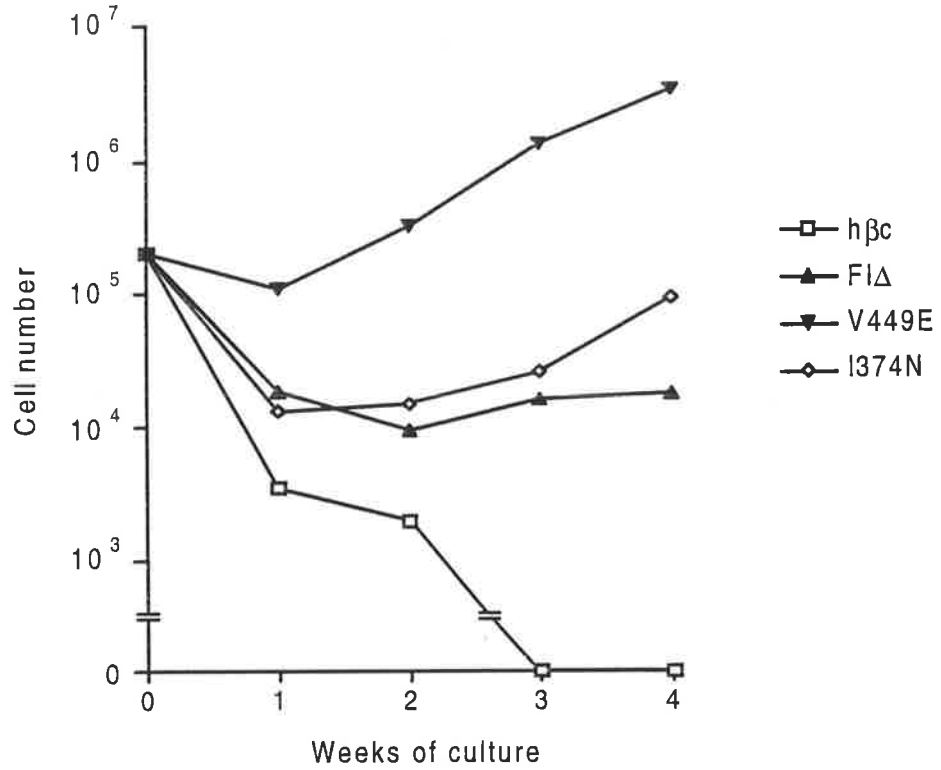
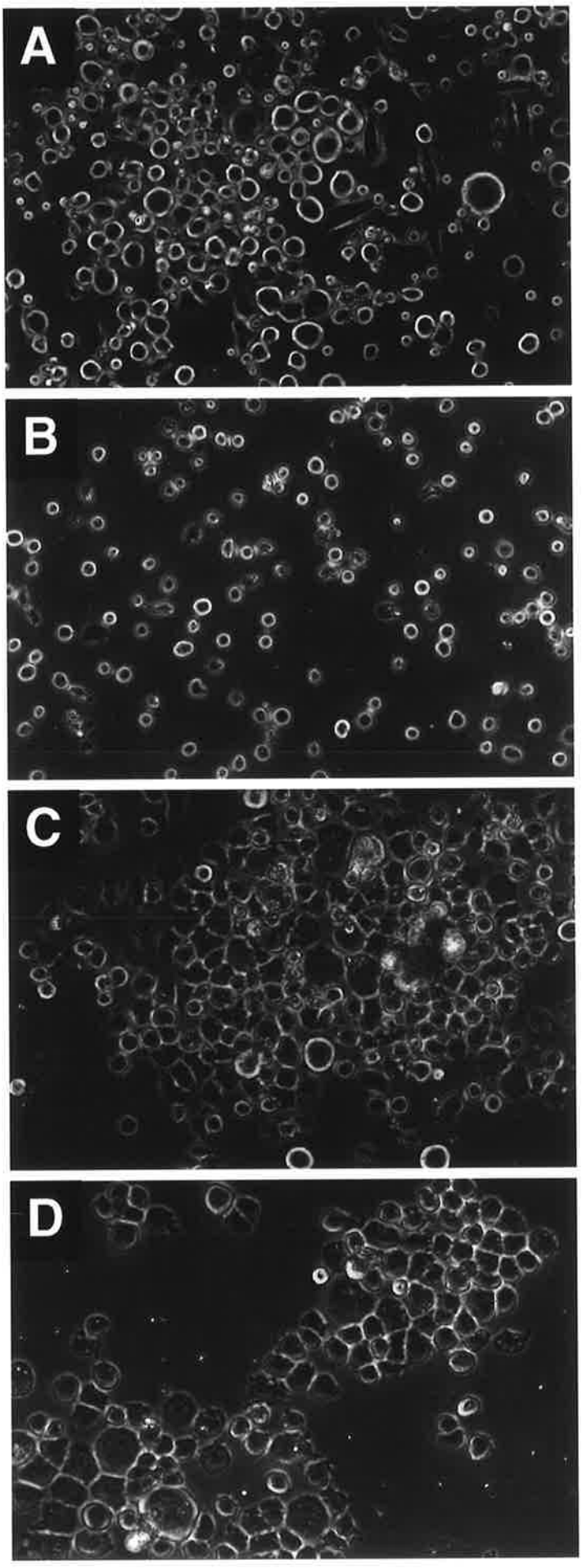

Cell number
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
0
0
1
2
3
4
Weeks of culture
hβc
FIΔ
V449E
I374N

Figure 3.2. *In situ* morphology of foetal liver liquid cultures.

(**A**) Cells infected with RufNeo-hβc grown in the presence of growth factors (mIL-3, mGM-CSF, hEpo). (**B**) Cells infected with RufNeo-V449E grown in the absence of growth factors. (**C**) Cells infected with RufNeo-FIΔ grown in the absence of growth factors. (**D**) Cells infected with RufNeo-I374N grown in the absence of growth factors. Photographs are at 170 × magnification, taken at the 14th day of culture.


A
B
C
D

leaving an exclusively adherent cell population which grew in colonies and morphologically resembled macrophages (Figure 3.2C and 3.2D). Hence an apparently mutually exclusive growth pattern was obtained from primary cells infected with constitutively active transmembrane and extracellular hβc mutants.

To confirm that the factor-independent cells obtained in these experiments did in fact express mutant hβc subunits, these cells were stained with the anti-hβc monoclonal antibody 4F3 (Woodcock et al., 1994) and flow cytometric analyses were performed. As shown in Figure 3.3A, hβc was detected on the surface of all factor-independent cells infected with the hβc mutants. To verify that the hβc subunits expressed on the factor-independent cells were the intended mutants, PCR products spanning the mutated region were obtained from the proviral hβc cDNAs in these cells and, where appropriate, digested with diagnostic restriction enzymes (Figure 3.3B) (Jenkins et al., 1995). As shown in Figure 3.3C, the V449E and I374N mutants were verified by their inherent additional *Bgl*II and lost *Bst*YI sites respectively, whereas the FIΔ mutant was distinguished by its increased size.

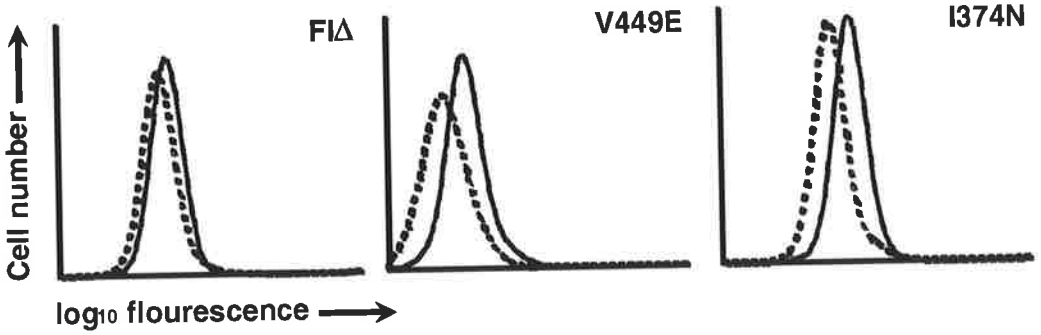
3.2.2 Characterisation of factor-independent foetal liver cells

In order to ascertain the factor-dependent and -independent cell types present in the liquid cultures, these cultures were agitated and cytocentrifuge preparations made at weekly intervals. As shown in Figure 3.4A and Table 3.1, cells grown in IL-3, GM-CSF and Epo consisted of all five myeloid lineages as well as erythroid cells and immature blast, promyelocyte and myelocyte cells. A similar range of cell types was seen in cells selected for RufNeo infection by culture in the presence of growth factors and G418 (Table 3.1). However, the factor-independent cells containing the FIΔ and I374N mutants were exclusively mature macrophages and neutrophils at day 7 of culture, the latter of which died after approximately 11 days culture leaving a monoculture of mature macrophages at day 21 (Figure 3.4B and 3.4D, Table 3.1). Whilst all the cell types obtained with added growth factors (GM-CSF, IL-3 and Epo) and G418 were present in factor-independent cell populations generated with V449E (without Epo), there was a significant decrease in the proportion of monocytic cells in the latter population at day 7 and day 21 (χ^2 tests, $P<0.005$ in each case). Conversely, V449E induced superior proliferation of immature cells types compared with cells

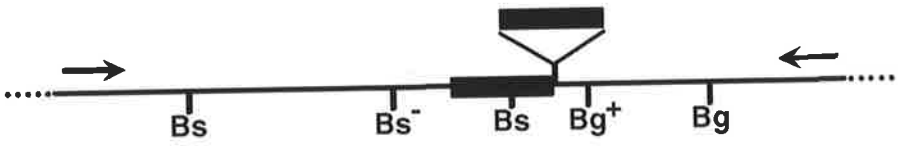
Figure 3.3. Expression and confirmation of identity of hβc mutants in factor-independent foetal liver cells.

(**A**) Flow cytometric analyses of mutant hβc expression. Dashed lines represent staining with an irrelevant isotype control antibody. Solid lines represent staining with an anti-hβc monoclonal antibody. (**B**) Map of hβc cDNA showing *Bst*YI (Bs) and *Bgl*II (Bg) restriction sites used to authenticate each form of hβc, as well as the region duplicated in FIΔ (indicated by boxes). The restriction sites affected by the point mutations are indicated as Bg^+ (gained in V449E) and Bs^- (lost in I374N). Arrows indicate the positions of PCR primers used to amplify hβc fragments from genomic DNA. (**C**) Electrophoretic analysis of PCR products generated from genomic DNA of factor-independent foetal liver cells infected with constructs containing the indicated hβc mutants. As a negative control, a reaction was performed containing no DNA (-). Lanes M contain DNA size standards (SPP-1 phage DNA digested with *Eco*RI). For comparison, PCR products were generated from a RufNeo-hβc plasmid (labelled 'wild-type'). PCR products were either not digested (lanes 1), digested with *Bgl*II (lanes 2), or *Bst*YI (lanes 3). Bands in each digest that differ between the mutants and the wild-type are indicated by asterisks.

A


FIΔ
V449E
I374N
Cell number
log10 flourescence

B


Bs
Bs-
Bs
Bg+
Bg

C

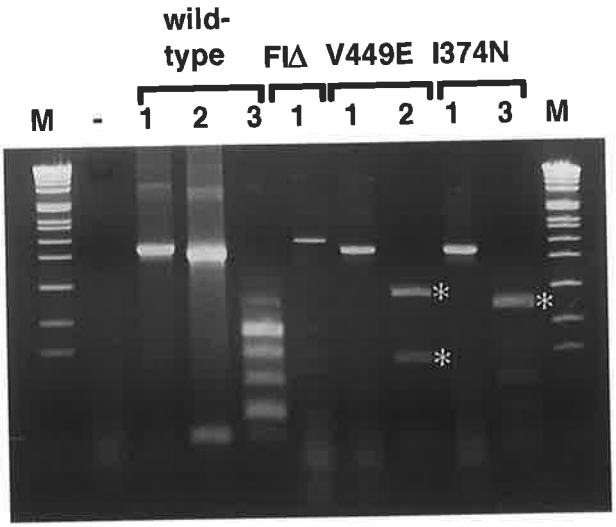

wild-type
FIΔ
V449E
I374N
M
-
1
2
3
1
1
2
1
3
M

Figure 3.4. Morphology of factor-dependent and -independent foetal liver cells in liquid suspension culture.

(**A**) Cells infected with RufNeo-hβc grown in IL-3, GM-CSF and Epo, (**B**) Cells infected with RufNeo-FIΔ grown in the absence of growth factors, (**C**) Cells infected with RufNeo-V449E grown in the absence of growth factors, (**D**) Cells infected with RufNeo-I374N grown in the absence of growth factors. Photographs are at 900 × magnification. Photographs were taken at the 21st day of culture.

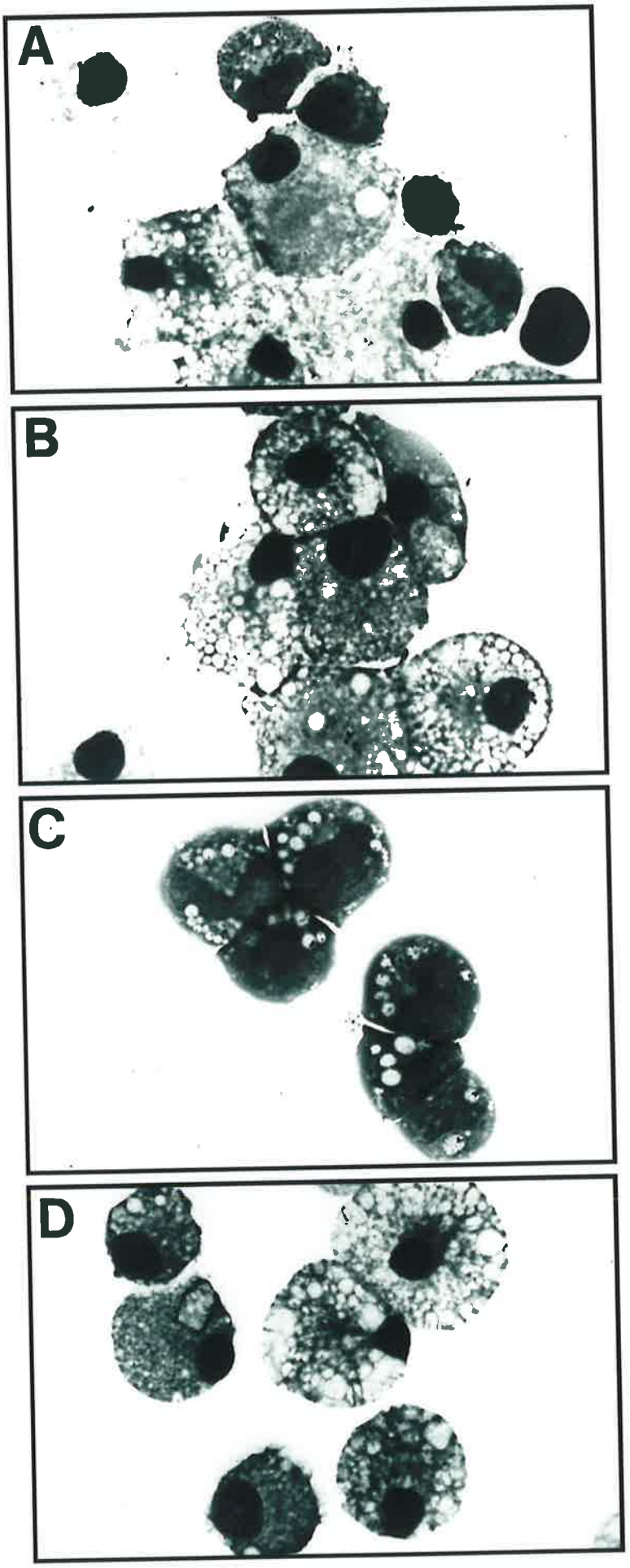

A
B
C
D

Table 3.1. Differential cell counts of factor-dependent and factor-independent foetal liver cells

RufNeo construct	Additions	Cell type (%)[a]							
		Eos	Mast	Neut	MK	M	Eryth	Myel	Blast
Day 7									
hβc	IL-3, GM-CSF, Epo	0.6	2.9	32.8	0.6	27.0	4.6	26.4	5.2
	IL-3, GM-CSF, Epo, G418	1.0	-	45.5	0.3	18.3	9.3	20.6	5.0
FIΔ	Epo	-	-	67.3	-	28.7	2.7	1.3	-
	-	-	-	69.7	-	30.3	-	-	-
V449E	Epo	0.3	1.3	47.8	0.3	4.3	10.0	32.6	3.3
	-	2.0	0.7	48.8	0.7	0.7	2.3	39.9	5.0
I374N	Epo	-	-	4.0	-	92.9	3.2	-	-
	-	-	-	11.2	-	88.8	-	-	-
Day 21									
hβc	IL-3, GM-CSF, Epo	-	9.0	-	-	41.0	41.0	9.0	-
	IL-3, GM-CSF, Epo, G418	-	1.0	0.3	-	79.3	13.3	6.0	-
FIΔ	Epo	-	-	-	-	99.3	0.7	-	-
	-	-	-	-	-	100.0	-	-	-
V449E	Epo	-	8.3	6.0	-	-	1.3	72.0	12.3
	-	-	0.7	2.7	-	-	-	93.3	3.3
I374N	Epo	-	-	-	-	100.0	-	-	-
	-	-	-	-	-	100.0	-	-	-

Numbers are percentages of 200 cells scored. Data are representative of three separate experiments.

[a]Eos- eosinophils, Mast- mast cells, Neut- neutrophils, MK- megakaryocytes, M- monocytes/macrophages, Eryth- erythroid cells, Myel- promyelocytes/myelocytes, Blast- blast cells.

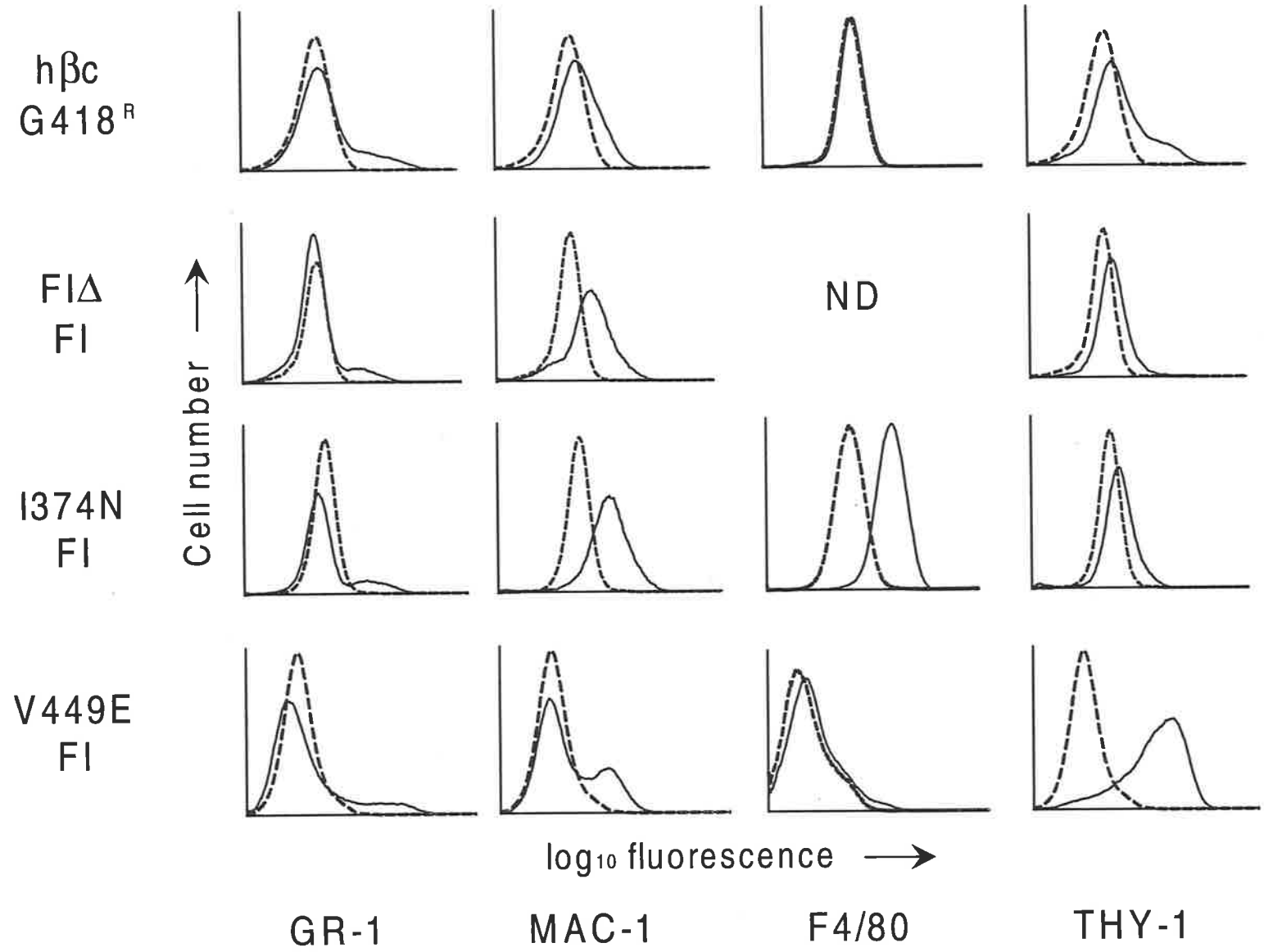
cultured in growth factors plus G418, with significant increases in the proportions of myelocytes at day 7 (χ^2 test, $P<0.005$) and both blast cells and myelocytes at day 21 (χ^2 tests, $P<0.005$ in each case), which at this stage constituted the majority of cells (Figure 3.4C, Table 3.1). Interestingly, factor-independent erythroid cells were obtained in the absence of Epo from cells containing V449E. Addition of Epo to factor-independent cultures containing V449E significantly increased the erythroid content at day 7 (χ^2 test, $P<0.005$). However, Epo did not significantly increase the numbers of erythroid cells in factor-independent cultures containing FIΔ or I374N above a small background which was obtained when Epo was added to mock-infected or RufNeo-hβc-infected cultures (data not shown).

Next, to verify the identities of the various cell types observed, the expression of haemopoietic cell lineage markers was examined on the 24th day of culture on factor-dependent and -independent cells by flow cytometry. As shown in Figure 3.5, a small fraction of G418-resistant cells infected with RufNeo-hβc, and factor-independent cells containing any of the hβc mutants, expressed the Gr-1 marker which is expressed on mature granulocytes and some granulocyte-macrophage precursors (Holmes et al., 1986). Factor-independent cells containing either extracellular hβc mutant expressed the granulocyte-macrophage specific marker Mac-1 (Springer et al., 1979), which is concordant with the mature macrophage morphology of these cells. However, only a fraction of factor-independent cells containing V449E expressed Mac-1, indicating that monocytic and neutrophilic cells make up only a fraction of this population (Figure 3.5). Accordingly, whilst all factor-independent cells containing I374N expressed the macrophage-specific cell marker F4/80 (Austyn and Gordon, 1981), only a small fraction of cells containing V449E were positive for this marker (Figure 3.5). Factor-independent cells containing V449E expressed the Thy-1 marker at high levels, unlike cells grown in growth factors or factor-independent cells containing either extracellular hβc mutant (Figure 3.5). Thy-1 is found on populations enriched for stem and early progenitor cells, hence its expression confirms the immature nature of these cells (Spangrude et al., 1988; Miller et al., 1985; Berman and Basch, 1985).

Taken together, these results indicate that the activated extracellular hβc mutants FIΔ and I374N can give rise to factor-independent neutrophil and macrophage cells only. In contrast, the transmembrane hβc mutant V449E can induce proliferation and

Figure 3.5. Expression of lineage-specific cell surface antigens on factor-dependent and -independent foetal liver cells.

Flow cytometric analyses were performed as described in Section 2.12.11.1. Dashed lines represent staining with an irrelevant isotype control antibody. Solid lines represent staining with monoclonal antibodies directed to the indicated cell surface markers. Note: F4/80 staining was performed on separate cell populations to those used to assess the other surface antigens. $G418^R$ - G418 resistant cell population. FI - factor-independent cell population.


hβc
G418R
FIΔ
FI
I374N
FI
V449E
FI
ND
Cell number
log10 fluorescence
GR-1
MAC-1
F4/80
THY-1

differentiation of all the lineages stimulated by its cognate ligands, IL-3, IL-5 and GM-CSF. Notably, though, V449E gives rise to a significantly lower proportion of macrophages and higher proportion of immature cells than does a combination of IL-3, GM-CSF and Epo.

3.2.3 Clonal analysis of factor-independent foetal liver cells

To further characterise the factor-independent response of foetal liver cells bearing mutant hβc subunits, I examined the proliferation of individual progenitors in colony assays. As shown in Figure 3.6, when foetal liver cells exposed to retroviruses encoding the various hβc mutants were cultured in semi-solid medium, 79-92% of progenitors were infected as assessed by the fraction of G418-resistant colonies obtained in the presence of growth factors. When cultured in the absence of growth factors, V449E induced colony formation by approximately half of the infected progenitors, whereas for FIΔ and I374N, approximately one quarter and one fifth of the infected progenitors were factor-independent respectively. Addition of Epo to cells infected with activated hβc mutants did not increase colony numbers beyond a small number of background colonies obtained when mock-infected or RufNeo-hβc infected cells were cultured in Epo alone, which is concordant with results obtained from liquid cultures (see above).

Factor-independent colonies containing V449E were of various types including neutrophil (G), eosinophil (Eo), mast cell (Mast), macrophage (M), granulocyte-macrophage (GM), mixed myeloid/erythroid (Mix) colonies containing granulocytes, erythroid cells, megakaryocytes and macrophages, and blast cell colonies (Table 3.2). In contrast, FIΔ and I374N gave rise to only G, M and GM colonies in the absence of growth factors. Addition of Epo did not increase numbers of BFU-E colonies above a small background which was obtained with mock-infected or RufNeo-hβc infected cells cultured in Epo alone (data not shown). However, Epo did increase the proportion of erythroid cells in factor-independent mixed myeloid/erythroid colonies containing V449E (data not shown), which is consistent with the fact that a significant increase in V449E-induced erythroid growth was obtained in liquid cultures containing Epo (see above). The lack of factor-independent BFU-E colonies induced by expression of V449E suggests that the factor-independent erythroid growth

Figure 3.6. Colony formation by mock-infected and infected foetal liver cells in methyl-cellulose.

Mock-infected foetal liver cells and cells infected with the indicated RufNeo constructs were plated in the indicated conditions in semi-solid methyl-cellulose medium and the resultant colonies scored after 7 days. The data shown are a combination of two separate experiments. In each experiment, duplicate dishes were counted in the case of those containing growth factors, whilst in the case of dishes containing Epo alone, or without added growth factors, eight dishes were counted. GFs - growth factors (IL-3, GM-CSF, Epo).

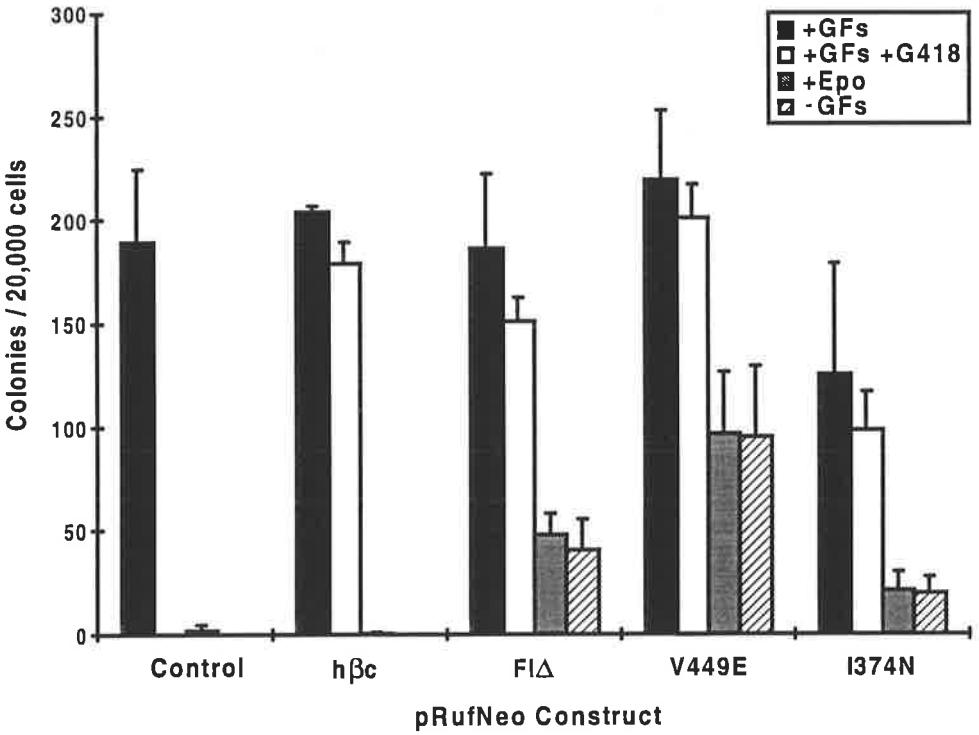

300
250
200
150
100
50
0
Colonies / 20,000 cells
+GFs
+GFs +G418
+Epo
-GFs
Control
hβc
FIΔ
V449E
I374N
pRufNeo Construct

Table 3.2. Factor-dependent and -independent colonies formed by foetal liver cells infected with wild-type and mutant hβc subunits

RufNeo construct	Additions	Colony types (%)[a]								Colonies examined
		G	Eo	Mast	M	GM	Mix	BFU-E	Blast	
hβc	IL-3, GM-CSF, Epo	12	1	4	39	33	8	4	-	103
	IL-3, GM-CSF, Epo, G418	18	3	6	38	26	5	5	-	109
FIΔ	Epo	34	-	-	24	36	-	6	-	80
	-	17	-	-	45	38	-	-	-	84
V449E	Epo	9	1	12	26	17	29	3	3	69
	-	13	8	7	30	20	18	-	5	61
I374N	Epo	4	-	-	79	15	-	1	-	73
	-	1	-	-	89	10	-	-	-	93

Data are a combination of two separate experiments

Colony types were determined at the 7th day of culture

[a] G- neutrophilic granulocyte, Eo- eosinophil, Mast- mast cell, M- macrophage, GM- granulocyte-macrophage, Mix- mixed myeloid/erythroid, BFU-E- erythroid burst-forming unit, Blast- blast cell

supported by this mutant in liquid cultures (Table 3.1) was derived from mixed myeloid/erythroid progenitors.

These results are concordant with those obtained in liquid culture and confirm the observations that whilst V449E can induce proliferation and differentiation of all the cell types examined in this study, FIΔ and I374N can do so to neutrophil and macrophage precursors only.

3.2.4 Isolation and characterisation of factor-independent cell lines

Cultures containing growth factors eventually gave rise to mast cell lines, as is frequently the case when murine haemopoietic cell populations are maintained in the presence of IL-3 (Figure 3.7A) (Schrader et al., 1981; Razin et al., 1984; Nagao et al., 1981). However, from two populations of factor-independent cells containing the V449E mutant (of 6 studied), continuously proliferating cell lines were established, termed RTVE1 and RTVE2.

Cells of the RTVE1 line grew in clumps and required cell contact for growth; when the clumps were disaggregated the resulting single cells rapidly died. These cells displayed ring nuclei characteristic of neutrophilic myelocytes (Figure 3.7B). Treatment with a myeloperoxidase stain revealed large primary granules expressing this enzyme, confirming that these cells are partially differentiated (Figure 3.8A).

Cells of the RTVE2 line were cytologically less mature than RTVE1 cells, with their development ostensibly arrested at the blast cell stage (Figure 3.7C), and did not require cell contact for growth. This line displayed some spontaneous neutrophil and macrophage differentiation, implying that it is a precursor for both of these lineages. Cells of this line did not express myeloperoxidase (Figure 3.8B), which is consistent with their myeloblast morphology, as this enzyme is induced at the promyelocyte stage (Lubbert et al., 1991; Austin et al., 1994).

To confirm that the RTVE1 cell line contained integrated V449E cDNA, genomic DNA was prepared from this line and used in a PCR reaction using primers spanning a 1091 bp region of the hβc cDNA which included the V449E mutation. As shown in Figure 3.9, a PCR product of the expected size was generated from RTVE1 genomic DNA, and the presence of an additional *Bgl*II site when compared to an identical PCR

Figure 3.7. Morphology of factor-independent foetal liver cell lines.

(**A**) For comparison, mast cells obtained from a foetal liver culture infected with RufNeo-hβc and cultured for 6 weeks in the presence of growth factors (IL-3, GM-CSF, Epo). (**B**), (**C**) RTVE1 and RTVE2 cell lines, respectively, obtained following infection of foetal liver cells with RufNeo-V449E. Photographs are at 900 × magnification.

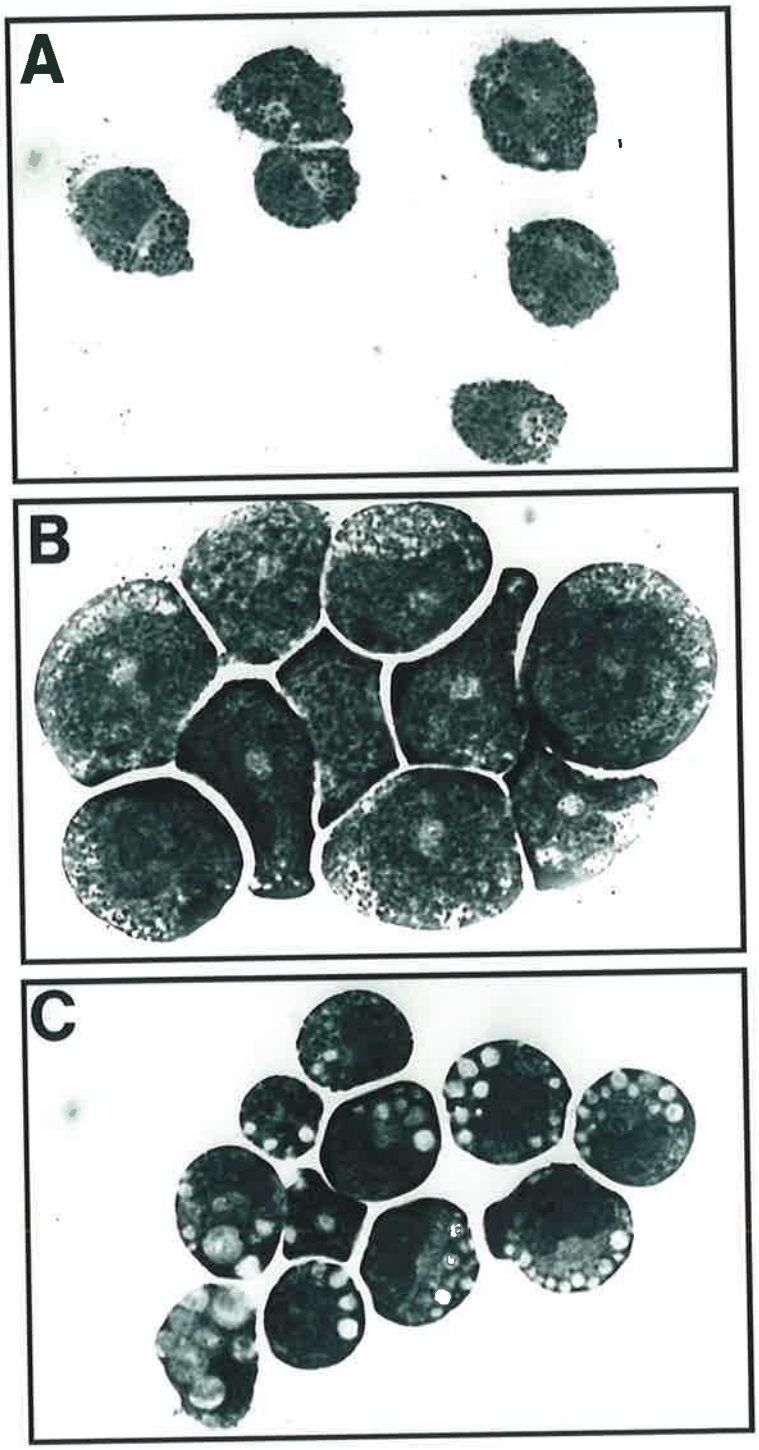
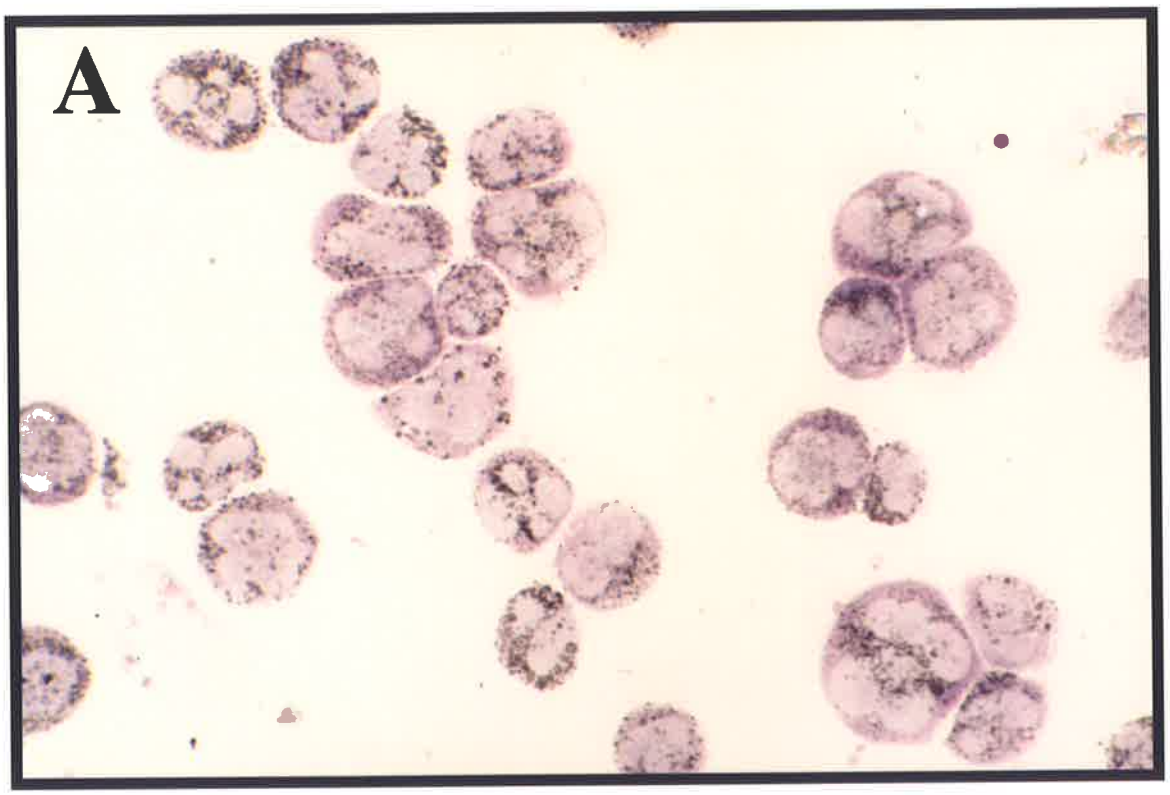

A
B
C

Figure 3.8. The RTVE1 cell line expresses the myeloperoxidase enzyme.

Cells from the RTVE1 (**A**) and RTVE2 (**B**) cell lines were cytocentrifuged, fixed and stained with a diaminobenzdine stain which reacts with the myeloperoxidase enzyme. Photographs are at 570 × magnification.


A

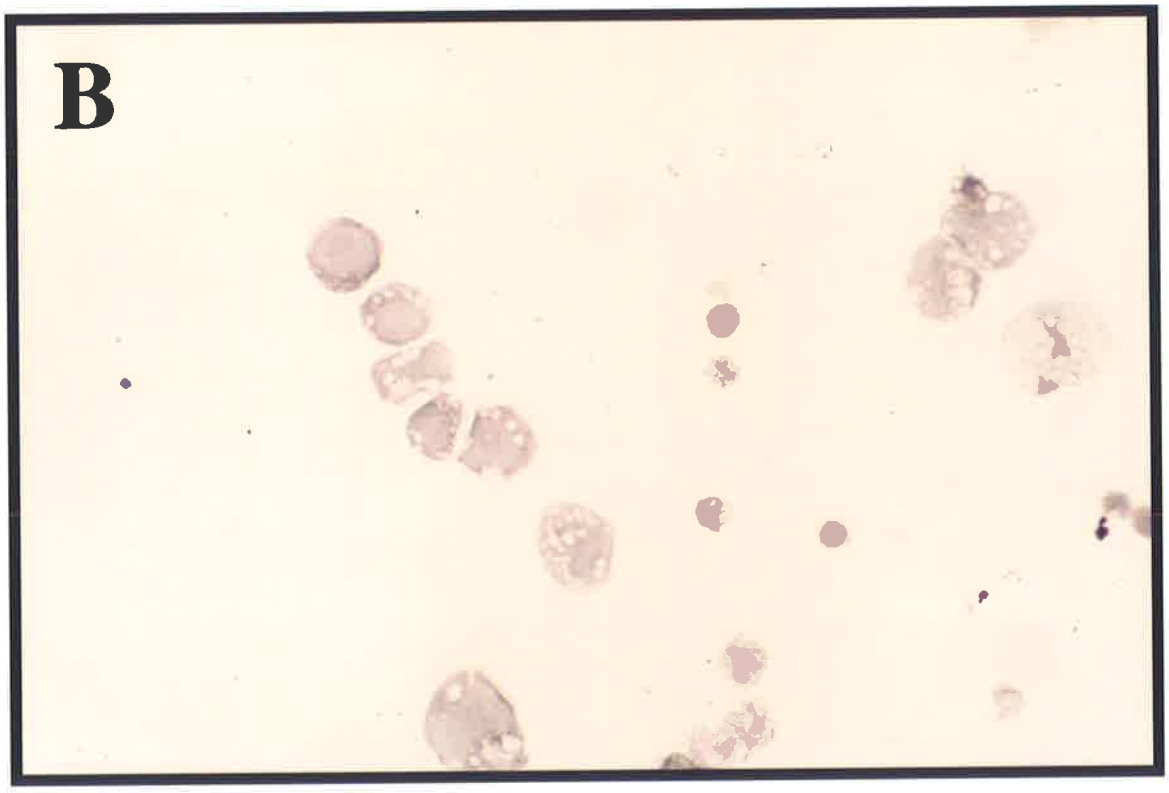
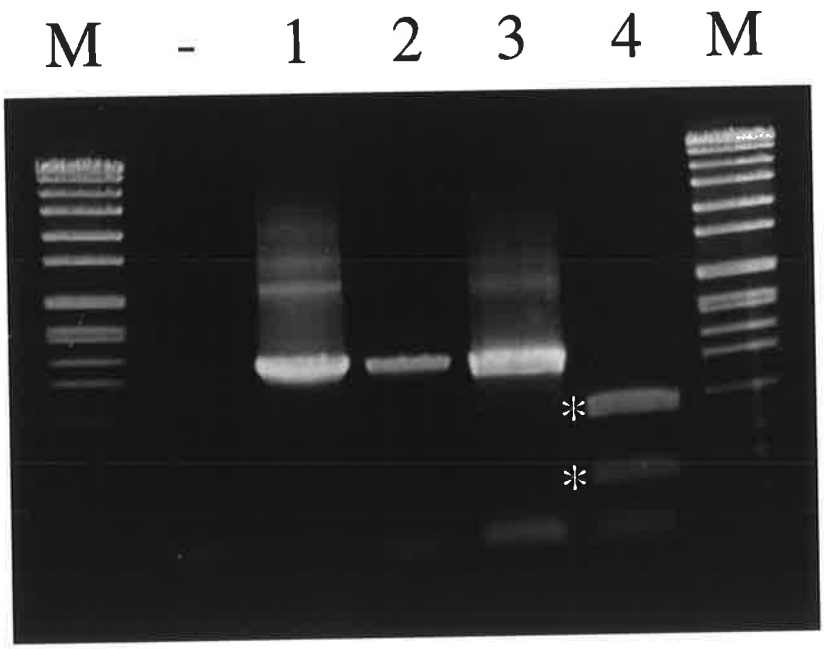

B

Figure 3.9. The RTVE1 cell line contains integrated V449E cDNA.

PCR products were derived from the genomic DNA of RTVE1 cells (2) or, as a positive control, RufNeo-hβc plasmid DNA (1) using hβc-specific primers. As a negative, control, a reaction was performed containing no DNA (-). The region spanned by the primers is that shown in Figure 3-3B. To confirm the identity of the V449E mutant, the PCR products derived from RTVE1 genomic DNA (4) or RufNeo-hβc plasmid DNA (3) were digested with the *Bgl*II restriction endonuclease. The presence of an additional *Bgl*II site introduced by the V449E mutant (see Figure 3-3B) was shown by the cleavage of the larger fragment in 3 to form two smaller products in 4 (indicated by asterisks). Lanes marked 'M' contain DNA size standards (SPP-1 phage DNA digested with *Eco*RI)


M
-
1
2
3
4
M

product derived from an hβc cDNA indicated that this DNA contained the V449E mutation.

The tumorigenicity of the factor-independent foetal liver cells lines was assessed by injecting mice subcutaneously with 5×10^6 cells. As a positive control, a Myb-transformed haemopoietic cell line expressing autocrine GM-CSF, which has been previously shown to be tumorigenic in mice, was used (MTHC-GMV) (Gonda et al., 1989). During a 16-week monitoring period, all eight mice injected with MTHC-GMV cells developed subcutaneous solid tumours, as expected (Table 3.3). Two of eight mice injected with RTVE1 cells developed tumours, whilst none of four mice injected with RTVE2 cells developed tumours. Cells isolated from the tumour of a mouse injected with RTVE1 retained displayed the ring nucleus characteristic of this cell line, confirming that the tumour was derived from injected RTVE1 cells (data not shown). The low frequency of tumours caused by RTVE1 imply that further mutations are required to confer tumorigenicity on this line. Nevertheless, the limited tumorigenicity of the RTVE1 cell line implies that the V449E mutant has oncogenic potential, and that this potential is not limited to previously immortalised growth factor-dependent lines such as FDC-P1 (Jenkins et al., 1995).

3.3 Discussion

To investigate the biological functions and leukaemogenic potential of three constitutively active mutants of the common β subunit for the human IL-3, IL-5 and GM-CSF receptors, I have introduced these mutants into primary murine haemopoietic cells. It was shown that activated forms of hβc can signal in primary haemopoietic cells, and differences were found in the biological effects of extracellular and transmembrane activated hβc mutants.

3.3.1 Implications for hβc signalling

Whereas the factor-independent transmembrane hβc mutant V449E could confer factor independence on all the cell types assessed in this study, the two extracellular mutants, FIΔ and I374N, showed a restricted cell specificity, conferring factor independence only on granulocyte-macrophage progenitors and their progeny. The different factor-independent cell and colony types generated by the transmembrane

Table 3.3. Tumorigenicity of factor-independent cell lines derived from murine foetal liver

Cell line	Experiment[a]	Tumour Incidence[b]	Mean Latency (days)
MTHC-GMV	1	4/4	39
	2	4/4	32
RTVE1	1	1/4	42
	2	1/4	57
RTVE2	2	0/4	-

5×10^6 cells cells from were washed three times in PBS and injected subcutaneously into CBA mice.

[a]Experiments 1 and 2 were performed on different days

[b]number of mice developing tumours within 16 weeks/number of mice injected

and extracellular mutants suggest signalling differences between these mutants. Signalling differences between the transmembrane and extracellular hβc mutants could be quantitative, i.e. reflect different "signal strengths", or qualitative, i.e. reflect different requirements for cell-type specific molecules for activation, or utilisation of different signal transduction pathways.

