The Role of γ-Catenin in Acute Myeloid Leukaemia

by

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2011

A thesis presented to Cardiff University in partial fulfilment of the requirement for the degree of Doctor of Philosophy

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Acknowledgements

I would like to acknowledge the following individuals for their invaluable contributions to this study:

- My supervisors Drs. Richard Darley and Alex Tonks for their expertise and unwavering patience.

- Professor Alan Burnett and the Medical Research Council for funding and allowing me to undertake this research within his department.

- Lorna Pearn and Dr. Kate Liddiard for technical support and laboratory expertise.
- UHW delivery suite and midwives for cord blood samples.
- Sarah Philips and the Welsh Bone Marrow Registry for bone marrow samples.
- Dr. Paul White and the NCRI-MRC AML trials for primary AML samples.
- Steve Couzens for use of UHW flow cytometry facilities.
- Dr. Chris Pepper for assistance with MoFlo FACS experiments.
- Professor Maurice Hallett and Dr. Chris George for technical support and guidance with Confocal Laser Scanning Microscopy.
- Professor Martin Rudhardt for the γ-catenin shRNA construct and Dr. James Matthews for the pRRLSIN.cPPT.PGK/GFP.WPRE construct.
- Dr. Robert Hills for assistance with statistical analyses.
- Drs. Steven Coles, Nader Omidvar, Paul Hole and Beth Walsby for moral support and laboratory banter.

- Finally, I wish to thank my family and friends for their continued support and kindness throughout this project. Especial thanks go to my wonderful fiancée Natasha for her unrelenting patience, love and support over this long and testing period.

Foreword

An electronic version of this document is provided on the accompanying DVD (see back sleeve). In the electronic version of the document, all cross-references act as hyperlinks.

A large number of confocal and morphology microscopy images are presented in this thesis which often suffer in quality when printed onto paper. Therefore, every image plus additional supplementary images/videos are supplied on the attached DVD disc. Whenever such an image/video is cross-referred in the thesis its exact location on the disc is described in an ***italic bold red font***.

Finally, *Chapter 5* contains references to specific time-points (days) of long-term experiments. Whenever such a day is referred to it will be highlighted in **bold blue font**.

Presentations and Publications

Publications

Morgan R, Pearn L, Burnett AK, Darley RL and Tonks A. γ-Catenin regulation is Distinct to that of β-Catenin in Normal Haematopoiesis and is Required for Monocyte/Macrophage Differentiation. (**In production**).

Morgan R, Pearn L, Burnett AK, Tonks A and Darley RL. γ-Catenin Expression is Dysregulated in Acute Myeloid Leukaemia and Consequently Stabilises Nuclear β-Catenin. (**In production**).

Morgan R, Pearn L, Liddiard K, Hills R, Burnett AK, Tonks A and Darley RL. Distinct Regulation of γ- and β-Catenin throughout Hematopoietic Development Contrasts with Their Cooperative Roles In Acute Myeloid Leukemia. *American Society of Haematology Annual Meeting*, December 2010.

Oral presentations

"The Role of γ-Catenin ('γ-Catastrophe') in Acute Myeloid Leukaemia". *Department of Haematology seminar series.* March 2008.

"The Role of γ-Catenin in Acute Myeloid Leukaemia". *Cancer IRG seminar series.* June 2008.

"γ-Catenin in Normal Haematopoiesis and AML". *Department of Haematology seminar series.* June 2009.

"The Role of γ-Catenin in Acute Myeloid Leukaemia". *Annual Workshop on RUNX Transcription Factors in Development and Disease, Oxford.* August 2009.
Travel sponsor awarded by Oxford University.

"The Role of γ-Catenin in Acute Myeloid Leukaemia". *24th Annual School of Medicine Postgraduate Research Day.* November 2009.
3rd prize awarded by judging panel and 1st prize awarded by audience vote.

"The Role of γ-Catenin in Normal Haematopoiesis and Acute Myeloid Leukaemia". *Cancer IRG seminar series.* May 2010.

"The Role of γ-Catenin in Normal Haematopoiesis and Acute Myeloid Leukaemia". *Annual Workshop on RUNX Transcription Factors in Development and Disease, Hiroshima, Japan.* July 2010.
Travel sponsor awarded by British Society of Haematology.

Poster presentations

"The Role of γ-Catenin in Acute Myeloid Leukaemia". *23rd Annual School of Medicine Postgraduate Research Day.* November 2008.

"A Distinct Role for the Wnt Signalling Protein γ-Catenin in Normal Haematopoiesis and its Dysregulation in Acute Myeloid Leukaemia". *2nd Wales Cancer Conference,* April 2010.

"Distinct Regulation of γ- and β-Catenin throughout Hematopoietic Development Contrasts with Their Cooperative Roles In Acute Myeloid Leukemia". *American Society of Haematology Annual Meeting*, December 2010.
Travel sponsors awarded by Elimination of Leukaemia Fund, Cardiff University Michael Banfill Fund, and ASH.

Abstract

Acute myeloid leukaemia (AML) is a heterogeneous clonal disorder of haematopoietic cells that primarily affects the elderly. Previously, our laboratory identified γ-catenin as significantly overexpressed in AML. γ-Catenin shares close structural and functional homology with the more intensively studied β-catenin. Both catenins have dual roles in cell adhesion complexes and in transcription. Their transcriptional role is regulated by Wnt signalling which is critical for normal development and is one of the most frequently dysregulated processes in AML. In spite of this, little is known regarding the specific role of γ-catenin in normal haematopoiesis or AML pathology. This study devised an intracellular flow cytometric staining assay to characterise the expression of γ-catenin in normal haematopoietic subsets. γ-Catenin exhibited a similar expression profile to β-catenin. Expression was relatively high in haematopoietic stem/progenitor cells (HSC/HPC) and showed increased expression in myeloid differentiated cells (granulocytes and monocytes) while expression was lower in lymphoid cells and undetectable in red blood cells. Studies of subcellular distribution by confocal imaging showed reciprocal localisation of catenins in $CD34^+$ cells, with β-catenin predominantly nuclear translocated and γ-catenin nuclear excluded. Conversely, in granulocytic and monocytic cells nuclear γ-catenin levels were relatively high whilst nuclear β-catenin levels were reduced. A small subset of the $CD14^+$ monocyte population exhibited heavily nuclear translocated γ-catenin. Subsequent knock-down studies of γ-catenin showed this protein to be required for normal haematopoietic development *in vitro*, evidenced by the inhibition of macrophage differentiation and apparent reprogramming of committed monocyte progenitors for granulocytic development. In AML patients, γ-catenin mRNA expression conferred a reduced complete remission rate arising from resistant disease, however discordance was found between mRNA and protein level, implying post-translational control of γ-catenin expression in AML. In primary AML blasts (undifferentiated) γ-catenin was aberrantly localised to the nucleus suggesting a transcriptional role in AML pathology. A correlation was identified between γ- and β-catenin protein expression in primary AML blasts and an association between nuclear levels of these proteins. To determine whether this association was causal, γ-catenin was ectopically expressed in normal human $CD34^+$ haematopoietic progenitor cells but had no significant influence on β-catenin expression or localisation even following subsequent differentiation. In contrast, overexpression of γ-catenin in leukaemic cell lines stabilised β-catenin protein and promoted its translocation to the nucleus suggesting that the influence of γ-catenin on β-catenin is a feature of leukaemic cells but not normal cells. Phenotypically, overexpression of γ-catenin had little effect on normal progenitor cells but was able to block agonist-induced differentiation of AML cell lines, probably via stabilisation of β-catenin. In summary, this study indicates a role for γ-catenin in the regulation of normal haematopoietic development and that its nuclear translocation is strictly regulated independently of β-catenin in normal haematopoiesis. In leukaemic cells, however, this control is dysfunctional allowing γ-catenin to promote the stabilisation and translocation of β-catenin. This relationship may represent a pathological mechanism active in AML blasts to block myeloid differentiation and promote a leukaemic phenotype.