The cellular proliferation caused by expression of V449E was far greater than that caused by expression of the extracellular mutants (Figure 3.1). This raises the question of whether the ability of V449E to confer factor independence on a greater variety of cell types than FIΔ or I374N is an effect of greater stimulation of common signalling pathways. Indeed, stimulation of murine bone marrow cells with GM-CSF induces, in order of increasing concentration, macrophage, neutrophil, granulocyte-macrophage, eosinophil, megakaryocyte and blast cell colonies from murine bone marrow (Robinson et al., 1987; Metcalf et al., 1986). Hence, whilst small doses of this factor produce exclusively macrophage, neutrophil and granulocyte-macrophage colonies, as do the extracellular hβc mutants (Table 3.2), larger doses induce formation of other colony types, as does the V449E mutant. However, the growth patterns of factor-independent foetal liver cells in liquid culture are incongruent with this possibility. There is an almost complete lack of macrophage development in factor-free foetal liver liquid cultures infected with the V449E mutant, whereas similar cultures infected with extracellular hβc mutants were exclusively of this cell type at the 21st day of culture (Table 3.1). Macrophage colony numbers were only slightly decreased in factor-free cultures containing V449E (Table 3.2), however these colonies were quite small (data not shown) implying a quantitative defect in signalling to this lineage. Thus the spectrum of cell types induced by each class of hβc mutant appear to be separate and complementary, rather than one encompassing and expanding upon the other.

Rather, the differences in the cell lineages induced by transmembrane and extracellular hβc mutants appear to be due to qualitative differences in signalling. Further evidence for this concept has come through studying these mutants in the IL-3 dependent murine pro-B cell line, BAF-B03. When expressed in this cell line, the transmembrane mutant, V449E, is capable of conferring factor-independence, whereas the extracellular mutants are not. This implies that the extracellular mutants require

the expression of cell type specific molecules for activation, which are not expressed in BAF-B03 cells but are expressed in the murine myelomonocytic cell line FDC-P1, in which these mutants were isolated. The identity of this cell type specific molecule has now been determined by the use of a screening approach in which a random cDNA library derived from FDC-P1 cells was used to complement the defective function of the I374N mutant in BAF-B03 cells. This showed that co-expression of the murine GM-CSF receptor α subunit (mGMRα) in BAF-B03 cells is sufficient to allow activity of the I374N mutant in this cell line (Jenkins et al., submitted). It has since been found that FIΔ also requires this subunit for function (R. D'Andrea, Hanson Centre for Cancer Research, Adelaide, South Australia, personal communication). The results in this chapter demonstrate that extracellular hβc mutants function in the neutrophil and macrophage lineages only. This suggests that the ability of FIΔ and I374N to confer factor-independence on the granulocyte-macrophage lineages is due to the restricted expression of mGMα, which is expressed primarily on the granulocyte-macrophage lineages (Wognum et al., 1994; Lanza et al., 1997; Jubinsky et al., 1994).

It has been shown that the I374N mutant can also confer factor-independence on a human GM-CSF-dependent cell line, UT-7 (Jenkins et al., submitted). The resultant factor-independent UT-7 cells show up-regulated hGMRα on the cell surface, suggesting that this subunit may be required for factor-independence in a manner analogous to mGMRα in murine cells. If this is the case, the extracellular hβc mutants have the potential to be involved in human disease in cells which express hGMRα. Since the expression of GMRα is likely to be similar in mouse and human, studies in the murine system should be useful as models for such disease states.

In contrast to the lineage-specificity of the extracellular mutants, V449E was able to confer factor independence on all five myeloid lineages, as well as erythroid cells. This indicates that V449E does not require mGMRα for function. Its wide-ranging functions, including the induction of terminal erythroid development, further suggests that this mutant does not require the presence of murine IL-3 or IL-5 receptors for function in primary cells. This is concordant with the hypothesis that V449E acts as a homodimer of hβc (Jenkins et al., 1995), by analogy to the V664E activating mutation

in the c-Neu tyrosine kinase receptor (Peles et al., 1991; Bargmann and Weinberg, 1988; Weiner et al., 1989; Yarden, 1990).

The stimulation of terminal erythroid development by V449E is interesting in that this is a function of hβc signalling not normally performed by its cognate growth factors, IL-3, IL-5 or GM-CSF. This is likely to be due to the retroviral expression of V449E in these experiments leading to ectopic expression in all haemopoietic cell types, including late erythroid precursors, which do not normally express receptors for IL-3, IL-5 or GM-CSF. This implies that hβc signalling has the potential to drive erythroid development if present in erythroid precursors. This result is similar to that of Nishijima et al. (1995), who showed that hGM-CSF can stimulate the erythroid development of bone marrow cells derived from transgenic mice bearing the hGMR.

In contrast to the extracellular hβc mutants, V449E caused extensive proliferation of early myeloid precursors, (myeloblasts, promyelocytes and myelocytes). These precursors comprised a larger proportion of factor-independent foetal liver cultures containing V449E relative to those grown in growth factors (IL-3, GM-CSF and Epo) (Table 3.1). There are at least two possible and non-exclusive explanations for this phenotype. Retroviral expression of V449E could lead to stimulation of early progenitors which do not normally express receptors for murine IL-3 or GM-CSF. This would lead to an increased proportion of immature cells at late stages of culture relative to cells grown in these growth factors. In fact, a similar result was obtained from transgenic mice expressing the high affinity hGM-CSF receptor (Nishijima et al., 1995). When bone marrow cells derived from these mice were stimulated with hGM-CSF, the numbers of mixed and blast cell colonies obtained were two to three fold higher than those obtained with mIL-3, suggesting that the hGMR was expressed in progenitors insensitive to mIL-3. Consistent with this, the proportion of mixed myeloid/erythroid colonies formed by V449E was 3-fold greater than that from cells stimulated with mIL-3 plus mGM-CSF (plus Epo), and blast colonies were only detected in factor-free cultures containing V449E. This may explain the persistence of immature cell types in factor-free liquid cultures containing V449E.

Alternatively, V449E may be deficient in some aspects of differentiative signalling to early myeloid precursors, and/or more effective in proliferative signalling, biasing their development towards self-renewal. In this regard, signalling through cytokine

receptors generally appears to be mediated by two separate regions of the cytoplasmic domain. The first, membrane-proximal region is necessary and sufficient to deliver a mitogenic signal, whilst a second, more membrane-distal region is required for differentiation signalling through the GM-CSF, Tpo and G-CSF receptors in murine myeloid cell lines (Smith et al., 1997; Sato et al., 1993; Sakamaki et al., 1992; Porteu et al., 1996; Fukunaga et al., 1993). The large proportion of immature cells produced by V449E at late stages of liquid culture suggests that this mutant may be deficient in differentiation signalling to myeloid progenitors, hence this mutant may not fully activate signal transduction pathways originating from the membrane-distal portion of hβc. One such signal transduction pathway is the Ras -Raf-MAPK pathway which has recently been implicated in the differentiation of both murine and human myeloid cell lines (Yen et al., 1998; Matsumura et al., 1998). However, it has been shown that V449E is capable of activating the Ras-Raf-MAPK pathway (Jenkins et al., 1998). It may be that this mutant is quantitatively defective in activation of this pathway, or that the timing of its activation is different to that of wild-type hβc signalling. Alternatively, it is possible that V449E may overstimulate pathways leading to proliferative signalling, similarly altering the balance between proliferation and differentiation. However, as mature cells of all myeloid lineages, as well as erythroid cells, were obtained with this mutant, any differences in differentiative signalling between V449E and the wild-type IL-3/GM-CSF receptors are likely to be quantitative.

3.3.2 Implications for the tumorigenic potential of hβc

The ability of constitutively active hβc mutants to deliver mitogenic signals to primary myeloid progenitors as well as to render the murine cell line FDC-P1 tumorigenic (Jenkins et al., 1995), raises the possibility that such mutants can contribute to human leukemia, as does autocrine production of GM-CSF (Young and Griffin, 1986). The results presented here suggest that of the three mutants studied, V449E would be predicted to have the greatest leukaemic potential. This mutant caused extensive proliferation of early myeloid precursors, (myeloblasts, promyelocytes and myelocytes) whereas extracellular hβc mutants did not (Table 3.1). Cells from myeloid leukemias such as acute myeloblastic leukemia (AML) generally resemble

blast cells, the proliferation of which was only observed with V449E (Table 3.1). Moreover, as discussed above, V449E may bias the development of these precursors towards self-renewal. This would be likely to increase the chance of a second mutational event blocking cell differentiation and/or immortalising these cells, both of which are required for leukaemogenesis.

Indeed, in this study two immortalised cell lines, termed RTVE1 and RTVE2, were derived from factor-independent foetal liver liquid cultures bearing the V449E mutant (Figure 3.7). It is possible that the retroviral infection *per se* of the foetal liver cells increased the frequency of immortalising events in these cultures. Retroviral integration has been previously documented to lead to transformation, which has generally occurred as a consequence of a retrovirus integrating near proto-oncogenes, leading to increased expression, or by insertional mutagenesis of proto-oncogenes, activating their leukaemic potential (for review see Athas et al., (1994)). In fact, during the course of this study, two factor-dependent cell lines were obtained from RufNeo-hβc infected foetal liver populations grown in growth factors, implying that immortalising events were not peculiar to factor-independent foetal liver cultures containing V449E. The isolation and characterisation of these cell lines is detailed in Chapter 5.

When the RTVE1 and RTVE2 cell lines were injected subcutaneously into syngeneic mice, only two of eight mice injected with RTVE1 cells developed tumours, whereas none of four mice injected with RTVE2 cells developed tumours (Table 3.3). These data imply that additional mutations are required to confer tumorigenicity on these lines. This is similar to the case for the activated form of the murine thrombopoietin receptor transduced by the myeloproliferative leukaemia virus (MPLV), *v-Mpl*, in that cell lines immortalised by MPLV require extended periods of *in vitro* culture (greater than 7 months) before becoming oncogenic in mice, implying a requirement for additional mutations (Souyri et al., 1990). These studies imply that, when combined with cellular growth factor independence, cellular transformation and oncogenicity are distinct steps in the oncogenic pathway. In contrast, several transforming events have been described which, when combined with cellular growth factor independence, result in full oncogenicity in mice. Examples are overexpression of the c-myb and Hox-B8 transcription factors (Perkins et al., 1990; Gonda et al.,

1989). Hence it appears that some transforming events are sufficient for oncogenicity when combined with cellular growth factor independence, whilst others are not.

The ability of V449E to signal in immature cell types and to give rise to transformed cell lines suggests that this mutant has greater oncogenic potential than the extracellular hβc mutants studied. However, it is worth noting that these observations relate only to the case where factor-independence is a primary oncogenic event, as was the case in this study. It has been shown previously that the FIΔ and I374N mutants can confer tumorigenicity on previously immortalised cells such as FDC-P1 (Jenkins et al., 1995; D'Andrea et al., 1994). Moreover, there is now abundant evidence that a constitutive GM-CSF signalling can contribute to leukaemic states, despite the fact that autocrine GM-CSF signalling does not in itself lead to leukaemia in murine models (Johnson et al., 1989; Lang et al., 1987). Hence it is possible that GM-CSF signalling, which appears to be mimicked by the extracellular hβc mutants, is more effective as an oncogenic event in previously transformed cells than in primary cells. With regard to factor independence in human leukaemia, there are not sufficient data relating to the ontogeny of these disorders to ascribe factor-independence as a primary or secondary event. The fact that overexpression of haemopoietic growth factors *in vivo* does not lead to acute leukaemia would suggest that factor independence follows cellular transformation in these disorders.

CHAPTER 4. Isolation and characterisation of factor-dependent multipotential cell lines

4.1 Introduction

Differentiation competent haemopoietic cell lines have provided defined systems in which to study cellular differentiation induced by a number of growth factors including G-CSF, Tpo, Epo and GM-CSF (Valtieri et al., 1987; Nagata et al., 1998; Just et al., 1991; Goncalves et al., 1998). The uniformity of these lines enables complex molecular and biochemical analyses of the signalling pathways involved in cellular differentiation, which are infeasible using primary cells. Here I describe isolation of two such cell lines which exhibit the unique properties of continuous growth in murine IL-3 alone and complete differentiation along the neutrophil and macrophage lineages in response to murine GM-CSF. These lines should be useful in studying the molecular aspects of GM-CSF induced myeloid differentiation.

4.2 Results

4.2.1 Isolation of factor-dependent cell lines

In the course of the experiments detailed in Chapter 3, murine foetal liver cells were infected with the RufNeo retrovirus bearing wild-type or mutant hβc cDNAs and cultured in liquid medium in the presence of high concentrations of IL-3, GM-CSF and Epo, with and without the presence of G418. Four such experiments were performed in total. In the normal situation, when cultured under the influence of such stimuli, murine haemopoietic cells terminally differentiate; the sole cell type that is perpetuated is the mast cell which, whilst apparently differentiated, continues to divide slowly. These have thus been termed "persisting cells" or simply "P-cells" (Schrader et al., 1981; Razin et al., 1984; Nagao et al., 1981). P-cell lines were indeed isolated in most instances in these experiments. However, from two such experiments (of four total) in which foetal liver cells were infected with RufNeo-hβc and cultured over a period of eight weeks in the absence of G418, permanently growing (transformed) cell lines arose. These lines are hereby designated FDB1 and FDB2, for factor dependent cell line expressing human beta common (hβc). An immortalised cell line was also obtained in one of four experiments in which RufNeo-I374N was the

infectious virus. Hence in total 3 of 16 foetal liver cell populations infected with RufNeo retroviruses and cultured in the 3-factor cocktail in the absence of G418 gave rise to transformed lines. No cell lines were obtained from the same 16 infected cell populations grown in the presence of G418, all of which gave rise to mast cell "lines". As detailed in Chapter 3, one of four cell populations containing RufNeo-V449E cultured in the absence of growth factors gave rise to a factor-independent transformed line, as did one of two cultured in Epo alone (this line was later found to be independent of this Epo for growth). These lines were termed RTVE1 and RTVE2.

4.2.2 Analysis of provirus integration and hβc expression

Both FDB1 and FDB2 cell lines are resistant to G418 even though they have not been selected with this antibiotic (data not shown), implying that they contain integrated RufNeo retrovirus. In order to confirm this, genomic DNA was prepared from the FDB2 cell line and Southern analysis performed using a cDNA probe versus the neomycin resistance cassette contained in this retrovirus. As shown in Figure 4.1A, the *Bam*HI restriction enzyme cuts the RufNeo provirus once; hence when FDB2 genomic DNA is digested with this enzyme, fragments are generated containing part of the RufNeo provirus encompassing the Neomycin resistance cassette and adjacent genomic DNA terminating at the nearest genomic *Bam*HI site (Figure 4.1A). As retroviruses integrate randomly, the location of this site will reflect the site of integration; hence each integrated provirus will generate a band of unique size on a Southern blot. As seen in Figure 4.1B, 8 bands are visible a MC1*Neo*-specific Southern blot of *Bam*HI-digested FDB2 genomic DNA, implying that at least 8 proviruses are present in this cell line. To determine if the FDB2 line is clonal, two clones of this line were generated by expansion of colonies grown in methyl-cellulose culture, termed FDB2-10 and FDB2-16, and a similar analysis was performed. These clones exhibited an identical banding pattern to the FDB2 cell line on the *Bam*HI genomic Southern blot, implying that the FDB2 cell line is clonal (Figure 4.1B).

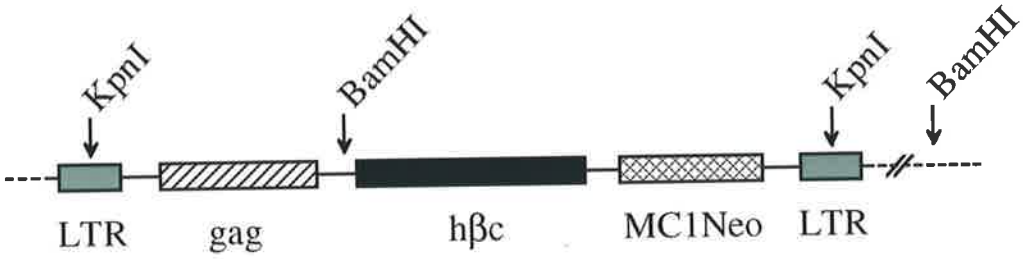
Next, in an attempt to quantify the number of proviral integrations in the FDB2 cell line, the same genomic DNA preparations were digested with *Kpn*I, which cuts at sites in the proviral long term repeats, releasing a fragment of identical size for each proviral integration. The intensity of the band in a MC1*Neo*-specific Southern blot is

Figure 4.1. The FDB2 cell line is clonal and contains multiple integrated RufNeo proviruses

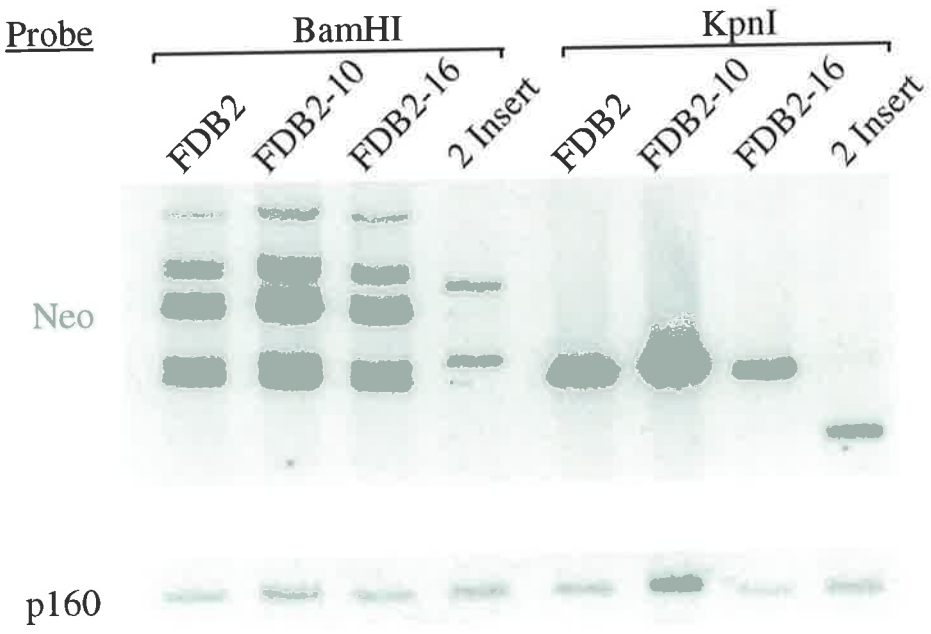
A. Schematic representation of an integrated RufNeo-hβc provirus showing the position of *Bam*HI and *Kpn*I restriction sites. Long terminal repeat sequences (LTR) are indicated.

B. Southern blot of genomic DNA isolated from the FDB2 cell line and two clones derived from this line, FDB2-10 and FDB2-16. For comparison, genomic DNA from a clone of the FDC-P1 cell line containing 2 copies of a wild-type RufNeo provirus (2 insert) was included (generously supplied by A. Zannettino, Department of Haematology, Hanson Centre for Cancer Research). *Bam*HI- and *Kpn*I-digested genomic DNA was analysed by southern blotting using a MC1*Neo* probe (Neo), before stripping and analysis using a probe against a genomic gene, *p160* (Tavner et al., 1998).

A


KpnI
BamHI
KpnI
BamHI
LTR
gag
hβc
MC1Neo
LTR

B


Probe
BamHI
KpnI
FDB2
FDB2-10
FDB2-16
2 Insert
FDB2
FDB2-10
FDB2-16
2 Insert
Neo
p160

thus dependent on the number of proviral integrations. An FDC-P1 clone containing two RufNeo proviral integrations was included as a reference (termed "2 insert"; Figure 4.1B). To compare loading the blot was stripped and probed with a random cDNA probe for an endogenous cellular gene, *p160* (Tavner et al., 1998). By comparing the intensities of the FDB2, FDB2-10 and FDB2-16 bands to this FDC-P1 clone in the MC1*Neo*-specific Southern blot and allowing for loading differences by comparison to the *p160* blot it was calculated that 11, 10 and 15 copies of RufNeo were present in FDB2, FDB2-10 and FDB2-16 respectively. Whilst this heterogeneity is likely to reflect the inaccuracy of the system used, the presence of more than 8 copies in each case suggests that the RufNeo proviral integration pattern observed in the *Bam*HI Southern blot is present in a clonal population and is not derived from subpopulations with distinct proviral banding patterns, providing further evidence that the FDB2 cell line is clonal.

The presence of a large number of RufNeo-hβc proviruses in the genome of FDB2 and the G418 resistance of FDB1 and FDB2 cell lines implies that these lines should express hβc protein. To confirm this, these cell lines were analysed by flow cytometry using an hβc-specific antibody, 4F3 (Woodcock et al., 1997). As shown in Figure 4.2 both FDB1 and FDB2 cell lines express surface hβc protein at high levels.

4.2.3 Growth factor requirements of FDB1 and FDB2 cells

As stated above, both FDB1 and FDB2 were isolated in a 3-factor cocktail of growth factors; IL-3, GM-CSF and Epo. To determine the growth factor requirements of these cell lines they were washed extensively and placed in culture with each one of these growth factors in isolation. When grown in the 3-factor cocktail both cell lines resembled blast cells and displayed a low level of spontaneous differentiation to mature neutrophil, macrophage and megakaryocyte cells (data not shown). Megakaryocyte differentiation was most pronounced in the FDB2 cell line. When deprived of growth factors cells from both lines rapidly died. However, when placed in culture with IL-3, the growth pattern and morphology of these cell lines was essentially unaltered from those in the 3-factor cocktail (Figure 4.3B, 4.4B). Surprisingly, when these cell lines were transferred to medium containing GM-CSF alone for 5 days the cells underwent synchronous differentiation to mature neutrophil

Figure 4.2. Surface expression of hβc on FDB cell lines

FDB1 and FDB2 cells were stained with an anti-hβc antibody, 4F3 (Woodcock et al., 1994) (solid lines), or an irrelevant isotype control antibody (dashed lines) followed by a streptavidin-conjugated anti-mouse monoclonal antibody and biotin-phycoerythrin.

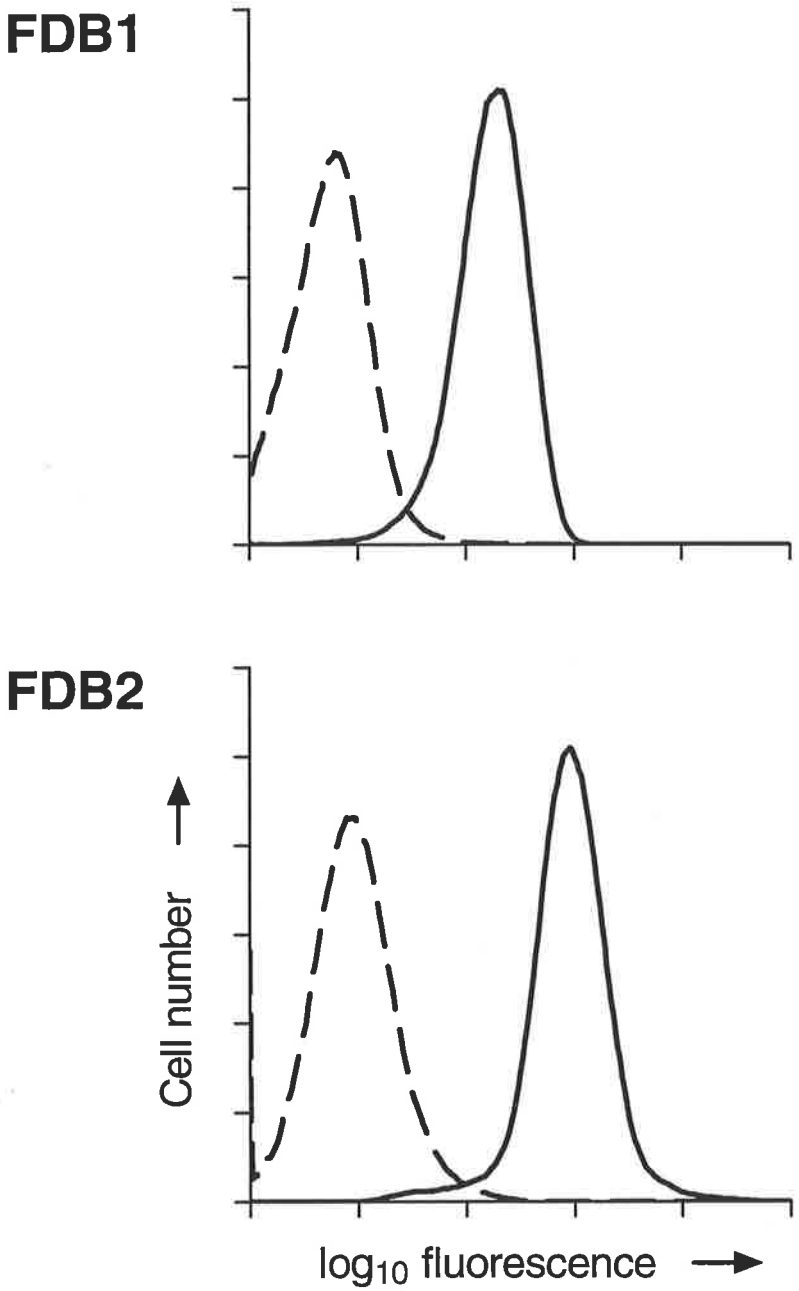

FDB1
FDB2
Cell number
log10 fluorescence

Figure 4.3. Morphology of the FDB1 cell line

FDB1 cells were washed 3 times in DMEM and cultured in the presence of IL-3 plus GM-CSF (**A**), IL-3 (**B**) or GM-CSF (**C**). After 5 days samples were cytocentrifuged and Wright-Giemsa stained. Photographs are at 450 × magnification.

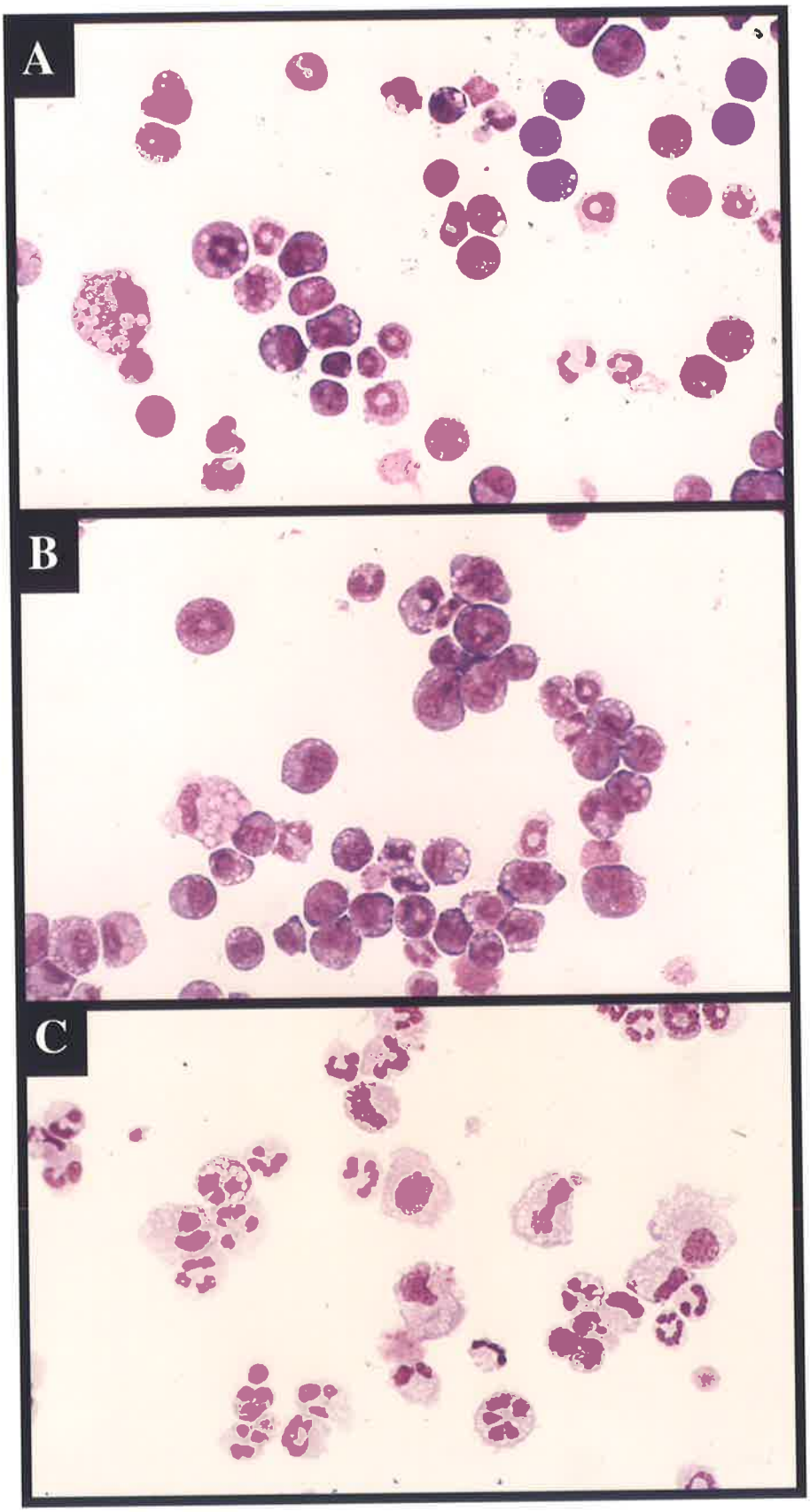
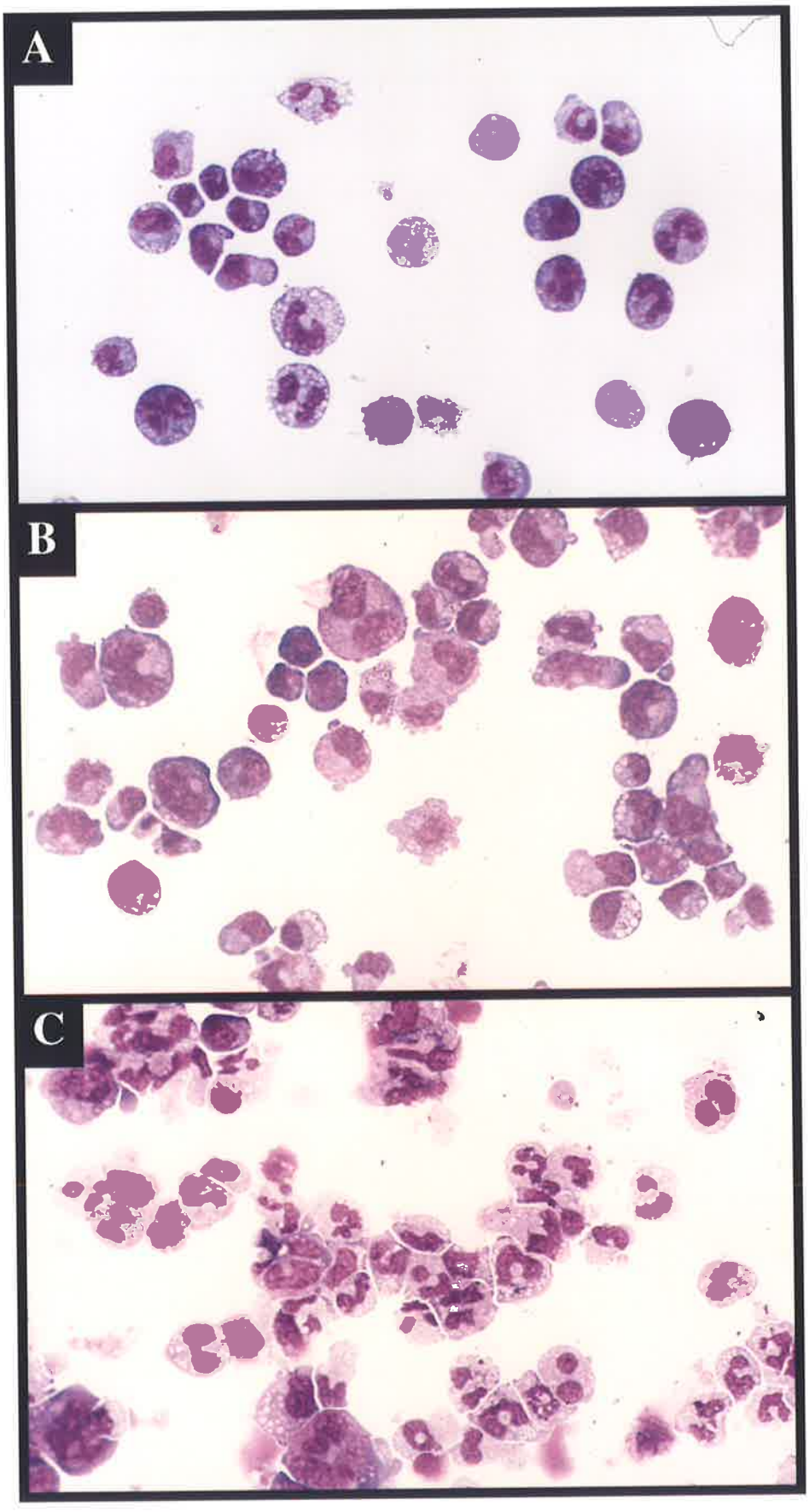

A
B
C

Figure 4.4. Morphology of the FDB2 cell line

FDB2 cells were washed 3 times in DMEM and cultured in the presence of IL-3 plus GM-CSF (**A**), IL-3 (**B**) or GM-CSF (**C**). After 5 days samples were cytocentrifuged and Wright-Giemsa stained. Photographs are at 450 × magnification.


A
B
C

and macrophage cells (Figures 4.3C, 4.4C). In the case of the FDB2 cell line, many of the resultant neutrophils were abnormal in appearance, containing a bilobulated nucleus rather than the normal ring or trilobulated form. As might be predicted from the growth of these cells in the initial 3-factor cocktail, the IL-3 signal was dominant over GM-CSF when FDB1 or FDB2 cells were placed in medium containing these factors in combination; that is the cells continued to grow without an apparent increase in the proportion of differentiated cells (Figures 4.3A, 4.4A). Neither FDB1 nor FDB2 cells responded to Epo alone or to a variety of other growth factors tested including IL-5, G-CSF, Tpo and M-CSF (data not shown). Interestingly, both lines grew in IMDM were incapable of growth in DMEM.

Hence FDB1 and FDB2 are cell lines which exhibit a novel growth factor-dependent differentiation switch. They are to my knowledge the first examples of cell lines which grow in IL-3 alone and differentiate completely in exogenous GM-CSF.

4.2.4 Timecourse of differentiation of FDB1 and FDB2 cell lines

Having determined that GM-CSF induced differentiation of the FDB1 and FDB2 cell lines, it was of interest to examine the nature of this differentiation with respect to its timing and associated effects on cell proliferation and viability. To achieve this, a detailed timecourse of differentiation was performed on each cell line and the proliferation, differentiation and viability of these lines was determined.

As shown in Figure 4.5C, GM-CSF-induced differentiation of the FDB1 cell line was rapid, peaking at 7-8 days of culture. At early timepoints the differentiated cells were predominantly neutrophils however at later timepoints monocytic cells were of equal or greater proportion, presumably due to death of the neutrophilic cells, which lead to a decrease in the overall viability (see below). Differentiation of FDB2 cells in GM-CSF was slower than that of FDB1 cells, with a lag phase of approximately 2 days before its onset (Figure 4.6C). Again the majority of differentiated cells were neutrophils, this being the case throughout the experiment until the thirteenth day of culture. IL-3 was again able to suppress GM-CSF-induced differentiation of both cell lines (Figures 4.5A, 4.6A)

Differentiation in medium containing GM-CSF was accompanied by rapid and complete growth arrest of FDB1 cells (Figure 4.7A). In the case of FDB2 cells there

Figure 4.5. Timecourse of differentiation of the FDB1 cell line

FDB1 cells were washed 3 times in DMEM and cultured in the presence of IL-3 plus GM-CSF (**A**), IL-3 (**B**) or GM-CSF (**C**). Samples were cytocentrifuged daily, Wright-Giemsa stained and the percentage of differentiated neutrophils (G), monocytes (M) megakaryocytes (MK), and the total percentage of differentiated cells (TOTAL) determined from at least 200 cells scored microscopically.

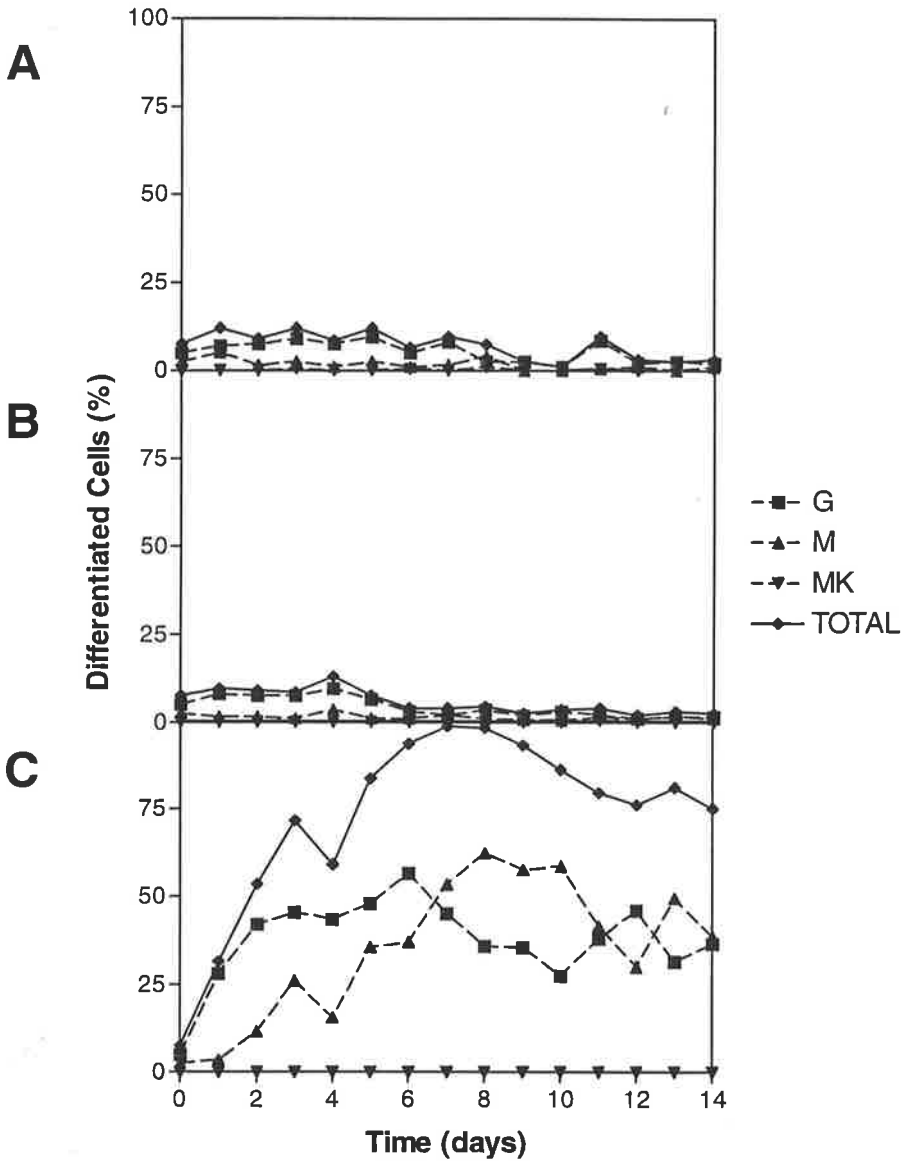

A
B
C
100
75
50
25
0
Differentiated Cells (%)
0
2
4
6
8
10
12
14
Time (days)
G
M
MK
TOTAL

Figure 4.6. Timecourse of differentiation of the FDB2 cell line.

FDB2 cells were washed 3 times in DMEM and cultured in the presence of IL-3 plus GM-CSF (**A**), IL-3 (**B**) or GM-CSF (**C**). Samples were cytocentrifuged daily, Wright-Giemsa stained and the percentage of differentiated neutrophils (G), monocytes (M) megakaryocytes (MK), and the total percentage of differentiated cells (TOTAL) determined from at least 200 cells scored microscopically.

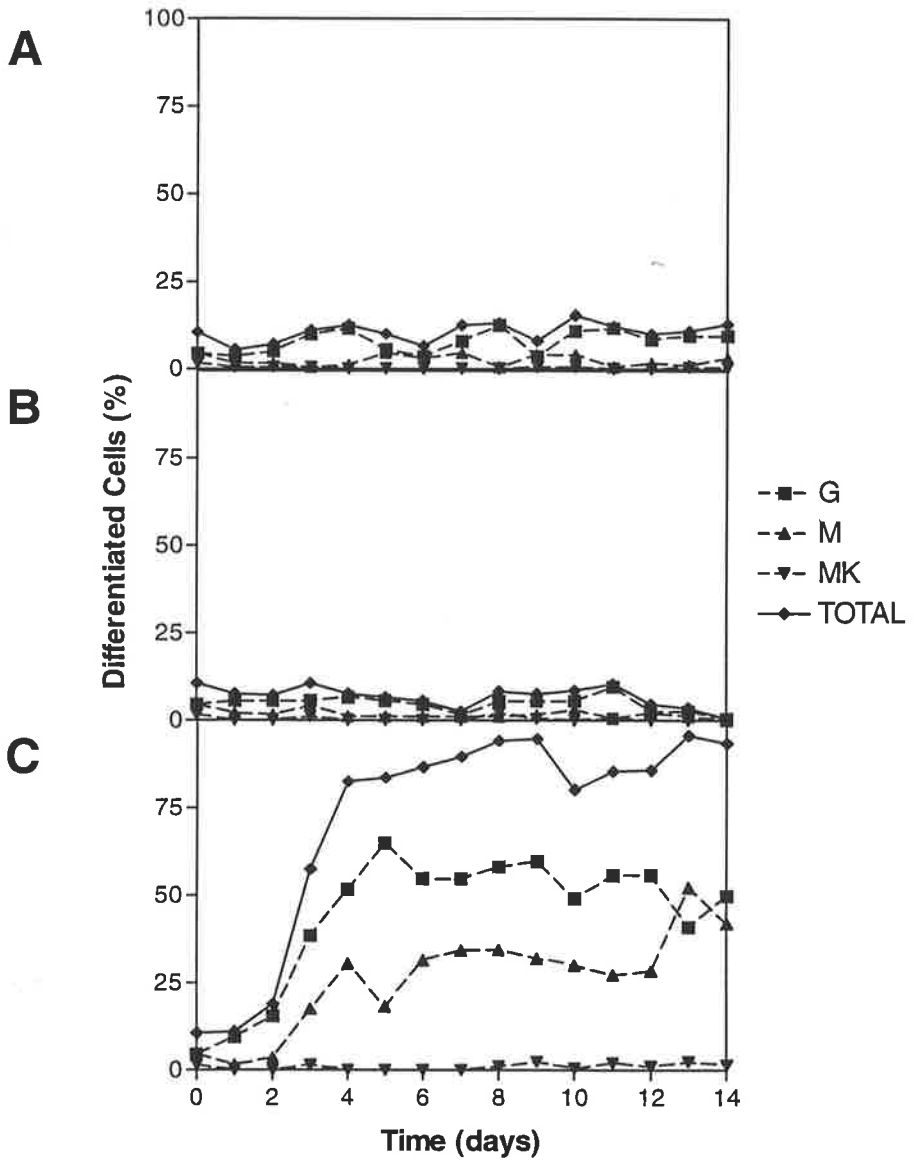
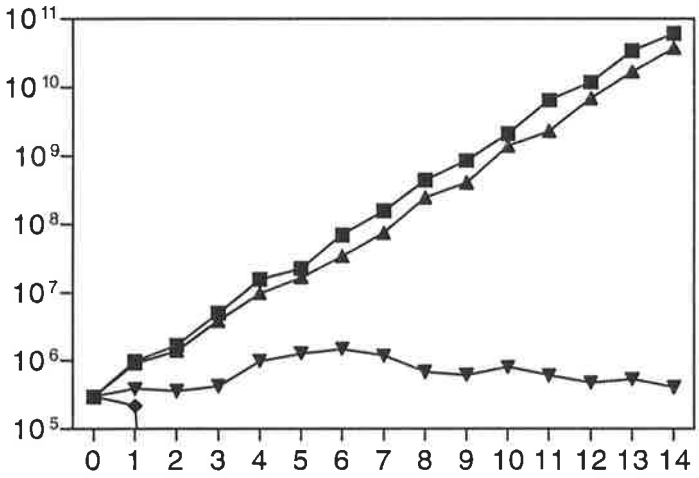

A
B
C
100
75
50
25
0
Differentiated Cells (%)
0
2
4
6
8
10
12
14
Time (days)
G
M
MK
TOTAL

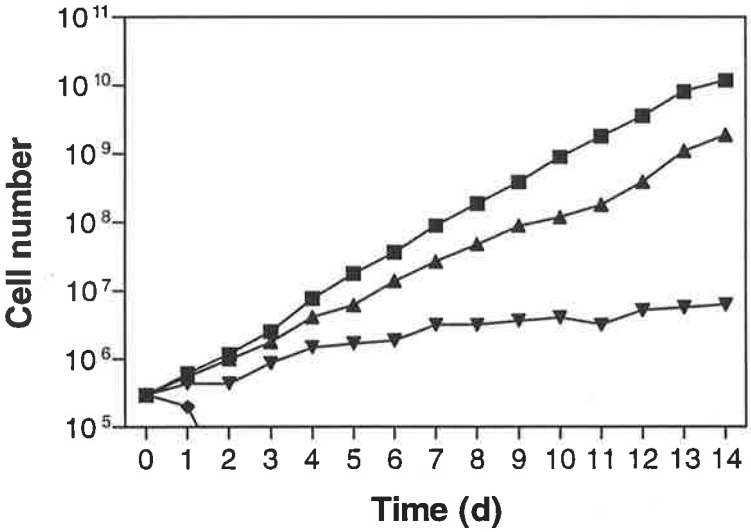
Figure 4.7. Proliferation of FDB cell lines.

FDB1 (**A**) and FDB2 (**B**) cell lines were washed 3 times in DMEM and cultured in the presence of IL-3 plus GM-CSF (■), IL-3 (▲), GM-CSF (▼) or in the absence of growth factors (◆). Viable cell counts were taken daily using a haemocytometer.

A


10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

B


Cell number
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (d)

was near-complete growth arrest, however the cells were able to proliferate slowly in GM-CSF in some experiments (Figure 4.7B). Addition of IL-3 and GM-CSF in combination to FDB1 cells gave a growth pattern similar to IL-3 alone (Figure 4.7A). However, in the case of FDB2 cells cultured in GM-CSF plus IL-3 grew at an increased rate compared to those in IL-3 alone (Figure 4.7B).

The viability of FDB1 and FDB2 cells cultured in IL-3 was generally greater than 90%, the non-viable cells present presumably being due to the low frequency of spontaneous terminal differentiation of these lines (Figure 4.8). Withdrawal of growth factor lead to rapid growth arrest and complete loss of viability by the third day of culture. The growth arrest of FDB1 cells in culture with GM-CSF was accompanied by a loss of viability (Figure 4.8A). This occurred primarily in two phases; an initial phase, when the cells were switched from IL-3 to GM-CSF, occurring over the initial two days, and a second phase occurring at days six to eight of culture. In cultures of FDB2 cells there was a small decrease in viability manifesting early in culture which was maintained over the course of the experiment (Figure 4.8B). When cultured in medium containing IL-3 and GM-CSF both cell lines showed similar viability to cells cultured in IL-3 alone.