Abbreviations

ABL	abelson murine leukemia viral oncogene homolog 1
AJ	adherens junctions
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
APC	adenomatous polyposis coli
APL	acute promyelocytic leukaemia
Axin-1	axis inhibition protein 1
BCL-9	B-cell lymphoma 9 protein
BM	bone marrow
bp	base pairs
BSA	bovine serum albumin
C	Celcius
CB	cord blood
CBF	core binding factor
CBP	CREB binding protein
CD	cluster of differentiation
CDC	catenin destruction complex
CEB	cytosolic extraction buffer
CEBP	CCAAT-enhancer-binding protein
CI	confidence intervals
CK-1	casein kinase 1
CLL	chronic lymphocytic leukaemia
CLP	common lymphoid progenitor
CLSM	confocal laser scanning microscopy
CML	chronic myeloid leukaemia
CMP	common myeloid progenitor
c-myc	see myc
COOH	carboxy (terminus)
CR	complete remission
CTTNB	β-catenin (gene)
CTTNG	γ-catenin (gene)
DFS	disease free survival
DKK-1	dickkopf 1
DNase	deoxyribonuclease
DVL	dishevelled
E-cad	E-cadherin
EDTA	ethylenediaminetetraacetic acid
EFS	event free survival
EPO	erythropoietin
ETO	eight twenty one (protein/gene)
EtBr	ethidium bromide
FAB	French-American-British (classification)
FACS	fluorescence activated cell sorting
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
FLT-3	FMS-like tyrosine kinase 3

FLT-3L	FLT-3 ligand
FLT3-ITD	FLT-3 with internal tandem duplication mutation
FLT3-TKD	FLT-3 with tyrosine kinase domain mutation
FZ	frizzled
G-CSF	granulocyte - colony stimulating factor
GF	growth factor
GFP	green fluorescent protein
GSK-3β	glycogen synthase kinase 3 beta
GM-CSF	granulocyte macrophage - colony stimulating factor
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HPC	haematopoietic progenitor cell
HSC	haematopoietic stem cell
IL	interleukin
IU	international units
JAK	janus associated kinase
JUP	junction plakoglobin (aka γ-catenin)
Kb	kilobase
KD	knockdown
kDa	kiloDalton
KO	knockout
LB	Luria Bertani
LEF	lymphoid enhancer factor
LIC	leukaemia intiating cells
LN_2	liquid nitrogen
LRP	lipoprotein receptor-related protein
LSC	leukaemic stem cell
LT-HSC	long-term HSC
MACS	magnetic-activated cell sorting
MAPK	mitogen-activated protein kinase
M-CSF	macrophage - colony stimulating factor
MCS	multiple cloning site
MDS	myelodysplastic syndrome
MFI	mean fluorescence intensity
miR	microRNA
MM	multiple myeloma
MPP	multi-potent progenitor
mRNA	messenger RNA
MRC	Medical Research Council
myc	myelocytomatosis oncogene
myc-T1	myc-target protein 1
N-Cad	N-cadherin
N/C	nuclear/cytosolic
N:C/M	nuclear:cytosolic/membrane (ratio)
NEB	nuclear extraction buffer
NES	nuclear export sequence
NH_2	amino (terminus)
NLS	nuclear localisation sequence
NOD-SCID	nonobese diabetic–severe combined immunodeficient (mice)

NPM-1	nucleophosmin 1
OS	overall survival
PBS	phosphate buffered saline
PML/RARα	promyelocytic leukaemia/retinoic acid receptor alpha
PMT	photomultiplier tube
PU.1	human homologue of murine Spi-1
qRT-PCR	quantitative Reverse Transcriptase - Polymerase Chain Reaction
RISC	RNA-induced silencing complex
ROI	region of interest
R-PE	R-Phycoerythrin
rpm	revolutions per minute
RR	relapse risk
RT	room temperature
RUNX-1	runt-related transcription factor 1
SB	staining buffer
SCF	stem cell factor
SD	standard deviation
Ser	serine
SF	serum-free
sFRP	soluble frizzled-related protein
shRNA	short hairpin RNA
SiRNA	small interfering RNA
Spi-1	spleen focus-forming virus proviral integration oncogene-1 (see PU.1)
SRC	SCID-repopulating cells
SSC	side scatter
STAT	signal transducer and activator of transcription
ST-HSC	short-term HSC
TCF	T-cell factor
TCR	T-cell antigen receptor
TF	transcription factor
Thr	threonine
UC	universal container
WBC	white blood cell
WHO	World Health Organisation
Wnt	Wingless-type MMTV integration site family
wt	wild type

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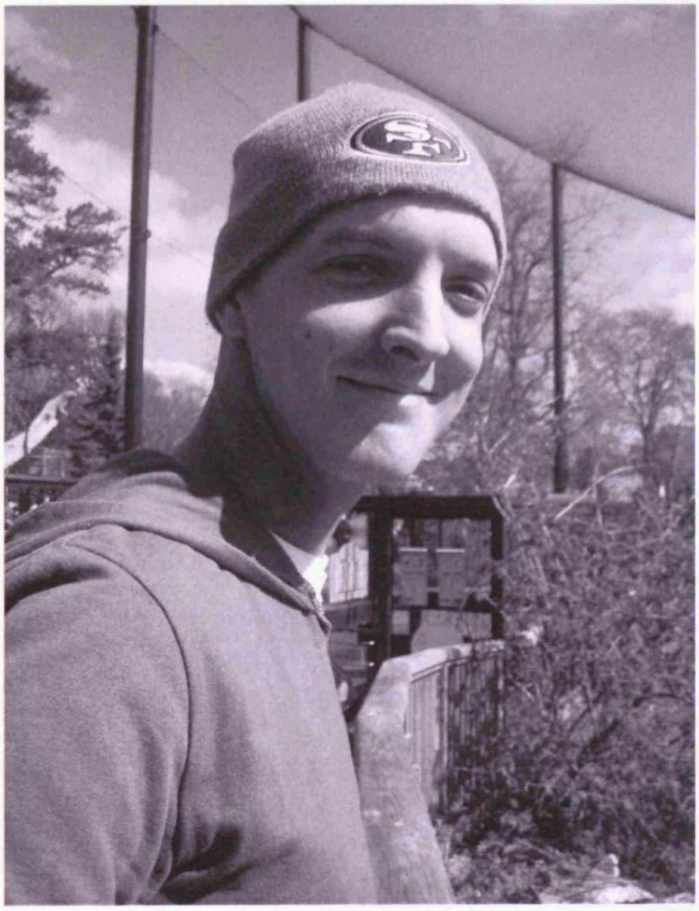
Summary of Tables

In loving memory of David John Morgan



1987-2009

For Tash and Alfie

1 - Introduction

1.1 Normal haematopoiesis

1.1.1 Overview of human haematopoiesis

Haematopoiesis is the life-long co-ordinated process of creating and maintaining all the cells that constitute blood. In the human embryo, the yolk sac is the main site for haematopoiesis up to 6 weeks gestation, before the liver and spleen take over for the following 6-7 months of foetal life (Hoffbrand *et al.*, 2005a). The bone marrow (BM) then becomes the most important site, and represents the only source of new blood cells in healthy childhood and adult life. Alternative sites of haematopoiesis such as the liver and spleen have been reported in adults but are generally only prevalent in times of haematopoietic stress or injury (Wilson and Trumpp, 2006). Otherwise, it is estimated that around $1x10^{12}$ blood cells are turned over daily in the healthy human body (Ogawa, 1993), with critical functions in host immunity (innate and adaptive), oxygen transport, blood coagulation and tissue repair.

Overwhelming evidence suggests the haematopoietic system is organised as a hierarchical structure, whereby rare pluripotent haematopoietic stem cells (HSC) with high potential for self-renewal, give rise to increasingly mature progeny with decreased self-renewal capacity but raised potential for differentiation (*Figure* 1.1). Although there remains some debate as to the intricate details of this organisation (reviewed by Rosenbauer and Tenen, 2007), the overall concept is a clear one: Increasing commitment to a particular lineage, leads to more restricted developmental capacity such that progeny eventually terminally differentiate into a single cell type with specialised function, structure and biochemical properties. For example, the small, smooth, enucleate, bi-concave morphology of the erythrocyte, and the high expression of haemoglobin, allows both maximum retention of oxygen and augmented navigation of the fine capillary networks to which it is delivered. Also, granulocytes (consisting of neutrophils, basophils and eosinophils) are able to fulfil their function of destroying pathogens and priming the adaptive immune system, through an extensive arsenal of

lysosomal granules, and a flexible, hyper-segmented nucleus that permits trans-endothelial migration through blood vessels into affected tissues.

Normal steady state haematopoiesis is regulated by a host of intrinsic and environmental influences. A complex network of signalling pathways, transcription factors, cytokines and growth factors synergise to influence the lineage fate, differentiation, proliferation and function of developing haematopoietic cells (see *1.1.3*). Although this process is tightly regulated, it must also adapt to the changing physiological demands of the body. In particular, erythropoiesis has been well studied in this context. For example, at times of haematopoietic injury, tissue hypoxia stimulates the secretion of erythropoietin (EPO) from the peritubular interstitial cells of the kidney. The presence of EPO in the BM biases developing HSCs down an erythroid path of differentiation. However, influencing the lineage fate of primitive HSC alone cannot solely replenish the haematopoietic system since more rapid responses are required. Rather, proliferation and apoptosis are are also critical regulators of a primed reservoir of more committed haematopoietic progenitors (De Haan *et al.*, 1996). Once again studies in erythropoiesis have demonstrated that a committed pool of erythroid progenitors reside in the bone marrow which can either; A) be rapidly induced by EPO to proliferate and terminally differentiate upon haematopoietic stress, or B) readily apoptose at times of excess during steady state erythropoiesis (De Maria *et al.*, 1999). The production of leukocytes is susceptible to similar regulatory mechanisms and can also be influenced by physiological demand, as evidenced by the increased pool of circulating neutrophils and lymphocytes prevalent during parasitic or viral invasion.

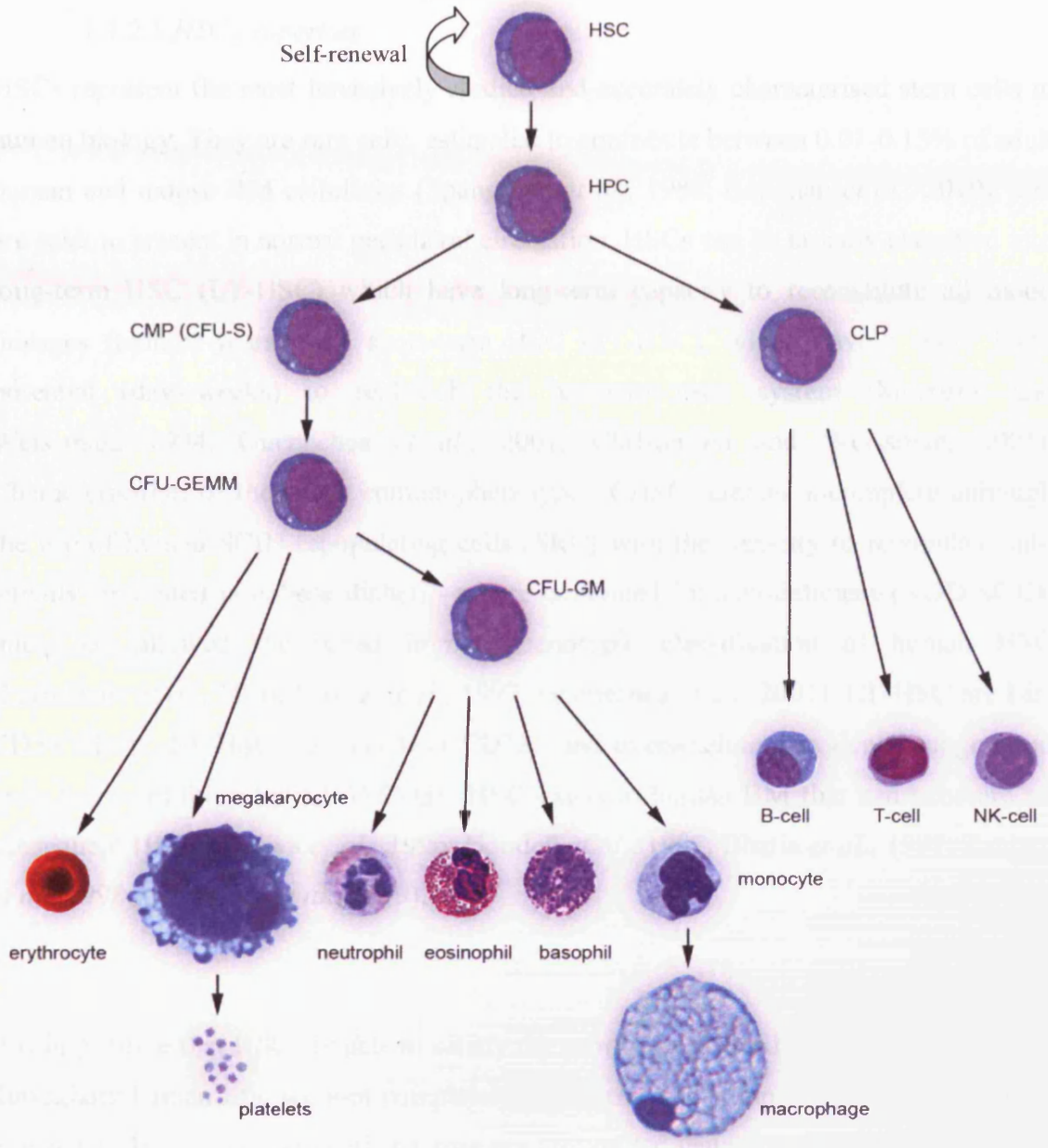


Figure 1.1 - The hierarchical arrangement of human haematopoietic development.