Hence transfer from medium containing IL-3 to that containing GM-CSF leads to decreased growth, loss of viability and differentiation of the FDB1 and FDB2 cell lines.

4.2.5 Cell surface antigen expression by FDB1 and FDB2 cell lines

After undergoing differentiation in the presence of GM-CSF, FDB1 and FDB2 cells resembled predominantly neutrophils and macrophages. To confirm the identity of these cells, FDB1 and FDB2 cells were placed in each growth factor in experiments identical to those described above, and the expression of various cell lineage markers was determined after 7 days by immunostaining and flow cytometry.

When FDB1 cells were switched from IL-3 to GM-CSF there was a decrease in cell size as measured by forward scatter and an increase in cell granularity as measured by the side scatter of the cells (Figure 4.9A). Both are consistent with the observed neutrophilic differentiation of the cells (above). There was a large induction of Gr-1 and Mac-1 expression on cells cultured in GM-CSF (Figure 4.9B). As these markers

Figure 4.8. Timecourse of viability of FDB cell lines

FDB1 (**A**) and FDB2 (**B**) cells were washed 3 times in DMEM and cultured in the presence of the IL-3 plus GM-CSF (■), IL-3 (▲), GM-CSF (▼) or in the absence of growth factors (◆). The proportion of cells excluding trypan blue was calculated daily using a haemocytometer. Numbers are percentages of at least 100 cells scored.

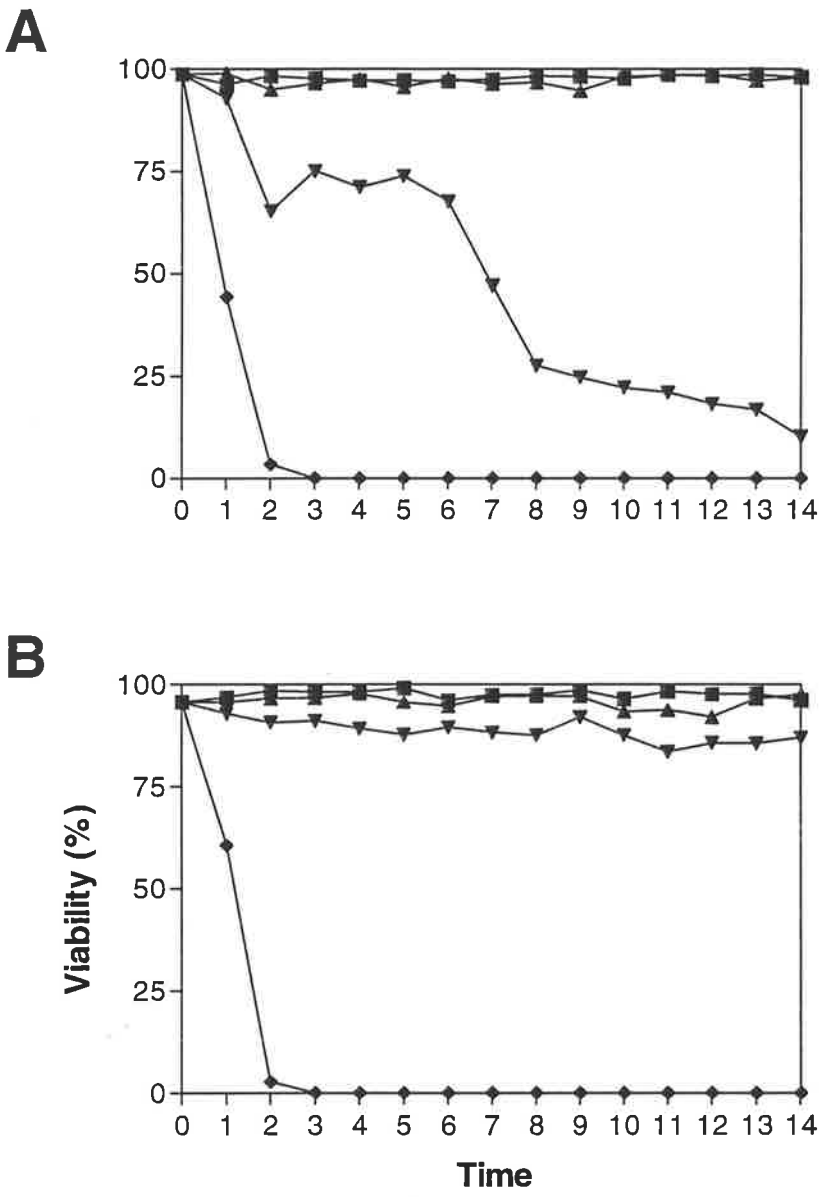
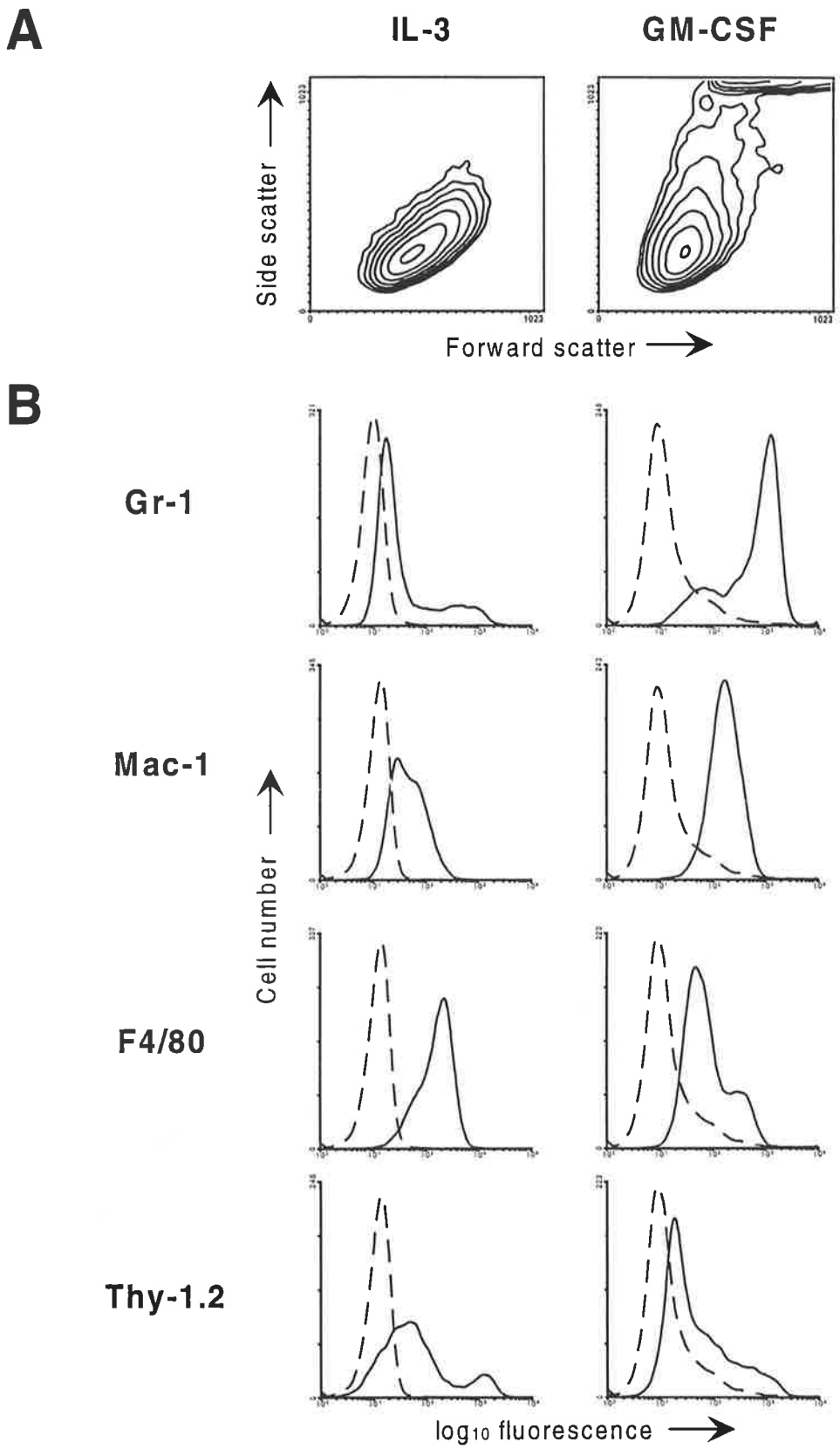

A
100
75
50
25
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
100
75
50
25
0
Viability (%)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time

Figure 4.9. Scatter profile and cell surface antigen expression of the FDB1 cell line

FDB1 cells were washed 3 times in DMEM and cultured in the presence of the indicated growth factors for 7 days. (**A**) Scatter profile of FDB1 cells (**B**) Flow cytometric analysis. Cells were stained with rat monoclonal antibodies specific for the indicated antigens (solid lines) or an irrelevant isotype control (dashed lines) followed by a FITC conjugated anti-rat immunoglobulin monoclonal antibody.


A
IL-3
GM-CSF
Side scatter
Forward scatter
B
Gr-1
Mac-1
F4/80
Thy-1.2
Cell number
log10 fluorescence

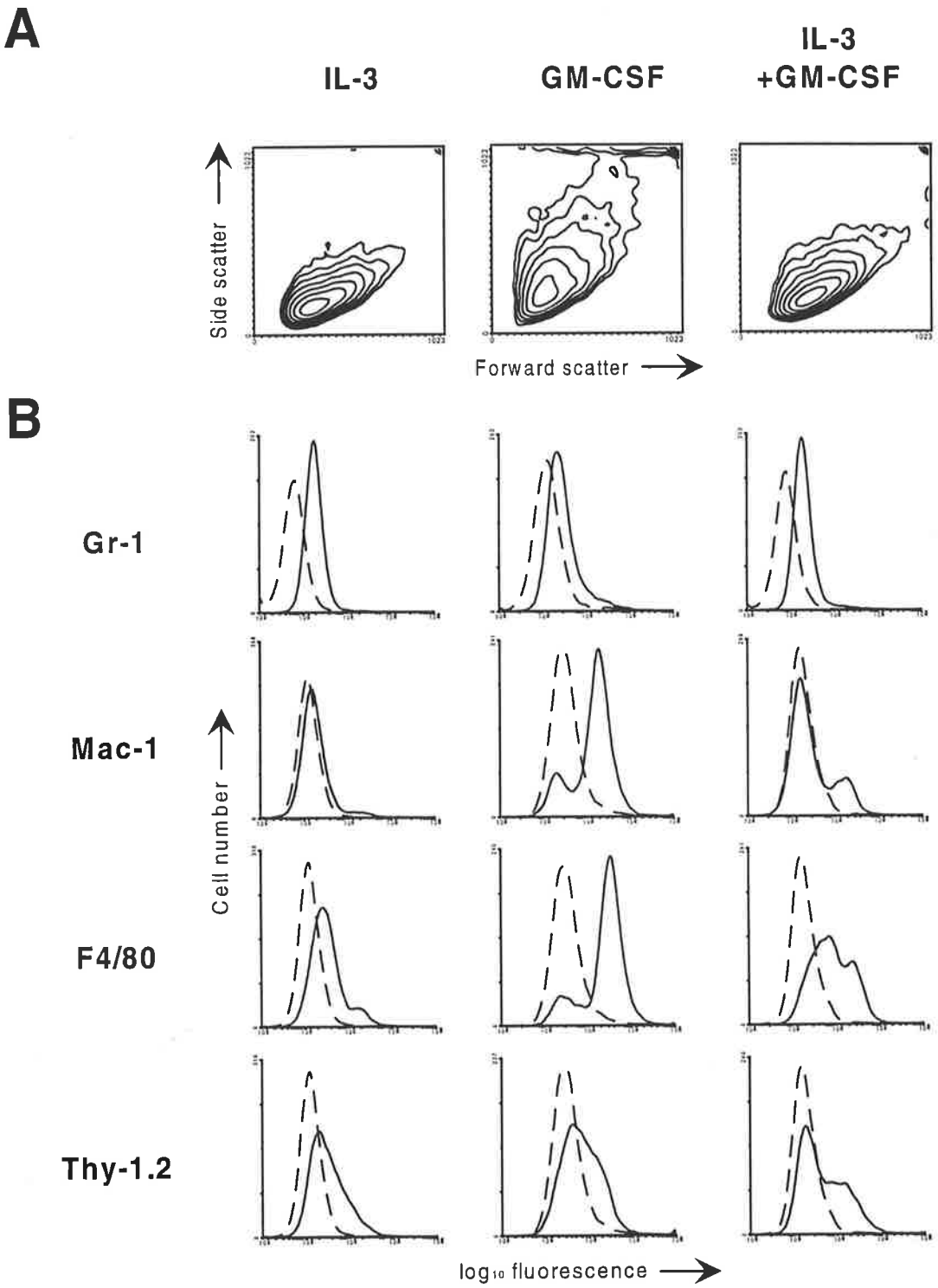
are restricted to granulocytic and monocytic cells (Springer et al., 1979; Holmes et al., 1986), this is consistent with the morphological observation that these cells resemble neutrophils and monocytes. Unusually, the macrophage marker F4/80 (Austyn and Gordon, 1981) was expressed on FDB1 cells grown in IL-3 and in fact decreased upon differentiation of this cell line in GM-CSF (although the resultant cells still expressed this marker) (Figure 4.9B). The endogenous expression of this marker on FDB1 cells cultured in IL-3 suggests that this line has myelomonocytic features in its proliferative state. The immature marker Thy-1.2 was also studied, and was found to be expressed on FDB1 cells growing in IL-3 (Figure 4.9B). Since this marker is found on stem cells and early haemopoietic progenitors, this is consistent with the immature morphology of this cell line (Spangrude et al., 1988; Miller et al., 1985; Berman and Basch, 1985). Thy-1.2 expression was down-regulated when these cells were switched to GM-CSF. This is consistent with the morphological observation that these cells differentiate to become mature myeloid cells, on which this marker is not expressed.

Like FDB1 cells, when FDB2 cells were switched to culture in GM-CSF they slightly decreased in size as measured by forward scatter and increased in granularity as measured by side scatter of the cells (Figure 4.10A). Unexpectedly, the Gr-1 marker was not induced when these cells were transferred to GM-CSF (Figure 4.10B). This suggests that in addition to their abnormal appearance (above), the "granulocytes" produced by culture in GM-CSF may be defective in some aspects of normal activation. However, in some experiments Gr-1 was induced on this cell line in response to GM-CSF (data not shown). Nevertheless, there were strong inductions of the granulocyte-macrophage marker Mac-1 and the macrophage marker F4/80 when these cells were switched from IL-3 to GM-CSF, consistent with granulocyte-macrophage differentiation of the cells (Figure 4.10B). The expression profile of the latter marker was again quite dissimilar to that found on FDB1 cells. Hence, despite their apparently similar growth factor responses, these two cell lines show marked differences with respect to Gr-1 and F4/80 antigen expression profiles. Thy-1.2 was expressed at low levels on FDB2 cells in the presence of IL-3 and its expression did not appear to be affected by transfer to medium containing GM-CSF (Figure 4.10B)

Culture of FDB2 cells in IL-3 plus GM-CSF had no significant effect on forward or side scatter or Gr-1 expression when compared to culture in IL-3 alone (Figure

Figure 4.10. Scatter profile and cell surface antigen expression of the FDB2 cell line

FDB2 cells were washed 3 times in DMEM and cultured in the presence of the indicated growth factors for 7 days. (**A**) Scatter profile of FDB2 cells (**B**) Flow cytometric analysis. Cells were stained with rat monoclonal antibodies specific for the indicated antigens (solid lines) or an irrelevant isotype control (dashed lines) followed by a FITC conjugated anti-rat immunoglobulin monoclonal antibody.


A
IL-3
GM-CSF
IL-3
+GM-CSF
Side scatter
Forward scatter
B
Gr-1
Mac-1
F4/80
Thy-1.2
Cell number
log10 fluorescence

4.10A). However, there were small increases in Mac-1 and F4/80 expression when compared to cells cultured in IL-3 alone. Thus whilst the IL-3 signal is apparently dominant to GM-CSF signalling in FDB2 cells in terms of their continued growth and lack of increased morphological differentiation (above), GM-CSF can still influence surface antigen expression on these cells in the presence of IL-3.

4.2.6 Effect of growth factor concentration on the FDB2 cell line

The experiments detailed above suggest a difference between the effects of murine IL-3 and GM-CSF on the FDB cell lines. To confirm that this was not peculiar to the concentration of cytokines used (500 U/ml in each case), the effect of various concentrations of IL-3 and GM-CSF on the FDB2 cell line was tested. When washed extensively and cultured in the presence of varying concentrations of IL-3 for six days, reduction of IL-3 concentrations lead to rapid granulocyte-macrophage differentiation of this cell line, with concomitant growth arrest (Table 4.1 and data not shown). However, very low concentrations of IL-3 (≤0.4 U/ml) were required to give a level of differentiation comparable to that seen when these cells are cultured in GM-CSF. In contrast, varying the concentration of GM-CSF had little effect on the granulocyte-macrophage development seen in this growth factor, except that neutrophilic differentiation appeared to be favoured at high concentrations of GM-CSF (Table 4.1).

4.3 Discussion

In this chapter the differentiation state and growth factor responses of two spontaneously arising murine IL-3-dependent cell lines, FDB1 and FDB2, have been determined. Morphologically and in terms of cell surface antigen expression, these cell lines resemble immature myeloid cells which show some spontaneous differentiation along the neutrophil, monocyte and megakaryocyte lineages. Furthermore, these cell lines demonstrate the novel property of terminal and complete differentiation in response to murine GM-CSF.

Cell lines displaying a mIL-3/mGM-CSF differentiation switch have been previously reported. When the FDCP-Mix cell line is infected with a retroviral vector expressing mGM-CSF, multipotent variants can be selected in IL-3 which have the properties of growth in IL-3 and differentiation upon IL-3 withdrawal due to autocrine

Table 4.1. Effect of growth factor concentration on FDB2 cells

Growth factor (U/ml)	Blast/pro	Myel/meta	Band/neut	Mono	MK
IL-3					
500	66	22	6	4	2
50	63	23	9	4	1
10	54	36	5	4	2
2	39	47	8	6	2
0.4	0	28	43	29	0
0.08	0	30	51	19	0
0.016	Non-viable[a]				
GM-CSF					
500	4	17	68	12	0
100	0	4	67	30	0
20	0	0	63	37	0
4	0	10	46	45	0
0.8	1	19	46	35	0
0.16	2	18	40	41	0
0.032	3	35	38	24	0

Cells were washed 3 times in DMEM and cultured in the presence of the indicated growth factors for six days, at which point samples were cytocentrifuged, Giemsa stained and the cell types determined microscopically. Numbers are percentages of blasts and promyelocytes (blast/pro), myelocytes and metamyelocytes (myel/meta), bands and mature neutrophils (band/neut), monocytes (mono) and megakaryocytes (MK) of 200 cells scored.

[a]No significant viability after 6 days culture.

GM-CSF stimulation (Just et al., 1991). However, uninfected FDCP-Mix cells respond poorly to exogenous GM-CSF unless low levels of IL-3 are supplied and the horse serum used for passage of this line is replaced by FCS (Heyworth et al., 1990). Also, a variant of the FDC-P1 cell line, WT19, which is dependent on IL-3 for growth, differentiates towards the monocyte lineage in response to GM-CSF (Matsuguchi et al., 1997; Bourette et al., 1995). However, this differentiation is partial and reversible, and does not lead to growth arrest of these cells. Furthermore, an IL-3 dependent cell line has been derived from a leukaemic mouse expressing IL-11 retrovirally, which has the property of differentiation when cultured in GM-CSF (Hawley et al., 1993). However, as this cell line also produces autocrine IL-11 the role of each growth factor in the growth and differentiation of this cell line remains obscure. Hence FDB1 and FDB2 are the first reported cell lines having the properties of growth in IL-3 alone and complete and terminal differentiation in response to exogenous GM-CSF. As such they have possible utility in determining the molecular characteristics of this unique differentiation switch.

4.3.1 Properties of the FDB1 and FDB2 cell lines

The FDB1 and FDB2 cell lines were isolated from murine foetal liver cells infected with the RufNeo-hβc retroviral construct and cultured long-term in a cocktail of growth factors consisting of mIL-3, mGM-CSF and hEpo. These lines are Neomycin resistant and express hβc protein on their cell surface, indicating that they have arisen from a cell(s) infected with the RufNeo-hβc construct. Moreover, Southern analysis of the FDB2 cell line indicates that this cell line contains at least 8 RufNeo proviruses. The protocol used for RufNeo-hβc infection in these experiments lead to an efficiency of infection of haemopoietic progenitors of approximately 85% (see Section 3.2.3). Hence the probability of a progenitor being infected with ≥8 copies of the RufNeo retrovirus is approximately $(0.85)^8$, or 27%. Such a high number of integrated proviruses suggests that the RufNeo provirus may be linked to the transforming element in these cell lines. Retroviral transformation of haemopoietic cells has been documented previously, and is generally a consequence of retroviruses integrating near cellular proto-oncogenes, leading to transcriptional activation, or by insertional mutagenesis of cellular proto-oncogenes, activating their latent transforming potential

(for review see Athas et al. (1994)). Moreover, I was unable to generate similar lines by long-term culture of uninfected foetal liver cells under identical conditions. However, the number of cultures examined thus (seven) were not great enough for this observation to be statistically significant.

Southern analysis of the FDB2 cell line suggests that this cell line is clonal (Figure 4.1). Clonality implies that this cell line is the progeny of a single transforming event. This cell line displays spontaneous differentiation along the neutrophil, macrophage and megakaryocyte lineages, indicating that the transformed cell must be quite immature, at the stage of a granulocyte-macrophage-megakaryocyte precursor or earlier.

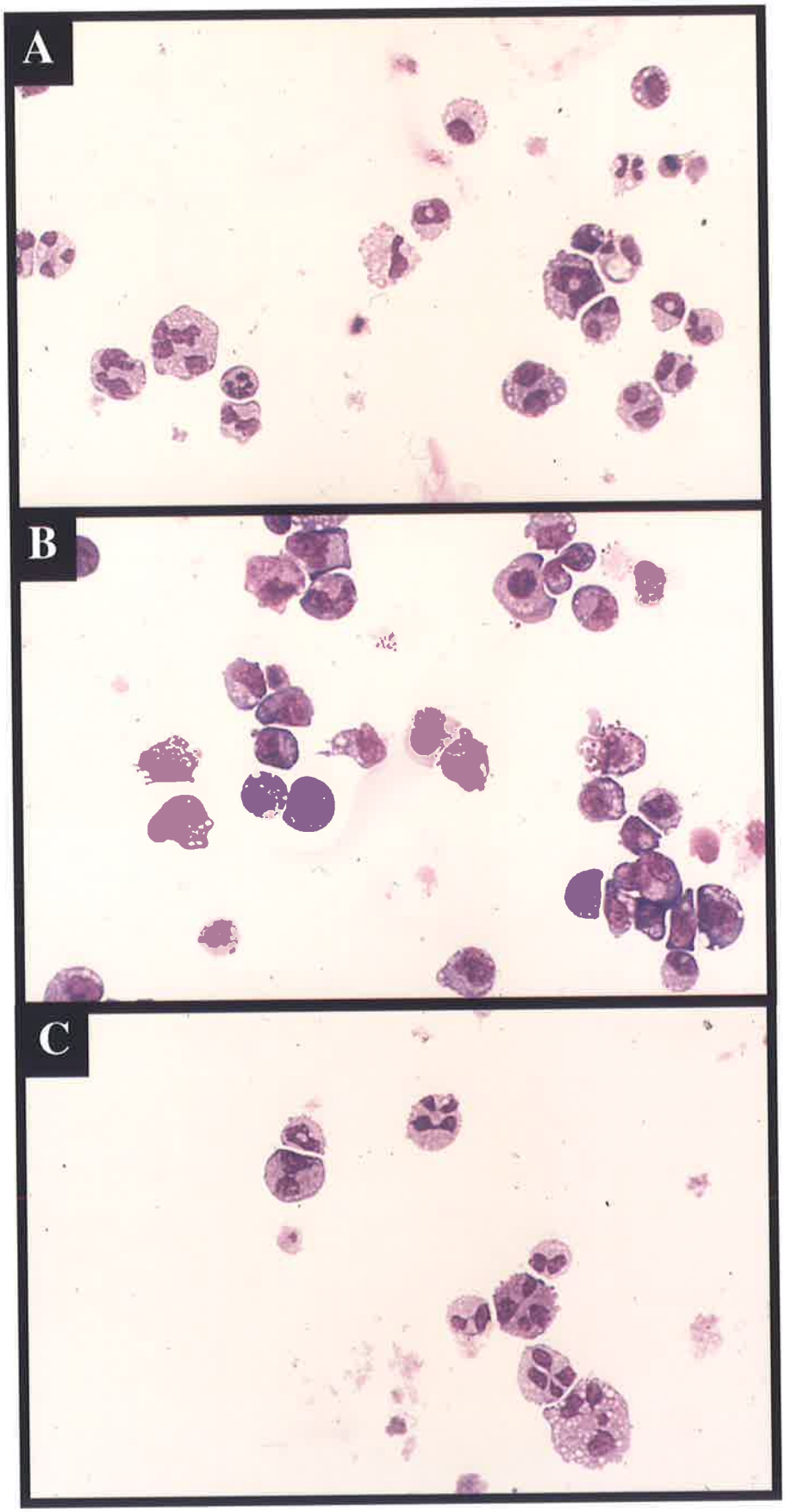
4.3.2 Murine IL-3 and GM-CSF deliver different signals to the FDB1 and FDB2 cell lines

Upon transfer to medium containing murine GM-CSF, FDB1 and FDB2 cells rapidly and terminally differentiated along the neutrophil and macrophage lineages. That this property is shared by both cell lines suggests that it may be a general feature of cell lines generated by this method; that is, retroviral infection followed by extended culture in IL-3, GM-CSF and Epo. If it is the case that transformation of these lines is a consequence of RufNeo integration, it may be possible to identify a common molecular lesion by identifying common sites of integration using a restriction fragment length polymorphism approach similar to that employed in Figure 5.1. The surrounding DNA could then be isolated by procedures such as the inverse PCR technique utilised by Pang and Knecht (1997) with the aim of identifying the gene(s) responsible for transformation.

Low concentrations of IL-3 lead to the differentiation of the FDB2 cell line, raising the possibility that the differentiation signal delivered by GM-CSF is a result of low level activation of the same signalling pathways activated on IL-3 stimulation (Table 4.1). However, this is unlikely to be the case as the very high level of GM-CSF used in differentiation experiments (500 U/ml) was much higher than the level of IL-3 required to prevent differentiation (2 U/ml, Table 4.1). Nevertheless it is possible that IL-3 is far more efficient at activating growth signal transduction pathways than GM-CSF. Regardless of whether the differences in the signalling caused by IL-3 and GM-

Figure 5.1. Morphology of factor-independent FDB2 cells containing constitutively active hβc mutants

Puromycin resistant FDB2 pools containing RufPuro retroviruses bearing the FIΔ (**A**), V449E (**B**) and I374N (**C**) hβc mutants were washed 3 times in DMEM and cultured without growth factor for five days, at which time samples were cytocentrifuged and Wright-Giemsa stained. Photographs are at 450 × magnification


A
B
C

CSF in this cell line are qualitative or quantitative, there are at least two possible explanations (models) for this phenomenon.

The unique α subunits bound by murine IL-3 and GM-CSF may have roles in cellular signalling. In this regard, the cytoplasmic domains of the α subunits are required for proliferative signalling through the human IL-3, GM-CSF and IL-5 receptors (Weiss et al., 1993; Takaki et al., 1994; Polotskaya et al., 1993; Barry et al., 1997). The required region of hGMRα has been further defined as a 29 amino acid membrane-proximal cytoplasmic region containing motifs conserved between all three receptors (Weiss et al., 1993; Polotskaya et al., 1994). Moreover, the same region of hGMRα is required for differentiation signalling through the hGMR in the WT19 cell line (Matsuguchi et al., 1997). Hence it is possible that the α subunits play a role in signalling through the mIL-3 and mGM-CSF receptors, with mGMRα inducing differentiation or mIL-3Rα blocking differentiative signalling through mβc. In support of the latter, mIL-3Rα appears to play an active and dominant role in inhibiting Epo-induced differentiation of the murine IL-3 dependant line Ba/F3 (Krosl et al., 1996).

Alternatively, it is possible that the different signalling properties of the mIL-3 and mGM-CSF receptors is due to the fact that they are composed of different β subunits. Hence whilst mGM-CSF signals through the mβc subunit only, mIL-3 utilises both mβc and $m\beta_{IL\text{-}3}$ subunits in signalling (Hara and Miyajima, 1992). It is therefore possible that the differentiation signal mediated by mGM-CSF signalling is a result of signalling through mβc alone, whereas in the case of IL-3 this signal is blocked by signalling through $m\beta_{IL\text{-}3}$. This is concordant with the observation that the proliferative signal delivered by mIL-3 is dominant to the mGM-CSF-mediated differentiation signal in the FDB cell lines.

Interestingly, no signalling difference has been found between mIL-3 and mGM-CSF in terms of substrate tyrosine phosphorylation in either human or murine systems (Duronio et al., 1992; Kanakura et al., 1990). However, these studies were performed in differentiation-arrested cell lines which give an identical growth response with either growth factor. It is possible that putative signalling differences will be observable only in cell lines which give distinct cellular responses to these two growth factors, such as the FDB lines described in this chapter.

CHAPTER 5. Function of activated hβc mutants in multipotential cell lines

5.1 Introduction

A qualitative difference in signalling has been observed when constitutively active hβc mutants are placed in the IL-3-dependent murine pro-B cell line BAF-B03, in that transmembrane but not extracellular mutants deliver a proliferative signal to this cell line (Jenkins et al., 1995). Moreover, as detailed in chapter 3, transmembrane and extracellular activated hβc mutants apparently deliver different signals to primary murine haemopoietic cells. That is, the extracellular mutants, FIΔ and I374N, could induce factor-independent proliferation and differentiation of neutrophil and monocyte cells only, whereas the transmembrane mutant, V449E, could do so to all the cell types of the myeloid and erythroid compartments. The response of these cells to FIΔ and I374N resembles that seen when cells are exposed to GM-CSF, whereas the broad effect of the transmembrane mutant, V449E, is more akin to that of IL-3. For these reasons it was of interest to determine if these two classes of activated hβc mutants would deliver different signals to the multipotential murine myeloid cell lines, FDB1 and FDB2, described in Chapter 4. This chapter describes the expression of all three hβc mutants in FDB1 and FDB2 cells, and the subsequent effects of factor-independent signalling on these cell lines.

5.2 Results

5.2.1 Expression of activated hβc subunits in FDB1 and FDB2 cell lines

To determine the effects of factor-independent signalling of constitutively active hβc mutants on the multipotential myelomonocytic cell lines, FDB1 and FDB2, wild-type hβc and three constitutively active mutants, FIΔ, I374N and V449E, were expressed in these cell lines. As both lines are constitutively neomycin resistant, these hβc subunits were expressed using the RufPuro retroviral expression vector, which contains a resistance gene for puromycin. As described in Section 2.12.6.3, FDB1 and FDB2 cells were co-cultivated with Ψ2 ecotropic retrovirus packaging cells producing RufPuro containing either a wild type or mutant hβc cDNA (as a control, parallel co-cultivations were carried out with untransfected Ψ2 cells). After two days, the FDB1 and FDB2 cells were harvested and placed in medium containing 1 μg/ml puromycin, to select for cells which had been infected with the RufPuro retrovirus. To limit the

effects of clonal variation, these puromycin resistant cell were maintained as stably infected pools. As FDB1 and FDB2 cells express wild-type hβc, it was impossible at this stage to determine the proportion of these pools expressing wild-type or mutant hβc subunits from the RufPuro retrovirus. However, by analogy with other infected cell lines, expression on approximately 50% of these stably infected cells could be expected.

When wild-type FDB2 cells and those containing RufPuro-hβc were washed and placed in culture without growth factors, the cells died within 3 days, as seen in previous experiments with these lines (data not shown; see Chapter 4). However, when FDB2 cells containing RufPuro constructs bearing each of the three activated hβc mutants, FIΔ, I374N and V449E, were washed and placed in culture without growth factors, viable, growth factor-independent cells were present after this time. After 5 days of culture, the surviving cells were cytocentrifuged and Wright-Giemsa stained and the cell types present were determined microscopically. As shown in Figure 5.1 and Table 5.1, factor-independent cells containing RufPuro-V449E were mostly immature, similar to FDB2 cells cultured in IL-3. However, factor-independent cells containing RufPuro-FIΔ and RufPuro-I374N were mostly neutrophils and monocytes, similar to FDB2 cells cultured in GM-CSF (Figure 5.1, Table 5.1). Hence whilst all three constitutively active hβc subunits were able to confer factor-independent survival on the FDB2 cell line, there was a difference in the effects of these mutants, with extracellular mutants delivering a mGM-CSF-like signal to FDB2 cells, whilst the transmembrane mutant, V449E, delivered an mIL-3-like signal. Essentially identical results were obtained from similar experiments conducted using the FDB1 cell line (data not shown).

5.2.2 Construction of epitope tagged hβc subunits

As high levels of IL-3 induce proliferation of FDB2 cells whilst low concentrations lead to differentiation (see Chapter 4), it could be argued that the apparent differences in signalling by transmembrane and extracellular activated hβc mutants in the FDB1 and FDB2 cell lines are due to differences in expression levels leading to stronger signalling by the transmembrane mutant. To address this question, wild type and mutant hβc subunits were joined to an epitope tag, termed FLAG, which is detectable by flow cytometry using a specific monoclonal antibody. The FLAG tag was placed 7

Table 5.1. Extracellular but not transmembrane activated hβc mutants induce differentiation of FDB2 cells

RufNeo Construct	Growth Factor	Blast/pro	Myel/meta	Band/neut	Mono	MK
-	IL-3	72	20	7	1	0
	GM-CSF	3	8	65	24	0
FIΔ	-	5	6	77	12	0
V449E	-	82	12	5	1	1
I374N	-	8	8	76	8	0

Cells were washed three times in DMEM and cultured in the presence of the indicated growth factors for five days, at which time samples were cytocentrifuged and Giemsa stained and the cell types determined microscopically. Numbers are the percentage of blasts and promyelocytes (blast/pro), myelocytes and metamyelocytes (myel/meta), bands and mature neutrophils (band/neut), monocytes (mono) and megakaryocytes (MK) of 200 cells scored. Data are representative of two separate experiments

amino acids from the N'-terminus of wild-type and mutant hβc proteins as described in Section 2.11.2, and the resultant RufPuro constructs containing epitope-tagged hβc cDNAs were sequenced to confirm the correct sequence and orientation of the FLAG tag.

5.2.3 Activity of epitope-tagged hβc mutants in FDC-P1 cells

To verify that the FLAG tag did not interfere with the expression or activity of the activated hβc mutants, the activity of these FLAG-tagged mutants was tested in the murine GM-CSF dependent myelomonocytic cell line, FDC-P1.

To achieve infection of FDC-P1 cells, pRufPuro constructs containing FLAG-tagged wild-type and mutant hβc cDNAs were transiently transfected into the murine ecotropic packaging cell line BOSC 23 as described in Section 2.12.6.4. These cells were subsequently co-cultivated with FDC-P1 cells for 2 days, after which the FDC-P1 cells were harvested and cultured in medium containing puromycin to select for cells containing the RufPuro retrovirus. Again, to limit the effects of clonal variation, the resulting puromycin-resistant cells were maintained as stably infected pools. To assess hβc expression, these pools were stained with a FLAG-specific monoclonal antibody, M2, and analysed by flow cytometry. As shown in Figure 5.2A, the majority of puromycin-resistant FDC-P1 cells expressed the FLAG epitope tag, indicating that FLAG-tagged hβc subunits were expressed on the surface of these cells. All four FLAG-tagged hβc subunits were expressed at similar levels on these cells.

To test the functional activity of the FLAG-tagged hβc subunits, FDC-P1 pools expressing these subunits were washed extensively and placed in a proliferation assay. As shown in Figure 5.2B, neither uninfected FDC-P1 cells nor those expressing FLAG-tagged wild-type hβc were able to grow in the absence of GM-CSF. However, FDC-P1 cells expressing any of the three hβc mutants were capable of factor-independent proliferation. This indicates that the presence of the FLAG tag did not interfere with the factor-independent signalling of the constitutively active hβc mutants.

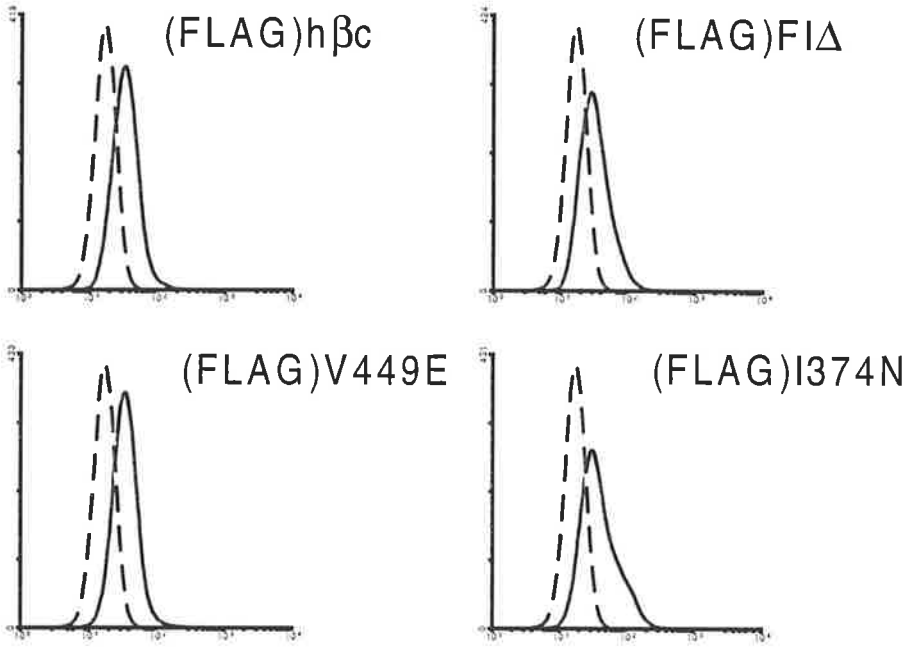
5.2.4 Activity of epitope-tagged hβc mutants in FDB1 cells

To assess the effect of FLAG-tagged hβc mutants, puromycin-resistant pools of FDB1 cells stably infected with RufPuro constructs containing FLAG-tagged wild-type and

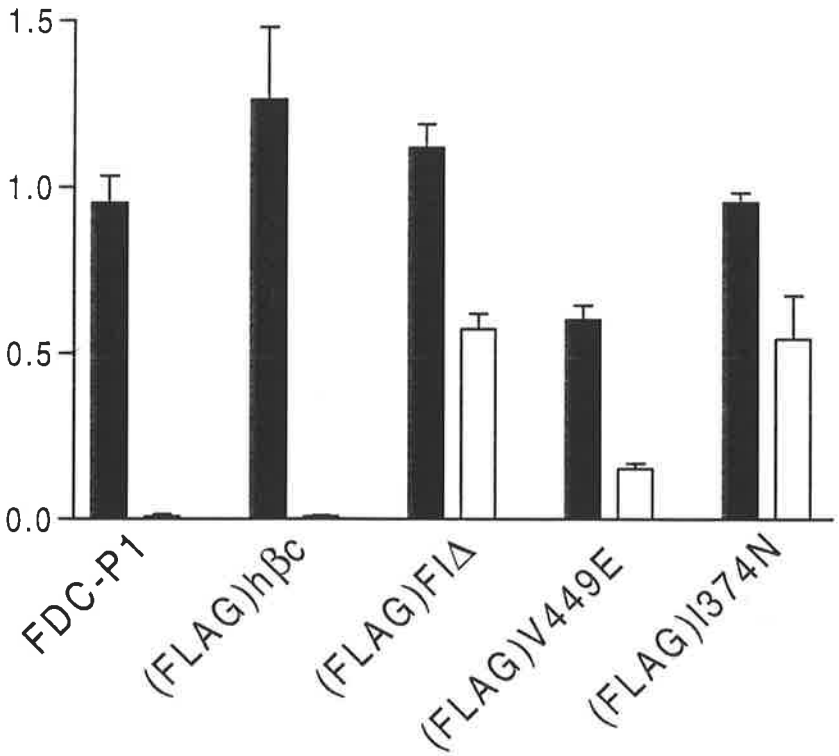
Figure 5.2. FDC-P1 cells expressing FLAG-tagged hβc mutants proliferate without growth factors

(**A**) Flow cytometric analysis. Puromycin-resistant pools of FDC-P1 cells infected with RufPuro bearing the indicated FLAG-tagged hβc cDNAs were stained with a monoclonal antibody, M2, directed against the FLAG epitope tag, followed by a FITC-conjugated anti-mouse immunoglobulin monoclonal antibody, represented by solid lines. The dashed lines indicate background staining of uninfected FDC-P1 cells. (**B**) Proliferation assay of puromycin-resistant FDC-P1 cells. Wild type FDC-P1 cells and the above puromycin-resistant pools were washed 3 times in DMEM and cultured with (filled bars) and without (open bars) 50 U/ml GM-CSF. After 3 days proliferation was measured as described in Section 2.12.12. Bars show the mean of triplicate samples whilst error bars show the Standard Deviation.

A


(FLAG)hβc
(FLAG)FIΔ
(FLAG)V449E
(FLAG)I374N

B


1.5
1.0
0.5
0.0
FDC-P1
(FLAG)hβc
(FLAG)FIΔ
(FLAG)V449E
(FLAG)I374N

mutant hβc cDNAs were generated, using identical methodology to that used for the above FDC-P1 infections. Infected FDB1 pools not expressing the FLAG tag on all cells were sorted for FLAG expression as described in Section 2.12.11.2. As shown in Figure 5.3, the majority of cells from the resulting FDB1 pools expressed the FLAG epitope at similar levels on the cell surface, indicating expression of the FLAG-tagged hβc subunits.

To study the response of FDB1 cells to the FLAG-tagged activated hβc mutants, these cells were washed extensively and placed in culture in the presence of IL-3, in the presence of GM-CSF and in the absence of growth factors.

As seen in the experiments performed in Chapter 4, there was a low level of differentiation of FDB1 cells cultured in IL-3 along the neutrophil, monocyte and megakaryocyte lineages (Figure 5.4). However, transfer to medium containing GM-CSF lead to rapid and synchronous differentiation along the neutrophil and monocyte lineages. This correlated with a decline in cell proliferation and a decrease in cell viability (Figures 5.5, 5.6). However, unlike earlier experiments using this cell line (see Chapter 4), this decline in cell proliferation was incomplete, and some cells were able to continue proliferating in GM-CSF, albeit slowly (Figure 5.5). This correlated with a recovery of cell viability and a decrease in the proportion of differentiated cells (Figures 5.6, 5.4). Hence there appeared to be a population within the FDB1 cell line which developed "resistance" to the differentiative effects of GM-CSF signalling. This apparent resistance was observed repeatedly and was particularly prominent in cells maintained for extensive periods. A similar phenomenon was observed with the FDB2 cell line, which appears to be clonal by Southern analysis (see below). Moreover, a clone of the FDB2 cell line, FDB2-20, developed a similar resistance to GM-CSF-induced differentiation (data not shown). Hence this effect appears not to be the result of clonal selection, but rather a mutability of the lines themselves. Acquired resistance to GM-CSF-induced differentiation correlated with a change in the growth pattern of these cell lines from small round cells growing chiefly in clumps to single, larger cells, and with the expression of myeloid differentiation antigens (data not shown). Hence this phenomenon appears to be due to a partial differentiation of the cell lines. Thus far it has been impossible to prevent this phenomenon through changes in IL-3 concentration or cell density in passaging. It may be that a

Figure 5.3. Flow cytometric analysis of puromycin resistant FDB cell pools

Infected FDB1 and FDB2 pools stably infected with RufPuro bearing the indicated FLAG-tagged hβc cDNAs were stained with a monoclonal antibody, M2, directed against the FLAG epitope tag, followed by a FITC-conjugated anti-mouse immunoglobulin monoclonal antibody, represented by solid lines. The dashed lines indicate background staining of uninfected cells.

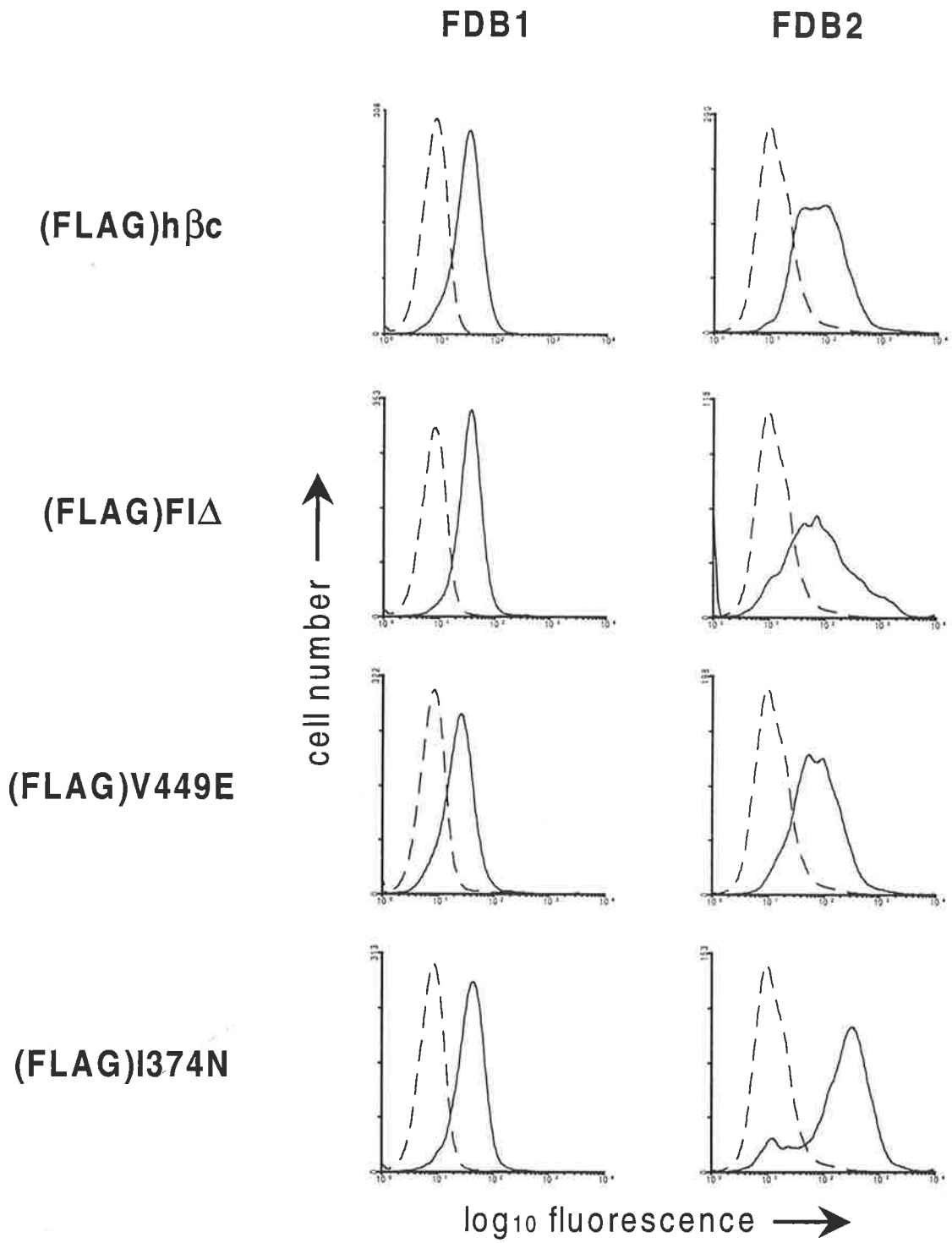

FDB1
FDB2
(FLAG)hβc
(FLAG)FIΔ
(FLAG)V449E
(FLAG)I374N
cell number
log10 fluorescence

Figure 5.4. Timecourse of differentiation of FDB1 cells expressing FLAG-tagged hβc mutants

Wild-type FDB1 cells and infected pools stably expressing the indicated FLAG-tagged hβc subunits were washed 3 times in DMEM and cultured in the presence of IL-3, GM-CSF or in the absence of growth factors (no factor). At the indicated timepoints samples were cytocentrifuged and Wright-Giemsa stained and the percentage of differentiated neutrophils (■), monocytes (▲), megakaryocytes (▼) and the total percentage of differentiated cells (◆) was determined from at least 200 cells scored. Dashed lines show differentiation along each lineage whilst solid lines represent total differentiation. Data are representative of two separate experiments.