The rare pluripotent HSC with increased capacity for self-renewal (curved arrow), gradually gives rise to mature progeny with reduced capacity for self renewal and increased differentiation potential (straight arrows). Eventually, cells terminally differentiate, where their morphology and physiology is adapted to their biological function. Abbreviations: HSC = haematopoietic stem cell; HPC = haematopoietic progenitor cell; CMP = common myeloid progenitor; CLP = common lymphoid progenitor; CFU-GEMM = colony forming unit – granulocyte-erythrocyte-monocyte-megakaryocyte; CFU-GM = colony forming unit – granulocyte-macrophage; NK cell = natural killer cell. Adapted from http://daley.med.harvard.edu.

1.1.2 Haematopoietic stem cells and the microenvironment

1.1.2.1 *HSC properties*

HSCs represent the most intensively studied and accurately characterised stem cells in human biology. They are rare cells, estimated to contribute between 0.01-0.15% of adult human and mouse BM cellularity (Spangrude *et al.*, 1988; Beerman *et al.*, 2010), and are seldom present in normal peripheral circulation. HSCs can be broadly classified into long-term HSC (LT-HSC) which have long-term capacity to reconstitute all blood lineages (months-years), and short-term HSC (ST-HSC), which have a more finite potential (days-weeks) to replenish the haematopoietic system (Morrison and Weissman, 1994; Guenechea *et al.*, 2001; Christensen and Weissman, 2001). Characterisation of the exact immunophenotype of HSC remains incomplete although the use of human SCID-repopulating cells (SRC) with the capacity to repopulate sub-lethally irradiated nonobese diabetic–severe combined immunodeficient (NOD-SCID) mice has allowed the broad immunophenotypic classification of human HSC (Larochelle *et al.*, 1996; Bhatia *et al.*, 1997; Guenechea *et al.*, 2001). LT-HSC are $Lin^-CD34^+CD38^-$, ST-HSC are $Lin^-CD34^+CD38^+$, and overwhelming evidence suggests an even more primitive $Lin^-CD34^-CD38^-$ HSC exists in human BM that can generate all subsequent HSCs (Osawa *et al.*, 1996; Goodell *et al.*, 1997; Bhatia *et al.*, 1998; Zanjani *et al.*, 1998; Nakamura *et al.*, 1999).

It is imperative that HSC are able to satisfy the enormous demand for mature blood cells throughout human life, without completely exhausting the comparatively small pool of pluripotent HSC. One stem cell can generate around 10^6 mature blood cells after 20 cell divisions, whilst preserving an exact copy (daughter) of itself. This considerable amplification is only achievable through a critical balance between self-renewal and differentiation. A number of models have been proposed to explain how HSCs co-ordinate this balance, and are summarised in *Figure 1.2* (reviewed by Roeder and Lorenz, 2006, Wilson and Trumpp, 2006 and Schroeder, 2007). It is likely that all models outlined are active to some degree during normal HSC division. The heterogeneity within this population make it probable that differentiation is a multi-step process where HSCs gradually lose their 'stem-ness'. This process is largely governed

by the transcription factors and the supporting network of cytokines, growth factors, signalling cascades described further in section *1.1.3*.

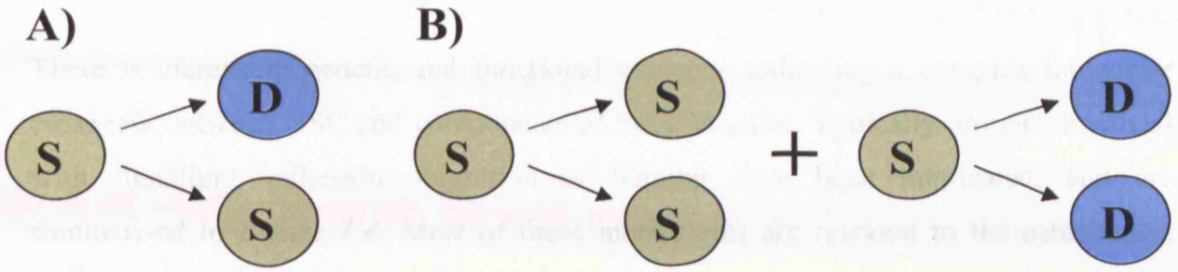


Figure 1.2 - Theoretical models of HSC division.

The two main models of stem cell division that have been proposed to explain the inherent ability of HSCs to continually replenish populations of mature cells whilst preserving the pool of uncommitted cells. **A)** The rigid *asymmetric* model whereby one HSC always generates one stem and one differentiated daughter cell. The probability of generating each type is fixed and ensures a continuous reservoir of stem cells. However, such a process is unlikely to represent the only type of division since a more dynamic expansion of the HSC pool is required at times of haematopoietic injury or myeloablation **B)** The more dynamic *symmetrical* or 'stochastic' theory where the HSC is able to produce either two identical stem daughter cells or two identical differentiated daughter cells according to physiological demand. Abbreviations: S = stem cell; D= differentiated cell. Adapted from Roeder and Lorenz, 2006.

1.1.2.2 *The HSC niche*

The instinctive ability of HSCs to home to the BM allowed the finding that stem cells are not randomly distributed through the marrow cavity, but instead occupy specialised niches close to blood vessels and the endosteum of the trabecular bone (Kopp *et al.*, 2005; Kiel *et al.*, 2005). The concept of a 'HSC niche' was originally coined by Schofield in 1978 following studies of colony forming units (CFU) in the spleen (Schofield, 1978). Earlier studies by Shackney had identified a development gradient in the BM whereby primitive cells were located along the endosteum, with developing cells moving towards the highly vascularised cavity (Shackney *et al.*, 1975). The BM niche itself (*Figure 1.3*) is composed of osteoblasts, osteoclasts, and various stromal cells including adipocytes, fibroblasts, reticulum cells, endothelial cells and macrophages, all enriched with a dense micro-vascular network. Stromal cells are able to generate an extracellular matrix by secreting molecules such as collagen,

glycoproteins (fibronectin and thrombospondin) and glycosaminoglycans (hyaluronic acid and chondroitin derivatives).

There is increasing genetic and functional evidence indicating a complex molecular cross-talk between HSC and components of the BM niche. Typically proteins involved with signalling, adhesion, migration or homing have been implicated, and are summarised in *Figure 1.4*. Most of these interactions are relevant to the osteoblastic niche, and human osteoblasts have been shown to support haematopoietic progenitor cells *in vitro* (Taichman *et al.*, 1996; Taichman *et al.*, 2001). This relationship was formally demonstrated *in vivo* through the pioneering studies of Zhang *et al.* (Zhang *et al.*, 2003), and Calvi *et al.* (Calvi *et al.*, 2003), who both increased osteoblast frequency through different means in mice, to the ultimate effect of increasing HSC number. This concept was reaffirmed by the reverse experiment by Visnjic *et al.* where conditional ablation of osteoblasts led to loss of normal haematopoiesis including a decline in HSC number (Visnjic *et al.*, 2004).