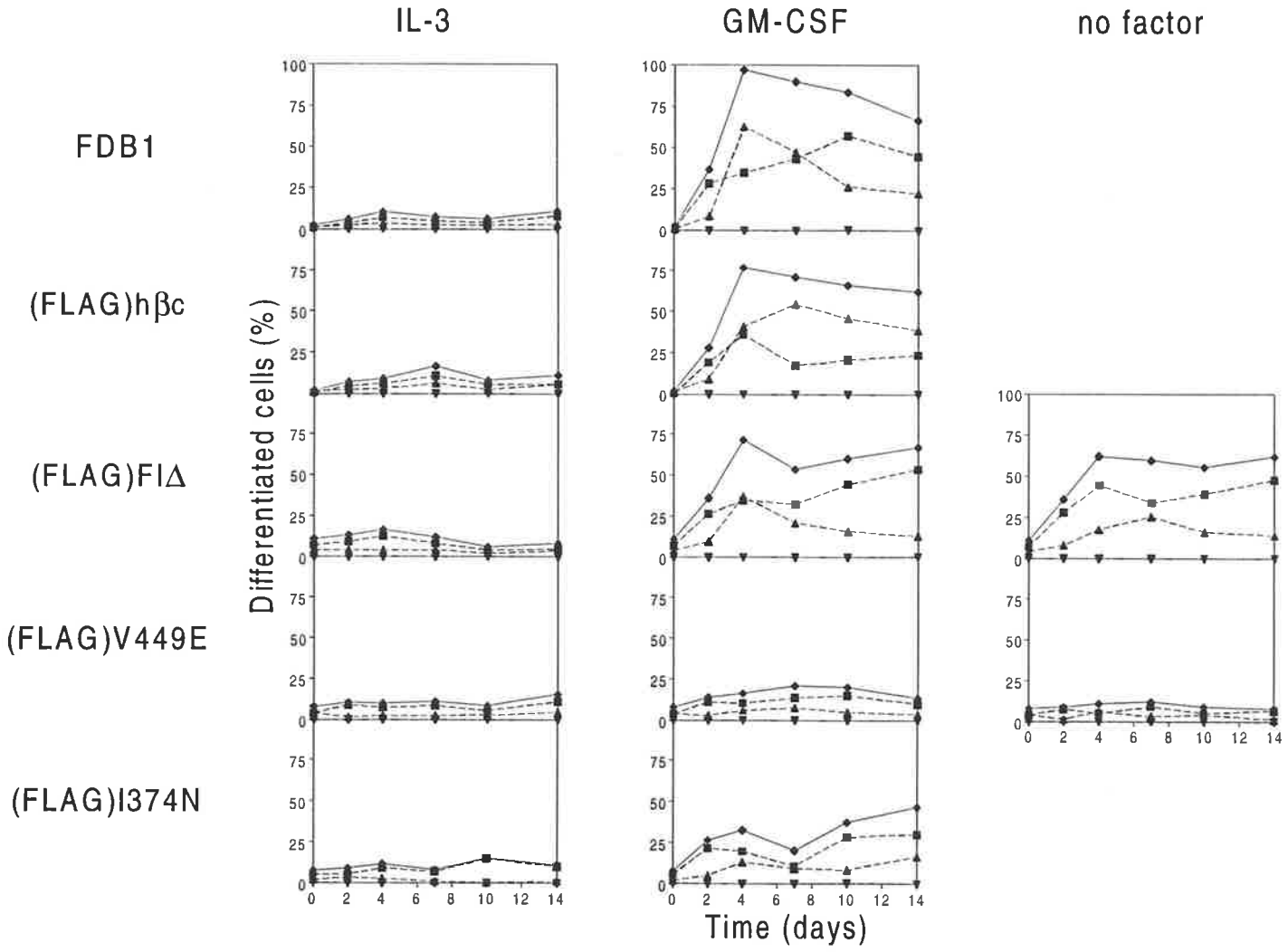

IL-3
GM-CSF
no factor
FDB1
(FLAG)hβc
(FLAG)FIΔ
(FLAG)V449E
(FLAG)I374N
Differentiated cells (%)
Time (days)
0
25
50
75
100
0 2 4 6 8 10 12 14

Figure 5.5. Proliferation of FDB1 cells expressing FLAG-tagged hβc mutants

Wild-type FDB1 cells and infected pools stably expressing the indicated FLAG-tagged hβc subunits were washed 3 times in DMEM and cultured in the presence of IL-3 (■), GM-CSF (▲) or in the absence of growth factor (▼). At the indicated timepoints, viable cell counts were taken using a haemocytometer. Data are representative of two separate experiments.

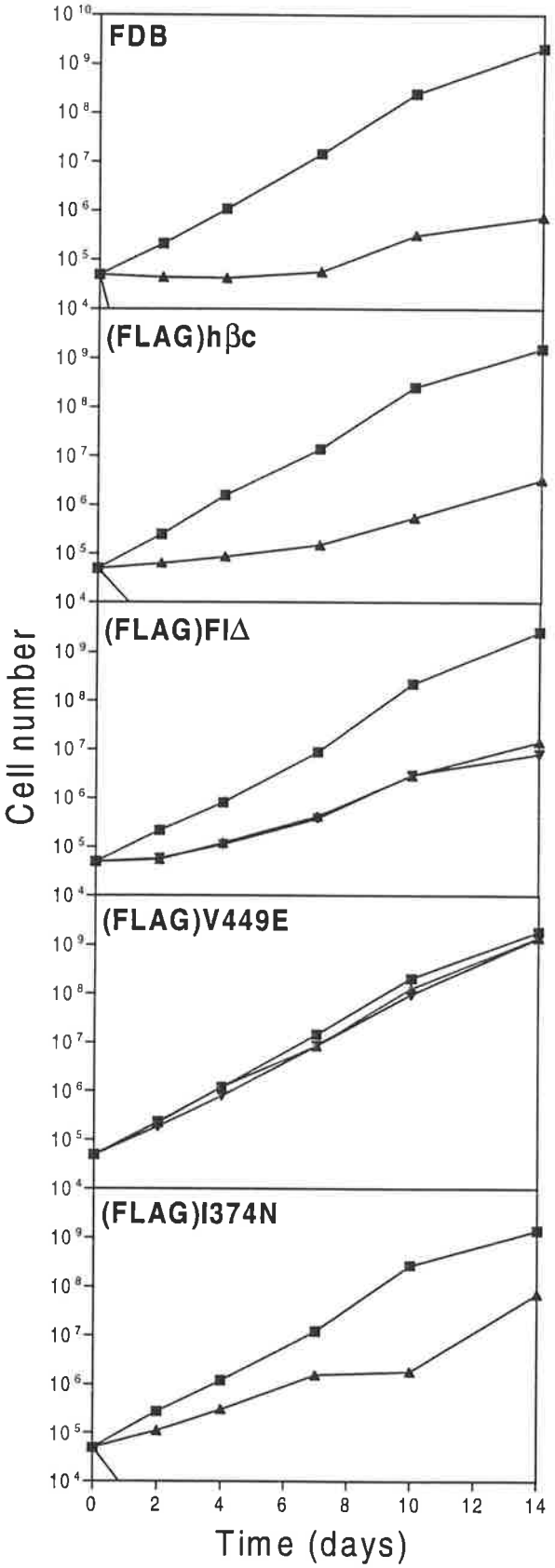
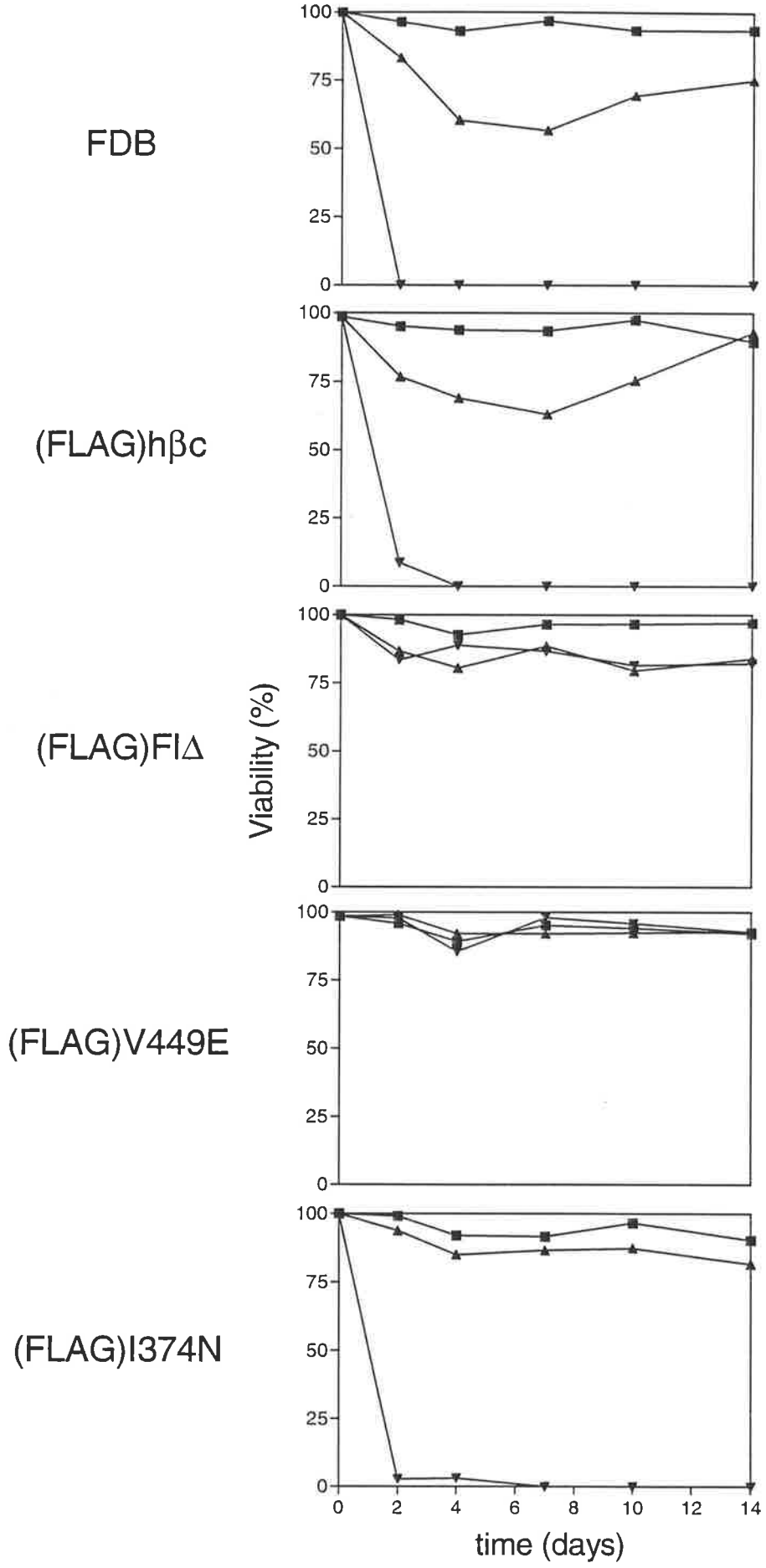

FDB
(FLAG)hβc
(FLAG)FIΔ
(FLAG)V449E
(FLAG)I374N
Cell number
Time (days)
0
2
4
6
8
10
12
14

Figure 5.6. Timecourse of viability of FDB1 cells expressing FLAG-tagged hβc mutants

Wild-type FDB1 cells and infected pools stably expressing the indicated FLAG-tagged hβc subunits were washed 3 times in DMEM and cultured in the presence of IL-3 (■), GM-CSF (▲) or in the absence of growth factors (▼). At the indicated timepoints, the percentage of cells excluding trypan blue was determined from at least 100 cells scored using a haemocytometer. Data are representative of two separate experiments.


FDB
(FLAG)hβc
(FLAG)FlΔ
(FLAG)V449E
(FLAG)I374N
Viability (%)
100
75
50
25
0
0
2
4
6
8
10
12
14
time (days)

combination of growth factors is required to maintain these cell lines long-term in the fully undifferentiated state.

FDB1 cells expressing FLAG-tagged hβc were unaltered in growth characteristics to the uninfected cell line. That is, in IL-3 the cells grew in an undifferentiated state with high viability, transfer to GM-CSF induced differentiation along the neutrophil and monocyte lineages, with decreased growth and some loss of viability, and when deprived of growth factors the cells rapidly died (Figures 5.4-5.6). However FDB1 cells expressing either (FLAG)FIΔ or (FLAG)V449E were capable of factor-independent survival. In the case of FDB1-(FLAG)FIΔ, the factor-independent response was similar to the response of FDB1 cells to GM-CSF, that is, neutrophilic and monocytic differentiation, decreased cell proliferation and a slight loss of viability (Figures 5.4-5.6). However, the factor-independent response of FDB1 cells expressing (FLAG)V449E was entirely distinct, and almost was identical to the response of these cells to IL-3 in terms of growth rate, viability and differentiation of the cells (Figures 5.4-5.6). These results confirmed those seen with non-tagged FIΔ and V449E mutants (above). Moreover, FDB1-(FLAG)V449E cells did not decrease in growth rate, lose viability or significantly morphologically differentiate when transferred to medium containing GM-CSF. Thus it appears that, like IL-3 signalling, the factor-independent signal delivered by (FLAG)V449E is dominant to GM-CSF-induced differentiation signalling in the FDB1 cell line.

Unexpectedly, expression of (FLAG)I374N did not confer factor-independence on the FDB1 line. This experiment was repeated using four independent infections of this line and three distinct bacterial clones of the pRufPuro-(FLAG)I374N construct, with identical results obtained in each case. This result is incongruous with the previous results showing that the non-tagged form of I374N could confer a GM-CSF-like signal to this cell line, and that the FLAG-tagged I374N protein was active in FDC-P1 cells (Table 5.1, Figure 5.2). It may be that the presence of the FLAG tag interferes with the function of I374N in FDB1 cells only.

5.2.5 Activity of epitope-tagged hβc mutants in FDB2 cells

To confirm the results obtained in the above studies using the FDB1 cell line, the FLAG-tagged wild-type and mutant hβc subunits were expressed in the FDB2 cell line, which, as shown in Chapter 4, has similar properties to the FDB1 cell line in

terms of its response to growth factors. Expression of these proteins in FDB2 cells was achieved using identical methodology to that detailed above for the FDB1 cell line. Again, stably infected FDB2 cells were maintained as puromycin resistant pools. To detect expression of the FLAG-tagged proteins, puromycin resistant pools of FDB2 cells were stained with the FLAG-specific monoclonal antibody M2 and expression detected by flow cytometry. As shown in Figure 5.3, the FLAG-tagged hβc proteins were detected on the majority of cells in these puromycin-resistant pools. Due to the high frequency of expression in these pools, cell sorting for the expression of the FLAG tag was deemed unnecessary.

To assess the function of the FLAG-tagged hβc subunits in FDB2 cells, the above puromycin-resistant pools were washed extensively and placed in culture with IL-3, GM-CSF, or no added growth factors. In the presence of GM-CSF, wild-type FDB2 cells and those expressing (FLAG)hβc morphologically differentiated, with concomitant decreases in proliferation and viability (Figures 5.7-5.9, Figure 5.10B). In the case of FDB2-(FLAG)hβc cells this differentiation was complete and terminal, however in the wild-type FDB2 cells used in this experiment there was a small population of cells resistant to GM-CSF-induced differentiation which continued to proliferate in the presence of GM-CSF, as seen for FDB1 cells (above) (Figure 5.8). When deprived of growth factors wild-type FDB2 cells and those expressing (FLAG)hβc died within 4 days of culture (Figure 5.9). However, cells expressing FLAG-tagged FIΔ and V449E proteins were capable of factor-independent survival after this period. In the case of cells expressing (FLAG)FIΔ, the cells morphologically differentiated in the absence of growth factors (Figure 5.7, 5.10C). This differentiation was terminal, leading to complete growth arrest and extensive loss of viability (Figures 5.8, 5.9). When FDB2-(FLAG)V449E cells were deprived of growth factors there some cell death was seen, suggesting that not all these cells expressed (FLAG)V449E at sufficient levels to confer factor-independence (Figure 5.9). However, after 2 days the remaining cells proliferated with complete survival and no morphological differentiation (Figure 5.7-5.9, Figure 5.10D). (FLAG)V449E signalling partially blocked the effects of GM-CSF on FDB2 cell differentiation (Figure 5.7). That this block was incomplete is consistent with the above observations that not all FDB2-(FLAG)V449E cells were capable of factor-independent growth.

Figure 5.7. Timecourse of differentiation of FDB2 cells expressing FLAG-tagged hβc mutants

Wild-type FDB2 cells and infected pools stably expressing the indicated FLAG-tagged hβc subunits were washed 3 times in DMEM and cultured in the presence of IL-3, GM-CSF or in the absence of growth factors (no factor). At the indicated timepoints samples were cytocentrifuged and Wright-Giemsa stained and the percentage of differentiated neutrophils (■) monocytes (▲), megakaryocytes (▼) and the total percentage of differentiated cells (◆) was determined from at least 200 cells scored microscopically. Dashed lines show differentiation along each lineage whilst solid lines represent total differentiation. Data are representative of two separate experiments.

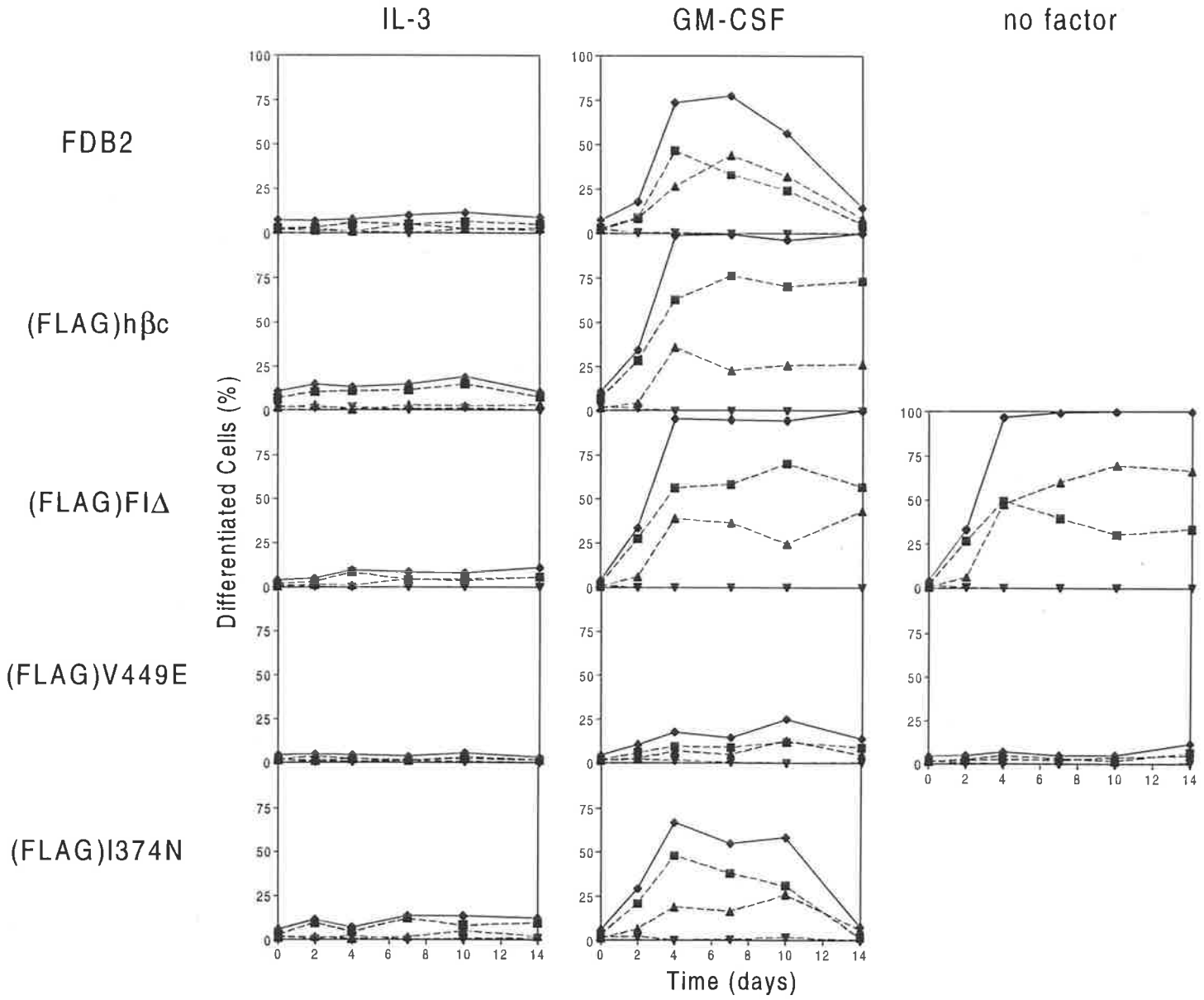

IL-3
GM-CSF
no factor
FDB2
(FLAG)hβc
(FLAG)FIΔ
(FLAG)V449E
(FLAG)I374N
Differentiated Cells (%)
Time (days)
0
25
50
75
100
0
2
4
6
8
10
12
14

Figure 5.8. Proliferation of FDB2 cells expressing FLAG-tagged hβc mutants

Wild-type FDB2 cells and pools stably expressing the indicated FLAG-tagged hβc subunits were washed 3 times in DMEM and cultured in the presence of IL-3 (■), GM-CSF (▲) or in the absence of growth factors (▼). At the indicated timepoints, viable cell counts were taken using a haemocytometer. Data are representative of two separate experiments.

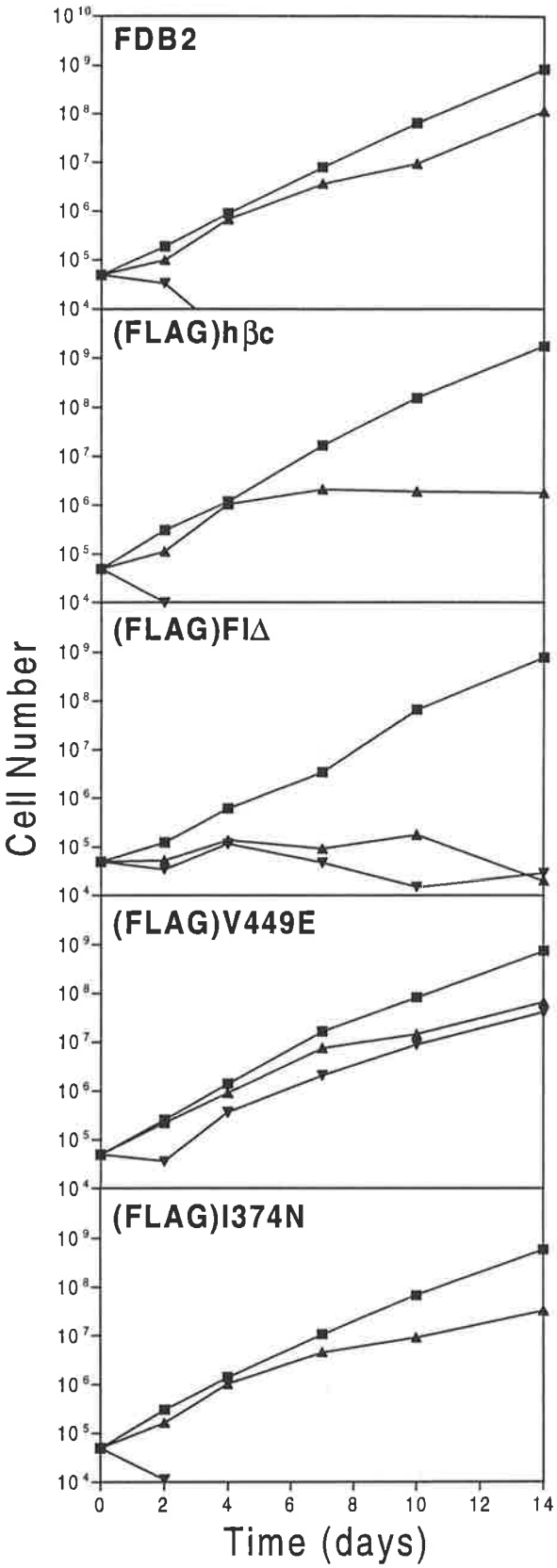

FDB2
(FLAG)hβc
(FLAG)FIΔ
(FLAG)V449E
(FLAG)I374N
Cell Number
Time (days)
0
2
4
6
8
10
12
14

Figure 5.9. Timecourse of viability of FDB2 cells expressing FLAG-tagged hβc mutants

Wild-type FDB2 cells and pools stably expressing the indicated FLAG-tagged hβc subunits were washed 3 times in DMEM and cultured in the presence of IL-3 (■), GM-CSF (▲) and in the absence of growth factors (▼). At the indicated timepoints, the percentage of cells excluding trypan blue was determined from at least 100 cells scored using a haemocytometer. Data are representative of two separate experiments.

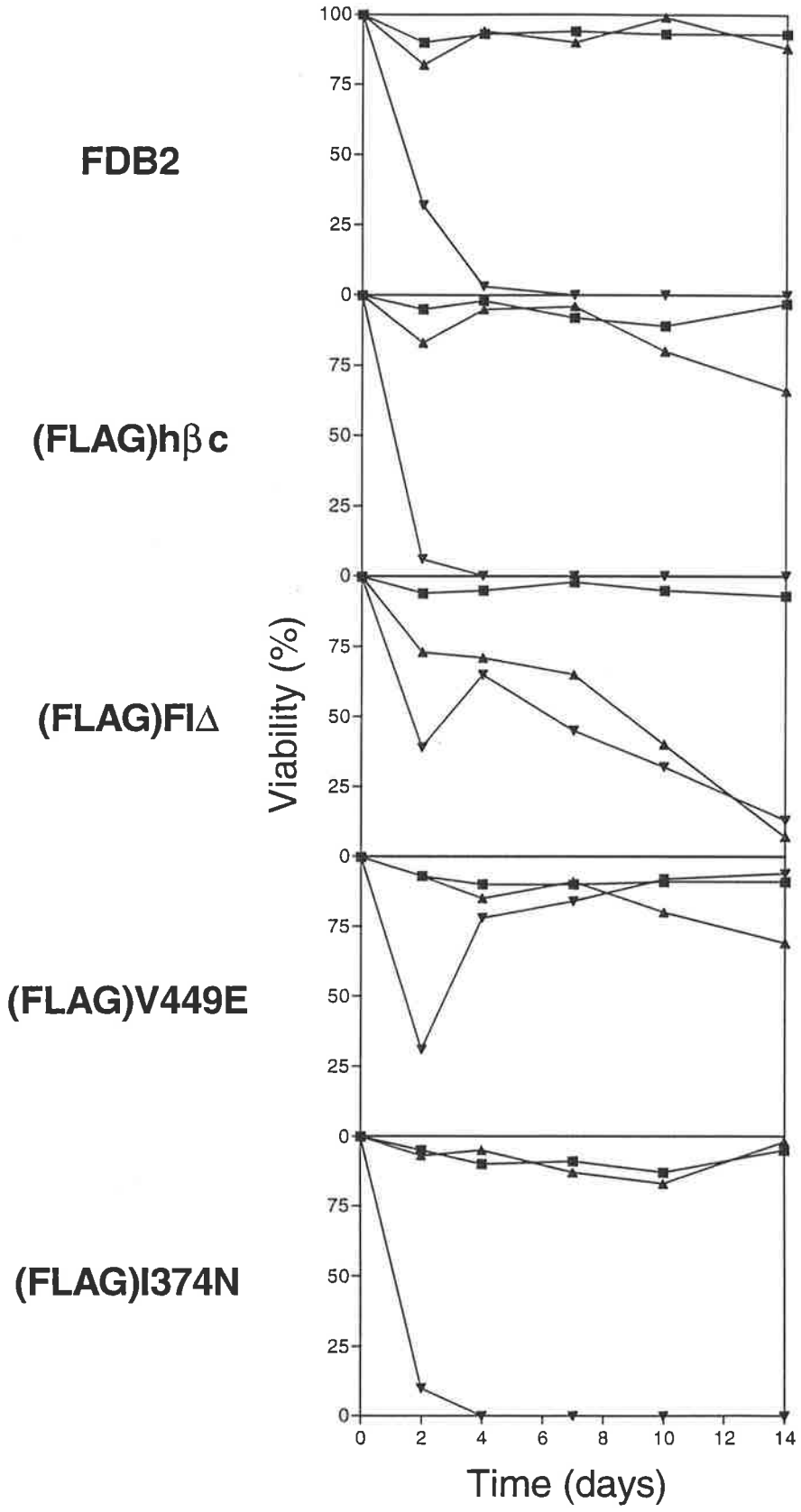
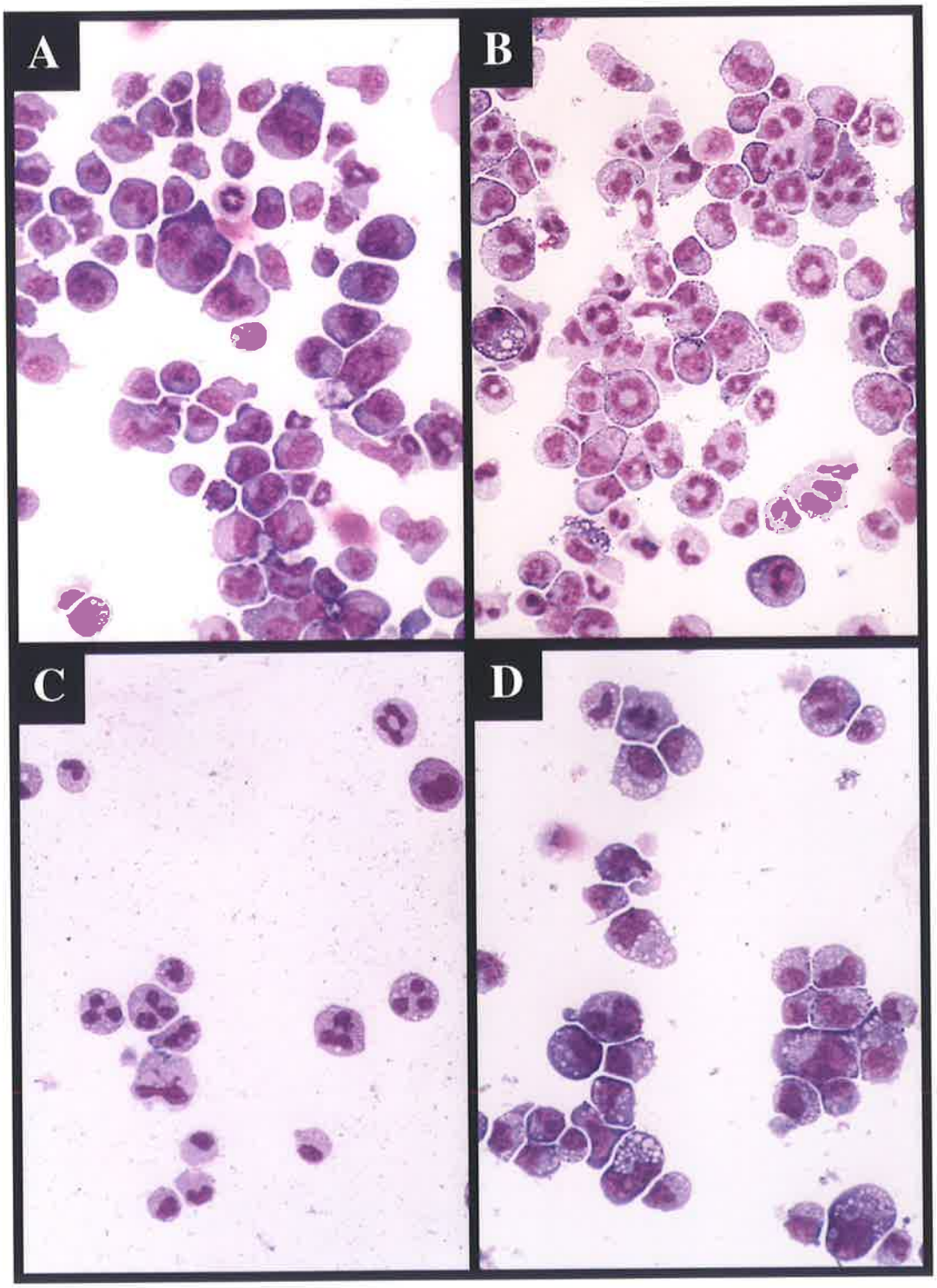

FDB2
(FLAG)hβc
(FLAG)FlΔ
(FLAG)V449E
(FLAG)I374N
Viability (%)
100
75
50
25
0
0
2
4
6
8
10
12
14
Time (days)

Figure 5.10. Morphology of factor-independent FDB2 cells expressing FLAG-tagged constitutively active hβc mutants

Morphology of puromycin resistant FDB2 pools expressing FLAG-hβc cultured in IL-3 (**A**), expressing FLAG-hβc cultured in GM-CSF (**B**) expressing (FLAG)FIΔ cultured without growth factors (**C**) and expressing (FLAG)V449E cultured without growth factors (**D**). Cells were washed 3 times in medium and cultured for five days, at which point samples were cytocentrifuged and Wright-Giemsa stained. Photographs are at 360 × magnification.


A
B
C
D

Hence, as was the case in FDB1 cells, (FLAG)V449E signalling was dominant over the differentiative signalling of GM-CSF. FDB2 cells expressing (FLAG)I374N were incapable of factor-independent survival indicating that, as was the case for FDB1 cells, (FLAG)I374N is not active in these cells.

5.2.6 Cell surface marker expression of factor-dependent and -independent FDB2 cells

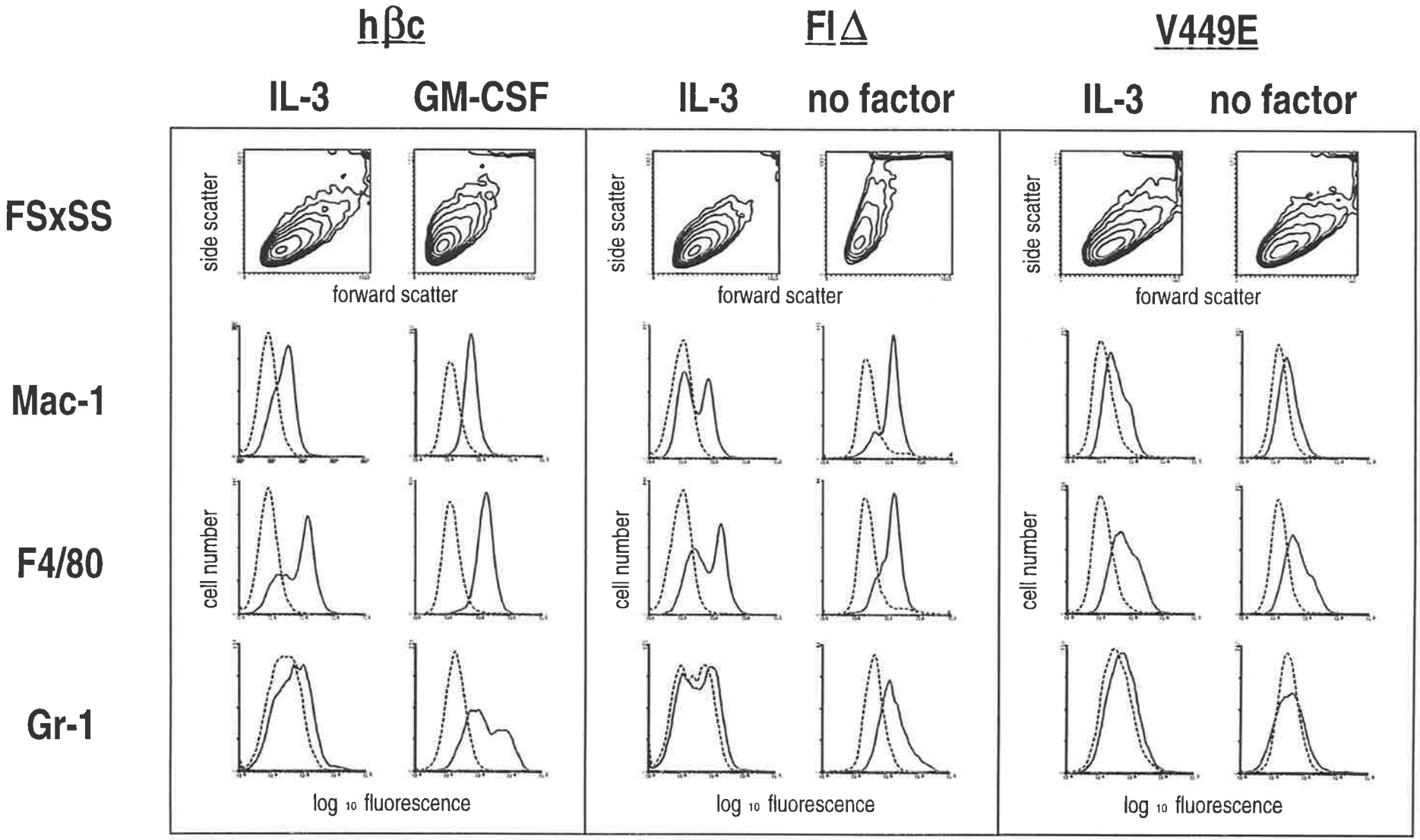
The above studies demonstrate morphological differences in the cell types produced by factor independent signalling of (FLAG)FIΔ and (FLAG)V449E in FDB2 cells. To verify the identities of the cell types observed, the expression of various cell surface markers on factor-independent FDB2 cells was determined by flow cytometry.

As a control, the cell surface marker expression of FDB2 cells expressing FLAG-tagged wild-type hβc was determined. Cells cultured in GM-CSF for 7 days were smaller than those cultured in IL-3, and more granular, as measured by forward and side scatter of the cells, respectively (Figure 5.11). When these cells were grown in IL-3, a proportion of the cells expressed the granulocyte-macrophage marker Mac-1 and the macrophage marker F4/80. However, upon culture in GM-CSF there was an induction of these two markers as well as the granulocyte-macrophage marker Gr-1. This induction of Gr-1 is the opposite to the previous studies in Chapter 4 showing a slight loss of this marker upon GM-CSF-induced differentiation of wild-type FDB2 cells. Notably the cells examined in Chapter 4 expressed Gr-1 in the presence of IL-3. It is possible that they had undergone a degree of myeloid differentiation due to the inherent variability in the differentiation state of this cell line (see Section 5.2.4).

Factor-independent cells containing (FLAG)FIΔ were smaller and more granular than cells grown in IL-3 as measured by forward scatter and side scatter of the cells, respectively (Figure 5-11). Similarly to cells expressing (FLAG)hβc, a proportion of FDB2 cells expressing (FLAG)FIΔ grown in IL-3 expressed Mac-1 and F4/80. However, there was an induction of these two markers and Gr-1 when these cells were cultured in the absence of growth factor for seven days. Hence the expression of these markers on factor-independent FDB2-(FLAG)FIΔ cells resembles that of FDB2-(FLAG)hβc cells cultured in GM-CSF. This increase in myeloid surface marker expression is consistent with the morphological differentiation of these cells (Figures 5.7, 5.10). In contrast, factor independent cells containing (FLAG)V449E were not

Figure 5.11. Scatter profile and cell surface antigen expression of FDB2 cells expressing FLAG-tagged hβc subunits

FDB2 cells expressing the indicated FLAG-tagged hβc subunits were washed 3 times in DMEM and cultured in the presence of IL-3, GM-CSF or in the absence of growth factors (no factor) as indicated for seven days. Cells were then stained with rat monoclonal antibodies specific for the indicated antigens (solid lines) or an irrelevant isotype control antibody (dashed lines), followed by a staining with a FITC conjugated anti-rat immunoglobulin monoclonal antibody. FS, forward scatter, SS, side scatter.


hβc
FIΔ
V449E
IL-3
GM-CSF
IL-3
no factor
IL-3
no factor
FSxSS
Mac-1
F4/80
Gr-1
side scatter
forward scatter
cell number
log 10 fluorescence

significantly different to cells grown in IL-3 with respect to forward and side scatter, and the expression of the Mac-1, F4/80 and Gr-1 myeloid cell surface markers (Figure 5.11).

Along with the previous studies relating to cell growth, viability and morphology, these studies demonstrate that FDB2 cells derived from factor-independent signalling of the constitutively active transmembrane hβc mutant, V449E, are analogous to FDB2 cells grown in IL-3. In contrast, cells derived from factor-independent signalling of the extracellular constitutively active hβc mutant, FIΔ, are equivalent to FDB2 cells cultured in GM-CSF in these respects, in that they undergo differentiation along the neutrophil and monocyte lineages. Hence, along with their previously documented ability to confer factor-independence on distinct spectra of haemopoietic cell types (see Chapter 3, Jenkins et al. (1995)), transmembrane and activated hβc mutants deliver functionally distinct signals to pluripotent haemopoietic progenitors, which must be taken into account when considering the biological effects of these mutants.

5.3 Discussion

In this chapter the function of two extracellular activated hβc mutants, FIΔ and I374N, and a transmembrane mutant, V449E, were compared in the multipotential murine myeloid cell lines, FDB1 and FDB2. It was found that when expressed in these cell lines, the extracellular mutants, FIΔ and I374N, induced granulocyte-macrophage differentiation of these cell lines in a manner analogous to mGM-CSF. Like the mGM-CSF signal, the differentiation signal transduced by the extracellular hβc mutants was overcome by proliferative signalling induced by mIL-3. In contrast, the transmembrane hβc mutant, V449E, lead to continuous proliferation of these cell lines in a manner analogous to mIL-3. Like mIL-3 signalling, the proliferative signal delivered by V449E was dominant over mGM-CSF-induced differentiation of these cell lines.

There are at least two possible explanations for the apparent signalling difference between transmembrane and extracellular hβc mutants in these cell lines. Firstly, it is possible that V449E delivers a stronger signal than the extracellular hβc mutants to these cell lines. It has been shown that low concentrations of mIL-3 lead to differentiation of the FDB2 cell line (see Section 4.2.6). It is possible that, in like

manner, the extracellular hβc mutants deliver a weaker form of the same signals induced by V449E signalling, leading to differentiation. However, this is unlikely to be the case, as the FLAG-tagged extracellular mutants were able to induce factor-independent proliferation of FDC-P1 cells at least as strongly as (FLAG)V449E (Figure 5.2), and there was no great difference in the expression levels of the FLAG-tagged extracellular mutants and (FLAG)V449E in the FDB1 and FDB2 cell lines (Figure 5.3).

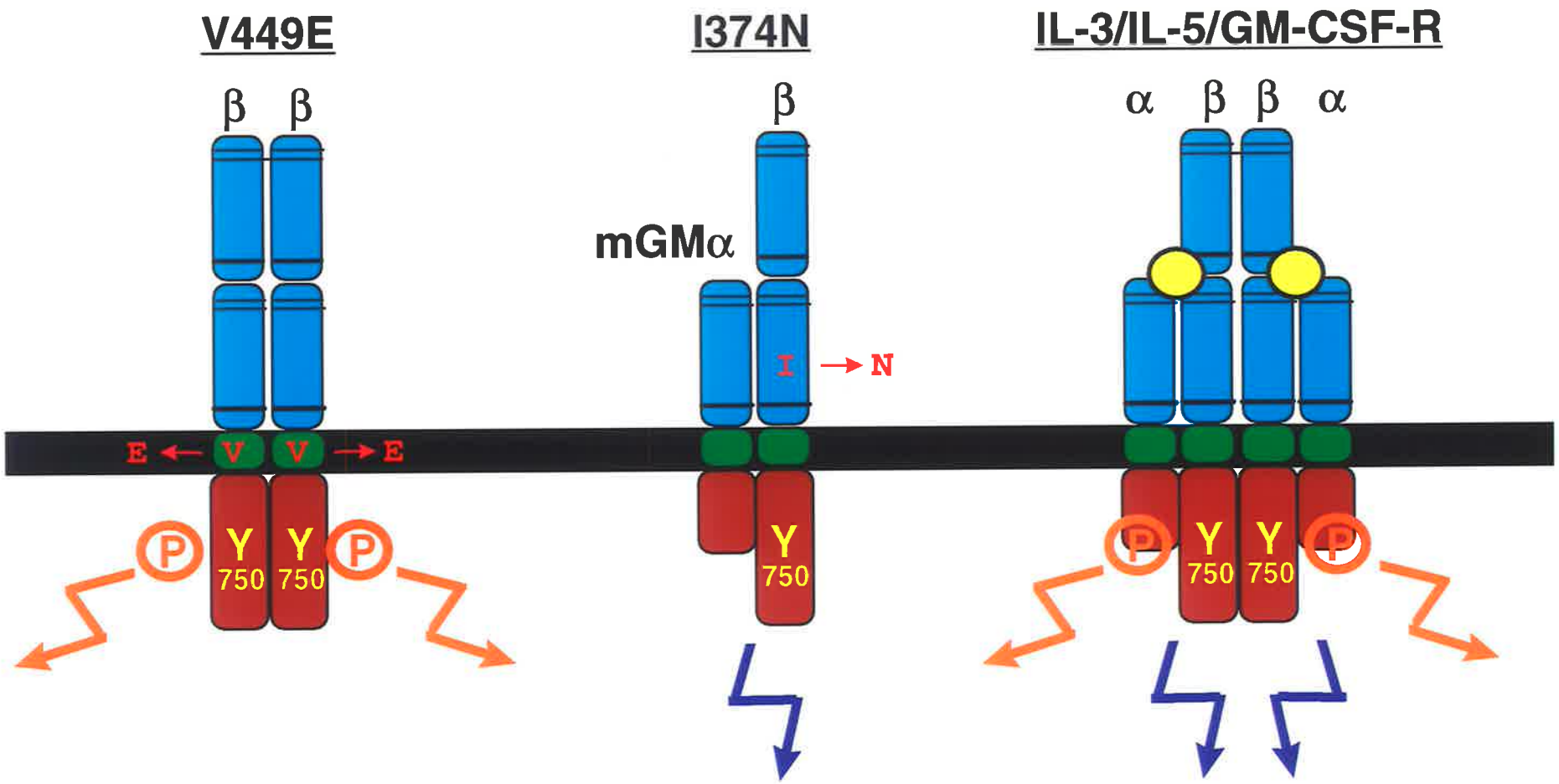
Rather, the different cellular outcomes elicited by transmembrane and extracellular hβc mutants in FDB cell lines is likely to be due to qualitative differences in signal transduction by these two classes of hβc mutant. This would be likely to result from distinct modes of activation, for which there is recent evidence. It has been demonstrated that factor-independent signalling of the I374N mutant requires the mGMRα subunit, whereas that of V449E does not (Jenkins et al, manuscript submitted). Furthermore, unlike V449E, I374N is deficient in receptor phosphorylation and phosphorylation of Shc and SHP2 proteins in the FDC-P1 cell line (Jenkins et al. (1998); T. Blake, personal communication). However this mutant is still capable of activating several signal transduction pathways normally activated by GM-CSF signalling including activation of JAK2, Stat5 and Erk1/2 (Jenkins et al., 1998), and induction of *c-myc* and *c-fos* transcription (T.Blake, personal communication). This implies that this mutant is only partially activated, even in a cell line which expresses mGMRα. Moreover, the V449E mutant fails to deliver a proliferative signal to the murine IL-2-dependent T cell line CTLL-2, unlike the wild-type human GM-CSF receptor (Jenkins et al., 1995). The reason for the apparent cell-type specificity of the V449E mutant is at present unknown. This finding implies that V449E is also deficient in some aspects of functional activation when compared to the wild-type human GM-CSF receptor, or requires cell-type specific molecules for function. In support of the former notion, this mutant has an unusually stringent requirement for the Tyrosine 750 residue of its intracellular domain for signalling. Mutation of this residue to Phenylalanine leads to almost complete abrogation of signalling through V449E in the FDC-P1 cell line, which is not the case when this residue is mutated in either the wild-type human GM-CSF receptor or the I374N mutant (T. Blake, personal communication).