Since this discovery, much work has been undertaken to identify the specific signalling proteins active in the niche and many receptor/ligand relationships have been characterised between osteoblasts and HSC including; stem cell factor (SCF)/c-kit, Jagged-1 (JAG-1)/Notch, Angiopoietin-1 (Ang-1)/ tyrosine kinase receptor 2 (Tie2) and Wingless-type MMTV integration site family (Wnt)/β-catenin. SCF has been found secreted and expressed from the osteoblast surface, and signalling through c-kit receptor expression in HSC is well known to promote the proliferation and survival of this cell type. Interestingly, this relationship has also been proposed to contribute an adhesive function (Kinashi and Springer, 1994). The apparent haematopoietic failure in mice caused by loss of SCF from the supporting environment would indicate a crucial function of this signalling axis in the niche (Bernstein *et al.*, 1991). Similarly, Notch receptors are expressed on HSC which can be bound by JAG-1 expression from surrounding stromal cells to expand the HSC pool (Calvi *et al.*, 2003). Another well characterised interaction is that between Ang-1 (expressed on osteoblasts) and Tie-2 (expressed on HSC) which has been formally demonstrated to maintain LT-HSC in a quiescent state (Arai *et al.*, 2004).

A host of adhesion molecules have been implicated in maintaining HSC interaction with the niche including N-cadherin(N-cad)/β-catenin, vascular cell adhesion molecule-1 (VCAM-1)/integrin, osteopontin (OPN)/β1-integrin and $Ca2^{+}$-sensing receptor (CaR) (reviewed by Yin and Li, 2006). Of particular interest, the adhesive interaction between N-cad and β-catenin has been previously demonstrated in a stem cell context within *Drosophila* (Song and Xie, 2002; Yamashita *et al.*, 2003). Both proteins have also been found symmetrically localised at the interface between HSCs and the osteoblastic niche, implying a heterotypic interaction maybe active in anchoring HSCs to the local stroma (Zhang *et al.*, 2003). Curiously this interaction may not be restricted to adhesion, and may instead negatively regulate β-catenin-mediated transcription of Wnt target genes. A study by Reiss *et al.* showed that N-cad cleavage by a disintegrin and metalloproteinase-10 (ADAM-10) leads to β-catenin redistribution to the cytoplasm thus increasing the pool of signalling competent β-catenin available to the nucleus (Reiss *et al.*, 2005). A similar re-localisation of β-catenin was observed upon cleavage of E-cadherin (E-cad) (Ito *et al.*, 1999). Despite the associated role of γ-catenin with cell adhesion (see *1.3.5.1*) no such role has ever been implicated for this molecule within the HSC BM niche.

Finally, a number of proteins crucial to the homing of HSCs to the niche are present within the BM microenvironment including stromal derived factor-1 (SDF-1), FGF-4, E- and P-selectins, integrins, very late antigen-4 (VLA-4) and lymphocyte function-associated antigen-1 (LFA-1) (Wilson and Trumpp, 2006). The most well characterised of these molecules, SDF-1 (also known as CXCL12), is constitutively released from endothelial and osteoblastic cells of the niche and the receptor, CXC chemokine receptor 4 (CXCR4) is present on the surface of HSCs (Peled *et al.*, 1999; Lapidot *et al.*, 2005; Kortesidis *et al.*, 2005). This chemokine is involved in the mobilisation, migration and retention of HSCs, and expression from stromal cells can be induced upon haematopoietic injury or myeloablation (Ponomaryov *et al.*, 2000; Ara *et al.*, 2003). Such properties have been exploited in modern BM transplant procedures.

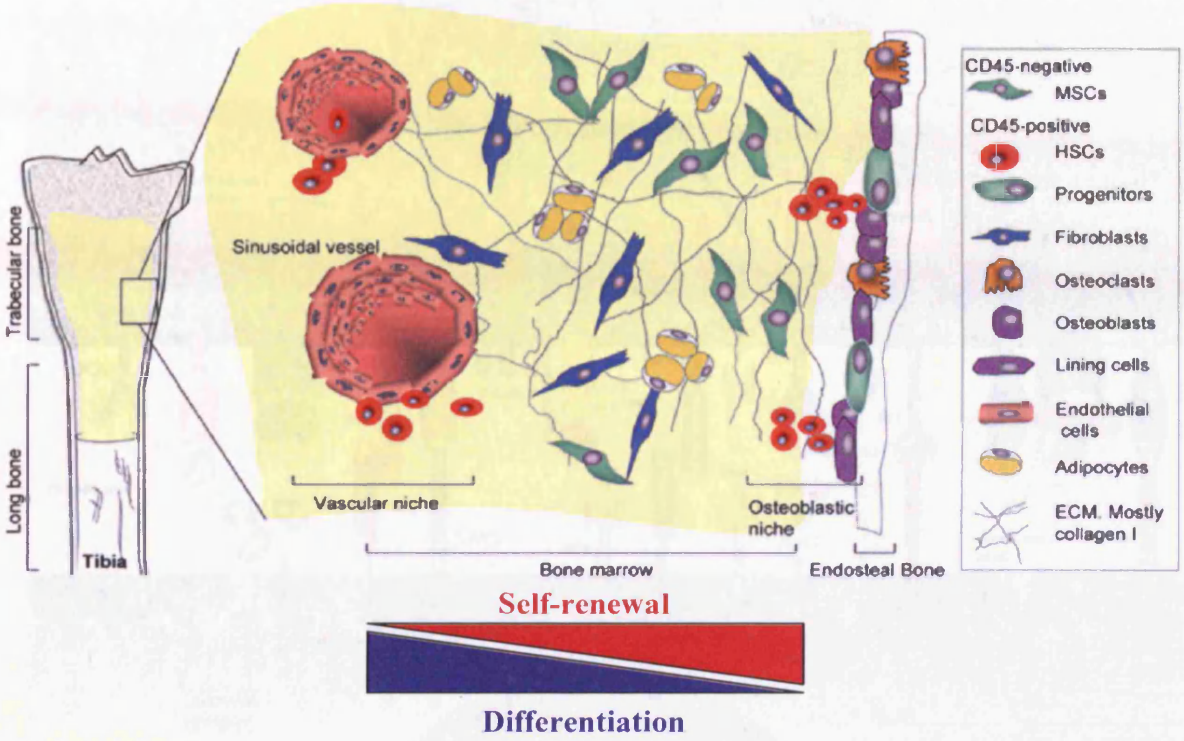


Figure 1.3 - The HSC bone marrow microenvironment.

Haematopoietic stem cells are immobilised in highly specialised BM niches. Two types of niche are believed to exist: the osteoblastic niche located near the endosteal bone and the vascular niche located on the endothelium of the sinusoidal vessels (reviewed by Yin and Li, 2006). Mesenchymal stem cells are located throughout the microenvironment and are able to continually replenish the stromal components of the niche (featured in right-hand legend box, reviewed by Uccelli *et al.*, 2008). Numerous adhesion and growth factors are responsible for anchoring and regulating the self-renewal/differentiation of HSC within the niche. A gradient of blood cell development is active in the BM. Primitive HSC, of which only 3.8% are estimated to be proliferating at any given time (Kiel *et al.*, 2005), are located near the endosteum. These gradually give rise to increasingly differentiated progeny with reduced self-renewal capacity, that are eventually released into the vasculature network upon terminal differentiation. This process is believed to be augmented by a co-increasing gradient of oxygen and growth factors. Abbreviations: MSCs = mesenchymal stem cells; HCSs = haematopoietic stem cells; ECM = extracellular matrix. Adapted from http://www.bioscience.org/2007/v12/af/2440/figures.htm.