Taking these results together with the findings detailed in this chapter, a model for activation of the GM-CSF, IL-3 and IL-5 receptors is proposed in Figure 5.12 which involves two modes of signalling. The V449E mutation is analogous to the V664E Valine to Glutamic Acid mutation in the transmembrane domain of the c-Neu receptor tyrosine kinase, which leads to ligand-independent aggregation and activation of this receptor (Peles et al., 1991; Bargmann and Weinberg, 1988; Weiner et al., 1989; Yarden, 1990). By analogy, it is likely that the V449E mutation acts by causing ligand-independent dimerisation of hβc. The model proposes that signalling through V449E is a result of hβc dimerisation in the absence of α chain association (Figure 5.12). This results in a partial signal involving phosphorylation of tyrosine 750 and possibly other tyrosine residues, which leads to phosphorylation of Shc and SHP2, and is predominantly one of self-renewal, leading to the immature phenotype of factor independent FDB1/2 cells and primary cells bearing this mutant (see Chapter 3) (shown in orange, Figure 5.12). In contrast, it is proposed that signalling through I374N is due to hβc functionally associating with an α subunit in the absence of functional hβc dimerisation (Figure 5.12). In the murine systems studied here the α chain responsible is mGMRα, however it has been shown that this mutant can also function in the murine megakaryoblastic cell line, UT-7, which expresses the hGMRα subunit, hence a similar mechanism is likely to occur in this line. Signalling through this heterodimeric complex does not require hβc phosphorylation or maximal phosphorylation of Shc or SHP2, and is predominantly a differentiation signal (shown in dark blue, Figure 5.12). However, since the extracellular activated hβc mutants induce factor-independent proliferation of FDC-P1 cells it is clear that this mode of signalling can also lead to extended proliferation in differentiation-arrested cell lines. It is therefore likely that both modes of signalling involve some elements of proliferation and differentiation signalling, but in different degrees of magnitude, leading to a bias towards proliferation or differentiation. Signalling through the wild-type IL-3/IL-5/GM-CSF receptors is proposed to involve both modes of signalling, leading to a balance of proliferative and differentiative signals (Figure 5.12).

A partial lack of differentiation signalling through the V449E mutant may explain the observation that this mutant causes abnormally extended proliferation of immature haemopoietic cells in culture (see Chapter 3). It would be of interest to determine

Figure 5.12. Model for cytokine receptor activation

A predicted stoichiometry of wild-type and mutant signalling complexes involving hβc is depicted. Extracellular regions are shown in light blue, transmembrane regions in green and intracellular regions in brown. The cell membrane is depicted as a black bar. Extracellular conserved cysteines and the conserved WSXWS box are shown in the extracellular region as black lines. Arrows indicate signalling. P- phosphate.


V449E
β β
E ← V V → E
P
Y 750
Y 750
P
I374N
β
mGMα
I → N
Y 750
IL-3/IL-5/GM-CSF-R
α β β α
P
Y 750
Y 750
P

whether co-expression of an extracellular mutant such as I374N would diminish this effect.

One unusual finding during the course of this study was the fact that whilst I374N was able to induce factor-independent survival and differentiation of FDB1/2 cells (Figure 5.1), (FLAG)I374N was not (Figures 5.5, 5.6, 5.8, 5.9). However, (FLAG)I374N was able to confer factor-independence on FDC-P1 cells (Figure 5.2), hence the defect appears to be specific to the FDB1/2 cell lines. The reason for this is unclear. It is possible that the FLAG tag leads to weaker signalling through I374N which is only apparent in the FDB1/2 cell lines, or that it interrupts some aspects of I374N signalling, but not others.

Finally, it is worth noting the potential utility of the system described in this Chapter in studying myeloid differentiation. Since the apparently proliferative and differentiative signals mediated by V449E and FIΔ/I374N in the FDB1/2 cell lines are presumably conveyed by the same signalling protein, hβc, further understanding of the molecular mechanism leading to the constitutive activity of these mutants, and of the signal transduction pathways utilised by transmembrane versus extracellular mutants, promises to provide valuable insights into the mechanics of myeloid differentiation.

6.1 Introduction

Whilst *in vitro* studies provide valuable information as to the effects of proteins on cell survival, proliferation and differentiation, a true assessment of a protein's oncogenic potential requires analysis of its effects *in vivo*. When choosing to study the effects of potentially dominant-acting proteins *in vivo* in mice, three techniques are available; systemic retroviral infection, transgenesis and bone marrow reconstitution. Bone marrow reconstitution is a technique pioneered by Williams et al. (1984). The technique involves lethal irradiation of "recipient" mice, which achieves near-complete depletion of their haemopoietic system. These mice are then rescued by injection of 5-FU-treated bone marrow from "donor" mice. Stem cells from this donor bone marrow will then go on to repopulate the entire haemopoietic system of the adult mouse. Genetic alteration of the donor bone marrow by retroviral infection can thus achieve expression of genes of interest in the entire haemopoietic system of the reconstituted mouse. This procedure has been used to demonstrate the oncogenic potential of a number of genes *in vivo*, for example the v-*src* oncogene and the gene fusions *bcr-abl* and *E2A-PBX* (Miller and Symonds, 1993; Kelliher et al., 1990; Keller and Wagner, 1989; Kamps and Baltimore, 1993; Elefanty et al., 1990; Daley et al., 1990). Furthermore, the biological effects of overexpression of a number of growth factors, including GM-CSF and IL-3, have been studied using this system (Johnson et al., 1989; Chang et al., 1989). Moreover, this technique has been used previously to study the biological effects of constitutively active forms of cellular growth factor receptors such as *v-fms* (Heard et al., 1987) and v-*erb*-B (von Ruden et al., 1992). Bone marrow reconstitution has advantages over transgenesis and retroviral infection when studying haemopoiesis in that it gives specificity of expression, with the expression of transduced genes being restricted to the haemopoietic system of the mouse. Furthermore, unlike infection with replication-competent retroviruses, this technique does not in itself lead to tumour formation, which simplifies results when dealing with transforming genes. Moreover the technique is simpler and more rapid to perform than transgenesis and several genes can be studied in parallel. For these

reasons I chose to study the *in vivo* effects of activated hβc mutants in mice using the technique of bone marrow reconstitution. The results of that study are presented here.

6.2 Results

6.2.1 Generation of bone marrow reconstituted mice containing constitutively active mutant hβc proteins

To express wild-type and mutant hβc proteins in the haemopoietic system of mice, 5-FU-treated murine bone marrow cells were infected with the RufNeo retroviral vector containing wild-type hβc and each of the three constitutively active hβc mutants, and injected into lethally irradiated recipient mice (as described in Section 2.13.4). As a control, mice were injected with cells infected with RufNeo. Mice were maintained in sterile conditions, in which most mice survived for over one year and were thus considered to be long-term reconstituted. For simplicity, bone marrow reconstituted mice will be referred to by the cDNA contained in the retrovirus infecting the reconstituting cells eg. 'hβc mice' for mice reconstituted with RufNeo-hβc-infected bone marrow cells. Mice reconstituted with RufNeo-infected bone marrow cells will be referred to as 'RufNeo mice'.

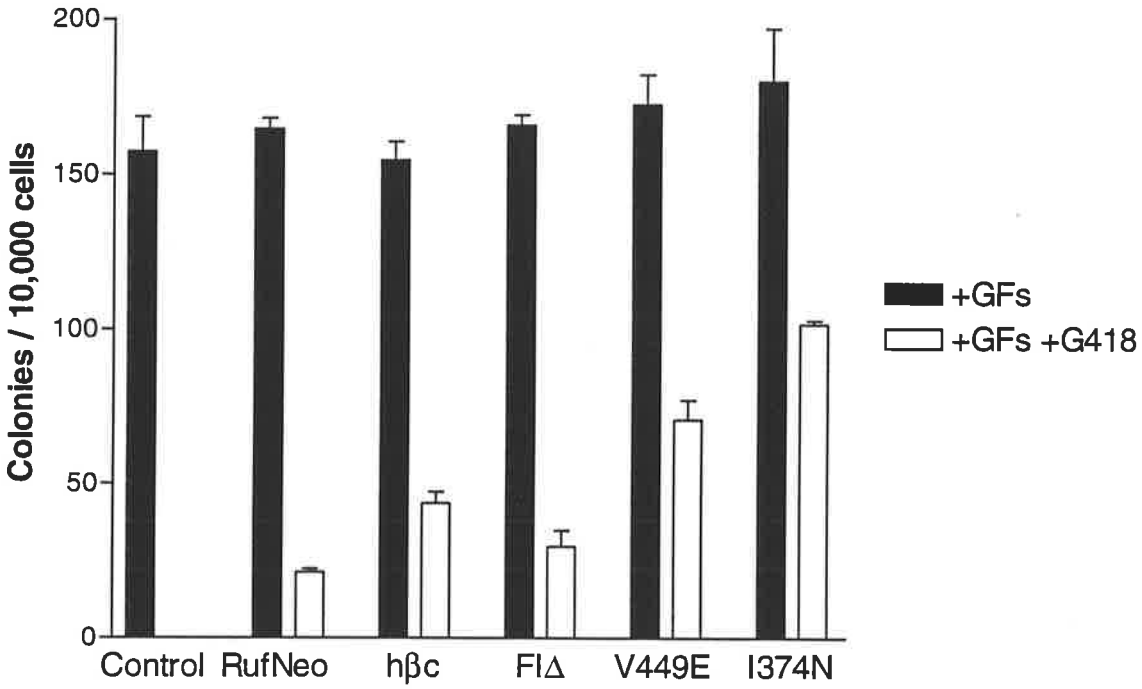
To assess the infection of 5-FU-treated bone marrow progenitors, samples of the infected donor bone marrow populations used for reconstitution were plated in methyl-cellulose and the frequency of infection of haemopoietic progenitors was determined by assessing resistance to geneticin (G418) which is transduced by the RufNeo retrovirus. Using this method it was determined that the efficiency of infection of haemopoietic progenitors ranged between 13% and 56% for each of the retrovirus constructs used (Figure 6.1).

6.2.2 Quantification of haemopoietic reconstitution

In order to assess the haemopoietic reconstitution of recipient mice quantitatively, donor and recipient mice used were of two congenic strains expressing antigenically distinct forms of the leukocyte common antigen, Ly5. Hence donor (BL/6) mice expressed the $Ly5^a$ isotype whereas recipient (B6.SJL) mice expressed the $Ly5^b$ isotype. Using antibodies specific for each isotype it is possible to estimate the proportion of donor and recipient cells in the peripheral white blood cell populations

Figure 6.1. Infection of haemopoietic progenitors from 5-FU treated bone marrow.

Following mock infection (Control), infection with RufNeo and infection with RufNeo containing the indicated hβc subunits, 10^4 5-FU treated bone marrow cells were cultured in 1 ml methyl-cellulose. Where indicated growth factors (GFs; 500 U/ml mIL-3, 10 ng/ml hIL-6, 50 ng/ml mSCF) and 1 mg/ml G418 were added. At the seventh day of culture colonies consisting of ≥ 50 cells were scored with the aid of a dissecting microscope. Results are representative of five separate experiments.


Colonies / 10,000 cells
200
150
100
50
0
Control
RufNeo
hβc
FlΔ
V449E
I374N
+GFs
+GFs +G418

of reconstituted mice. Therefore, white blood cells were purified from the peripheral blood of reconstituted mice at 4-8 weeks post-reconstitution, stained with antibodies directed to the $Ly5^a$ and $Ly5^b$ isoforms, and flow cytometric analyses performed. To achieve homogeneous staining of the cells, the flow cytometric analysis was gated to detect lymphocytes only, which constitute approximately 80% of the white blood cells of normal mice, and which should serve as a reliable indicator of the reconstitution of the entire haemopoietic system. Using this approach it was determined that on average 84% of peripheral blood lymphocytes of reconstituted mice were of donor origin, and that the percentages of reconstitution were similar for all groups (Table 6.1).

6.2.3 Detection of integrated hβc cDNAs in the genomic DNA of bone marrow reconstituted mice

Having confirmed that reconstituted mice contained a majority of donor-derived cells, it was necessary to determine whether the haemopoietic system of reconstituted mice contained integrated retroviral constructs containing hβc cDNAs. To achieve this genomic DNA was isolated from peripheral white blood cells of reconstituted mice and PCR performed using hβc-specific primers. As positive controls, PCR was performed on the same genomic DNA samples using primers specific for an endogenous cellular gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). As shown in Figure 6.2, all genomic DNA samples gave PCR products with GAPDH-specific primers, indicating that genomic DNA was present. Neither a negative control nor genomic DNA samples derived from the blood of 5 RufNeo mice gave PCR products using hβc-specific primers, as expected. However, 3 of 3 hβc mice, 3 of 5 FIΔ mice, 4 of 6 V449E mice and 2 of 3 I374N mice gave products with hβc-specific primers, indicating that hβc cDNAs were integrated in the peripheral blood of these mice (Figure 6.2).

6.2.4 Haemopoietic disorders induced by hβc expression in mice

To monitor the reconstituted mice for haemopoietic abnormalities, mice were bled monthly and their peripheral blood analysed for perturbations in white blood cell, erythrocyte and platelet levels and white blood cell profiles. Mice were also monitored at least weekly for any signs of disease and at first signs of illness the mice were

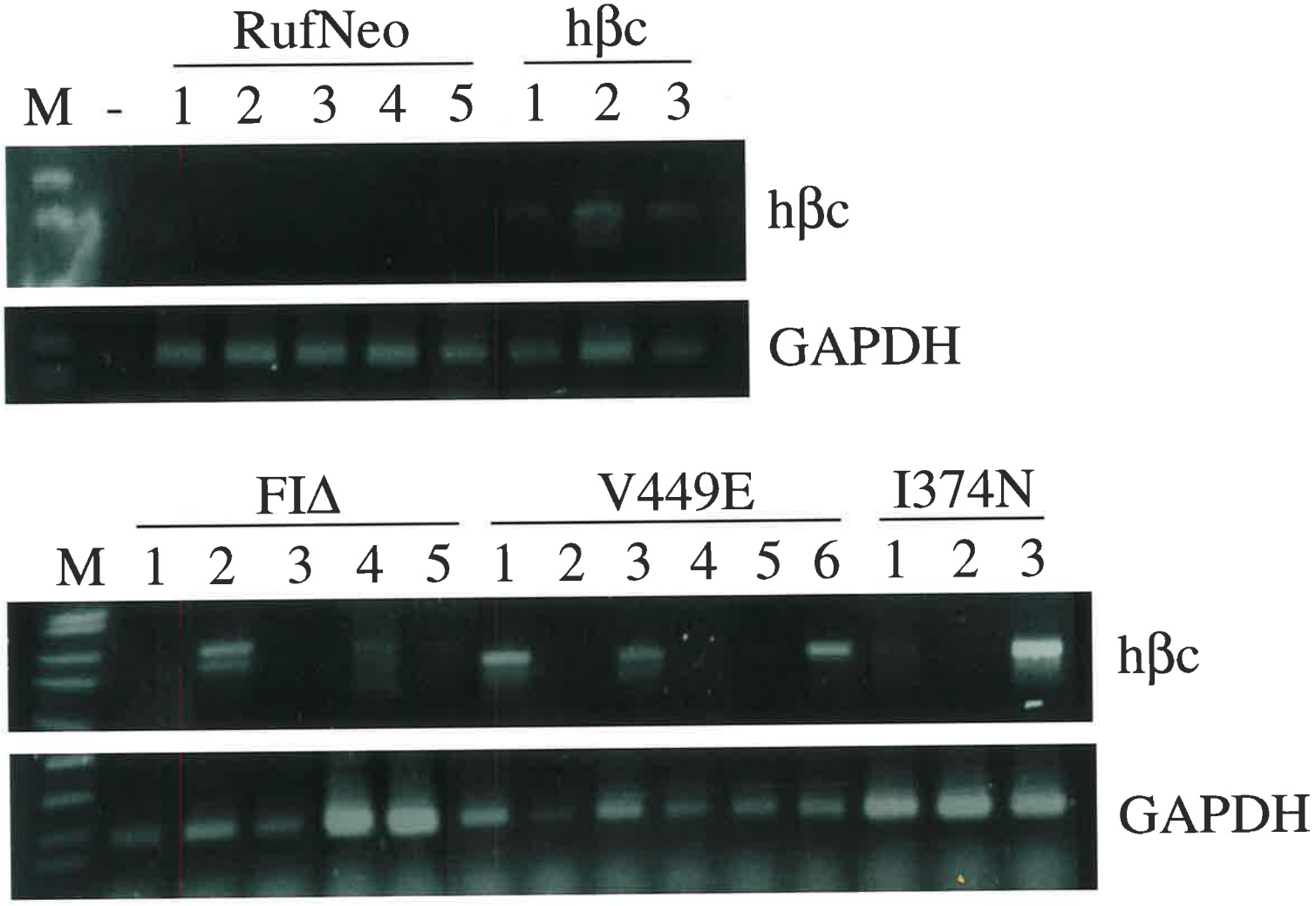
Table 6.1. Quantification of reconstitution of bone marrow reconstituted mice.

Group	Number	Ly5.1+ (%)[a]	Ly5.2+ (%)
BL/6 (donor)	1	98.7	1.0
B6.SJL (recipient)	1	0.9	99.2
RufNeo	7	87.4 ± 8.1	12.4 ± 4.6
hβc	7	87.6 ± 8.3	9.7 ± 3.4
FIΔ	6	80.5 ± 16.2	16.1 ± 13.8
V449E	17	87.3 ± 9.3	11.9 ± 9.1
I374N	6	72.1 ± 18.3	19.5 ± 9.8

[a]Percentage of peripheral blood lymphocytes expressing Ly5.1

Figure 6.2. Integration of wild-type and mutant hβc cDNAs in the genomic DNA of bone marrow reconstituted mice.

Genomic DNA was derived from the peripheral white blood cells of the indicated bone-marrow reconstituted mice and used in PCR reactions using primers directed against a central 1091 bp fragment of hβc (marked hβc) or against an approximately 400 bp fragment of the endogenous gluteraldehyde-3-phosphate dehydrogenase gene (marked GAPDH). Lanes marked '-' contain no DNA. Lanes marked 'M' contain DNA size standards (SPP-1 phage DNA digested with *Eco*RI).


RufNeo
hβc
M - 1 2 3 4 5 1 2 3
hβc
GAPDH
FIΔ
V449E
I374N
M 1 2 3 4 5 1 2 3 4 5 6 1 2 3
hβc
GAPDH

sacrificed and their haemopoietic organs analysed. As shown in Table 6.2, none of seven RufNeo mice and none of nine hβc mice developed haemopoietic disorders and/or illness within an 8 month monitoring period. However, five of nine FIΔ mice, five of seventeen V449E mice and six of six I374N mice became ill during this period, all of which experienced haemopoietic disorders marked by abnormalities in one or more of the above peripheral blood attributes (see below). Mice experiencing haemopoietic disorders to the point of becoming moribund will be referred to as "diseased" mice.

Reconstituted mice were of similar weight to non-reconstituted female B6.SJL control mouse (Figure 6.3). There was no significant alteration in the weight of diseased mice at the time of sacrifice.

Of the diseased mice, three out of five FIΔ mice, five out of six V449E mice and none of six I374N mice showed elevated white blood cells (Figure 6.4). Those FIΔ mice showing elevated white blood cells did so over a period of several months, however white blood cell elevations in V449E mice were acute in onset (Figure 6.5). Analysis of blood smears and white blood cell preparations revealed a dysregulation of the proportions of the various haemopoietic lineages comprising the peripheral leukocytes of all diseased mice, including those with normal white blood cell levels (data not shown). All diseased FIΔ and I374N mice displayed an elevated proportion of neutrophils in the blood, with corresponding decreases in the proportion of peripheral blood lymphocytes (Figure 6.6, Table 6.3). Diseased I374N mice showed a significant increase in the proportion of monocytes in the blood (Student's t-test, $p<0.05$). Whilst the total number of monocytes was elevated in 3 of 5 diseased FIΔ mice, the overall increase in this group was not statistically significant (data not shown). Peripheral blood from diseased V449E mice displayed an altogether distinct white blood cell profile. Whilst the proportion of neutrophils in the blood of these mice was elevated in some cases, blast cells were also present in the blood of all five diseased mice (Figure 6.6, Table 6.3). The presence of blast cells in the peripheral blood is reminiscent of human leukaemias such as acute myeloid leukaemia.

All five diseased FIΔ mice, and three of six diseased I374N mice displayed peripheral blood erythrocytosis as compared to control RufNeo and hβc mice (Figure

Table 6.2. Incidence of haemopoietic disorders in bone marrow reconstituted mice.

Group	Incidence[a]
RufNeo	0/7
hβc	0/9
FIΔ	5/9
V449E	5/17
I374N	6/6

[a]number of mice developing haemopoietic disorders within 8 months/number of successfully reconstituted mice.

Figure 6.3. Weight of control and bone marrow reconstituted mice.

(**A**) Mean and (**B**) individual weights of control and bone marrow reconstituted mice at the time of sacrifice. For mice containing hβc mutants (FIΔ, V449E, I374N) only mice experiencing haemopoietic disorders at the time of sacrifice are shown. Error bars show one standard deviation of the mean. Dashed lines span the range containing the control (B6.SJL, RufNeo and hβc) animals.

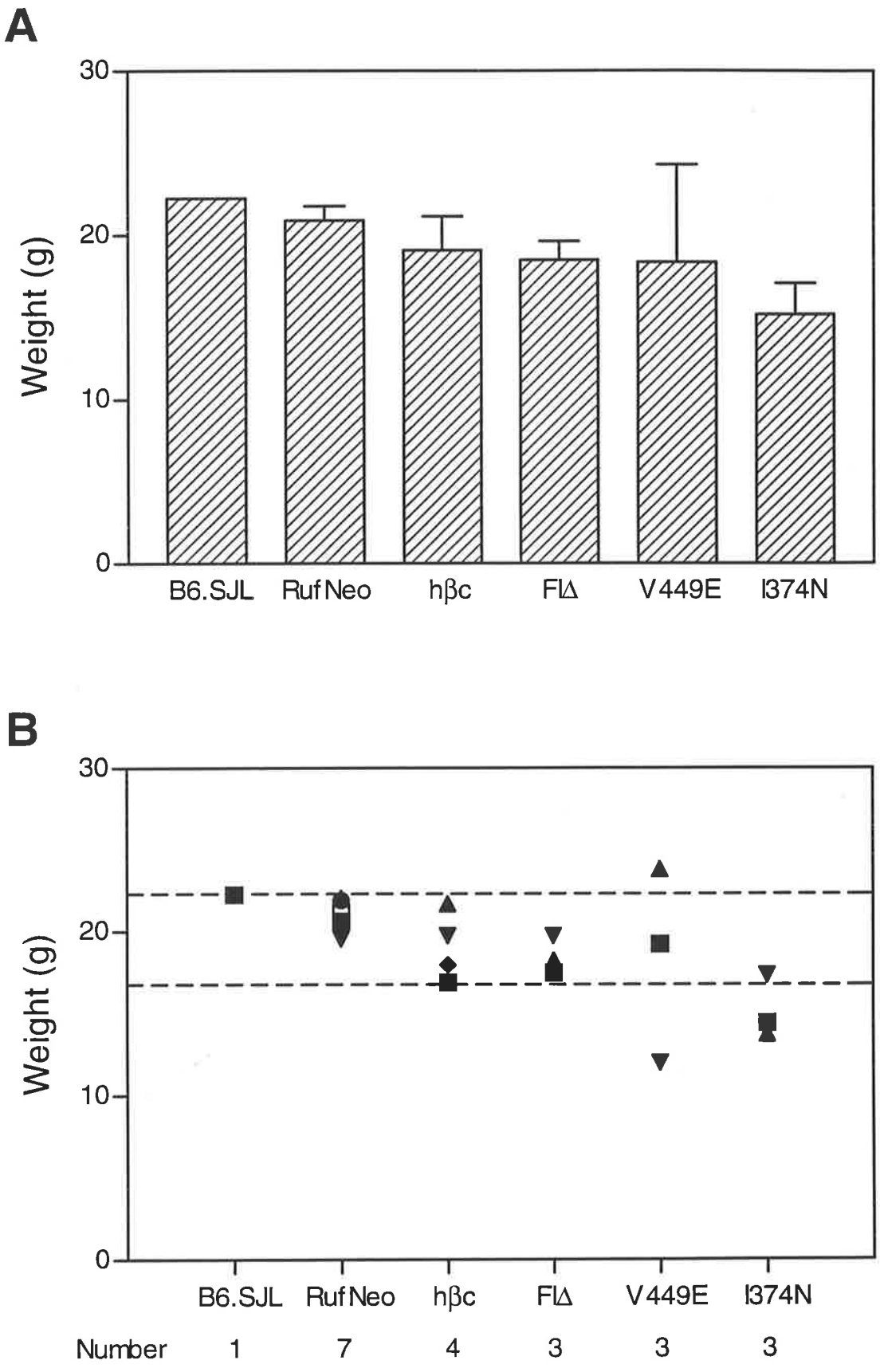

A
30
20
10
0
Weight (g)
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
B
30
20
10
0
Weight (g)
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
Number
1
7
4
3
3
3

Figure 6.4. White blood cell levels of control and bone marrow reconstituted mice.

(**A**) Mean and (**B**) individual white blood cell counts of control and bone marrow reconstituted mice at the time of sacrifice. For mice containing hβc mutants (FIΔ, V449E, I374N) only mice showing haemopoietic disorders at the time of sacrifice are shown. Error bars show one standard deviation of the mean. The dashed line shows the maximum value of control (BL/6, B6.SJJL, RufNeo and hβc) animals.

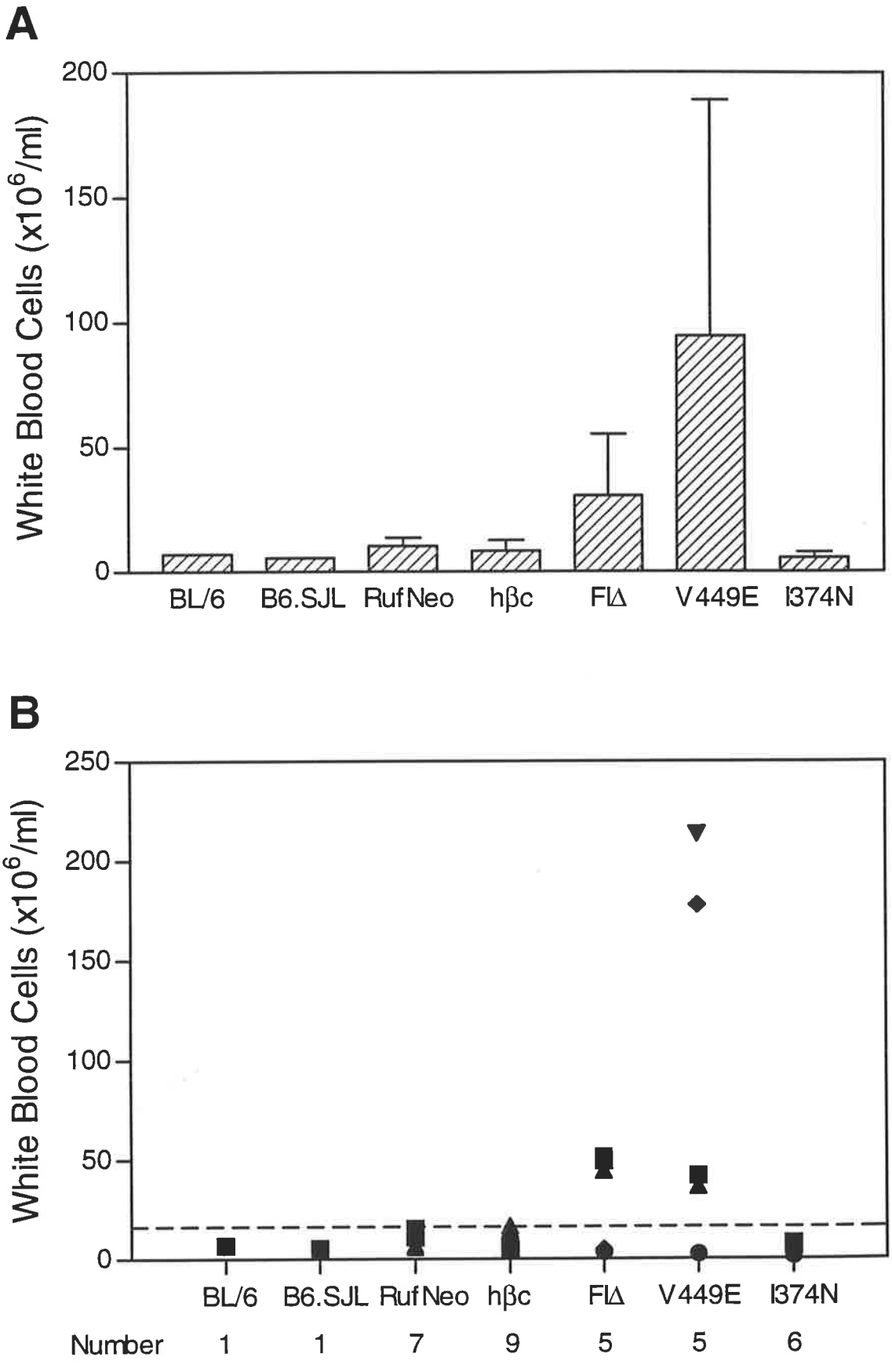

A
White Blood Cells (x10^6/ml)
200
150
100
50
0
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
B
White Blood Cells (x10^6/ml)
250
200
150
100
50
0
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
Number
1
1
7
9
5
5
6

Figure 6.5. Timecourse of white blood cell levels of bone marrow reconstituted mice.

Each line represents serial leukocyte counts from a single animal. The dashed line shows the maximum count reached by control (RufNeo and hβc) mice. The '+' symbol indicates the timepoint at which a mouse became moribund and was sacrificed.

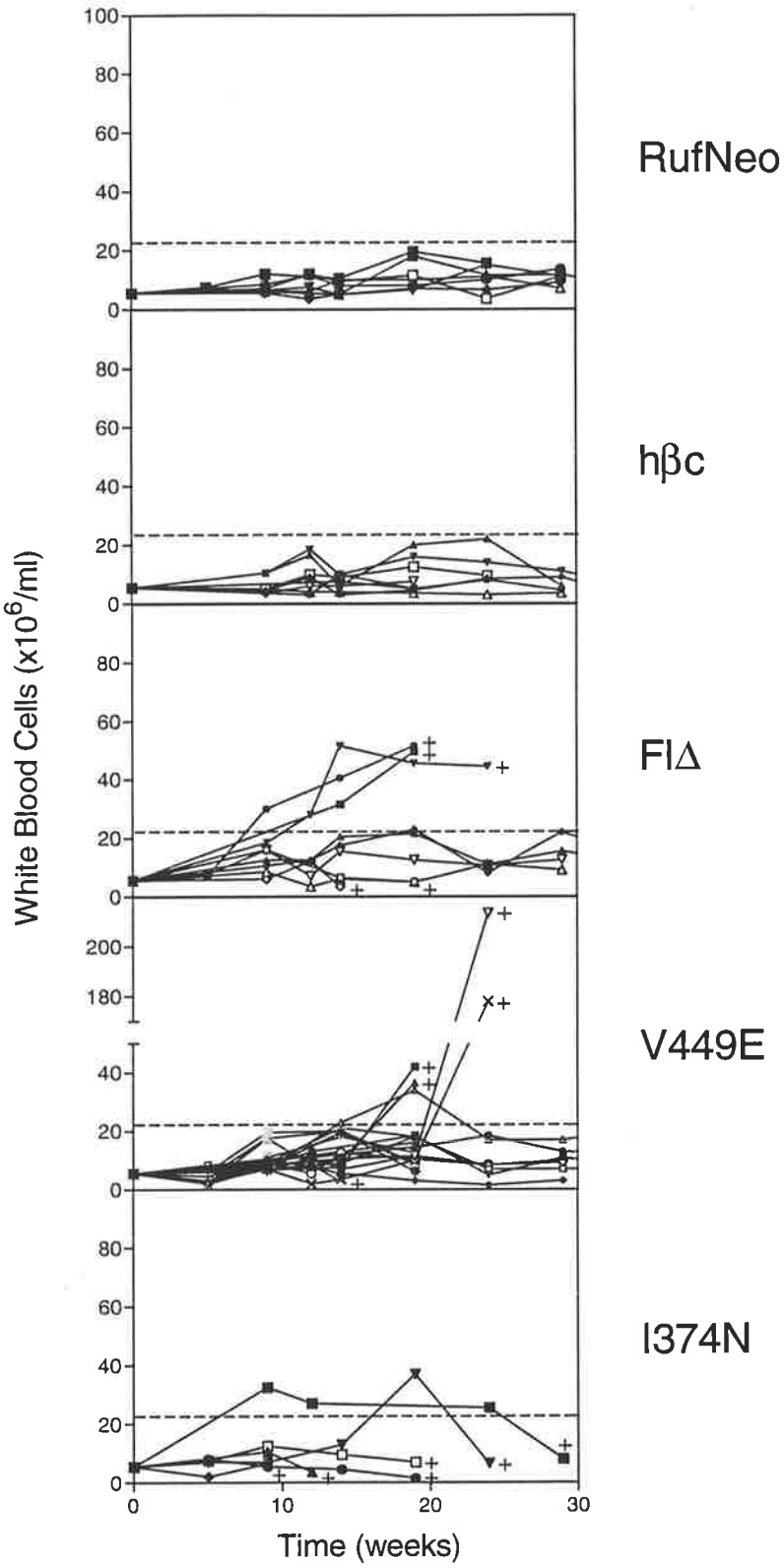

White Blood Cells (x10^6/ml)
Time (weeks)
RufNeo
hβc
FlΔ
V449E
I374N

Figure 6.6. Morphology of white blood cells derived from bone marrow reconstituted mice.

White blood cells were purified from the peripheral blood of bone marrow reconstituted mice as described in Section 2.12.2.5., cytocentrifuged and Wright-Giemsa stained. Representative lymphocyte (l), neutrophil (n), monocyte (m) and blast (b) cells are indicated with arrows. Photographs are at 350 × magnification.

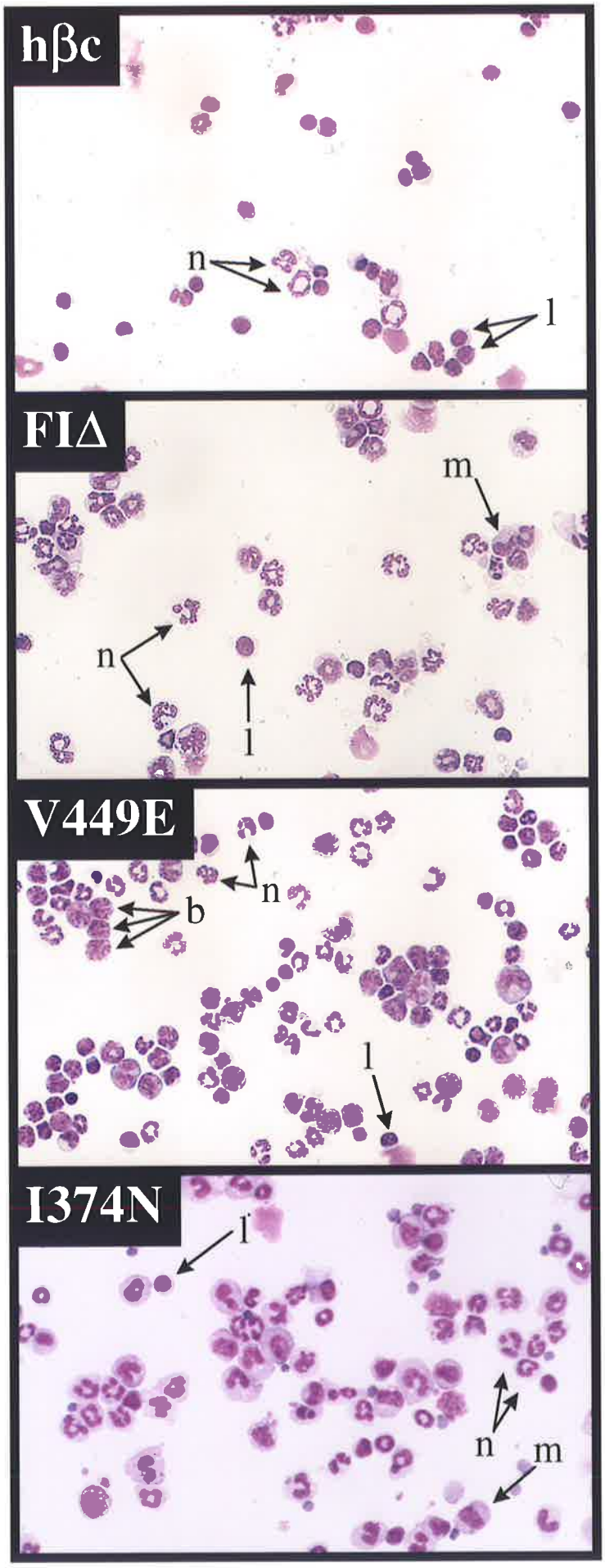

hβc
n
l
FIΔ
m
n
l
V449E
n
b
l
I374N
l
n
m

Table 6.3. Differential cell counts of haemopoietic cells derived from bone marrow reconstituted mice.

Group	Number	Cell types (%)							
		Blast	Myelocyte	Erythroid	Lymphoid	Eosinophil	Neutrophil	Basophil	Monocyte
Peripheral Blood [a]									
B6.SJL female	1	-	-	-	80	1	17	-	2
RufNeo	7	-	-	-	84 ± 6	2 ± 2	13 ± 5	-	2 ± 1
hβc	6	-	-	-	75 ± 6	1 ± 2	21 ± 5	-	3 ± 1
V449E	5	44 ± 26	5 ± 8	1 ± 2	7 ± 7	-	40 ± 21	-	2 ± 2
FIΔ	4	-	0 ± 1	7 ± 15	13 ± 3	2 ± 1	73 ± 13	-	5 ± 2
I374N	4	-	1 ± 2	12 ± 10	14 ± 8	1 ± 1	60 ± 12	-	12 ± 6
Bone Marrow									
B6.SJL female	1	2	4	39	19	2	30	-	4
RufNeo	7	3 ± 1	8 ± 3	29 ± 4	16 ± 3	4 ± 2	35 ± 5	-	4 ± 3
hβc	6	2 ± 1	7 ± 3	34 ± 8	12 ± 7	1 ± 1	40 ± 9	-	3 ± 1
V449E	5	59 ± 30	11 ± 5	7 ± 5	2 ± 1	0 ± 1	19 ± 24	-	1 ± 1
FIΔ	4	1 ± 1	9 ± 4	21 ± 15	2 ± 1	2 ± 1	64 ± 19	-	1 ± 1
I374N	4	1 ± 1	6 ± 6	38 ± 19	2 ± 1	1 ± 2	42 ± 14	1 ± 2	9 ± 5
Spleen									
B6.SJL female	1	-	1	3	91	-	3	-	3
RufNeo	7	-	-	6 ± 3	91 ± 4	-	2 ± 1	-	1 ± 1
hβc	6	0 ± 1	1 ± 1	12 ± 5	83 ± 6	-	2 ± 1	-	2 ± 1
V449E	5	61 ± 33	6 ± 3	14 ± 12	7 ± 10	1 ± 2	10 ± 15	-	1 ± 1
FIΔ	4	-	2 ± 1	75 ± 4	6 ± 5	1 ± 1	15 ± 3	-	1 ± 1
I374N	4	1 ± 1	10 ± 20	70 ± 30	7 ± 9	0 ± 1	10 ± 11	-	3 ± 2

Single cell suspensions were prepared from the indicated organs, cytocentrifuged, Wright-Giemsa stained and the cell types determined microscopically. Numbers are mean ± Standard Deviation of the percentages of at least 200 cells scored.

[a] Nucleated cell preparations prepared from peripheral blood were used.

6.7). In mice displaying erythrocytosis peripheral blood haemoglobin and packed cell volume levels were correspondingly increased (Figure 6.8, Figure 6.9). This indicates that the erythrocytes in these mice were of normal volume and haemoglobinisation. In contrast, none of five diseased V449E mice displayed elevated peripheral blood erythrocytes and in fact three were anaemic, with correspondingly decreased haemoglobin levels (Figure 6.7, 6.8). The packed cell volumes of two of these mice were correspondingly decreased, however that of one mouse fell within the normal range (represented by ◆; Figure 6.9B). This is likely to be due to the extremely high white blood cell level of this mouse increasing the packed cell volume of the blood, which is usually determined primarily by blood erythrocyte levels (Figure 6.4B). In support of this notion, another V449E mouse with extremely high white blood cell levels showed a packed cell volume higher than would be predicted from its erythrocyte and haemoglobin levels (represented by ▼; Figures 6.4, 6.7-6.9). Thus it can be reasonably concluded that the haemoglobinisation and volume of the erythrocytes in diseased V449E mice was normal. Anaemia is a common feature of acute leukaemia, and has been attributed to the perturbation of erythropoiesis in the bone marrow by the presence of leukaemic blasts at this site (Miller, 1991).

Elevations in erythrocyte levels in FIΔ and I374N mice were seen at the earliest timepoint measured - 5 weeks post-reconstitution (Figure 6.10). In fact, at early timepoints (9 weeks post-reconstitution or earlier) all reconstituted FIΔ and I374N mice showed erythrocytosis and concomitant elevations in peripheral blood haemoglobin and packed cell volume (Figure 6.10, data not shown), including those which did not later develop disease. At later timepoints these levels either persisted and in some cases continued to rise or returned to normal levels. In FIΔ mice increased erythrocyte levels persisted in those mice which went on to develop disease, however erythrocyte counts returned to normal levels in those mice which did not. Similarly, in I374N mice, erythrocyte levels would either continue to rise or subsequently decline, however mice with declining erythrocyte levels also went on to develop haemopoietic disorders and illness. In contrast, the erythrocyte levels of V449E mice remained in the normal range prior to an acute decline in those mice developing anaemia associated with a leukaemia-like syndrome (Figures 6.10).

Figure 6.7. Red blood cell levels of control and bone marrow reconstituted mice.

(**A**) Mean and (**B**) individual peripheral blood erythrocyte counts of control and bone marrow reconstituted mice at the time of sacrifice. For mice containing hβc mutants (FIΔ, V449E, I374N) only mice experiencing haemopoietic disorders at the time of sacrifice are shown. Error bars show one standard deviation of the mean. Dashed lines span the range containing the control (BL/6, B6.SJL, RufNeo and hβc) animals.

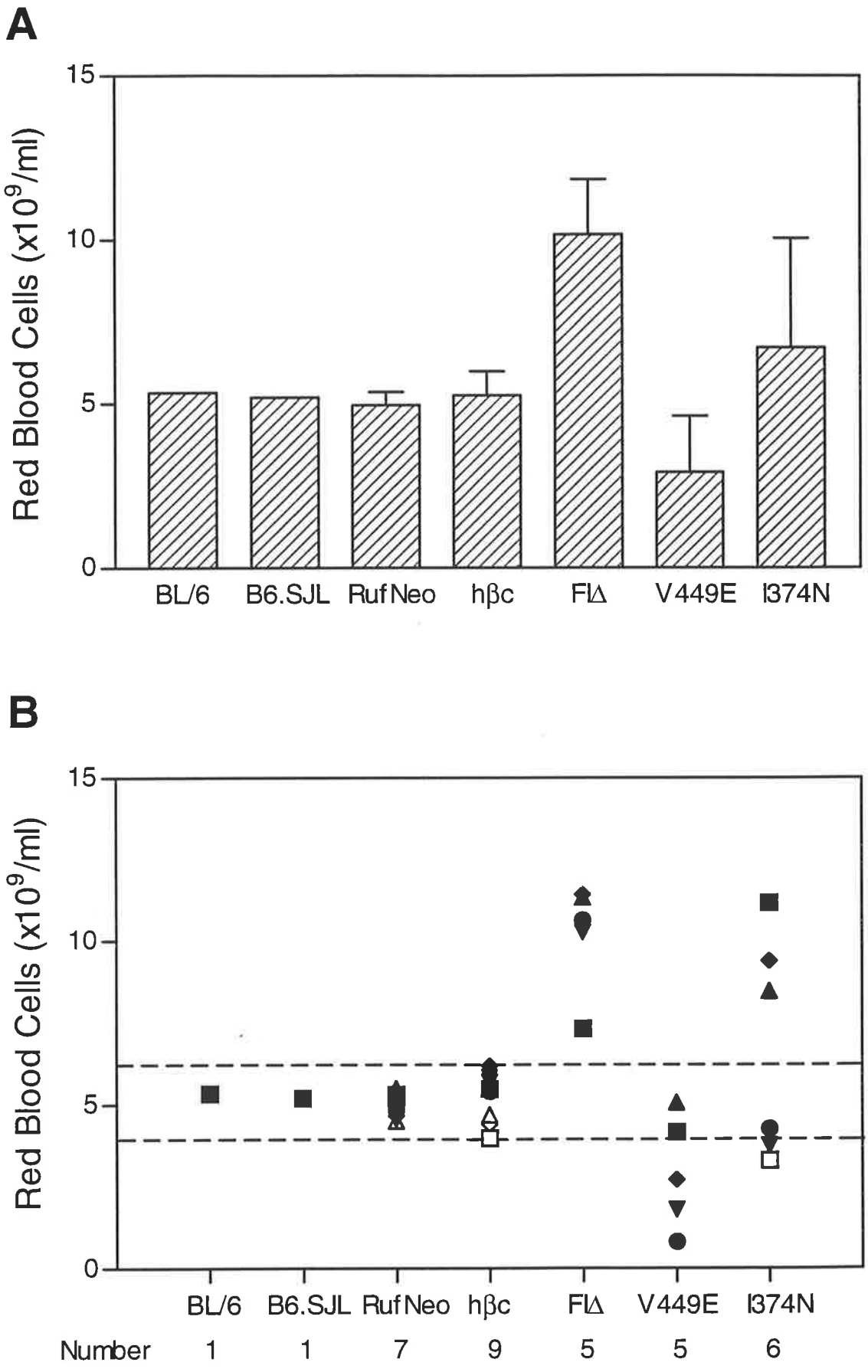

A
15
10
5
0
Red Blood Cells (x10^9/ml)
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
B
15
10
5
0
Red Blood Cells (x10^9/ml)
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
Number
1
1
7
9
5
5
6

Figure 6.8. Peripheral blood haemoglobin levels of control and bone marrow reconstituted mice.

(**A**) Mean and (**B**) individual peripheral blood haemoglobin levels of control and bone marrow reconstituted mice at the time of sacrifice. For mice containing hβc mutants (FIΔ, V449E, I374N) only mice experiencing haemopoietic disorders at the time of sacrifice are shown. Error bars show one standard deviation of the mean. Dashed lines span the range containing the control (BL/6, B6.SJL, RufNeo and hβc) animals.

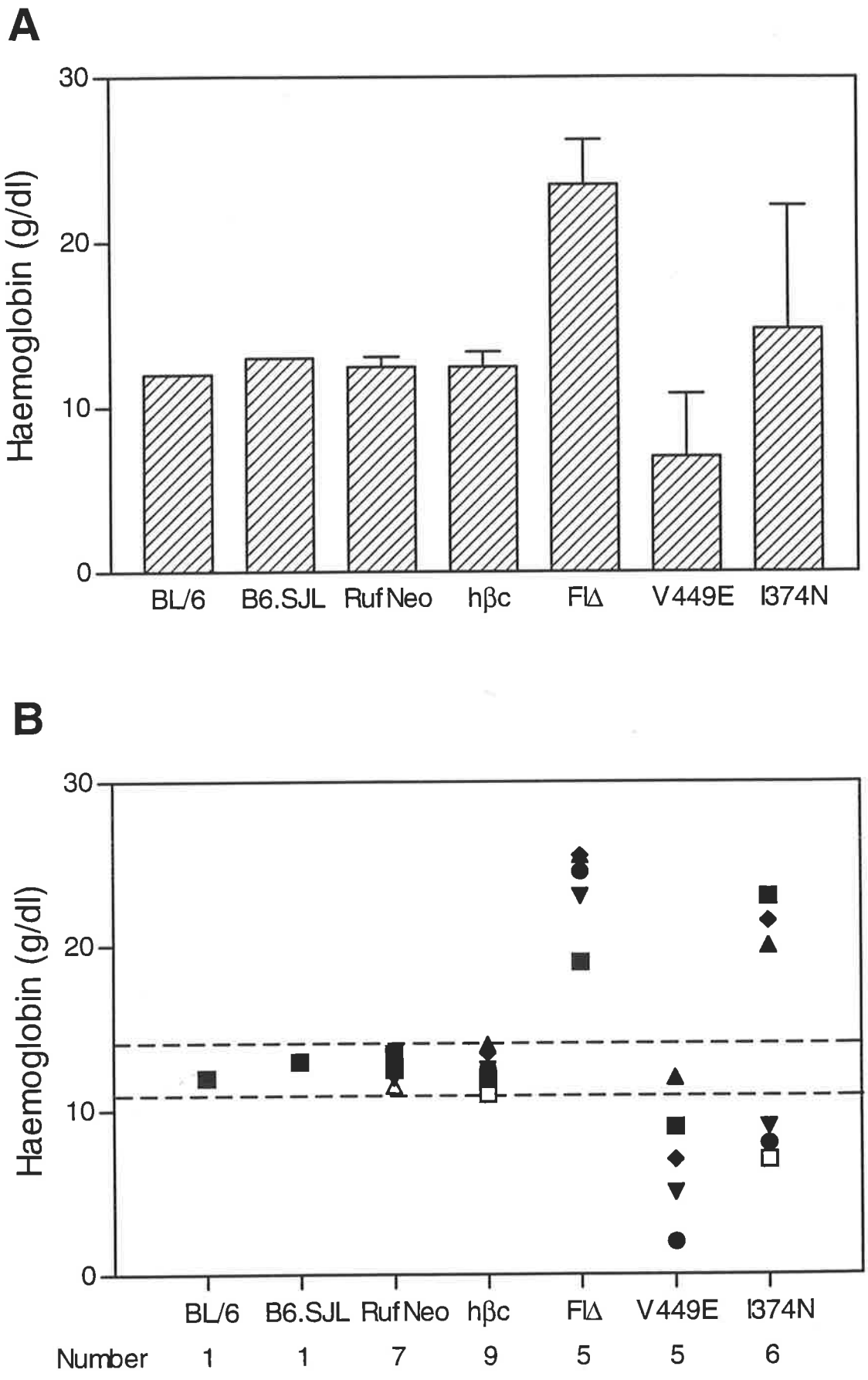

A
Haemoglobin (g/dl)
30
20
10
0
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
B
Haemoglobin (g/dl)
30
20
10
0
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
Number
1
1
7
9
5
5
6

Figure 6.9. Packed cell volume of peripheral blood from control and bone marrow reconstituted mice.

(**A**) Mean and (**B**) individual peripheral blood packed cell volumes of control and bone marrow reconstituted mice at the time of sacrifice. For mice containing hβc mutants (FIΔ, V449E, I374N) only mice experiencing haemopoietic disorders at the time of sacrifice are shown. Error bars show one standard deviation of the mean. Dashed lines span the range containing the control (BL/6, B6.SJL, RufNeo and hβc) animals.

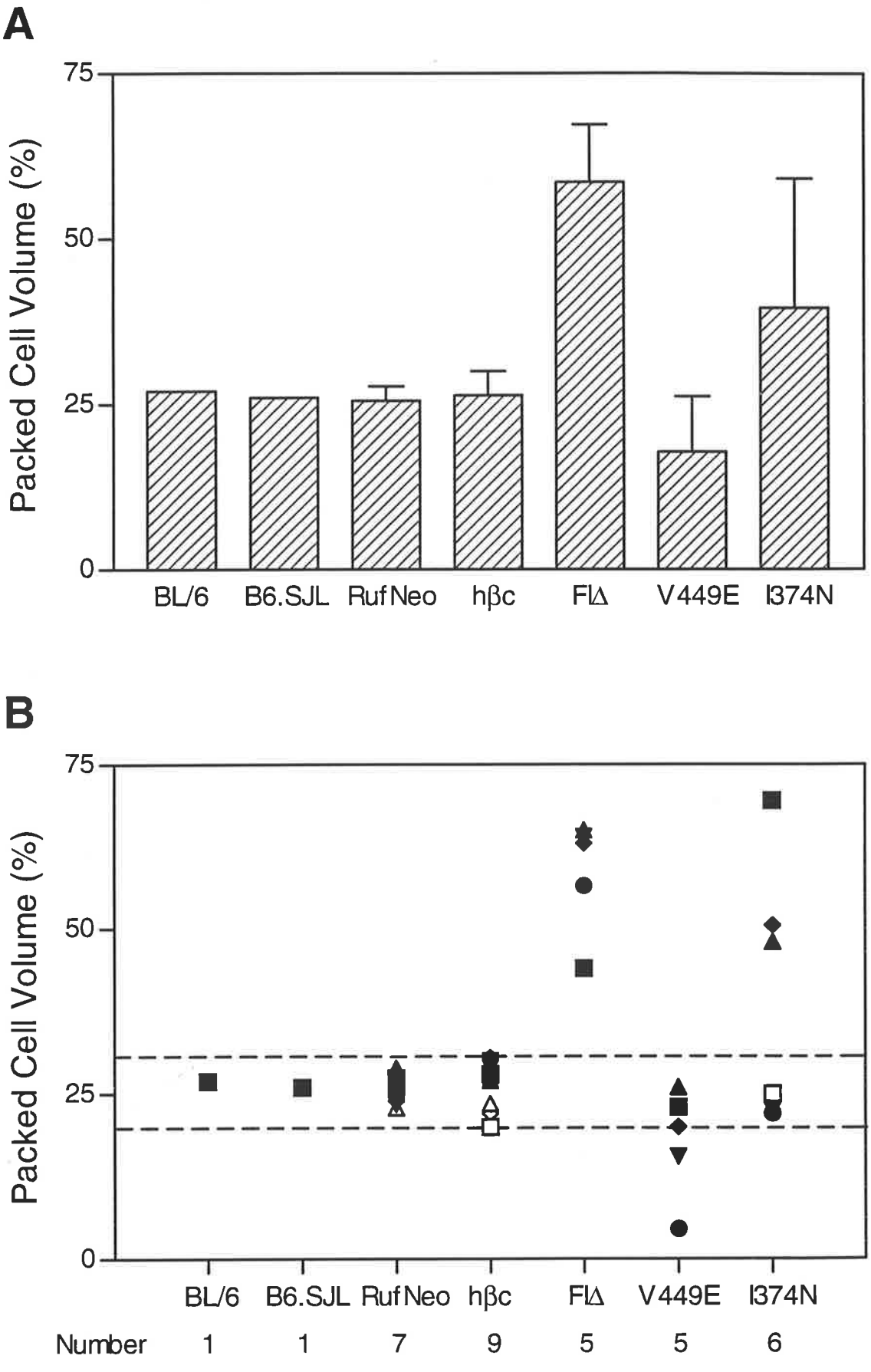
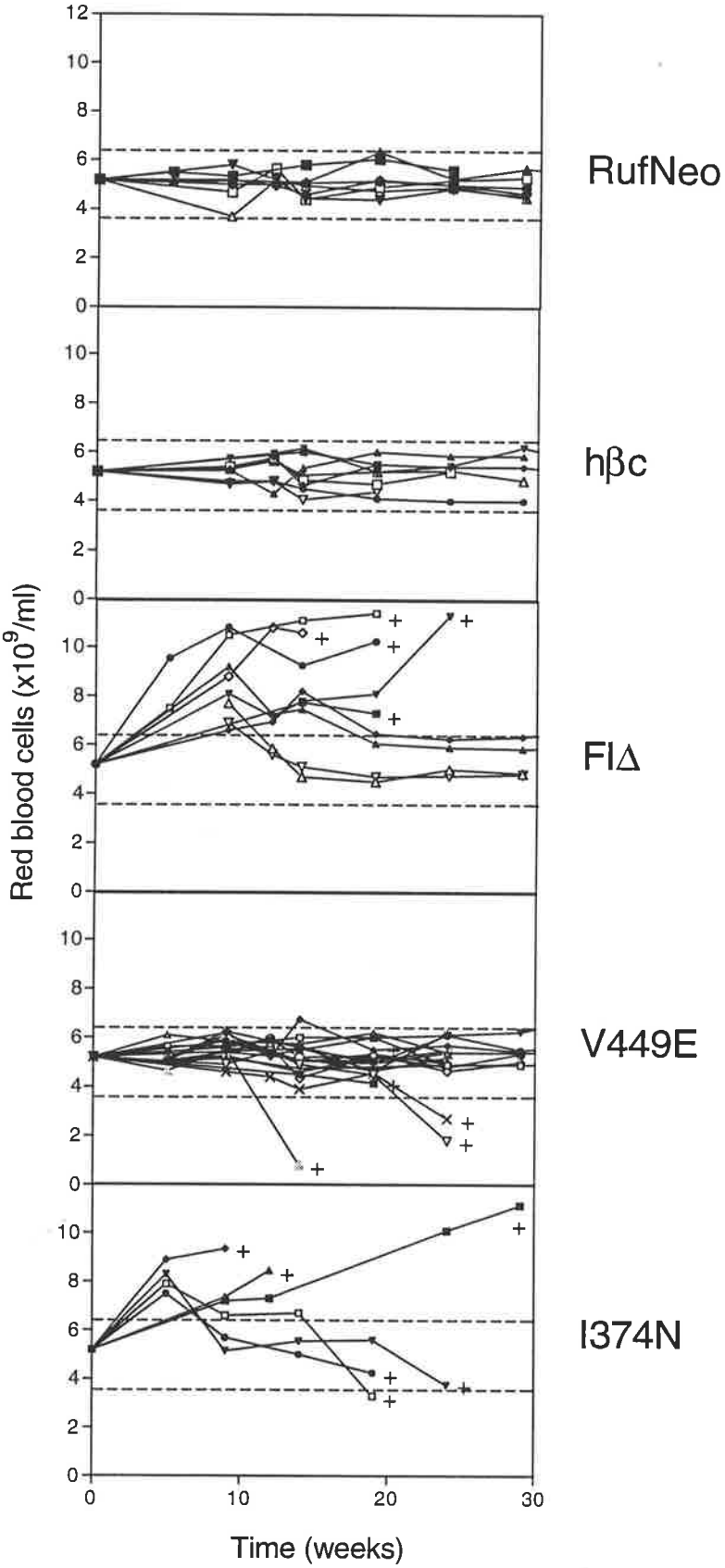

A
Packed Cell Volume (%)
75
50
25
0
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
B
Packed Cell Volume (%)
75
50
25
0
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
Number
1
1
7
9
5
5
6

Figure 6.10. Timecourse of peripheral red blood cell levels of bone marrow reconstituted mice.

Each line represents serial erythrocyte counts from a single animal. The dashed lines span the range containing the control (RufNeo and hβc) mice. The '+' symbol indicates the timepoint at which a mouse became moribund and was sacrificed.


RufNeo
hβc
FIΔ
V449E
I374N
Red blood cells (x10^9/ml)
Time (weeks)
0
10
20
30

Several mice bearing constitutively active hβc mutants also showed dysregulated platelet production. Four of five diseased FIΔ mice and two of six diseased I374N mice displayed thrombocytosis in the peripheral blood (Figure 6.11). In contrast, four of five diseased V449E mice were thrombocytopaenic. Four out of six diseased I374N mice were thrombocytopaenic also, indicating that elevations in neutrophils, erythrocytes and platelets are separable components of I374N-induced haemopoietic disorders. Thrombocytosis in the diseased FIΔ and I374N mice was again a chronic disorder, however thrombocytopaenia in V449E mice was acute (Figure 6.12). Similarly to the erythrocytosis seen in mice containing the extracellular hβc mutants, some FIΔ and I374N mice which were did not exhibit thrombocytosis at the time of sacrifice did so at early timepoints (≤9 weeks), however this phenomenon was not as uniform as that seen in the case of the erythrocytosis (Figure 6.12).

In summary, diseased mice containing the extracellular hβc mutants (FIΔ and I374N mice) suffered from a chronic myeloproliferative disorder characterised by overproduction of neutrophils often with concurrent expansion of the erythroid and platelet lineages. In contrast, mice containing the transmembrane mutant (V449E mice) developed an acute leukaemia-like syndrome characterised by anaemia, thrombocytopaenia and blast cells in the blood.

To further define the phenotypes seen in the blood of diseased mice bearing constitutively active hβc mutants, the major haemopoietic organs, bone marrow and spleen, were analysed. Whilst bone marrow cellularity was not significantly altered in diseased mice containing the extracellular hβc mutants, the majority of diseased V449E mice showed reduced cellularity at this site (Figure 6.13). Bone marrow cell preparations revealed significant decreases in the proportion of lymphocytes in the bone marrow of diseased mice containing all three hβc mutants (Table 6.3, student's t-test, $p<0.01$). The bone marrow of diseased V449E mice, but not diseased FIΔ or I374N mice, also contained a significantly decreased proportion of erythroid cells (student's t-test, $p<0.01$). Accordingly, the proportion of blast cells was significantly increased in diseased V449E mice (student's t-test, $p<0.05$), which is consistent with the white blood cell profile of these mice.

Figure 6.11. Peripheral blood platelet levels of control and bone marrow reconstituted mice.

(**A**) Mean and (**B**) individual peripheral blood platelet counts of control and bone marrow reconstituted mice at the time of sacrifice. For mice containing hβc mutants (FIΔ, V449E, I374N) only mice experiencing haemopoietic disorders at the time of sacrifice are shown. Error bars show one standard deviation of the mean. Dashed lines span the range containing the control (BL/6, B6.SJL, RufNeo and hβc) animals.

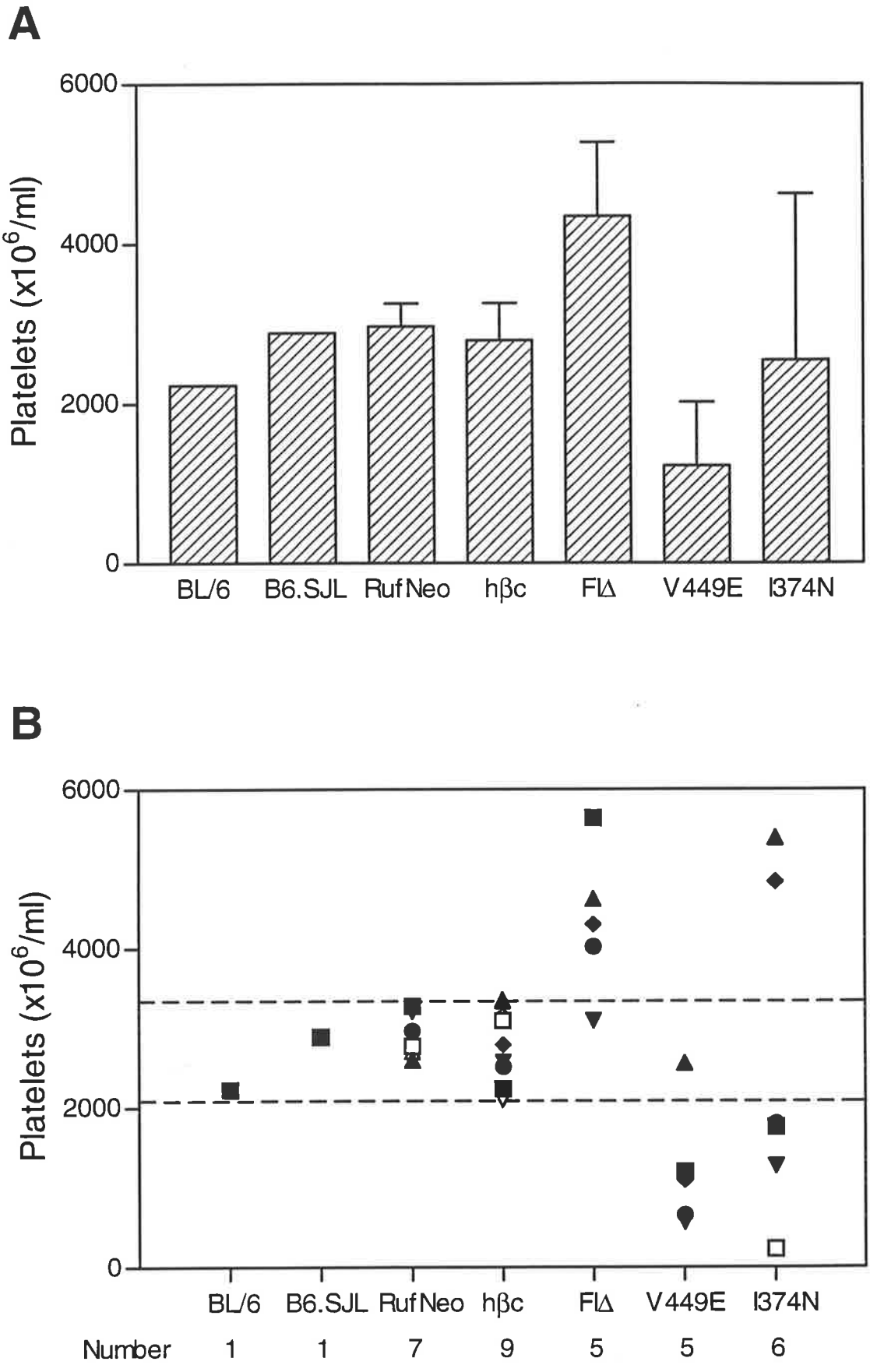

A
6000
4000
2000
0
Platelets (x10^6/ml)
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
B
6000
4000
2000
0
Platelets (x10^6/ml)
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
Number
1
1
7
9
5
5
6

Figure 6.12. Timecourse of peripheral blood platelet levels of bone marrow reconstituted mice.

Each line represents serial erythrocyte counts from a single animal. The dashed lines span the range containing the control (RufNeo and hβc) mice. The '+' symbol indicates the timepoint at which a mouse became moribund and was sacrificed.

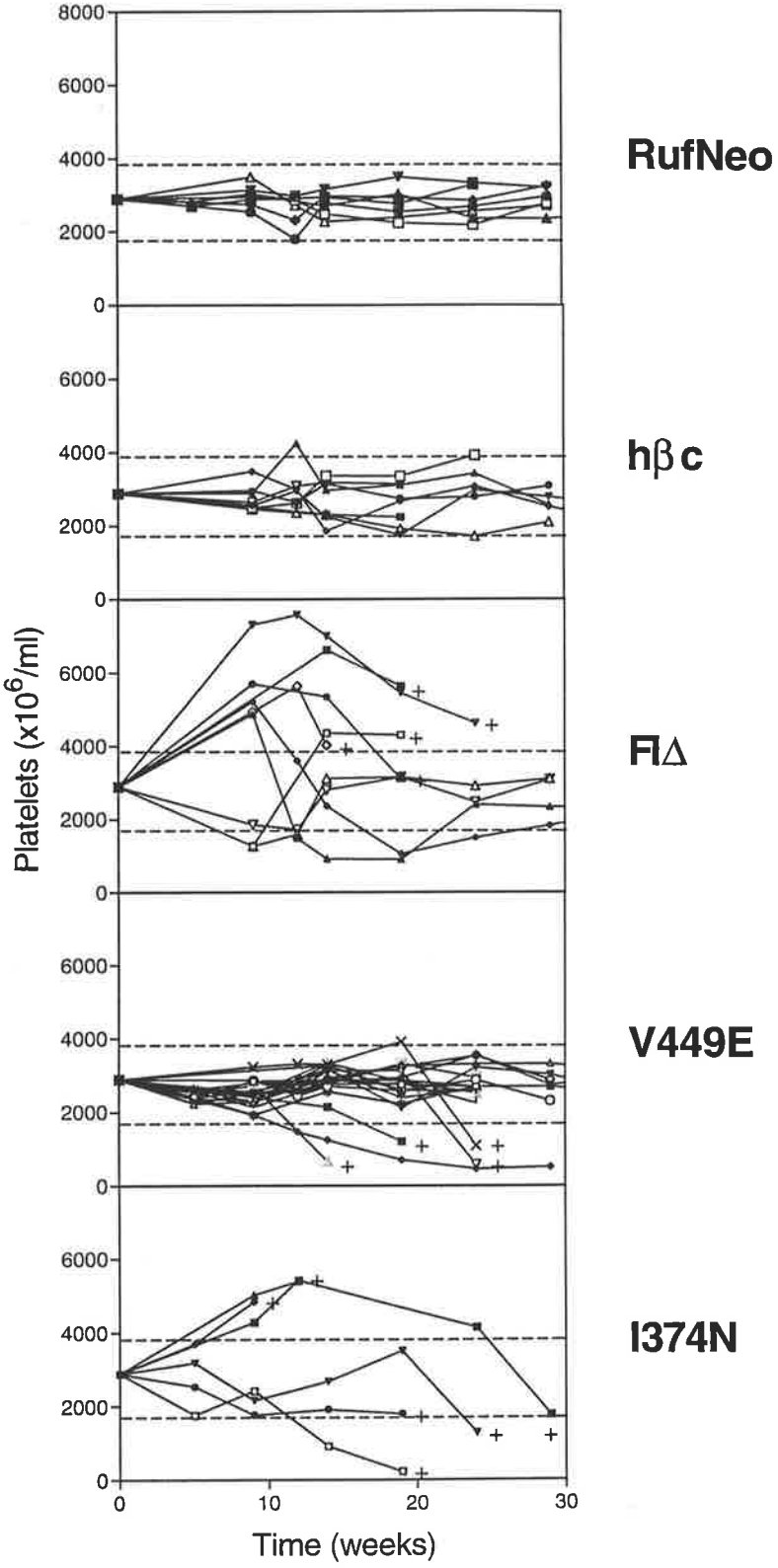
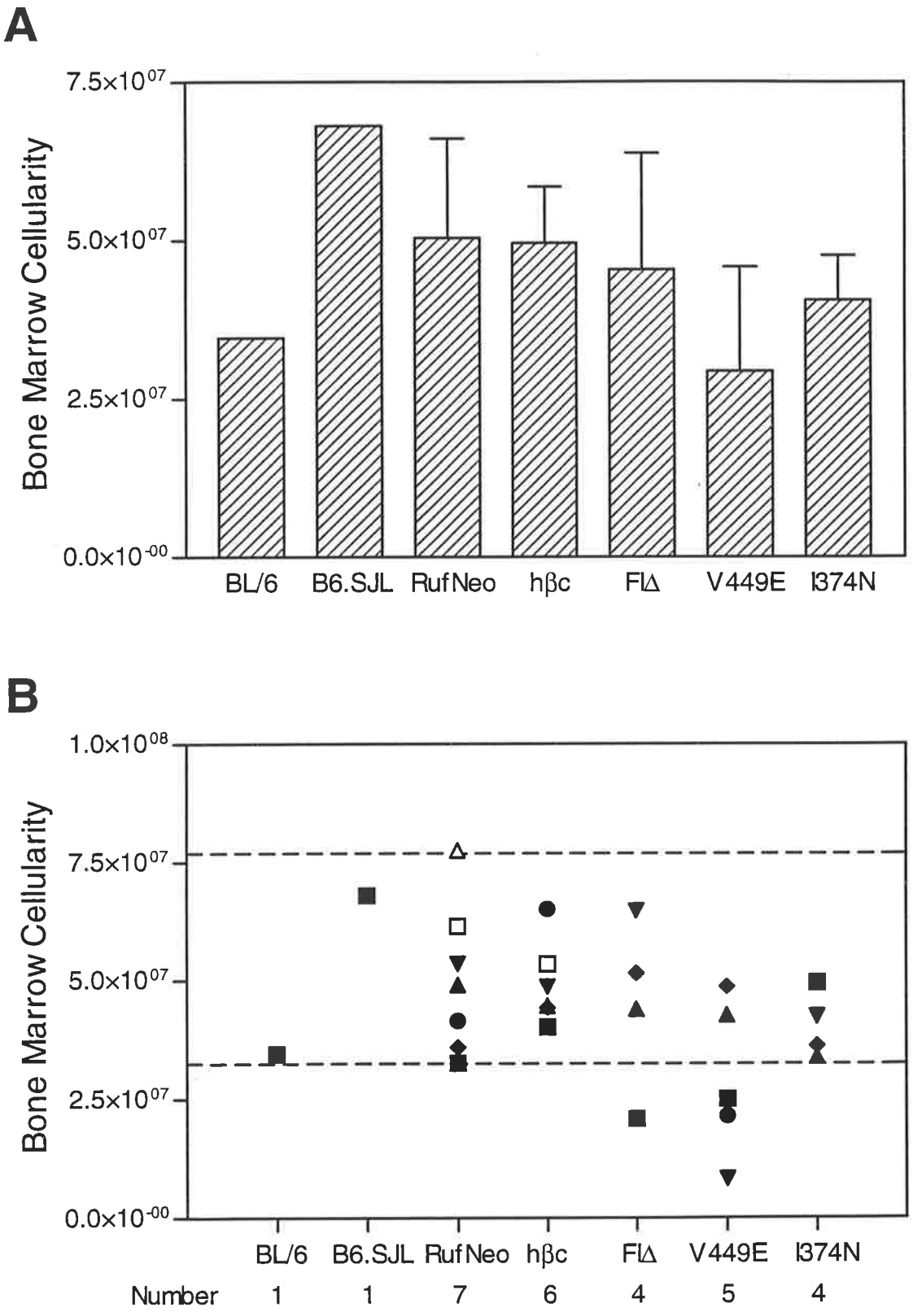

RufNeo
hβc
FlΔ
V449E
I374N
Platelets (x10^6/ml)
Time (weeks)
8000
6000
4000
2000
0
0
10
20
30

Figure 6.13. Bone marrow cellularity of control and bone marrow reconstituted mice.

(**A**) Mean and (**B**) individual counts of bone marrow cells derived from two femurs of control and bone marrow reconstituted mice at the time of sacrifice. For mice containing hβc mutants (FIΔ, V449E, I374N) only mice experiencing haemopoietic disorders at the time of sacrifice are shown. Error bars show one standard deviation of the mean. Dashed lines span the range containing the control (BL/6, B6.SJL, RufNeo and hβc) animals.


A
7.5×10^07
5.0×10^07
2.5×10^07
0.0×10^-00
Bone Marrow Cellularity
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
B
1.0×10^08
7.5×10^07
5.0×10^07
2.5×10^07
0.0×10^-00
Bone Marrow Cellularity
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
Number
1
1
7
6
4
5
4

All diseased FIΔ and V449E mice autopsied, and 2 of 4 diseased I374N mice, displayed splenomegaly at the time of sacrifice (Figure 6.14). Spleen cellularity was correspondingly increased in mice exhibiting splenomegaly, except in the case of one diseased V449E mouse (marked ●, Figures 6.14, 6.15). The reason for this was determined by histopathological analysis of this organ, which showed the presence of fibrous lesions displacing the spleen cells (data not shown). Similar lesions were found in the bone marrow of these mice. This phenomenon, known as myelofibrosis, is a condition associated with a number of acute and chronic leukaemias (McCarthy, 1985). The presence of myelofibrosis in a V449E mouse is further evidence that mice bearing V449E suffer from a leukaemia-like syndrome.

The spleens of diseased mice containing all three hβc mutants contained a significantly decreased proportion of lymphoid cells compared to control mice containing hβc (Table 6.3, student's t-test, $p<0.01$). In diseased V449E mice there was a corresponding increase in blast cells, consistent with that seen in the bone marrow and blood (Table 6.3, student's t-test, $p<0.05$). In contrast, in diseased mice containing the extracellular hβc mutants, FIΔ and I374N, there was no significant increase in the proportion of blast cells in the spleen. Rather, the proportion of erythroid cells in the spleen was significantly increased in these mice (Table 6.3, student's t-test, $p<0.01$ and $p<0.05$ respectively), unlike diseased V449E mice. There were also increases in the proportion of neutrophils at this site in mice containing all three hβc mutants, which in the case of FIΔ mice was significant (Table 6.3, student's t-test, $p<0.05$). Diseased I374N mice which did not exhibit splenomegaly (Figure 6.14) displayed similar spleen cell populations to those mice which did show increased spleen weight (data not shown).

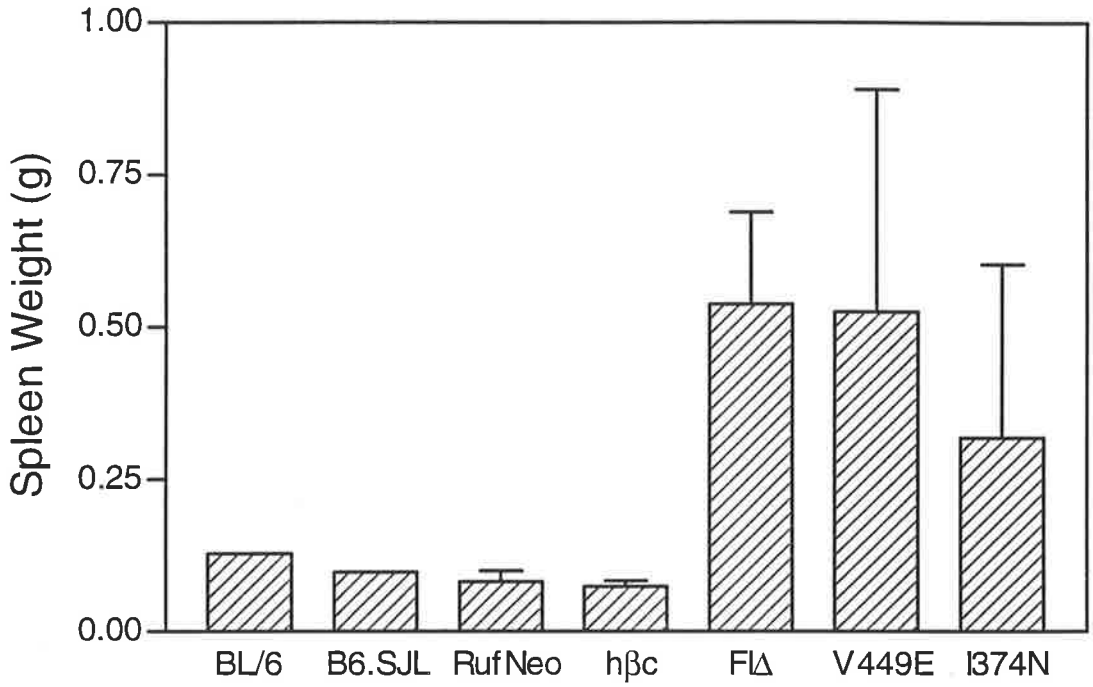
In addition to abnormalities of the blood, bone marrow and spleen, one diseased V449E mouse (mouse 5E4) contained a grossly enlarged thymus (weight 0.525 g, 3.3×10^8 cells). Cells prepared from this organ were almost solely blast cells of similar morphology to those seen in the blood, bone marrow and spleen (data not shown).

6.2.5 Cell surface antigen expression on spleen cells of bone marrow reconstituted mice

Figure 6.14. Spleen weight of control and bone marrow reconstituted mice.

(**A**) Mean and (**B**) individual spleen weights of control and bone marrow reconstituted mice at the time of sacrifice. For mice containing hβc mutants (FIΔ, V449E, I374N) only mice experiencing haemopoietic disorders at the time of sacrifice are shown. Error bars show one standard deviation of the mean. The dashed line shows the maximum value of the control (BL/6, B6.SJL, RufNeo and hβc) animals.

A


Spleen Weight (g)
1.00
0.75
0.50
0.25
0.00
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N

B

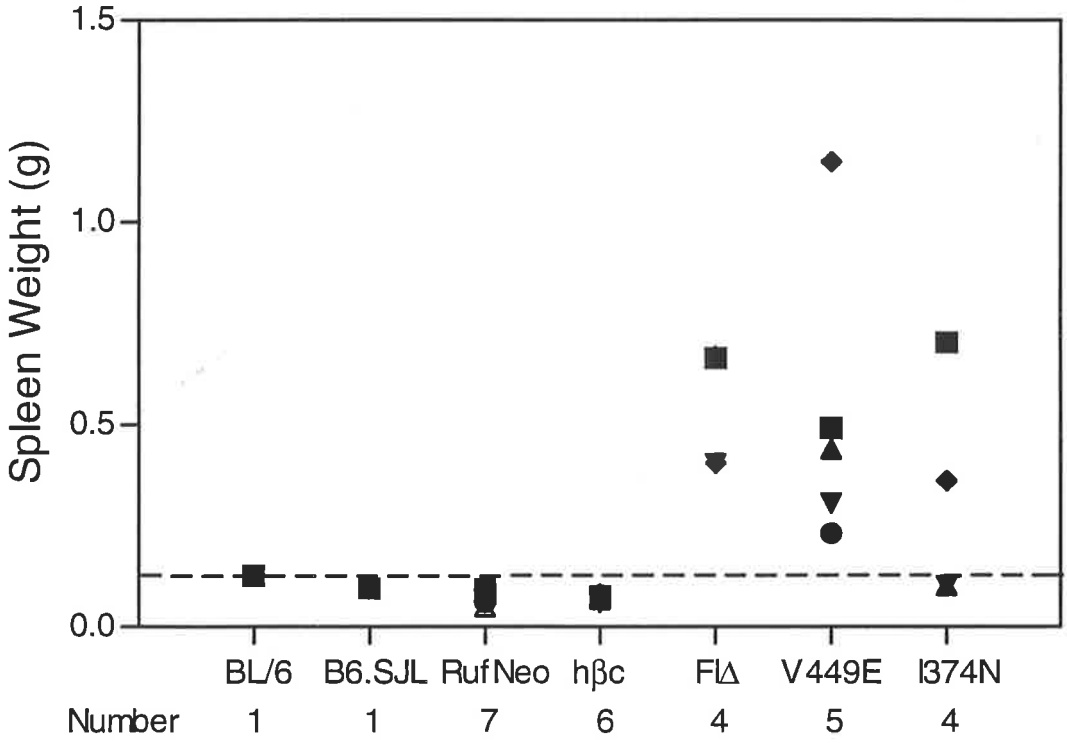
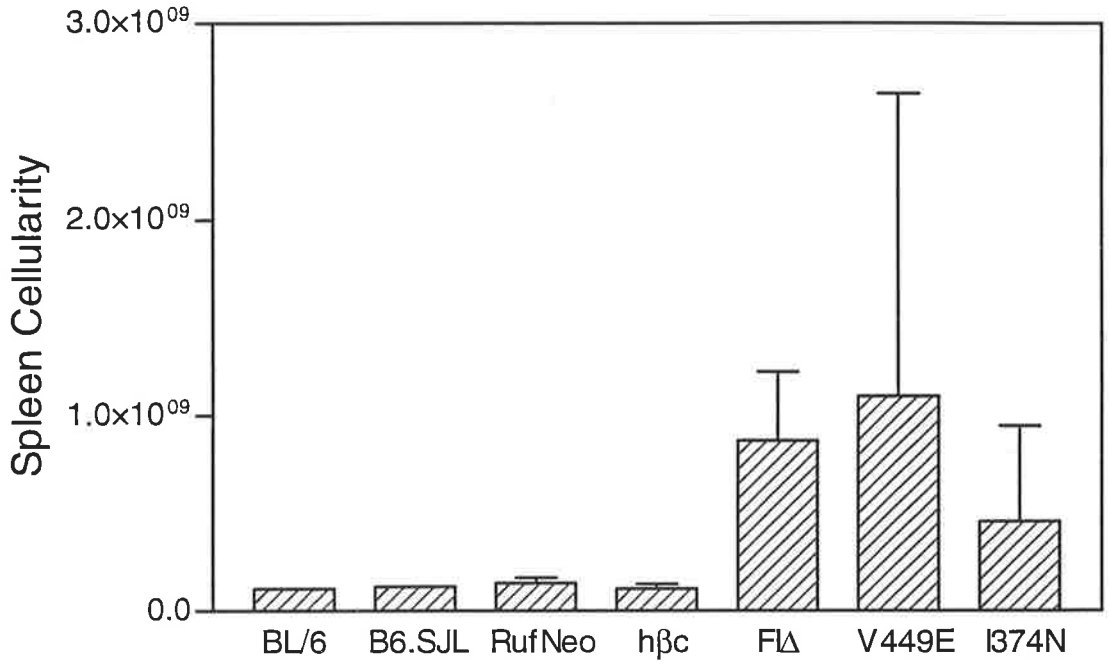

Spleen Weight (g)
1.5
1.0
0.5
0.0
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
Number
1
1
7
6
4
5
4

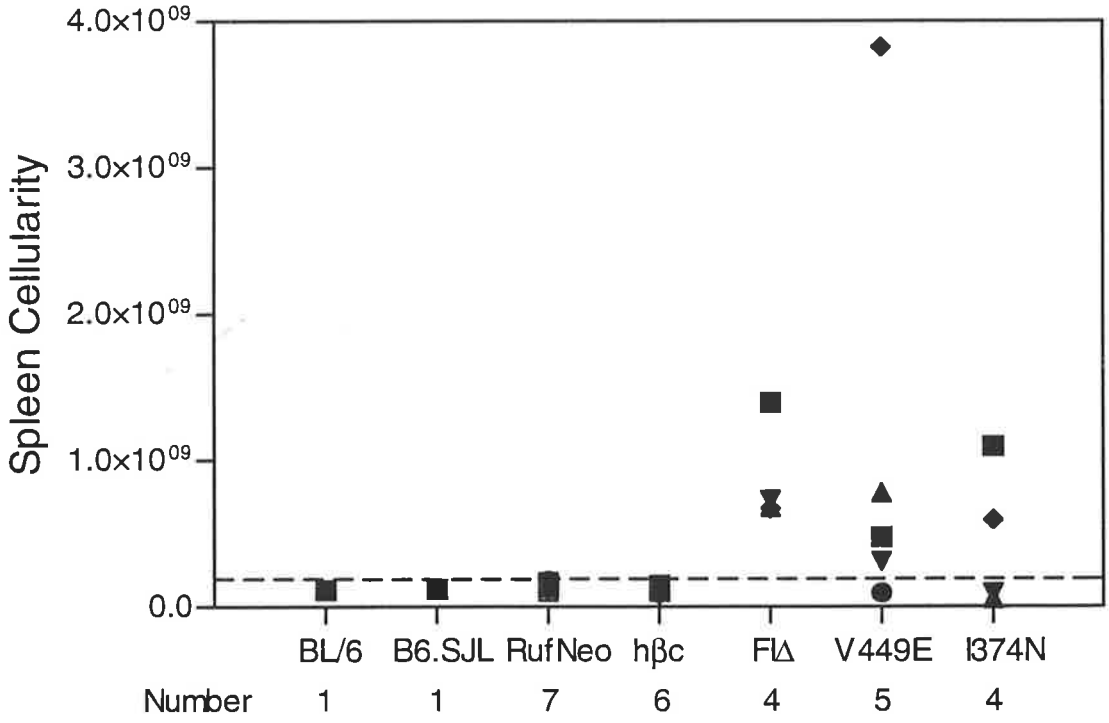
Figure 6.15. Spleen cellularity of control and bone marrow reconstituted mice.

(**A**) Mean and (**B**) individual numbers of cells derived from the spleens of control and bone marrow reconstituted mice at the time of sacrifice. For mice containing hβc mutants (FIΔ, V449E, I374N) only mice experiencing haemopoietic disorders at the time of sacrifice are shown. Error bars show one standard deviation of the mean. The dashed line shows the maximum value of the control (BL/6, B6.SJL, RufNeo and hβc) animals.

A


3.0x10^09
2.0x10^09
1.0x10^09
0.0
Spleen Cellularity
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N

B


4.0x10^09
3.0x10^09
2.0x10^09
1.0x10^09
0.0
Spleen Cellularity
BL/6
B6.SJL
RufNeo
hβc
FlΔ
V449E
I374N
Number
1
1
7
6
4
5
4

In order to verify the identities of the cell types observed, the expression of several cell surface antigens on the spleen cells of disease mice was studied using flow cytometry. Spleen cells were chosen because the spectrum of cell types observed in this organ showed the greatest deviation from control mice. For this analysis, a V449E mouse was chosen which had a very high proportion of blast cells in the spleen (96%) in order to study these cells in near-homogeneity.

To detect lymphocytes, spleen cells were stained with antibodies detecting the B-cell lineage marker B-220 (Coffman and Weissman, 1981) and the T-cell lineage markers CD4 and CD8 (Ledbetter and Herzenberg, 1979; Dialynas et al., 1983). The majority of spleen cells from control mice (B6.SJL, RufNeo and hβc) were positive for one of these markers, verifying their identity as lymphocytes (Figure 6.16 and data not shown). In contrast, the majority of spleen cells from diseased mice containing any of the three constitutively active hβc mutants were negative for all three lymphocyte markers, verifying that there is a large decrease in lymphocytes in the spleens of these mice (see Table 6.3).

In the spleens of control mice (B6.SJL, RufNeo and hβc) there was little expression of the murine erythroid lineage marker Ter-119 (Ogawa et al., 1991) (Figure 6.17 and data not shown). However, a large proportion of spleen cells from diseased mice containing the extracellular hβc mutants, FIΔ and I374N, expressed this marker, verifying that erythroid cells were present in the spleens of these mice. In contrast, spleen cells from a V449E mouse were negative for this marker, confirming that there was no increase in erythroid cells in the spleen of this mouse.

The expression of two myeloid lineage markers was also studied. The Mac-1 and Gr-1 antigens are expressed on mature granulocytes and macrophages and their precursors (Springer et al., 1979; Holmes et al., 1986). Cells from the spleens of control mice (B6.SJL, RufNeo and hβc) were negative for this marker indicating that there were few myeloid cell present in these spleens (Figure 6.17). In contrast the majority of spleen cells from a diseased FIΔ mouse were positive for these markers indicating that there is a large infiltrate of myeloid cells in the spleen of this mouse. Cells from the spleen of a diseased I374N mouse were largely Gr-1 negative. This is unsurprising as only 1% of these cells were morphologically neutrophils (data not shown). However, there was an increase in Mac-1-expressing cells relative to the

Figure 6.16. Expression of lymphoid cell surface antigens on spleen cells derived from bone marrow reconstituted mice.

Flow cytometric analyses were carried out as described in Section 2.12.11.1. Dashed lines represent staining with irrelevant isotype control antibodies. Solid lines represent staining with monoclonal antibodies detecting the indicated cell surface markers.

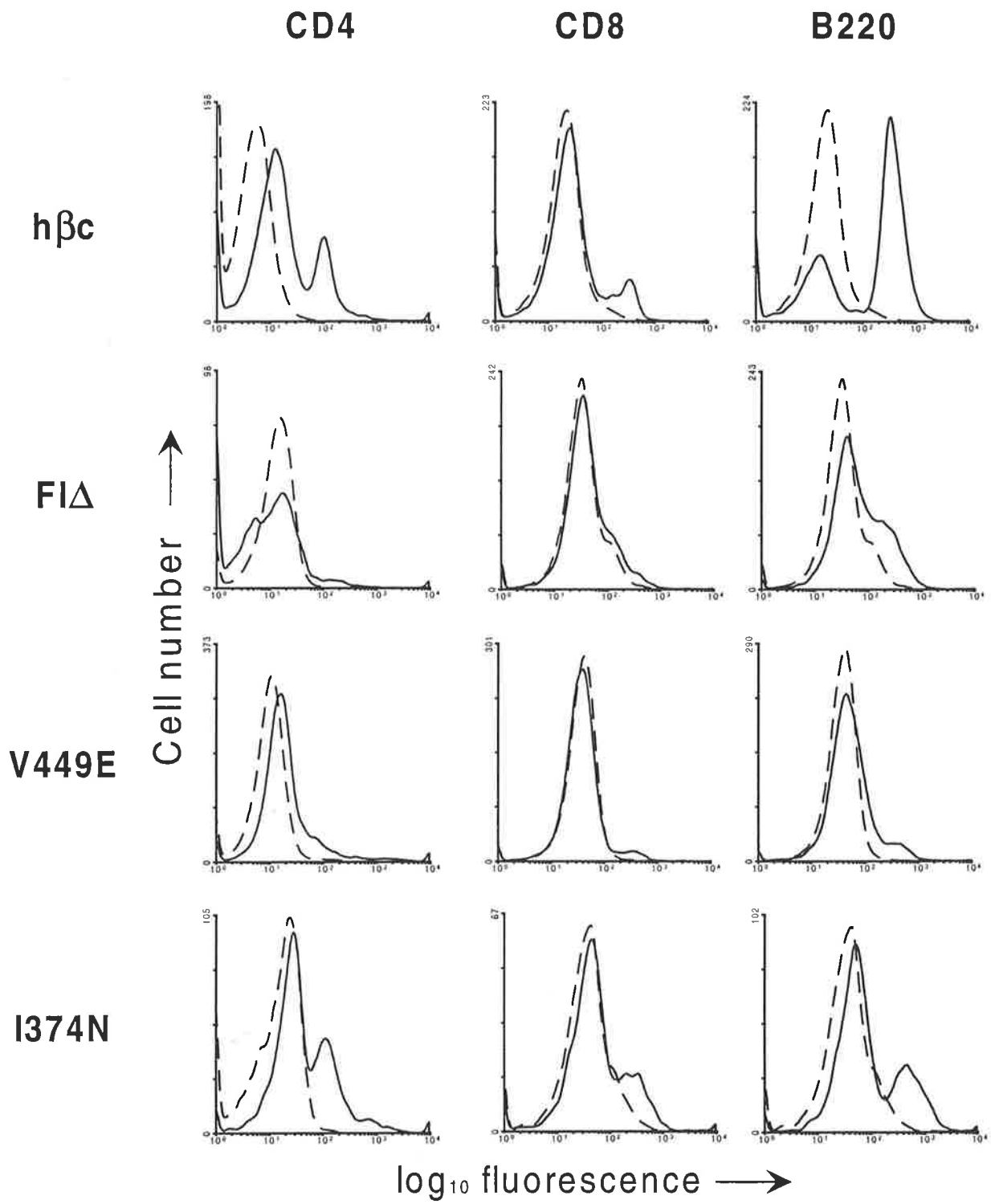
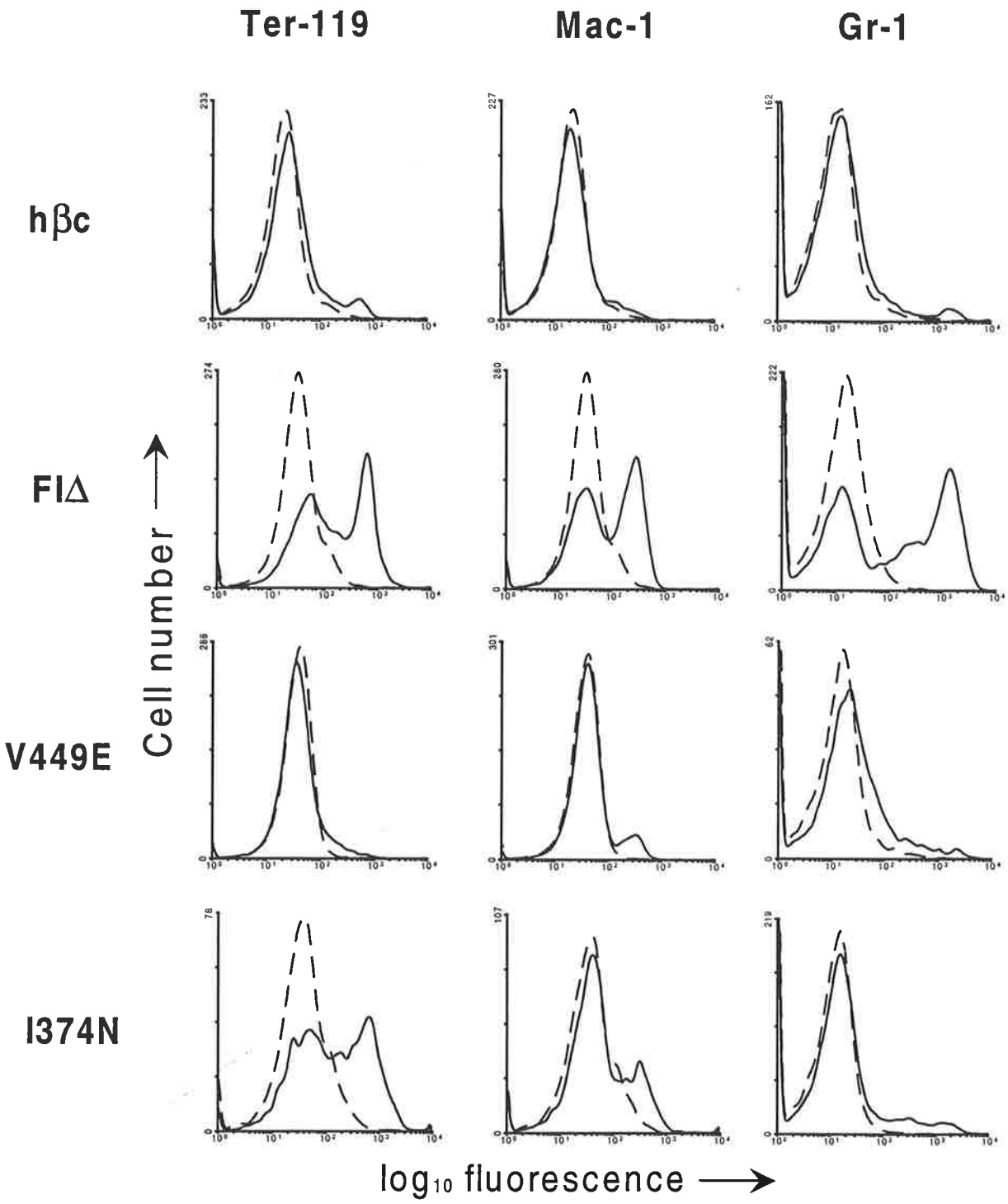

CD4
CD8
B220
hβc
FIΔ
V449E
I374N
Cell number
log10 fluorescence

Figure 6.17. Expression of erythroid and myeloid cell surface antigens on spleen cells derived from bone marrow reconstituted mice.