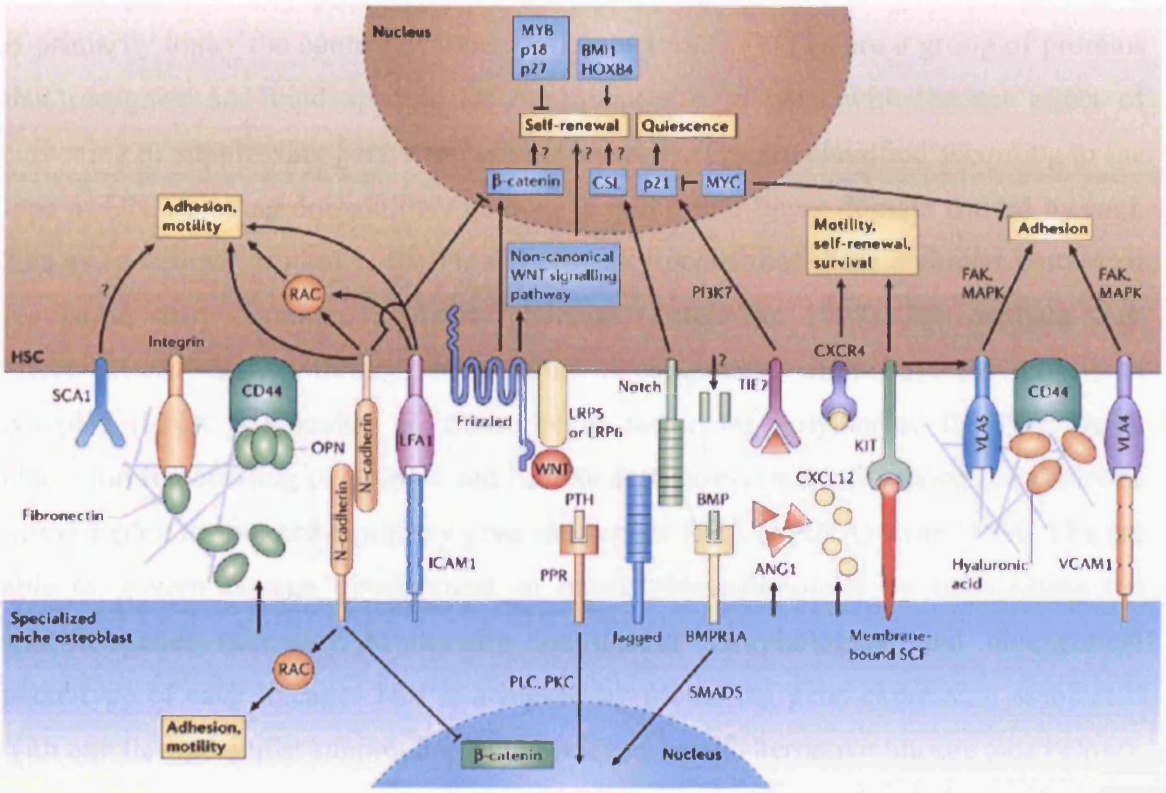


Figure 1.4 - The multitude of adhesion, signalling and homing molecules present in the endosteal HSC bone marrow niche.

The complexity of molecular cross-talk proposed to be active between HSC and niche components. Abbreviations: ANG1= angiopoietin-1; BMI1= polycomb repressor; BMP= bone morphogenetic protein; BMPR1A= BMP receptor 1A; CSL= CBF1 suppressor of Hairless and LAG1; CXCL12= CXC chemokine ligand 12; CXCR4= CXC-chemokine receptor 4; FAK= focal adhesion kinase; HOXB4= homeobox B4; HSC= haematopoietic stem cell; ICAM1= intercellular adhesion molecule 1; LFA1= lymphocyte function-associated antigen-1; LRP= low-density-lipoprotein-receptor-related protein; MAPK= mitogen-activated protein kinase; OPN= osteopontin; PI3K= phosphatidylinositol-3 kinase; PLC= phospholipase C; PKC= protein kinase C; PPR= PTH/PTH-related protein receptor; PTH= parathyroid hormone; SCF= stem-cell factor; SMADS= mothers against decapentaplegic-related homologue; SNO= spindle-shaped N-cadherin-expressing osteoblast; TIE-2= tyrosine kinase receptor 2; VCAM-1= vascular cell-adhesion molecule 1; VLA4= very late antigen 4; '?' = molecules and/or interactions for which only indirect or contradictory evidence is available. Adapted from Wilson and Trumpp, 2006.

1.1.3 The transcriptional control of haematopoiesis

The determination of lineage fate and subsequent differentiation of haematopoietic cells is primarily under the control of transcription factors (TF). TFs are a group of proteins that recognise and bind specific DNA sequences of a gene with the net effect of activating or suppressing gene expression. Generally, TFs are classified according to the type of DNA binding domain they harbour (e.g. the zinc finger domain carried by such TFs as specificity protein 1; SP-1), and typically genes that share a similar pattern of regulation share common regulatory elements (Latchman, 1997). TFs mediate their effects on gene activity through interaction with components of the basal transcriptional complex (RNA polymerase II, transcription factor for polymerase II (TFII) A-H, chromatin remodelling complexes and histone acetylases) thus influencing the ability of this complex to transcribe primary gene messenger RNA (mRNA) from DNA. TFs are able to govern lineage development in normal haematopoiesis by transcribing the specific genes necessary to develop the distinct morphological and biochemical phenotype of each lineage. This is achieved by promoting gene expression associated with one lineage whilst suppressing genes relevant to an alternative lineage (see below). The importance and intricate regulation of TFs in normal haematopoietic development is affirmed by the prevalence of TF abnormalities in haematological malignancies such as AML (see section *1.2.2*).

A plethora of TFs have been identified for their role in directing lineage-specific commitment (reviewed by Shivdasani and Orkin, 1996 and Tenen *et al.*, 1997) and some of the best characterised are outlined in *Table 1.1*. Of particular interest for myeloid development, is the complex relationship between GATA-binding protein 1 (GATA-1) and PU.1 (also known as spleen focus-forming virus proviral integration oncogene-1; Spi-1) which govern the transcriptional 'check-point' between erythroid and myeloid commitment respectively. GATA-1 is a zinc-finger domain transcription factor that is able to bind and repress PU.1 in order to suppress myeloid differentiation and promote erythropoiesis (Nerlov *et al.*, 2000). Consistent with this GATA-1 expression is up-regulated in erythroid cells and concomitantly suppressed in committed myeloid precursors (Sposi *et al.*, 1992). Furthermore, loss of GATA-1 expression both *in vitro* (Weiss *et al.*, 1994; Pevny *et al.*, 1995; Weiss and Orkin, 1995), and *in vivo* (Pevny *et al.*, 1991; Simon *et al.*, 1992) leads to a loss of erythropoiesis. GATA-1 is

able to mediate erythropoiesis through transcription of genes indispensable for red cell development, such as the upregulation of globin proteins (Orkin, 1992) and the EPO receptor (Zon *et al.*, 1991). Interestingly, GATA-1 is able to maintain these developmental stimuli on a continuous automated feedback loop through self-transactivation of its own promoter (Tsai *et al.*, 1991).