Flow cytometric analyses were carried out as described in Section 2.12.11.1. Dashed lines represent staining with irrelevant isotype control antibodies. Solid lines represent staining with monoclonal antibodies detecting the indicated cell surface markers.


Ter-119
Mac-1
Gr-1
hβc
FIΔ
V449E
I374N
Cell number
log10 fluorescence

control mice, indicating that like the FIΔ mouse studied, there is also a myeloid infiltrate in the spleen of this mouse. Interestingly, there was some Mac-1 expression in the spleen of the diseased V449E mouse, indicating that in addition to blast cells there were more mature myeloid cells in the spleen of this mouse.

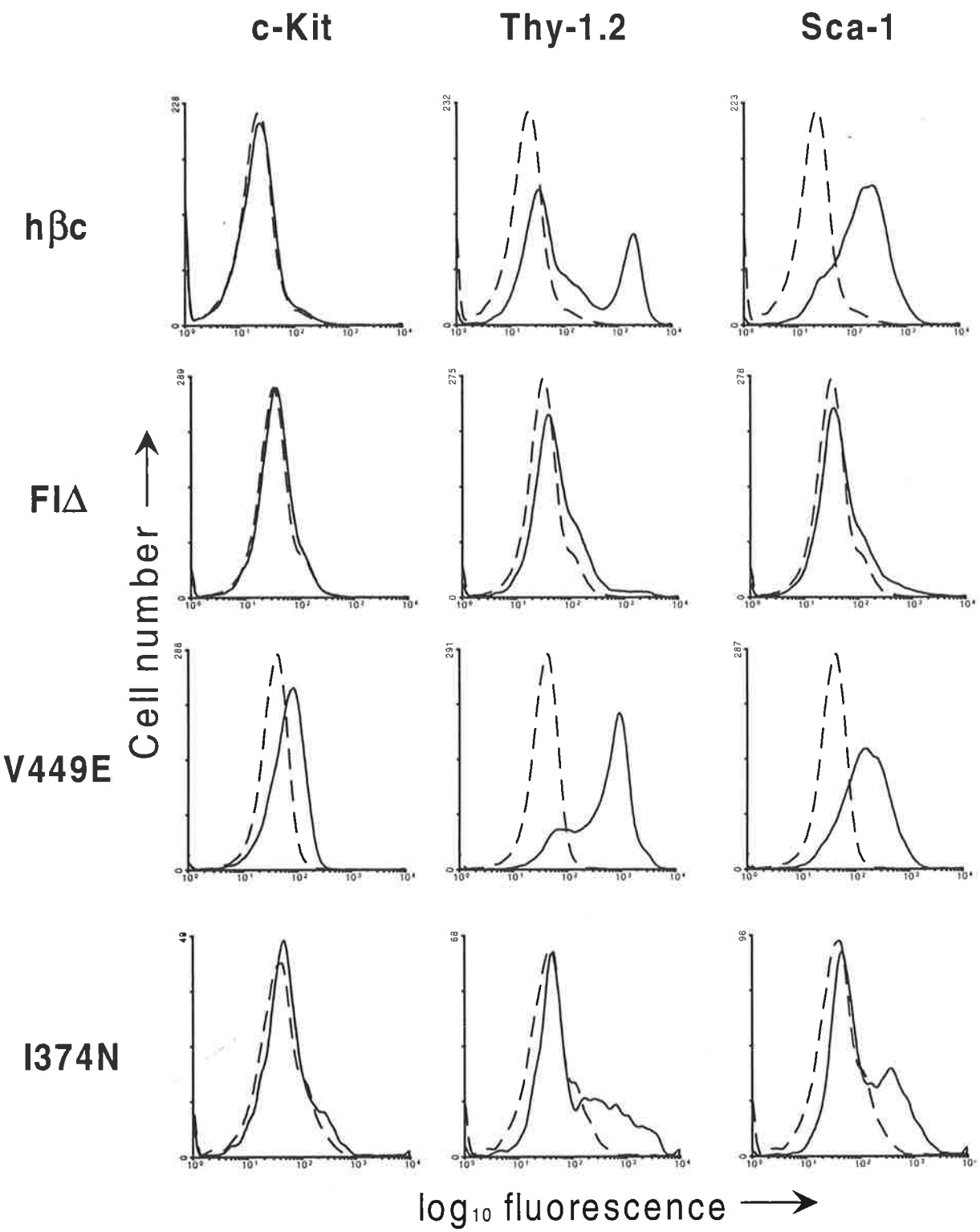
Several primitive cell markers were analysed. The Stem Cell Factor receptor (c-kit) is expressed on immature murine haemopoietic cells including the majority of haemopoietic progenitors (Ogawa et al., 1991). Spleen cells from control mice and diseased mice containing the extracellular hβc mutants were negative for c-kit, however spleen cells from a diseased mouse containing V449E expressed c-kit, albeit at low levels (Figure 6.18). This is consistent with the immature morphology of these cells.

The spleen cells from the V449E mouse studied also expressed Thy-1.2 (Figure 6.18). This is consistent with the immature morphology of these cells since this marker has been found on populations enriched for haemopoietic stem cells and on early progenitor cells (Spangrude et al., 1988; Miller et al., 1985; Berman and Basch, 1985). This marker is also expressed on mature T-cells, hence its expression was found on a subset of spleen cells from a hβc control mouse (Ledbetter and Herzenberg, 1979). Thy-1+ spleen cells from the V449E mouse express lower levels of Thy-1 than those from the hβc control mouse, resembling the Thy-1^{lo} population which contains the murine haemopoietic stem cells (Uchida and Weissman, 1992). Moreover, these cells are CD4- and CD8- hence they are not mature T cells (Figure 6.16). Mice bearing the extracellular hβc mutants, FIΔ and I374N, were largely negative for this marker.

Sca-1 is a marker found on immature cells of the haemopoietic system (Spangrude et al., 1989). Its expression further subdivides haemopoietic cells which are negative for haemopoietic lineage markers (lin-) and are c-kit positive, the Sca-1 positive fraction being less mature and containing the haemopoietic repopulating cells (Okada et al., 1992). Hence the expression of Sca-1 on the spleen cells of the diseased V449E mouse studied is further evidence that these cells are very immature, since they are negative for lymphoid, myeloid and erythroid lineage antigens and express c-kit (Figures 6.16-6.18). This marker is not informative of the spleen cells from the other mice as they are in the main positive for haemopoietic lineage markers.

Figure 6.18. Expression of stem cell antigens on spleen cells derived from bone marrow reconstituted mice.

Flow cytometric analyses were carried out as described in Section 2.12.11.1. Dashed lines represent staining with irrelevant isotype control antibodies. Solid lines represent staining with monoclonal antibodies detecting the indicated cell surface markers.


c-Kit
Thy-1.2
Sca-1
hβc
FIΔ
V449E
I374N
Cell number
log10 fluorescence

It can be concluded from these flow cytometric analyses that the surface antigen profile of the majority of cells present in the spleen of a V449E mouse is c-kit+, Thy-1^{lo}, lin-, Sca-1+, which is identical to the population in murine bone marrow which contains the haemopoietic stem cells (Uchida and Weissman, 1992; Okada et al., 1992). This is consistent with the immature (blast cell) morphology of these cells. There is also a small fraction of more mature myeloid cells present. In contrast, cells from mice bearing extracellular hβc mutants, FIΔ and I374N were predominantly a mixture of mature erythroid and myeloid cell types. In both cases the spectrum of cell types observed was highly abnormal when compared to spleen cells from control mice, which were mostly lymphocytes. This is consistent with the morphological observations of these cells (see Section 6.2.4 above).

6.2.6 Histopathology of organs derived from diseased mice bearing constitutively active hβc mutants

Histopathological analyses were carried out on several organs from control mice and diseased mice bearing constitutively active hβc mutants. Spleens from hβc control mice displayed normal splenic architecture with clearly defined areas of red and white pulp (Figure 6.19). However, in the spleens of diseased mice bearing any of the activated hβc mutants this organisation was completely disrupted, resulting in a homogeneous arrangement with no defined red and white pulp (Figure 6.19). In several of the FIΔ and I374N mice there were large numbers of megakaryocytes discernible in the spleen, indicating that this organ may be a site of origin of the thrombocytosis seen in the peripheral blood of these mice (Figure 6.19, see section 6.2.4). In the spleen of one diseased V449E mouse were fibrous lesions, leading to a reduced cell count in the spleen of this animal (see section 6.2.4).

The liver of diseased mice containing the extracellular hβc mutants contained infiltrates of myeloid and erythroid cells, chiefly around the blood vessels but often extending into the main tissue of the organ (Figure 6.20). Similar lesions were observed in the liver of diseased V449E mice, however in this case the infiltrating cells were blast cells and in some cases neutrophils.

Lesions were also found in the lungs of diseased mice bearing all three constitutively active hβc mutants (Figure 6.21). These infiltrated the alveolar spaces

Figure 6.19. Spleen histopathology of bone marrow reconstituted mice.

Haematoxylin and eosin-stained sections of spleens derived from bone marrow reconstituted mice containing the indicated hβc subunits. The hβc panel shows normal splenic architecture with clearly defined areas of red (R) and white (W) pulp. In spleens from mice containing mutant hβc subunits there is expansion of the red pulp, absence of lymphoid germinal centres and, in the case of FIΔ and I374N, the presence of megakaryocytes (MK). Photographs are at 90 × magnification.

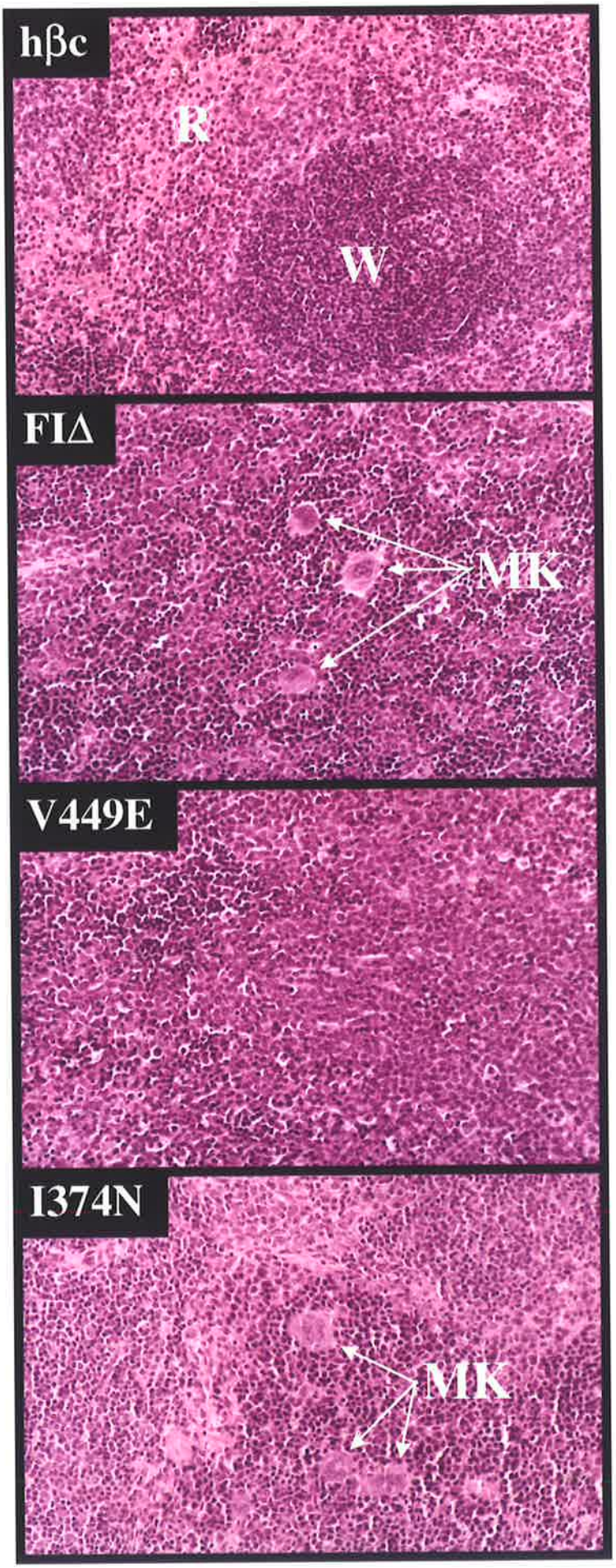

hβc
R
W
FIΔ
MK
V449E
I374N
MK

Figure 6.20. Liver histopathology of bone marrow reconstituted mice.

Haematoxylin and eosin stained sections of livers derived from bone marrow reconstituted mice containing the indicated hβc subunits. Representative capillary regions are shown, showing normal liver histology in the hβc mouse and typical lesions in FIΔ, V449E and I374N mice. In FIΔ and I374N mice basophilic erythroid precursors are the predominant infiltrating type, with neutrophils containing ring nuclei also visible in the I374N panel. In the V449E panel large blast cells and neutrophils with ring nuclei are visible. Photographs are at 350 × magnification.

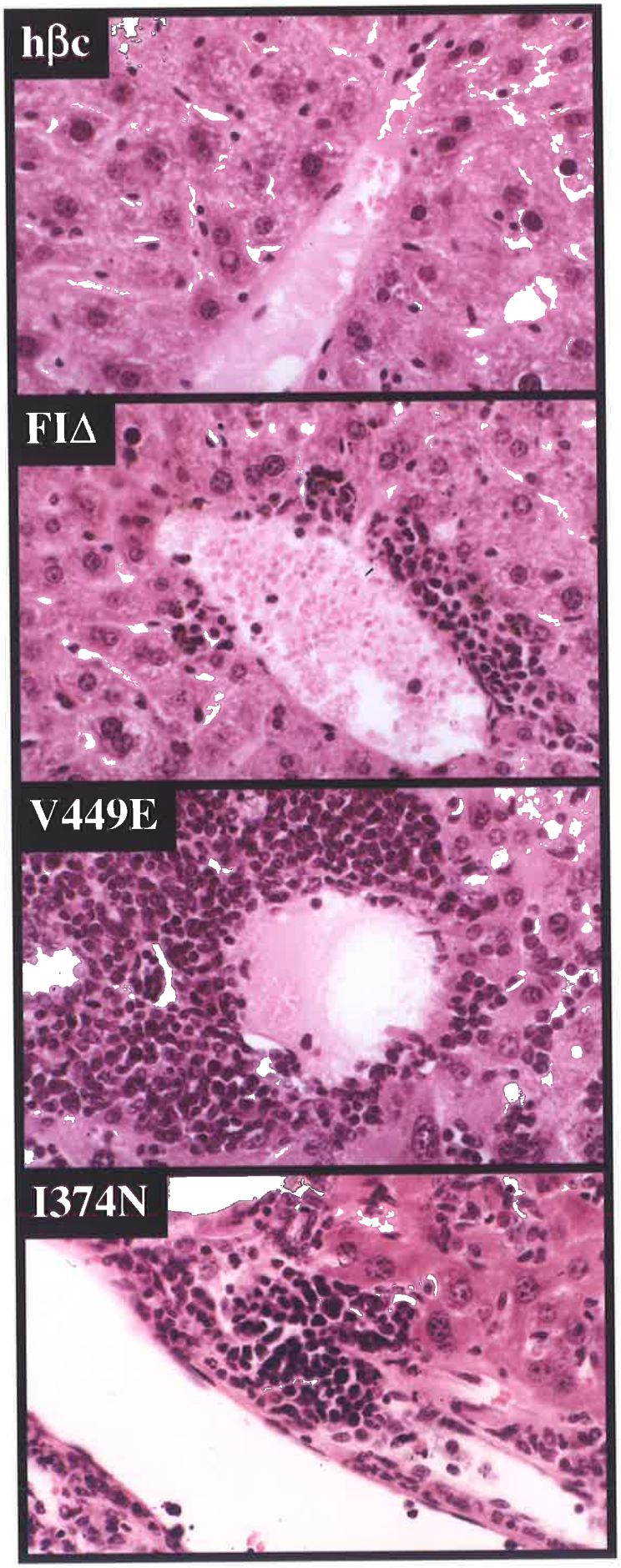
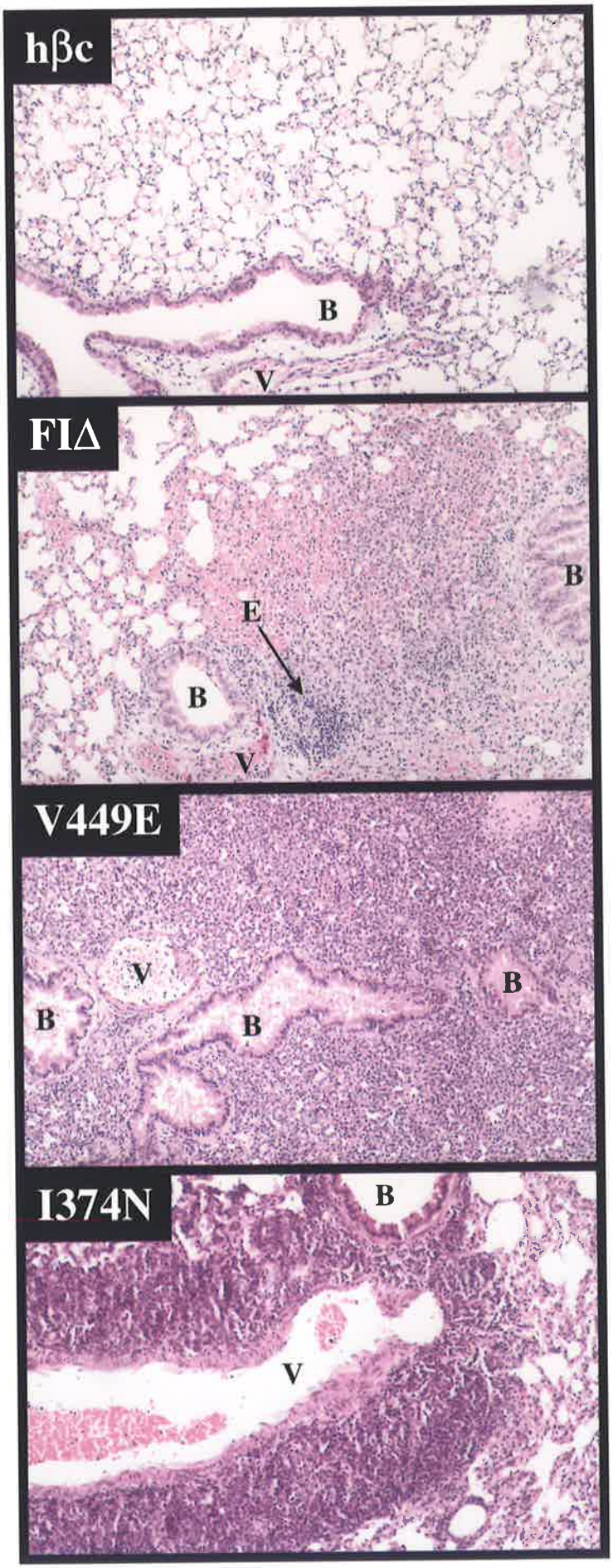

hβc
FIΔ
V449E
I374N

Figure 6.21. Lung histopathology of bone marrow reconstituted mice.

Haematoxylin and eosin stained sections of lungs derived from bone marrow reconstituted mice, showing normal lung histology in the hβc mouse and occlusion of the spongiform tissue in mice containing hβc mutants due to infiltration of haemopoietic cells. Bronchioles (B) and blood vessels (V) are marked. An focus of basophilic erythroid cells (E) is indicated in the FIΔ panel by and arrow. Extravascular erythrocytes showing pale pink staining are visible in the FIΔ panel, indicative of pulmonary haemorrhage. Photographs are at 90 × magnification.


hβc
B
V
FIΔ
B
E
B
V
V449E
V
B
B
B
I374N
B
V

and consisted of the same cell types as those observed in the liver of the same animals; erythroid cells and neutrophils in the case of FIΔ and I374N mice and blast cells and neutrophils in the case of V449E mice. The lungs of several mice containing all three constitutively active hβc mutants contained extravascular erythrocytes indicative of pulmonary haemorrhage (seen for FIΔ in Figure 6.21 and data not shown).

No pathology was found in skeletal muscle, brain or heart of the same reconstituted mice bearing constitutively active hβc mutants. Taken together, these histopathological analyses indicate that the haemopoietic diseases suffered by mice bearing hβc mutants were aggressive in their tissue infiltration, which may be a cause of their lethality.

6.2.7 Colony formation by haemopoietic cells derived from bone marrow reconstituted mice

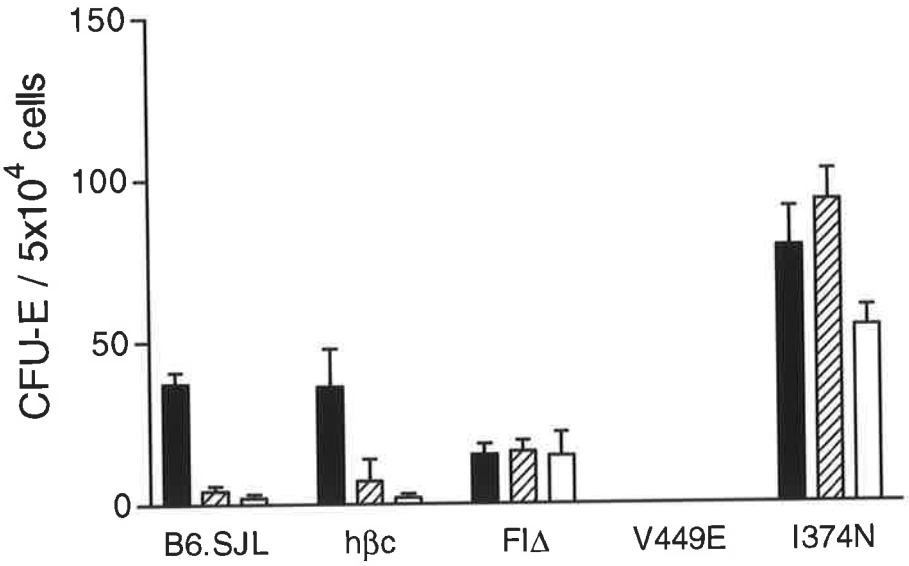
To further understand the effects of constitutively active hβc mutants on murine haemopoiesis *in vivo*, haemopoietic progenitor cell colony assays were carried out in methyl-cellulose using cells derived from the bone marrow and spleen of diseased bone-marrow reconstituted mice bearing these mutants. The I374N mouse used for this analysis was one of three showing erythrocytosis at the time of sacrifice (29 weeks post-reconstitution) (see Figure 6.7).

Firstly, the numbers of erythroid colony-forming units (CFU-E) were determined following culture in the presence of Stem Cell Factor (SCF) and Epo for two days. The number of CFU-E was slightly reduced in the bone marrow of a diseased FIΔ mouse and elevated 2-fold in that of a diseased I374N mouse, when compared to levels in the marrow of control B6.SJL and hβc mice (Figure 6-22A). A small proportion (11%) of CFU-E in the bone marrow of a control B6.SJL mouse were capable of formation in the presence of growth factors and G418. The presence of G418-resistant CFU-E colonies in the bone marrow of the B6.SJL control mouse indicates that G418 is unable to completely suppress colony formation by uninfected CFU-E at the concentration used (1 mg/ml). Nevertheless, the presence of G418 had no effect on the growth of CFU-E from FIΔ and I374N mice, indicating that these colonies contained integrated RufNeo retrovirus. In the absence of growth factors, only a small proportion of the CFU-E in the bone marrow of control mice were

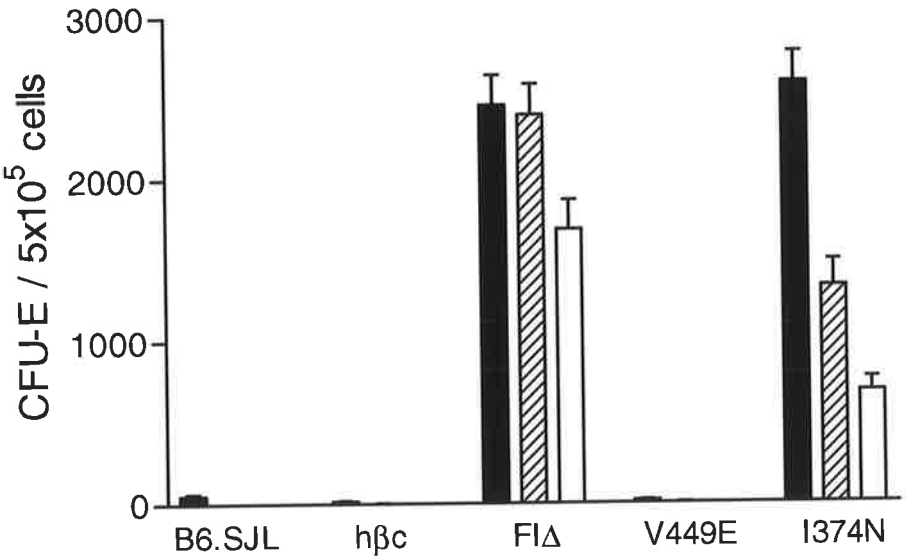
6.22. Mice bearing extracellular hβc mutants show factor independent erythropoiesis.

Numbers of CFU-E in the bone marrow (**A**) and spleen (**B**) of control and bone marrow reconstituted mice. Cells were plated in methyl-cellulose alone (open bars) or in the presence of growth factors (50 ng/ml mSCF plus 2 U/ml hEpo) (filled bars) or growth factors plus 1 mg/ml G418 (dashed bars). Numbers of CFU-E were determined after 2 days incubation. Numbers are the means of triplicate determinations. Error bars show the standard deviation of the mean. (**C**) *In situ* morphology of representative benzidine-stained CFU-E derived from the spleen of control and bone marrow reconstituted mice in the presence (+SCF, Epo) or absence (-GFs) of growth factors. Photographs are at 150 × magnification.

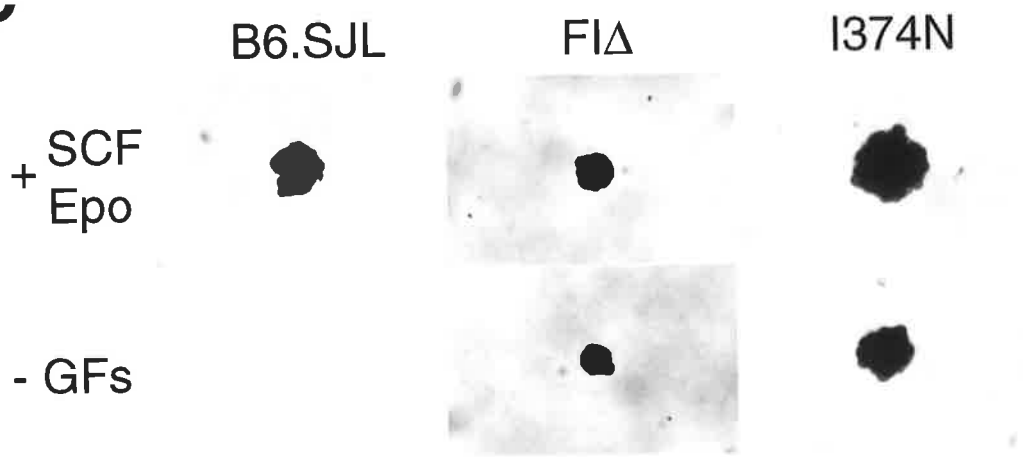
A


150
100
50
0
CFU-E / 5x10^4 cells
B6.SJL
hβc
FIΔ
V449E
I374N

B


3000
2000
1000
0
CFU-E / 5x10^5 cells
B6.SJL
hβc
FIΔ
V449E
I374N

C


B6.SJL
FIΔ
I374N
+ SCF
Epo
- GFs

capable of colony formation (5% and 6% for B6.SJL and hβc mice respectively). Unexpectedly, however, the majority of bone-marrow-derived CFU-E from FIΔ and I374N mice were capable of factor-independent growth (Figure 6-22A).

CFU-E were absent in the bone marrow of a diseased V449E mouse, which is consistent with the observation that erythroid precursors were significantly decreased in the bone marrow of diseased V449E mice (Table 6.3).

There was a vast (approximately 50-fold) increase in CFU-E in the spleens of diseased FIΔ and I374N mice when compared to the B6.SJL control (Figure 6-22B). As was seen in the case of CFU-E derived from the bone marrow of these mice, the CFU-E derived from the spleen of an FIΔ mouse were G418-resistant indicating that they contained integrated RufNeo retroviruses, whereas approximately one half of the splenic CFU-E from an I374N mouse were G418-resistant. Factor-independent CFU-E were present in the spleens of both FIΔ and I374N mice. In contrast, there was no increase in CFU-E in the spleen of the V449E mouse, and the CFU-E present were neither G418-resistant nor factor-independent. Factor-dependent and -independent CFU-E derived from the spleen of an FIΔ mouse were of similar size to those derived from a B6.SJL control mouse cultured in the presence of SCF and Epo, whereas those derived from an I374N mouse were somewhat larger (Figure 6-22C). These data suggest that the erythrocytosis observed in FIΔ and I374N mice is due at least in part to excessive and factor-independent erythropoiesis occurring in the bone marrow and spleen of these mice.

Next the numbers of various myeloid and erythroid colonies in the bone marrow and spleen of diseased bone-marrow reconstituted mice were assessed by plating in methyl-cellulose for 7 days. To maximally stimulate colony formation by a variety of myeloid and erythroid progenitors, saturating concentrations of IL-3, GM-CSF and Epo were used as stimuli. As shown in Table 6.4, the number of colony-forming units present in the bone marrow of a diseased FIΔ mouse was significantly reduced when compared to control (B6.SJL and hβc) mice (Student's t-test, $p<0.05$). There was a slight reduction in colony formation in the bone marrow of a diseased I374N mouse which was not significant. In contrast, there were large increases in haemopoietic progenitors in the spleen of diseased mice bearing all three constitutively active hβc

Table 6.4. Colony formation by bone marrow and spleen cells derived from control and bone marrow-reconstituted mice.

Group	+ GFs[a]	+ GFs + G418	-	- (HD)[b]
Bone Marrow				
B6.SJL	200 ± 15	0 ± 0	0 ± 0	0 ± 0
hβc	122 ± 23	0 ± 0	0 ± 0	0 ± 0
FIΔ	20 ± 1	12 ± 1	2 ± 1	7 ± 1
V449E	259 ± 13	4 ± 2	0 ± 0	0 ± 0
I374N	95 ± 9	40 ± 3	0 ± 0	1 ± 1
Spleen				
B6.SJL	25 ± 4	0 ± 0	0 ± 0	
hβc	24 ± 5	0 ± 0	0 ± 0	
FIΔ	123 ± 3	111 ± 3	6 ± 4	
V449E	259 ± 25	37 ± 5	0 ± 0	
I374N	150 ± 23	58 ± 10	0 ± 0	

Bone marrow cells were cultured at 5×10^4 cells/ml and spleen cells at 5×10^5 cells/ml in 1 ml methyl-cellulose medium containing the indicated additives. Colonies were scored at the seventh day of culture. Numbers are mean ± standard deviation of triplicate determinations.

[a]GFs, Growth factors: IL-3, GM-CSF, Epo.

[b]HD, High density: 2.5×10^5 cells/ml.

mutants. Thus there appears to be a general shift of haemopoietic progenitors from the bone marrow to the spleen in mice bearing the extracellular hβc mutants.

The majority of haemopoietic progenitors in the bone marrow and spleen of the FIΔ and I374N mice contained the RufNeo retrovirus as assessed by G418-resistance (Table 6.4). Factor-independent colonies were obtained from both bone marrow and spleen of the FIΔ mouse tested (Table 6.4), and were found in the bone marrow of a further 3 mice tested in a similar assay (data not shown). These colonies represented a small proportion of the G418-resistant progenitors (17% in the bone marrow and 5% in the spleen) and were smaller than similar colonies obtained in growth factors (Figure 6-23). In contrast, factor-independent colonies were not obtained from an I374N mouse except for a small number of colonies when bone marrow cells were plated at high density (Table 6.4), which were morphologically macrophage colonies (data not shown). Moreover, factor-independent colonies were not obtained from another two I374N mice tested in a similar assay (data not shown).

In contrast to the FIΔ and I374N mice, the number of progenitors in the bone marrow of a diseased V449E mouse was increased (Table 6.4). There was also an approximately 10-fold increase in the number of progenitors present in the spleen of this mouse when compared to B6.SJL and hβc control mice. Similar increases in colony formation were found in the bone marrow and spleen of a further 3 V449E mice in similar assays (data not shown). Whilst G418-resistant progenitors were present in the bone marrow and spleen of this mouse, factor-independent colonies were not. These data indicate that the leukaemic blast cells present in these tissues are incapable of factor-independent growth in this culture system.

The types of haemopoietic progenitors obtained in the presence of growth factors from the bone marrow of the FIΔ and I374N mice were similar to a hβc control mouse (Table 6.5). Whilst there was a significant increase in the proportion of BFU-E in the spleen of the FIΔ mouse when compared to a B6.SJL control (χ^2 test, $p<0.01$), this was not the case in the I374N mouse assessed. In contrast to the various colony types obtained in the presence of growth factors, factor-independent colonies obtained from the bone marrow or spleen of the FIΔ mouse were neutrophil, macrophage and granulocyte-macrophage colonies only. Hence factor-independent signalling by FIΔ

Figure 6.23. *In situ* morphology of colonies formed by FIΔ bone marrow in methyl-cellulose.

Representative granulocyte-macrophage colonies grown in the presence (**A**) and absence (**B**) of growth factors. Photographs are at 50 × magnification, taken using a phase-contrast microscope.

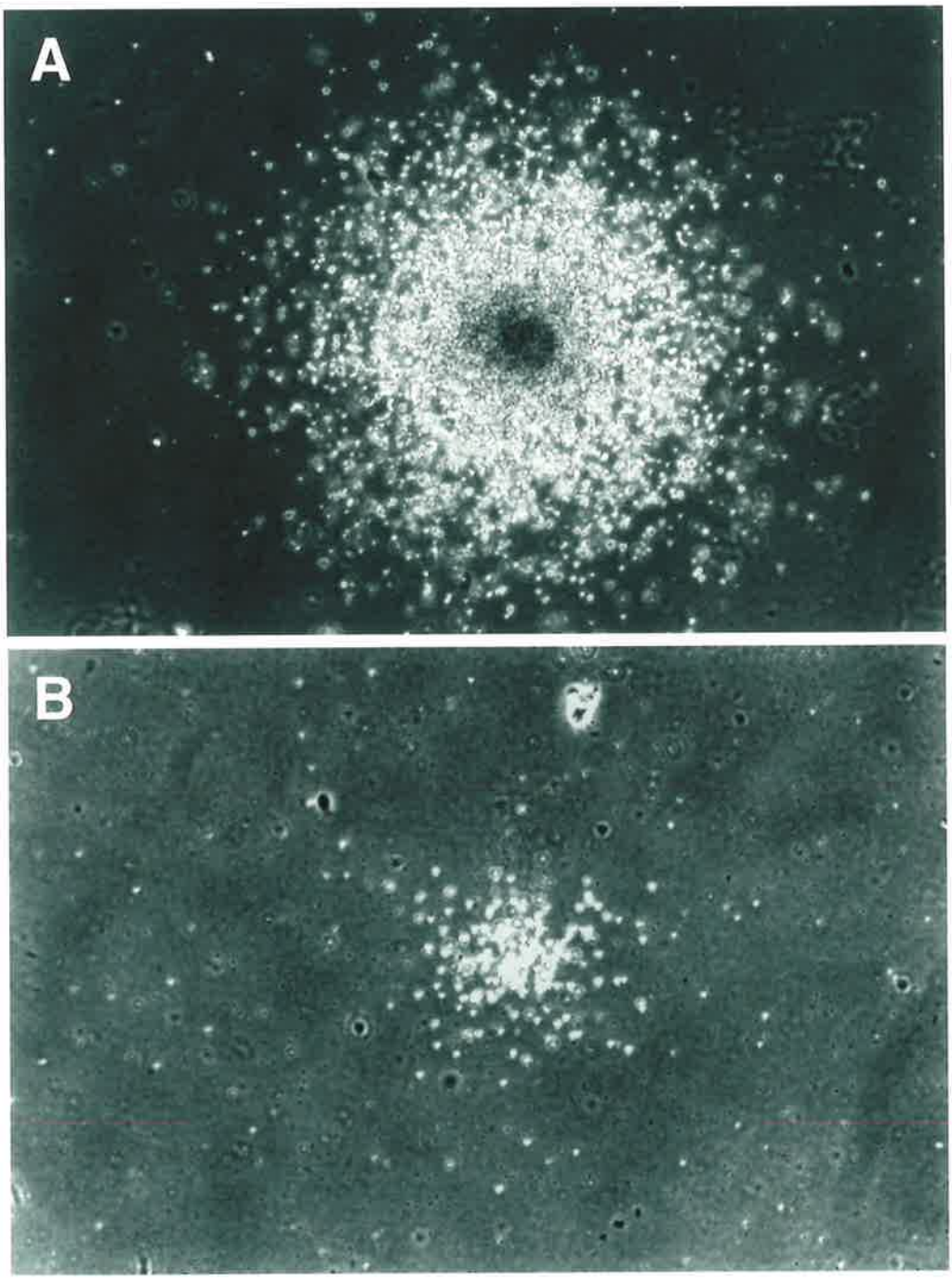

A
B

Table 6.5. Factor-dependent and -independent colonies formed by bone marrow and spleen cells derived from control and bone marrow-reconstituted mice.

Group	Additions	Colony types (%)								Colonies examined
		G	Eo	Mast	M	GM	Mix	BFU-E	Blast	
Bone Marrow										
hβc	IL-3, GM, Epo	12	1	7	28	33	14	3	2	102
FIΔ	IL-3, GM, Epo[a]	16	0	7	44	20	11	0	2	61
	-	18	0	0	51	31	0	0	0	65
V449E	IL-3, GM, Epo	3	0	0	35	16	21	21	4	107
I374N	IL-3, GM, Epo	10	1	3	43	36	6	2	0	103
Spleen										
B6.SJL	IL-3, GM, Epo	6	0	9	26	26	28	4	2	98
hβc	IL-3, GM, Epo	15	0	3	26	26	21	6	3	34
FIΔ	IL-3, GM, Epo	15	0	1	9	15	30	27	4	103
	-	38	0	0	25	38	0	0	0	16
V449E	IL-3, GM, Epo	11	0	2	37	7	26	13	5	122
	IL-3, GM, Epo, G418	50	0	2	2	20	25	0	0	44
I374N	IL-3, GM, Epo	7	1	6	35	31	13	8	0	101

Colony types were determined at the 7th day of culture.

Abbreviations: G, neutrophilic granulocyte; Eo, eosinophil; Mast, mast cell; M, macrophage; GM, granulocyte-macrophage; Mix, mixed myeloid/erythroid, BFU-E, erythroid burst-forming unit; Blast, blast cell

[a]To gain sufficient numbers, colonies grown with and without G418 are combined

appears to be restricted to granulocyte, macrophage, granulocyte-macrophage and CFU-E colonies. As mentioned above, there were not sufficient factor-independent colonies in the bone marrow or spleen of I374N mice to allow a similar analysis to be performed.

There was an increased proportion of BFU-E in the bone marrow and spleen of a V449E mouse (Table 6.5) (χ^2 tests, $p<0.01$ and $p<0.05$ respectively), however as those in the spleen were not G418 resistant this increase is unlikely to be a direct consequence of V449E signalling. The presence of various colony types in the spleen of this mouse, together with the overall 10-fold increase in colony formation in this organ (Table 6.4) indicate that there is an increase in all colony types in the spleen of the V449E mouse tested.

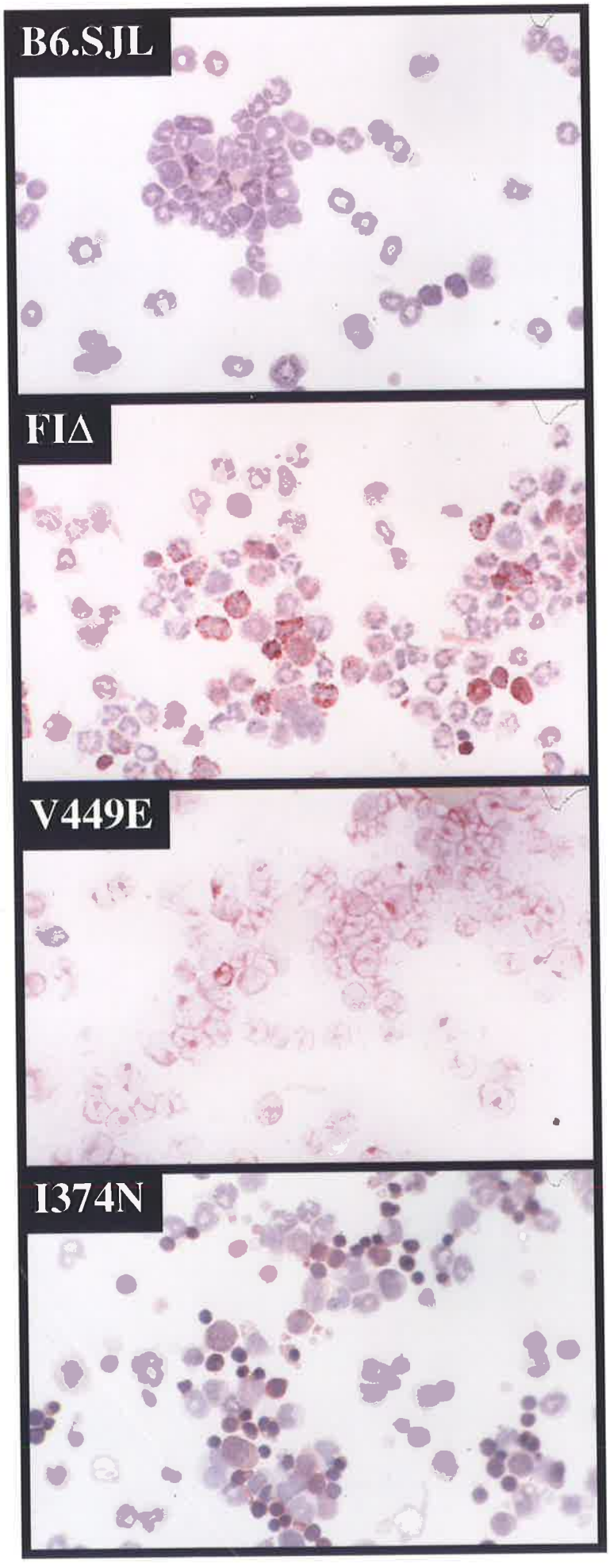
6.2.8 hβc protein expression in reconstituted mice

To verify that mice experiencing haemopoietic disorders did in fact contain the hβc proteins used in this study, bone marrow and spleen cell preparations from control and diseased mice were stained with an anti-hβc monoclonal antibody which was detected using an immunohistochemical approach. As detailed in Section 2.13.9, the approach used employed an enzymatic alkaline phosphatase reaction leading to cells expressing hβc staining red. Using this approach, no expression was found on bone marrow cells derived from control B6.SJL mice or RufNeo mice (Figure 6.24 and data not shown). However, expression of hβc protein was detected on both bone marrow and spleen cells of FIΔ, V449E and I374N mice (Figure 6.24 and data not shown). Expression was found on a variety of cell types; on myeloid and erythroid cells in the FIΔ and I374N mice and on blast cells and neutrophils in V449E mice. Surprisingly, expression of hβc protein was not seen in the tissues of hβc mice, including those which had appeared positive by the genomic PCR screen (data not shown, see section 6.2.3). It is possible that wild-type hβc protein was expressed at very low levels in the absence of a functional selection, such as factor-independence, and that the assay used was not sensitive enough to detect its expression.

6.3 Discussion

Figure 6.24. Expression of hβc proteins in the bone marrow of bone marrow reconstituted mice.

Bone marrow cells from a control mouse (B6.SJL) and bone marrow reconstituted mice containing the indicated hβc subunits were cytocentrifuged and hβc protein expression was detected using an immunohistochemical approach which stains expressing cells red (as described in Section 2.13.9). Cells were counterstained with haematoxylin. Photographs are at 180 × magnification.