For HSCs to commit to myeloid development the converse is the case; GATA-1 expression is suppressed whilst PU.1 is upregulated (Lee *et al.*, 1991; Sposi *et al.*, 1992; Voso *et al.*, 1994; Pevny *et al.*, 1995). Consistent with this, enforced expression of GATA-1 in multi-potent progenitor (MPP) cells leads to a block in myeloid development (Kulessa *et al.*, 1995). PU.1 is a member of the Ets family of TFs (Klemsz *et al.*, 1990) and its elevated expression is observed in various myeloid cells including monocytes, macrophages and granulocytes (Chen *et al.*, 1995b). Loss of PU.1 itself causes haematopoietic deficiencies *in vivo*, and mice die shortly before birth with a lack of monocytes and granulocytes (Scott *et al.*, 1994). Like GATA-1, PU.1 is able to positively regulate its own expression, and thus that of other myeloid-specific genes, through its ability to transactivate its own promoter (Chen *et al.*, 1995a). PU.1 is in transcriptional control of a number of genes critical for early myeloid differentiation cues including the receptors for macrophage - colony stimulating factor (M-CSF) (Zhang *et al.*, 1994a), granulocyte macrophage - colony stimulating factor (GM-CSF) (Hohaus *et al.*, 1995), and granulocyte - colony stimulating factor (G-CSF) (Smith *et al.*, 1996), as well as markers of mature myeloid cells such as CD14 (Zhang *et al.*, 1994b), CD11b (Pahl *et al.*, 1992) and the macrophage scavenger receptor (Horvai *et al.*, 1995). Therefore, the interaction between GATA-1 and PU.1 represents a well characterised mechanism of how transcriptional activation and suppression are necessary for haematopoietic lineage-commitment and development. A summary of this concept encompassing the influence of growth factor signalling is shown in *Figure* 1.5.

Table 1.1 - Characterised transcription factors involved in lineage commitment.

Transcription factor	Lineage-direction
GATA-1	Erythroid
PU.1	Myeloid (monocyte and macrophage) and lymphoid
CCAT/enhancer binding protein (CEPBα)	Myeloid (granulocytes)
Homeobox protein A10 (HoxA10)	Myeloid
Erythroid Kruppel-like factor (EKLF)	Erythroid
E2A immunoglobulin enhancer binding factors E12/ E47 (E2A)	B-cell lymphocytes
Octamer-binding transcription factor 2 (Oct-2)	B-cell lymphocytes
Retinoic acid receptor α (RARα)	Myeloid (granulocytes)
Nuclear factor erythroid derived-2 (NF-E2)	Megakaryocytic
Ikaros	Lymphoid
Runt-related transcription factor 1 (RUNX-1)	Myeloid and lymphoid
T-cell factor/lymphoid enhancer factor (TCF/LEF)	Lymphoid

1.1.3.1 *Cytokines and growth factors*

Haematopoietic growth factors (GF) and cytokines are soluble or membrane-bound glycoproteins that direct TFs in mediating proliferative and developmental changes in cells. They are also vital to the continued functionality of terminally differentiated cells. It was generally appreciated that GFs and cytokines played a supplementary role to TFs in mediating lineage selection, however, emerging evidence suggests these proteins are also capable of initiating lineage fate in otherwise uncommitted cells (Rieger and Schroeder, 2009; Schroeder, 2010). GFs and cytokines are predominantly secreted from stromal cells and many types of mature and primitive leukocyte, except for EPO and thrombopoietin (TPO) which are produced in the kidney and liver, respectively. These

proteins are able to mediate their effects through specific receptors on target cells at very low concentrations in a para-, endo- or auto-crine fashion. For example, stem cell factor (SCF) and FMS-like tyrosine kinase 3 ligand (FLT-3L) act predominantly on pluripotent HSCs, CMP's and CLP's to mediate proliferation of these subsets (Hoffbrand *et al.*, 2005a). GFs and cytokines are also capable of working synergistically with one another in order to generate effects on target cells. An example of this would be the ability of G-CSF and TPO to augment the effects of SCF, FLT-L, and GM-CSF on the survival and proliferation of MPPs. GFs and cytokines mediate their effects on transcription via signalling cascades.

1.1.3.2 *Overview of cell signalling pathways in haematopoiesis*

Signal transduction is an evolutionary conserved mechanism allowing an immediate genetic response to changes from the external environment. Invariably, a common mechanism is shared between pathways whereby transmembrane receptor and ligand interactions result in the phosphorylation and conformational change of downstream cytosolic proteins, with the ultimate effect of DNA binding by TFs. In haematopoiesis, the pathway by which GFs and cytokines mediate their developmental effects has been well characterised (Robb, 2007). Typically, these proteins bind the extracellular domain of class-I or class-II cytokine receptors resulting in a conformational change. The alteration in the intracellular domain of the receptor causes activation of bound tyrosine kinases, like the janus associated kinase family (JAK). JAK then phosphorylate members of the signal transducer and activator of transcription (STAT) family, resulting in dimerisation and nuclear translocation, where they serve as TFs for lineage-specific genes. Alternatively, receptor tyrosine kinases such as FLT-3 and c-kit (SCF receptor) exist which are capable of directly phosphorylating downstream targets such as phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) (reviewed by Doepfner *et al.*, 2007) when bound to the relevant ligand. JAK family members may also induce anti-apoptotic and proliferative responses. For example, JAK-mediated activation of protein kinase B (PKB), results in an anti-apoptotic response through phosphorylation and inactivation of BAD (pro-apoptotic) protein. Alternatively, JAKs also initiate a proliferative response by stimulation of Rat sarcoma (RAS), RAF and MAPK to induce myelocytomatosis oncogene (myc) and FBJ murine osteosarcoma viral oncogene homolog (FOS) TFs (Hoffbrand *et al.*, 2005a).

Aside from JAK/STAT signalling, additional well-established pathways and their effector molecules have been implicated in the normal development of haematopoietic cells. These include Wnt, Notch, Sonic hedgehog (SHH), mothers against decapentaplegic-related homologue (Smad) and epidermal growth factor (EGF) signalling pathways and have been reviewed by others (Rizo *et al.*, 2006; Blank *et al.*, 2008; Campbell *et al.*, 2008). Components within these cascades communicate in much the same manner as previously described but utilise distinct TFs to activate/suppress target genes. Typically, these pathways can autoregulate themselves either positively by transcribing promoter molecules, or negatively by transcribing inhibitory molecules. These signalling cascades regulate important processes in haematopoietic cells including survival, proliferation, self-renewal and differentiation. Specifically, the Wnt pathway has been shown to be important for the self-renewal of HSCs (Reya *et al.*, 2003), whilst SHH signalling appears to be vital for lymphoid differentiation (El *et al.*, 2006). The regulation of haematopoietic cells by signalling pathways is complicated by the finding that several pathways can interact to mediate a developmental phenotype. Such molecular cross-talk was identified between the Wnt and notch pathways which could converge to maintain the HSC pool (Duncan *et al.*, 2005). The overall importance of signal transduction networks in normal haematopoietic development is underlined by the observation that so many, like *runt-related transcription factor 1* (RUNX-1), are dysregulated in haematological malignancies such as AML (Majeti *et al.*, 2009) (see also section *1.2.2.1*).