B6.SJL
FIΔ
V449E
I374N

This study has shown the results of expression of three activated mutants of the human GM-CSF receptor β subunit in mice. In summary, irradiated mice receiving bone marrow cells infected with RufNeo-FIΔ or RufNeo-I374N developed chronic, lethal myeloproliferative syndromes characterised by neutrophilia, erythrocytosis, thrombocytosis and often monocytosis in the blood, splenomegaly, and myeloid and erythroid infiltration of the spleen, liver and lung. In the case of I374N mice this disease was variable, the only aspects present in all diseased mice being peripheral blood neutrophilia and myeloid and erythroid infiltrates of the spleen and other organs. However, disease in FIΔ mice was more uniform, with diseased mice exhibiting all the above features prior to illness.

In contrast, irradiated mice receiving bone marrow cells infected with RufNeo-V449E developed acute, lethal leukaemia-like disorders characterised by neutrophilia and blast cells in the blood, anaemia, splenomegaly and infiltration of blast cells and neutrophils into the spleen, liver and lung.

6.3.1 Implications for involvement of FIΔ and I374N in disease

The clinical features of FIΔ and I374N mice are similar, which is supportive of the previous hypothesis that these mutants signal via similar mechanisms (Jenkins et al., 1995). Similar clinical features were observed in transgenic mice expressing FIΔ; these mice also develop a trilineage disorder characterised by expansion of the neutrophil, erythrocyte and platelet lineages, splenomegaly and erythroid precursors in the spleen (D'Andrea et al., 1998). This implies that the biological effects of FIΔ in mice are not limited to the bone marrow reconstitution system used here. However, transgenic FIΔ mice also exhibited ataxia due to an accumulation of macrophage cells in the brain. This neurological phenotype was not observed here. The reason for this discrepancy is likely to reflect the different experimental systems used. Transgenesis integrates the gene of interest into the genome of cells in all tissues of the mouse, including the brain, whereas bone marrow reconstitution causes leads to expression in the haemopoietic system only. Hence whilst FIΔ was expressed in the brain of FIΔ transgenic mice, haemopoietic cells bearing FIΔ in this study would be unlikely to pass the blood-brain barrier and hence would not be expected to cause a similar pathology.

Many clinical features of the FIΔ and I374N mice were also observed in mice transgenic for the α and β subunits of the hGM-CSF receptor when treated with hGM-CSF (Nishijima et al., 1997). These mice also developed neutrophilia and monocytosis in the blood, and enlarged spleens containing erythroid precursors. However, these mice also displayed lymphocytosis in the blood and spleen, which was not observed here. Explanation for this comes from molecular studies of the signalling of the extracellular mutants, which have shown that I374N associates with the mGMRα subunit and that this mutant as well as FIΔ require co-expression of mGMRα for function in murine factor-dependent cell lines (B. Jenkins et al., manuscript submitted and R. D'Andrea, Hanson Centre for Cancer Research, Adelaide, South Australia, personal communication). If so, these mutants could only signal *in vivo* to cells which express mGMRα. In humans, hGMRα is not expressed on lymphocytes except for mature B cells (Till et al., 1996; Kurata et al., 1995; Jubinsky et al., 1994). Whilst a similar analysis has not been performed in mice, mGM-CSF does not induce lymphocyte development in mice hence these cells are unlikely to express mGMRα. Hence FIΔ and I374N would not be active in lymphoid cells of reconstituted mice, in contrast to the case of hGM-CSF transgenic mice.

The above studies showing a requirement of mGMRα for the function of FIΔ and I374N in murine factor-dependent cell lines correlate well with the neutrophilia and monocytosis observed in diseased FIΔ and I374N mice. The ability of the FIΔ and I374N mutants to signal in haemopoietic cells expressing the mGMRα subunit provides a molecular explanation for the factor-independent granulocyte-macrophage colonies formed by these mutants *in vitro* (see section 3.2.3) and found *in vivo* in the bone marrow and spleen of diseased FIΔ mice in this study. Hence the neutrophilia and monocytosis seen in these mice are likely to be due to the formation of active heterodimeric complexes of FIΔ/I374N with mGMRα in the granulocyte-macrophage lineages. In contrast to FIΔ mice, factor-independent granulocyte-macrophage colonies were not obtained from I374N mice bar a small number of macrophage colonies obtained when bone marrow cells were plated at high density. Hence it appears that the actions of I374N on the granulocyte-macrophage lineages *in vivo* are weaker than those of FIΔ, which is consistent with the fact that less severe neutrophilia was present

in the blood of these mice (Figure 6.4, Table 6.3). Interestingly, this difference was not seen in previous *in vitro* experiments using these mutants (see Chapter 3).

In addition to neutrophilia and monocytosis, FIΔ and I374N mice develop early-onset chronic erythrocytosis (Figure 6.7, Figure 6.10). This appears to be due to high levels of factor-independent CFU-E in the bone marrow and spleen of these mice (Figure 6.22). This unexpected result does not fit with the above model of FIΔ/I374N signalling being restricted to cells which express the mGMRα subunit. Using flow cytometric analyses, de Jong et al. (1997) were unable to detect expression of the GMRα subunit on developing erythroid cells in rhesus monkey bone marrow cells. Moreover, mGM-CSF does not effect erythroid development of murine haemopoietic cells *in vitro* or *in vivo* (Metcalf et al., 1986a; Metcalf et al., 1986b; Metcalf et al., 1987b) and mice reconstituted with retroviruses bearing mGM-CSF do not develop erythrocytosis despite moderate increases in erythroid progenitors in the spleen (Johnson et al., 1989). Hence murine erythroid cells are unlikely to express mGMRα. This then implies that the factor-independent erythroid development in FIΔ/I374N mice is likely to involve a mechanism other than that of constitutive activity of FIΔ/I374N due to the formation of heterodimeric complexes of these mutants with the mGMRα subunit. One possible explanation for this has come from work involving these mutants studied in the murine IL-3-dependent cell line BAF-B03. As stated above, FIΔ and I374N are unable to deliver proliferative signals to this cell line, however expression of the mGMRα subunit is able to rescue factor-independent signalling by these mutants. Recent work has identified other molecules which are able to complement the signalling defect of FIΔ and I374N in these cells. These include the human and murine Epo receptors and the murine TpoR (T. Blake, Hanson Centre for Cancer Research, Frome Road, Adelaide, South Australia, personal communication). Moreover, it has been shown that I374N leads to phosphorylation of the hEpoR in the human megakaryoblastic cell line UT-7, demonstrating a functional interaction (B. Jenkins, Hanson Centre for Cancer Research, Frome Road, Adelaide, South Australia, personal communication). The data obtained in BAF-B03 cells indicate that FIΔ and I374N can transduce proliferative signals in cells expressing mEpoR in the absence of mGMRα. Hence this is a likely mechanism of factor-independent erythroid development in FIΔ and I374N mice. If so, this represents the

first instance in which interaction of haemopoietic growth factor receptors are implicated in the pathophysiology of a haemopoietic disorder. Further work is required to test whether there is an *in vivo* interaction between FIΔ/I374N and the mEpoR in these mice.

Whilst factor-independent erythroid development was not detected in the previous study of the function of these mutants in primary haemopoietic cells *in vitro* (see Chapter 3), CFU-E were not specifically studied in these experiments, which were scored at day 7 hence would only have detected factor-independent BFU-E. Future studies should aim to demonstrate factor-independent erythroid development by these mutants *in vitro* in order to demonstrate that this is a direct consequence of FIΔ/I374N signalling.

Factor-independent BFU-E were not detected in diseased FIΔ/I374N mice nor in *in vitro* studies (see Chapter 3). Hence it appears that the actions of these mutants in erythroid development are at the level of CFU-E. The reason for this is likely to relate to the growth factor requirements of BFU-E versus those of CFU-E. Whilst Epo is required and sufficient for the development of murine CFU-E, Epo is not required for development of BFU-E to form CFU-E (Wu et al., 1995), and the hEpoR is not expressed on early human BFU-E (Sawada et al., 1988). Rather, the maturation of BFU-E to form CFU-E requires the activities of other haemopoietic growth factors such as IL-3, GM-CSF or SCF (Mitjavila et al., 1989; Anderson et al., 1990; Sonoda et al., 1988; Sieff et al., 1985; Emerson et al., 1985; Donahue et al., 1985). Hence the ability of FIΔ and I374N to function in cells expressing the mEpoR would not in itself lead to the formation of factor-independent BFU-E formation. However, since GM-CSF can stimulate BFU-E and together with Epo can induce their terminal maturation, it might be predicted that the combined signalling of FIΔ and I374N in cells which express the mGMRα and, later, the mEpoR, would lead to the complete development of BFU-E in the absence of growth factors. The reason this does not occur is not obvious. It may be that the signalling of FIΔ and I374N is deficient in some aspects when compared to signalling through wild-type GM-CSF or Epo receptors, or that the actions of these mutants which mimic GM-CSF and Epo in the absence of growth factors cannot occur simultaneously. Alternatively, FIΔ and I374N may require high

levels of mGMRα for function, or other cell type-specific molecules in addition to mGMα or mEpoR.

The functional interaction observed between I374N and the mTpoR in BAF-B03 cells (T.Blake, personal communication) may provide a molecular explanation for the thrombocytosis seen in some FIΔ and I374N mice (Figure 6.11). Increased thrombopoiesis was not observed in mice expressing GM-CSF in transgenesis or bone marrow reconstitution models, hence the thrombocytosis observed in FIΔ and I374N mice in this study is unlikely to be an effect of these mutants signalling in conjunction with mGMRα (Lang et al., 1987; Johnson et al., 1989). The increased thrombopoiesis in these mice may therefore be due to activity of these mutants in cells which express the TpoR. Again, megakaryocyte colony formation was not specifically addressed in the *in vitro* experiments performed using FIΔ and I374N detailed in Chapter 3, nor in the colony assays described in this chapter. Future experiments should aim to discern whether factor-independent megakaryocyte colonies are present in murine haemopoietic cells expressing these mutants both *in vitro* and *in vivo*.

The erythrocytosis observed in FIΔ and I374N mice was present in all reconstituted mice at early timepoints, including those which did not develop lethal haemopoietic disorders. In some mice this abnormality worsened to a lethal state, however in others it cleared. Clearance of the erythrocytosis in some mice may be due to loss of reconstituting stem cell clones expressing these mutants. It has been shown previously that after bone marrow reconstitution the majority of the haemopoietic system of a successfully reconstituted mouse is derived from one or two clones (Snodgrass and Keller, 1987; Lemischka et al., 1986). However, at about 4 to six months post-reconstitution new clones often emerge to supersede those originally present in a phenomenon known as clonal succession (Jordan and Lemischka, 1990). Accordingly, several FIΔ mice in which peripheral erythrocyte levels returned to normal did not develop disease. However, some I374N mice which cleared the initial erythrocytosis developed neutrophilia and disease. Erythroid cells were present in the spleens of these mice, and colony assays showed the presence of factor-independent CFU-E in the spleen of one such mouse (data not shown). The reason for the lack of erythrocytosis in these mice is unclear. It may be that basal erythropoiesis is perturbed in these mice.

6.3.2 Implications for involvement of V449E in disease

Mice reconstituted with V449E suffered from an acute disease characterised by accumulation of blast cells in the blood, spleen, liver and lung. This disorder is distinct from that seen in FIΔ and I374N mice, which is consistent with previous studies implying that transmembrane and extracellular mutants signal via different mechanisms (McCormack and Gonda, 1997; Jenkins et al., 1995). This is the first example of different mutants of a single haemopoietic growth factor receptor causing distinct myeloproliferative disorders.

The more restricted phenotype of V449E mice opposite what might be predicted from the *in vitro* studies using these mutants, in which FIΔ and I374N stimulate development of neutrophilic and monocytic cells only, whereas V449E can signal to all myeloid and erythroid cell types (see Chapter 3). Since V449E causes much greater factor-independent proliferation than FIΔ and I374N in these *in vitro* experiments, the lack of chronic myeloproliferative disorders in mice bearing V449E is unlikely to be due to this mutant signalling more weakly than the extracellular hβc mutants. Moreover, the disorder of V449E mice is unlike that seen when IL-3 and GM-CSF- which activate mouse βc- are expressed in mice via a similar protocol (Johnson et al., 1989; Chang et al., 1989). These mice develop lethal non-neoplastic myeloproliferative syndromes without leukaemia. There are at least two possible explanations for this discrepancy. Firstly, the receptors for mIL-3 and mGM-CSF may not be expressed on certain immature subpopulations of murine haemopoietic cells which are susceptible to leukaemic transformation by factor-independent signalling. However, since myeloproliferative and/or erythroproliferative disorders do not precede acute leukaemia in V449E mice, this is unlikely to be the only difference between V449E and IL-3/GM-CSF signalling. Alternatively, the different biological effects of V449E and IL-3/GM-CSF *in vivo* may be explained by the unique signalling effects of V449E identified *in vivo* (see Chapter 3). When expressed in fetal liver cells, V449E exhibits a partial defect in signalling macrophage development and leads to abnormally extended proliferation of immature haemopoietic cells. Hence it appears V449E is defective in some aspects of differentiative signalling and/or superior at signalling self-renewal, when compared to IL-3/GM-CSF signalling, which may explain the lack of mature myeloid and erythroid development observed in

V449E mice. This would also be predicted to increase the leukaemogenic potential of this mutant.

The reason for these putative signalling differences may relate to the stoichiometry of the active V449E complex. A similar mutation to V449E has been found in the c-*Neu* tyrosine kinase oncogene of rats, which leads to a valine to glutamic acid substitution in the transmembrane domain of this molecule and activation of its tyrosine kinase activity (Bargmann et al., 1986a). It has been proposed that this mutation leads to constitutive dimerisation of c-Neu and activation of its tyrosine kinase activity (Weiner et al., 1989; Sternberg and Gullick, 1989). By analogy, it is likely that the V449E mutation leads to factor-independent functional dimerisation and activation of hβc, which may occur in the absence of α chain binding. As signalling through wild type GM-CSF/IL-3/IL-5 receptors occurs via complexes consisting of both α and β subunits, this would be an abnormal situation (Woodcock et al., 1997). It is possible that there is a requirement for the α subunit for some aspects of differentiative signalling through these receptors, which are therefore not transduced by the V449E mutant. The nature of any putative qualitative signalling differences between V449E and wild-type IL-3/GM-CSF/IL-5 receptors is not presently obvious, as V449E has been shown to mediate most known signalling functions of these receptors, including activation of the JAK2 kinase and STAT transcription factors, phosphorylation of Shc, SHP2 and the hβc receptor subunit itself, activation of Erk1 and Erk2 and induction of c-myc and c-fos expression (Jenkins et al. (1998), T. Blake, Hanson Centre for Cancer Research, Adelaide, South Australia, personal communication).

Disease in V449E mice occurred after a long and variable latency (mean 20 weeks), and in a minority of reconstituted mice (5/17). The long latency and acute onset of disease in these mice imply that further mutations are required in addition to V449E signalling, consistent with the multi-step model of leukaemogenesis. This is similar to the previous findings that factor-independent cell lines generated by expression of RufNeo-V449E in fetal liver cells were tumorigenic in only a proportion of mice, implying that subsequent mutations were required for full oncogenicity (see Chapter 3).

The V449E protein is almost certainly involved in the generation of the leukaemic phenotype in V449E mice. If the disease phenotype observed were due to factors other than the V449E protein, the probability of the 16 control (RufNeo and hβc) mice remaining disease free is less than 2% (χ^2 test). Furthermore, other investigators have found that of more than 300 control mice bone-marrow reconstituted with retrovirally-infected cells using similar methodology to that employed in this study, none developed leukaemia (Hawley et al., 1993). Moreover, hβc protein expression was detected on bone marrow and spleen cells of some leukaemic V449E mice using immunohistochemical staining (Figure, 6.24). This implies that V449E is involved in the generation of the leukaemic phenotype in V449E mice. However, whilst there were virally-infected progenitors in the bone marrow and spleen of these mice as measured by G418 resistance, these were not factor-independent (Table 6.4, Table 6.5). Moreover, transformed cell lines were not obtained from the bone marrow of V449E mice by culture in the absence of growth factors or in the presence of various growth factors including IL-3, GM-CSF, Epo or SCF (data not shown). Whilst there were increases in BFU-E and mixed myeloid/erythroid colonies in the bone marrow and spleen of V449E mice (Table 6.5), the relationship of these colonies to the leukaemic blasts is uncertain. Hence the exact mechanism causing the leukaemic proliferation of blast cells in V449E mice remains obscure. It is possible that an additional signal(s) is required for proliferation of the leukaemic blasts in addition to V449E signalling. Alternately, the leukaemic blast cells in the diseased V449E mice may be incapable of growth in the tissue culture systems employed here. Future efforts should be made to culture these blast cells *in vitro*, perhaps using other early-acting cytokines and/or bone marrow stromal feeder layers as stimuli. A further understanding of the contribution of V449E to the leukaemic phenotype of V449E mice may require a more detailed analysis of these mice in the early, asymptomatic phase. It is possible that V449E signalling causes an increase in immature haemopoietic precursors in the bone marrow, similar to that seen in *in vitro* studies using this mutant, and that this population is susceptible to second events required for leukaemogenesis.

In summary, I have shown that activated mutants of the hβc subunit cause haemopoietic disorders in mice, and that the nature of the disorder depends on the

location of the activating mutation. Whilst the extracellular activated mutants, FIΔ and I374N, cause a lethal myeloproliferative disorder of the erythrocyte, monocyte, neutrophil and platelet lineages, a transmembrane activated mutant, V449E, causes an acute leukaemia-like disease. Future studies aimed at further clarifying signalling differences between these two classes of hβc mutants may provide important information regarding the types of signals leading to chronic myeloproliferative disorders and leukaemia.

This study has shown that constitutively active transmembrane and extracellular hβc mutants differ in their signalling properties in three separate experimental systems: (1) in their *in vitro* effects in foetal liver cells, in which the transmembrane mutant, V449E, can confer factor independence on all the haemopoietic cell types assayed, whereas extracellular hβc mutants could signal to the granulocyte-macrophage lineages only; (2) in their signalling *in vitro* to the factor-dependent murine differentiation-competent cell lines, FDB1 and FDB2, in which V449E causes factor-independent proliferation however extracellular mutants lead to granulocyte-macrophage differentiation; and (3) in their effects *in vivo* in bone marrow reconstituted mice, in which V449E causes an acute late-onset leukaemia-like disorder whereas the extracellular mutants lead to an early onset, chronic multilineage myelo- and erythro-proliferative disorder. This is the first demonstration of different activated forms of a cytokine receptor having different biological effects. These findings provide important information relating to both cytokine receptor signal transduction and the oncogenic potential of the cytokine receptor superfamily.

7.1 Implications for cytokine receptor signal transduction

A combination of the data obtained in the three separate systems employed in this study suggests that there are signalling differences between constitutively active transmembrane and extracellular hβc mutants. Are the modes of signalling employed by the two classes of hβc mutant separate and complementary, or does one encompass and expand upon the other? Two separate lines of evidence suggest the former possibility. Firstly, the spectra of factor-independent cell types induced by these mutants in foetal liver cell liquid cultures appear distinct. The extracellular mutants strongly induce macrophage development, whereas V449E induces this lineage poorly. In addition, V449E induces extensive proliferation of immature cell types (blast cells, promyelocytes and myelocytes), whereas the extracellular hβc mutants do not. Secondly, the phenotypes of mice reconstituted with transmembrane and extracellular hβc mutants are distinct. Whilst mice bearing extracellular hβc mutants develop an early onset, chronic multilineage myeloproliferative disorder, mice bearing

the V449E mutant do not. Conversely, mice bearing the transmembrane mutant, V449E, develop acute leukaemia, again in contrast to those expressing the extracellular mutants.

There are two possible and non-exclusive mechanistic bases for these differences. Transmembrane and extracellular mutants may signal to different progenitor cell types, or they may deliver distinct signals to the same progenitor cell types. It is clear from the findings detailed in Chapter 3, which show that extracellular hβc mutants are restricted to the granulocyte-macrophage lineages whereas the transmembrane mutant is not, that the former possibility is true. However, the studies in Chapter 5 demonstrating that transmembrane and extracellular mutants deliver distinct signals to multipotential cell lines show that these mutants can deliver qualitatively different signals to the same progenitor cell type. Hence a combination of these data suggests that the differences in biological effects of transmembrane and extracellular hβc mutants are probably due to a combination of these two possibilities.

The nature of the putative signalling differences between extracellular and transmembrane hβc mutants has been studied in the FDC-P1 cell line. It has been found that extracellular constitutively active hβc mutants are deficient in receptor phosphorylation, Shc phosphorylation and activation and phosphorylation of SHP2, but show normal activation of JAK2, STAT proteins, Erk1 and Erk2, and induction of the *c-myc* and *c-fos* genes (Jenkins et al. (1998) T. Blake, Hanson Centre for Cancer Research, Adelaide, South Australia, personal communication). Defects in Shc and SHP2 phosphorylation may be secondary to the defective receptor phosphorylation of this mutant, since these proteins bind to phosphorylated residues in the hβc cytoplasmic domain and recent studies show a requirement of hβc phosphorylation for phosphorylation of these proteins by the hGMR (Okuda et al., 1997; Itoh et al., 1996; Durstin et al., 1996). This implies that hβc phosphorylation (and the aspects of hβc-mediated signal transduction which require hβc phosphorylation) is not required for proliferation in the FDC-P1 cell line, and in combination with the results of this study suggests that hβc phosphorylation is not required for terminal granulocyte-macrophage development *in vitro* or *in vivo*.

In contrast, the transmembrane hβc mutant, V449E, is able to activate all of the above signalling responses in FDC-P1 cells. It is interesting that no defect in signal transduction was found in studies using this mutant, as several lines of evidence suggest that such defects should exist. Firstly, this mutant does not cause chronic myeloproliferative disorders *in vivo* in mice, unlike the extracellular mutants or a signal delivered through the high affinity hGMR (Nishijima et al., 1997). This result is remarkable in light of the fact that V449E causes much greater factor-independent proliferation of foetal liver cells *in vitro* than the extracellular hβc mutants (see Chapter 3). Secondly, this mutant is unable to deliver a proliferative signal to the murine IL-2-dependent T cell line, CTLL-2, unlike the wild-type hGMR or the I374N mutant co-expressed with the mGMRα subunit (Jenkins et al. (1995), B.J. Jenkins et al. (submitted)). This implies that cell-type specific molecules are required for the function of the V449E protein, or that V449E is unable to activate some signal transduction pathways required for the proliferation of CTLL-2 cells. Thirdly, this mutant has an unusual requirement for Y750 for function, in that when this residue is mutated, signalling through V449E in FDC-P1 or FDB2 cells is perturbed to a far greater extent than Y750 mutants of I374N or hβc in the context of a high affinity hGMR (T. Blake, personal communication). Together with the results of this study, this suggests a role for signal transduction through Y750 primarily in proliferation, whilst other regions of hβc are required for maximal differentiation signalling. It has been suggested previously that Y750 contributes to the maintenance of cellular viability in response to hGM-CSF and is partially responsible for Shc phosphorylation (Inhorn et al., 1995). The results of this study suggest that, in the context of the V449E mutant, signals originating from this residue are involved in proliferative signalling but are unlikely to be involved in cellular differentiation. This is concordant with the observation that the extracellular hβc mutant, I374N, is defective in receptor phosphorylation (including Y750) and Shc phosphorylation, but shows no defect in differentiation signalling *in vitro* or *in vivo* in the various assays employed in this study.

Future studies should aim to identify the putative signalling defects of the V449E mutant. One approach to achieve this would be to attempt to complement the defective signalling of this mutant in CTLL-2 cells by introducing cDNA libraries

derived from a permissive cell type such as FDC-P1 or BAF-B03. It is also possible, however, that CTLL-2 cells express the components required for growth but that V449E is unable to activate one or more signalling pathways, for example due to an inability to associate with an α subunit. This model requires that activation of different signalling pathways is required for proliferation of different cell types. Deficient activation of cellular signal transduction pathways may also be responsible for the inability of V449E to maximally signal differentiation *in vitro* and *in vivo*. An alternative approach, then, would be to intuitively select signalling pathways implicated in cellular differentiation and activate them in CTLL-2 cells which express the V449E mutant, also with the aim of complementing its defective proliferation signalling in this cell line. Signal transduction components implicated in haemopoietic differentiation include $p21^{waf1}$ (Liu et al., 1996), C/EBP (Westendorf et al., 1998), STAT5 (Woldman et al., 1997; Wakao et al., 1997), PKC (Durkin et al., 1992; Whetton et al., 1994), the Ras-Raf-MAPK pathway (Yen et al., 1998; Matsumura et al., 1998) and p53 (Soddu et al., 1996).

7.2 Implications for the involvement of hβc mutations in disease

7.2.1 Extracellular mutants

This study has shown that the extracellular constitutively active hβc mutants FIΔ and I374N can confer cellular growth-factor independence on the granulocyte-macrophage lineages *in vitro* and *in vivo* and can deliver a functionally analogous signal to mGM-CSF in the absence of growth factors to the murine myeloid cell lines FDB1 and FDB2. The cell-type specificity of these mutants previously observed in myeloid cell lines (Jenkins et al., 1995) and seen in this study in primary haemopoietic cells has recently been explained by the finding that these mutants require the mGMRα subunit for function in the murine cell line BAF-B03 (B.J. Jenkins et al., manuscript submitted).

The requirement for mGMRα and similarity to mGM-CSF signalling suggests that the potential of extracellular hβc mutants to contribute to neoplastic disease might be similar to that of GM-CSF. In this regard, hGM-CSF has been shown to play a role in a number of disorders including AML, ALL and JCML (Gualtieri et al., 1989; Freedman et al., 1993; Young et al., 1987). It is therefore possible that the

extracellular hβc mutants could contribute to such disease states through a non-autocrine mechanism. If so, mutations such as these are likely to be events secondary to cellular transformation, as neither production of GM-CSF *in vivo* (Johnson et al., 1989; Lang et al., 1987) nor the expression of extracellular hβc mutants leads to the development of acute leukaemia in mice.

Several studies have examined the structure of hβc in AML. Brown et al. (1993) studied the structure of hβc and hGMRα in a number of human leukaemias by southern blotting. Whilst no rearrangements were found, the technique used would only detect structural alterations hence point mutations such as those examined in this study may have been missed. In a separate study Freeburn et al. (1995) surveyed the intracellular domain of hβc derived from several AML patients for activating point mutations using the technique of single-stranded conformational polymorphism (SSCP). None were found, however since only the intracellular domain was screened, activating mutations such as V449E or I374N which lie in the transmembrane and extracellular domains of hβc, respectively, again would not have been detected.

In contrast to its apparent granulocyte-macrophage specificity in signalling to primary haemopoietic cells *in vitro*, FIΔ and I374N lead to expansion of these lineages as well as the erythrocyte and megakaryocyte lineages *in vitro*. As described in Chapter 6, this multilineage signalling may be due to functional interactions with other related cytokine receptors including the mEpoR and the mTpoR. Function in cells expressing these receptors as well as in those expressing mGMRα would then broaden the range of cell types permissive to factor-independent proliferative signalling by these mutants. This implies that in addition to their GM-CSF-like effects on haemopoiesis, FIΔ and I374N have the potential to be involved in disorders of dysregulated erythropoiesis and thrombopoiesis.

The multilineage phenotype of FIΔ and I374N mice is in many ways similar to the human disease Polycythaemia Vera (PV). This disease is characterised by increased red cell mass often with associated thrombocytosis and neutrophil leucocytosis (reviewed in Pearson and Messinezy, (1996)). Whilst the molecular basis for PV is not well understood, it has been hypothesised that PV is caused by an alteration in a multipotential progenitor common to these lineages leading to clonal multilineage

expansion (Hinshelwood et al., 1997). Since hβc is expressed on multilineage progenitors (Sato et al., 1993a) and extracellular hβc mutants induce a PV-like disease *in vivo*, these mutants are candidates for lesions leading to PV. However, whilst the phenotype of this disease is quite similar to that of mice bearing FIΔ and I374N, the behaviour of progenitor cells from PV patients differs somewhat from those of FIΔ and I374N mice. Bone marrow from the majority of patients show factor-independent erythroid colony growth, however unlike FIΔ and I374N mice, BFU-E are generally factor-independent in addition to CFU-E (reviewed in Reid et al., (1987)). Moreover, whilst granulocyte-macrophage progenitors from PV patients are hypersensitive to both IL-3 and GM-CSF, they are not completely factor-independent (Dai et al., 1992), unlike those of FIΔ mice. However, the levels of factor-independent colonies are low in these mice, and there was no significant colony formation from some I374N mice which showed PV-like symptoms, even though these mutants were expressed at high levels from a retroviral promoter. It would be useful, in future studies, to test the sensitivity of granulocyte-macrophage progenitors to GM-CSF and IL-3, to examine whether they are hypersensitive to these factors similarly to those from PV patients. Factor-independent megakaryocyte colonies are also frequently present in PV (Li et al., 1994). Whilst these were not assessed in FIΔ and I374N mice, the thrombocytosis of some of these mice suggests factor-independent stimulation of the megakaryocyte lineage in these mice. Due the similarity of the phenotype of FIΔ/I374N mice and PV, these mice may provide a useful model for studying this disease.

The finding that FIΔ and I374N cause factor-independent erythroid development *in vivo*, together with the finding that these mutants can co-operate with the mEpoR to lead to factor independent signalling in BAF-B03 cells (T. Blake, personal communication), suggests that interactions between growth factor receptors, and in particular hβc and the EpoR, occur *in vivo* and could have important implications in disease states. In considering the involvement of this interaction in normal and abnormal haemopoiesis, it is important to consider the expression of hβc and the EpoR, since their interaction can only occur in cells in which both proteins are expressed. Using semiquantitative PCR analyses, Ashihara et al., (1997) showed that both mβc and EpoR are barely expressed in the most primitive murine haemopoietic cells. However, after subsequent culture, the EpoR is expressed on both erythroid and

myeloid cells. The expression of this receptor in myeloid cells permits the possibility that the EpoR may interact with the GM-CSF receptor in certain myeloid cell subsets. In the erythroid lineage, the EpoR is induced during the BFU-E stage and its expression continues until the late basophilic erythroblast stage, at which point the cells are no longer factor-dependent for continued maturation (Sawada et al., 1988; Sawada et al., 1990; Koury and Bondurant, 1988). Whilst the expression of hβc in this lineage has not been studied, it is known that both IL-3 and GM-CSF regulate the transition of BFU-E to form CFU-E (Mitjavila et al., 1989). Hence there appears to be an overlap in the expression of hβc and the EpoR in the erythroid lineage, which would permit an interaction. However, the GM-CSF and IL-3 receptors are lost at the CFU-E stage, although it is not known if this is due to loss of the α subunits, hβc or both. If hβc is lost, a functional heterodimeric complex of an activated hβc mutant and the EpoR would not normally be able to form late in erythroid development leading to terminal maturation of this lineage. This would contrast with the situation of mice expressing FIΔ and I374N retrovirally, in which expression of hβc was enforced in erythroid cells throughout maturation. Further study is required to elucidate the extent of expression of hβc in this lineage and its role if any in erythroid development.

As mentioned in Chapter 6, the I374N and the mTpoR are able to functionally interact to allow factor-independent signalling to the BAF-B03 cell in the absence of mGMRα, providing a possible molecular explanation for the thrombocytosis seen in some FIΔ and I374N mice. Similarly to the case for the interaction of hβc with the EpoR outlined above, it is possible that this interaction could play a role in normal and abnormal haemopoiesis. Both hβc and hTpoR are expressed in early haemopoietic cells providing an opportunity for these receptors to interact *in vivo* (Zeigler et al., 1994; Vigon et al., 1992; Sato et al., 1993). Moreover both GM-CSF and Tpo can drive complete megakaryopoiesis, however Tpo and not GM-CSF can effectively induce the final stages of maturation up to and including platelet shedding (Norol et al., 1998; Kaushansky et al., 1994; Metcalf et al., 1986). Perhaps due to this, neither transgenic nor bone marrow reconstituted mice expressing GM-CSF develop thrombocytosis (Lang et al., 1987; Johnson et al., 1989). The thrombopoiesis of FIΔ and I374N mice suggests that, unlike mGM-CSF, these mutants are capable of inducing the terminal stages of megakaryopoiesis, implying that such mutants may

contribute to disorders involving thrombocytosis, such as PV. However, it is possible that the actions of FIΔ and I374N on this lineage simply render megakaryocyte precursors to other growth factors, such as Tpo. Future experiments should aim to identify the role of cytokine receptor interaction in the disease phenotype of FIΔ and I374N mice.

7.2.2 V449E

The results detailed in Chapter 6 show that, when expressed *in vivo*, the constitutively active transmembrane hβc mutant, V449E, causes acute leukaemia in mice without a detectable prior haemopoietic disorder. This suggests that V449E has the potential to play a role in the genesis of human leukaemia. Furthermore, together with the results obtained from FIΔ and I374N mice, these results imply that different types of hβc mutants can lead to chronic myeloproliferative disorders or acute leukaemia. Since transmembrane and extracellular mutants have distinct signalling properties, implicit in these findings is that different types of cytokine receptor-induced signalling may be responsible for these two types of haemopoietic disorders.

That the disease states induced by dysregulated GM-CSF/IL-3 stimulation and V449E signalling differ correlates with the unusual signalling properties of V449E outlined in Chapter 3, in which immature (promyelocyte and myelocyte) cells predominate during the course of factor-independent culture of foetal liver cells expressing this mutant, unlike culture of foetal liver cells in the presence of growth factors (IL-3, GM-CSF and Epo). This outgrowth of immature cell types was rapid, and by the third week of culture cells containing V449E were almost entirely immature myeloid cell types. The loss of mature cell types in favour of more immature precursors may provide an explanation for the lack of ostensible myeloproliferative disorders in mice containing V449E, since these immature cell types are not normally found in the peripheral blood and so would be expected to remain in the bone marrow. This could be investigated in future studies by examining the haemopoietic organs of V449E mice in the pre-leukaemic state.

Both chronic myeloproliferative disorders and acute leukaemia have been observed previously in murine models of dysregulated growth factor stimulation. Whilst IL-3 and GM-CSF induce chronic, non-neoplastic myeloproliferative disorders (Johnson et

al., 1989; Chang et al., 1989), dysregulation of early-acting growth factors such as Flt3 and IL-11 induces leukaemic predisposition in mice without prior chronic myeloproliferative disorders, similarly to V449E (Hawley et al., 1998; Hawley et al., 1993). In these cases, however, the biological effects induced by overexpression of these factors is more akin to their biological functions *in vitro*, which are predominantly stimulation of immature haemopoiesis in synergy with other growth factors (reviewed in Lyman and Jacobsen (1998)) (Tsuji et al., 1992; Musashi et al., 1991), which is distinct from the case of V449E mice. Like the diseases induced by stimulation with these early-acting growth factors, the late onset and low frequency of disease in V449E mice implies that further mutations are required for the generation of leukaemia in these mice. I have found that V449E can confer factor-independence on *myb*-transformed haemopoietic cells as well as on cell lines transformed by *Hox-B8* (formerly *Hox-2.4*) (data not shown). Since factor-independence has been previously shown to confer tumourigenicity on these two cell lines, activations in these two transforming transcription factors are candidates for second events leading to leukaemia (Perkins et al., 1990; Gonda et al., 1989).

On the other hand, leukaemic blasts from V449E do not exhibit factor-independent growth in culture. It may be that these blasts are simply unable to grow in the *in vitro* culture systems used, or that additional growth signals are required for their growth *in vitro* in addition to V449E signalling. Such signals may be provided in a paracrine manner *in vivo* and their provision may be second events required for leukaemogenesis. Paracrine growth stimuli have been previously implicated in human leukaemia, for example some AML samples produce IL-1, a factor which induces expression of GM-CSF from bone marrow stroma (Griffin et al., 1987).

7.3 Future studies

7.3.1 Studies using the FDB cell lines

The studies in Chapter 5 demonstrate isolation and characterisation of the FDB1 and FDB2 cell lines, which have the unique properties of growth in murine IL-3 and growth arrest and complete granulocyte-macrophage differentiation in murine GM-CSF. Future studies should address the mechanism the different signalling of mIL-3 and mGM-CSF in this cell line. With regard to their cognate receptors, there are two

important differences which may play a role. Firstly, each binds to a specific α subunit. As the cytoplasmic region of these subunits is required for signal transduction through the IL-3 and GM-CSF receptors, it is possible that they play active and non-identical roles in signalling, however the role of these subunits in signalling is still ill-defined (Polotskaya et al., 1993; Barry et al., 1997). With regard to the differentiation switch of the FDB cell lines, the observation that IL-3 signalling is dominant to that of GM-CSF suggests that the most likely scenario is that the cytoplasmic domain of mIL-3Rα transduces signals which block mGM-CSF-induced differentiation. One way to demonstrate this would be the construction of chimaeric proteins consisting of the extracellular and transmembrane domains of mGMRα and the intracellular domain of mIL-3Rα. If the above hypothesis were true, the expression of such chimaeric proteins in FDB cells followed by stimulation with GM-CSF should allow continuous proliferation without differentiation.

The other fundamental difference between mIL-3 and mGM-CSF receptors is that whilst the mGM-CSF receptor utilises a single β subunit, mβc, the mIL-3 receptor utilises both mβc and an IL-3 specific β subunit $m\beta_{IL\text{-}3}$ (Hara and Miyajima, 1992; Gorman et al., 1990). Hence a block in GM-CSF-induced differentiation may be transduced by the $m\beta_{IL\text{-}3}$ protein. Again, since the mIL-3 signal is dominant, this hypothesis could be tested by the construction of chimaeric proteins, in this case consisting of the extracellular and transmembrane regions of mβc fused to the intracellular region of $m\beta_{IL\text{-}3}$. Expression of such proteins in the FDB cell lines would, if this hypothesis was correct, allow mGM-CSF to deliver a continuous proliferative signal to these cell lines.

As they exhibit a growth factor-dependent differentiation switch, the FDB cell lines have potential utility in screening for genes whose expression influences, or is influenced by, myeloid differentiation. An approach to achieve this would be to study the gene expression in FDB1/2 cells in the presence of IL-3 versus that of cells in GM-CSF. There are a number of approaches which could achieve this, including differential display PCR, representational difference analysis and cDNA library subtraction techniques.

Finally, as the FDB cell lines completely differentiate in mGM-CSF, these cell lines may provide a useful system to screen for genes which block GM-CSF-induced differentiation. One approach would be to introduce expressed genes from a cell line which is blocked in mGM-CSF-induced differentiation, such as FDC-P1, using a cDNA library screening approach. The identification of such genes would be interesting from the point of view of leukaemogenesis, as several types of leukaemia including AML, JCML and ALL have been shown to express GM-CSF in an autocrine loop, and do not exhibit normal differentiation in response to this factor (Gualtieri et al., 1989; Freedman et al., 1993; Young et al., 1987). Hence genes which block GM-CSF-induced differentiation may play a role in the genesis of these types of leukaemia.

7.3.2 Studies relating to activated hβc mutants

The *in vitro* and *in vivo* studies using constitutively active hβc mutants detailed in Chapters 3 and 6, respectively, imply that constitutively active hβc mutants have the potential to be involved in human myeloproliferative disorders and leukaemia. Several aspects of this work await further investigation.

Firstly, the mechanism behind the chronic erythropoiesis and thrombopoiesis in FIΔ and I374N mice warrants examination, in particular as to the putative requirement for cytokine receptor interaction. Most immediately, *in vitro* studies using retroviral infection of primary murine haemopoietic cells should be used to confirm that the extracellular hβc mutants can confer factor independence on erythroid and megakaryocyte colonies, thereby verifying that the effects of FIΔ and I374N on the erythrocyte and platelet lineages *in vivo* are direct. If so, the putative requirement for cytokine receptor interactions awaits further study. One possible approach to confirm this would be the use of antisense oligonucleotides to the EpoR to block factor-independent CFU-E formation by haemopoietic cells derived from FIΔ or I374N mice.

As the blast cells in V449E-induced leukaemia do not grow factor-independently *in vitro*, the exact mechanism leading to their leukaemic proliferation is unclear. Future work should further examine these mice in the pre-leukaemic phase, in particular studying the bone marrow and spleen of these mice to examine whether V449E is delivering factor-independent signals to haemopoietic cells in these mice and, if so, to

which types. These studies could provide important information relating to the state of the haemopoietic system in the pre-leukaemic phase of acute human leukaemias such as acute myelogenous leukaemia.

As it is clear that the extracellular hβc mutants require interaction with murine cytokine receptor components, and the exact mechanism of V449E signalling is at present unclear, the function of these mutants in human haemopoietic cells warrants investigation. An approach similar to that employed in Chapter 3, that involves amphotropic retroviral infection of human haemopoietic cells followed by culture in the absence of growth factors, could be employed.

Finally, based on the *in vitro* and *in vitro* activities identified in this study, screening of human diseases for activating mutations in hβc is warranted. Candidate diseases for extracellular activated mutants are chronic disorders such as chronic myeloid leukaemia and polycythemia vera, whereas V449E may play a role in acute leukaemias such as acute myeloid leukaemia.

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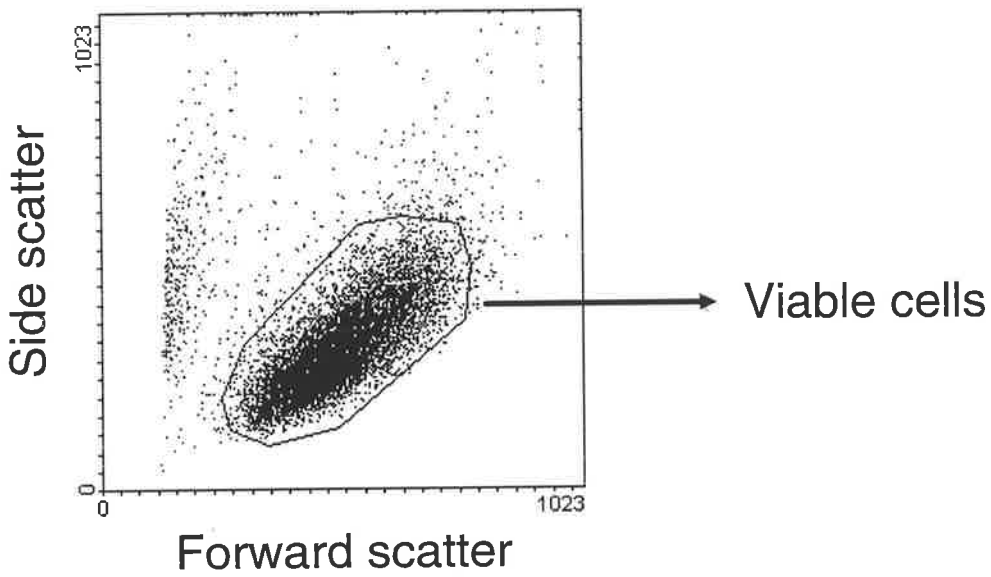
Amendments to PhD thesis entitled "The biological effects of constitutively active mutants of the common β subunit of the human IL-3, IL-5 and GM-CSF receptors" by Matthew Paul McCormack

General Points

- **Chapter 3, pg. 58, Chapter 4, pg. 73.** Whilst expression of the cell surface antigen Thy-1 is characteristic of immature haemopoietic cells, it may also be expressed by mature cells of various lineages. In the experiments described in **pg. 58**, Thy-1 positive cells were negative for myeloid markers (**Figure 3.5**) and were clearly not lymphoid or erythroid by morphology (**Figure 3.4**) hence in conjunction with these observations, Thy-1 expression is suggestive of an immature phenotype. In those described in **pg. 73**, cells cultured in IL-3 expressing Thy-1 may have been maturing myeloid cells, however absence of this marker on cells cultured in GM-CSF suggests that very immature cells were not present in this population.

- **Chapter 2.12.11, pg. 51.** For flow cytometric analyses viable cells only were analysed by gating on scatter profile (forward scatter versus side scatter). An example of this is provided below.



- **Chapter 2.12.11.2, pg. 51.** For cell sorting, identically stained uninfected (non-expressing) cells of an identical line were used as negative controls. Fluorescence

gates were set such that 0.0% of these cells fell within the gate. Where more than 10% of the cells to be sorted fell within this region, 5% of cells were taken typically. Following expansion, flow cytometric analysis confirmed that all cells from the resulting sorted pools expressed the desired antigen.

- **Chapter 2.12.11, pps. 50-51.** Where indicated, isotype controls of known protein concentration were used. When possible, isotype controls were used at a similar protein concentration to monoclonal antibodies used for analysis of cell surface antigens. When protein concentration was not known, both isotype controls and monoclonal antibodies used to detect cell surface antigens were used at previously determined optimal concentrations.

- **Chapter 2.12.11, pps. 50-51.** For each flow cytometric analysis (both scatter profiles and cell surface antigen analysis) 10^4 viable cells (gated as described above) are depicted.

Specific Points

- **Chapter 2.12.1, pg. 43.**
  - Ψ2 cells were generally passaged at a split ratio of 1:20
  - BOSC-23 cells were generally passaged at a split ratio of 1:5
  - FDC-P1 cells were generally passaged at a split ration of 1:40
  - The FDB1 and FDB2 cell lines were generally passaged at a split ratio of 1:10. Care was taken not to overgrow the cells, which promoted spontaneous differentiation. In differentiation assays care was taken not to decrease cell density below 10^5/ml where possible.

- **Chapter 2.12.11.2, pg. 51. line 8.** "maintenance medium" should read "the medium used to maintain the individual cell lines as described in section 2.12.1.

- **Chapter 2.13.5, pg. 53 and Chapter 6, pg. 91.** It is stated that "to achieve homogeneous staining...the flow cytometric analysis was gated to detect lymphocytes only". Obviously such gating would not affect the *staining* of the cells, but rather allows fluorescence to be observed on a near-homogeneous cell population, which simplifies a quantitative analysis such as that shown in **Table**

6.1. In fact repopulation as judged by the relative levels of Ly5.1 isotopes was similar on lymphocytes, monocytes and neutrophils (data not shown), however as each of these cell populations expresses slightly different levels of Ly5.1, this analysis is best performed by studying each population in isolation.

Minor textual errors

Chapter 1

Pg. 5, line 26 should read "...It also affects development ..."

Pg. 6, line 11 should read "...Hence IL-3 can affect production..."

Pg. 8, line 23 should read "...at the level of the eosinophil progenitor, leading to..."

Pg. 17, line 8 should read "...whilst a region between..."

Pg. 21, line 13 should read "...bone marrow stroma and by inducing"

Pg. 21, line 18 should read "...is by mutation of the receptors..."

Chapter 2

Pg. 35, 3rd last line should read "...L-Agar containing 100 μg/ml..."

Pg. 36, line 4 should read "...2.5 μg/ml fungizone..."

Chapter 3

Pg. 61, line 11 should read "...injected with RTVE1 displayed the ring nucleus..."

Chapter 5

Pg. 79, line 1 should read "...these puromycin resistant cells were maintained..."

Pg. 83, line 26 should read "some cell death was seen..."

Chapter 6

Pg. 94, line 10 should read "...mice which did not exhibit..."

Pg. 103, line 27 should read "...bone marrow reconstitution leads to expression..."

Chapter 7

Pg. 117, line 20 should read "...Due to the similarity of the phenotype..."

Pg. 123, line 10 should read "...based on the *in vitro* and *in vivo* activities ..."

McCormack, M. P., and Gonda, T.J., (1997) Expression of activated mutants of the human interleukin-3/interleukin-5/granulocyte-macrophage colony-stimulating factor receptor common β subunit in primary hematopoietic cells induces factor-independent proliferation and differentiation.
Blood, v. 90 (4), pp. 1471-1481.