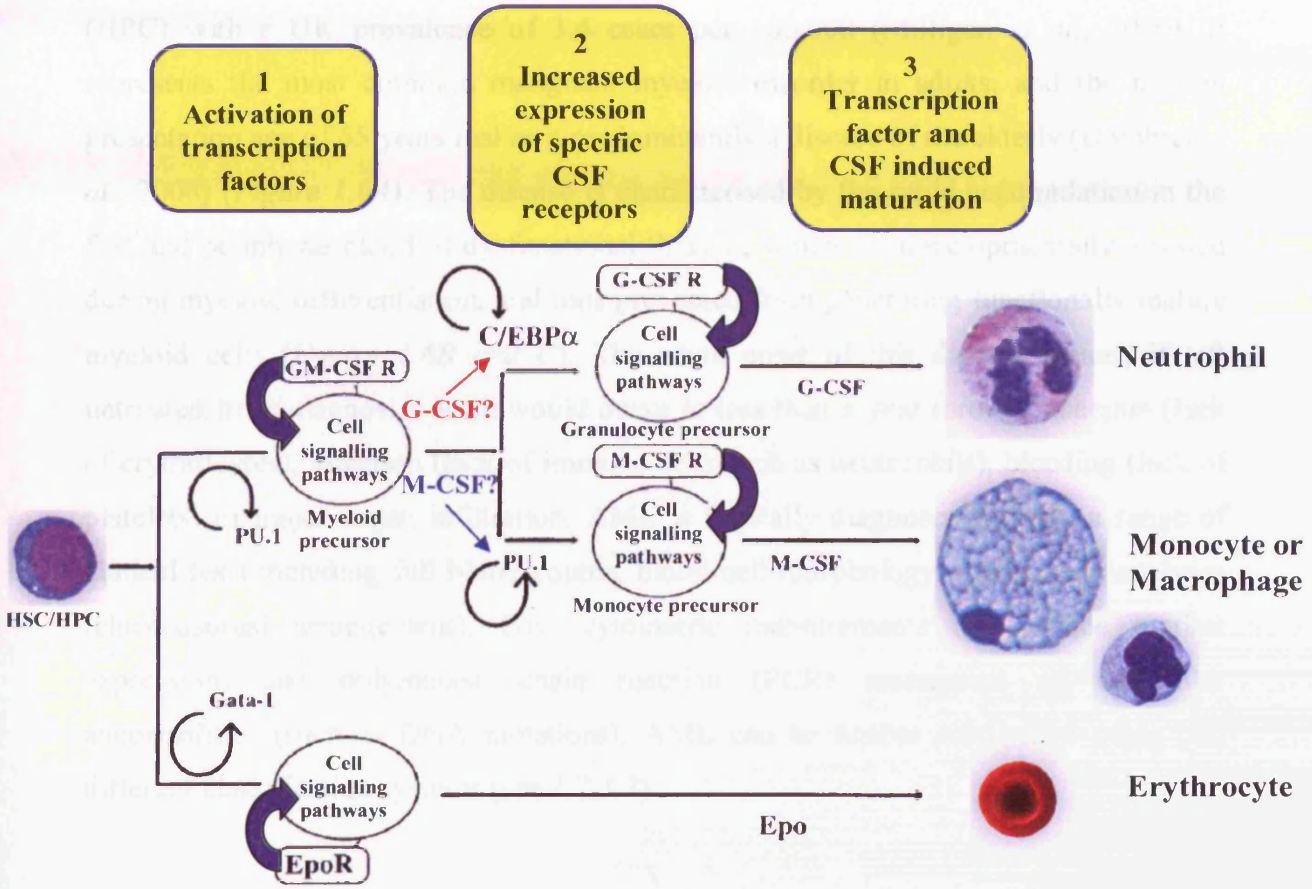


Figure 1.5 - The molecular control of haematopoiesis.

Schematic summarising the factors involved in haematopoietic development, as exemplified by myeloid differentiation. Lineage fate is primarily driven by powerful intrinsic transcription factors such as GATA-1 or PU.1 which can direct erythroid or myeloid commitment, respectively, by transcribing developmentally relevant genes such as growth factor receptors (e.g. EPO or GM-CSF). Recent evidence would suggest that specific growth factors (featured in red and blue writing) can also determine lineage fate of uncommitted downstream progenitor cells (Rieger and Schroeder, 2009). Ultimately, growth factors initiate cell signalling pathways, through the relevant receptor, which activates/suppresses additional transcription factors necessary for terminal differentiation. Adapted from Tenen *et al.*, 1997.

1.2 Acute myeloid leukaemia

1.2.1 Overview

AML is a heterogeneous clonal disorder of HSC and haematopoietic progenitor cells (HPC) with a UK prevalence of 3.4 cases per 100,000 (Milligan *et al.*, 2006). It represents the most common malignant myeloid disorder in adults, and the median presentation age of 65 years makes it predominantly a disease of the elderly (Dombret *et al.*, 2008) (*Figure 1.6A*). The disease is characterised by the rapid accumulation in the BM and peripheral blood of dysfunctional 'blasts', which are developmentally arrested during myeloid differentiation, and thus prevented from generating functionally mature myeloid cells (*Figure 1.6B and C*). The acute onset of this disease means, if left untreated from diagnosis, death would occur in less than a year through anaemia (lack of erythrocytes), infection (lack of immune cells such as neutrophils), bleeding (lack of platelets) or major organ infiltration. AML is typically diagnosed through a range of clinical tests including full blood counts, blood cell morphology, cytogenetic analyses (chromosomal arrangement), flow cytometric measurements (of surface marker expression) and polymerase chain reaction (PCR) assessment of molecular abnormalities (such as DNA mutations). AML can be further subdivided using two different classification systems (see *1.2.3.2*).

Risk factors for AML acquisition include exposure to benzene, ionising radiation and cytotoxic chemotherapy (Estey and Dohner, 2006). Indeed Japanese survivors of the atomic bombs (Nakanishi *et al.*, 1999), workers in the nuclear industry (Cardis *et al.*, 1995) and flight crews of commercial aircraft (Gundestrup and Storm, 1999), have all been associated with an increased susceptibility to AML. Genetic predisposition is a further risk factor for AML that is beyond human control. AML can either be primary (*de novo*) or secondary, developing through existing haematological disorders (e.g. myelodysplastic syndrome; MDS) or through previous chemotherapeutic treatment of cancer (10-15%). Current treatment strategies in AML (see *1.2.4.1*) generally involve the infusion of cytotoxic agents with the aim of achieving a complete remission (CR) through reduction of the blast count, and restoration of normal circulating neutrophils and platelets.

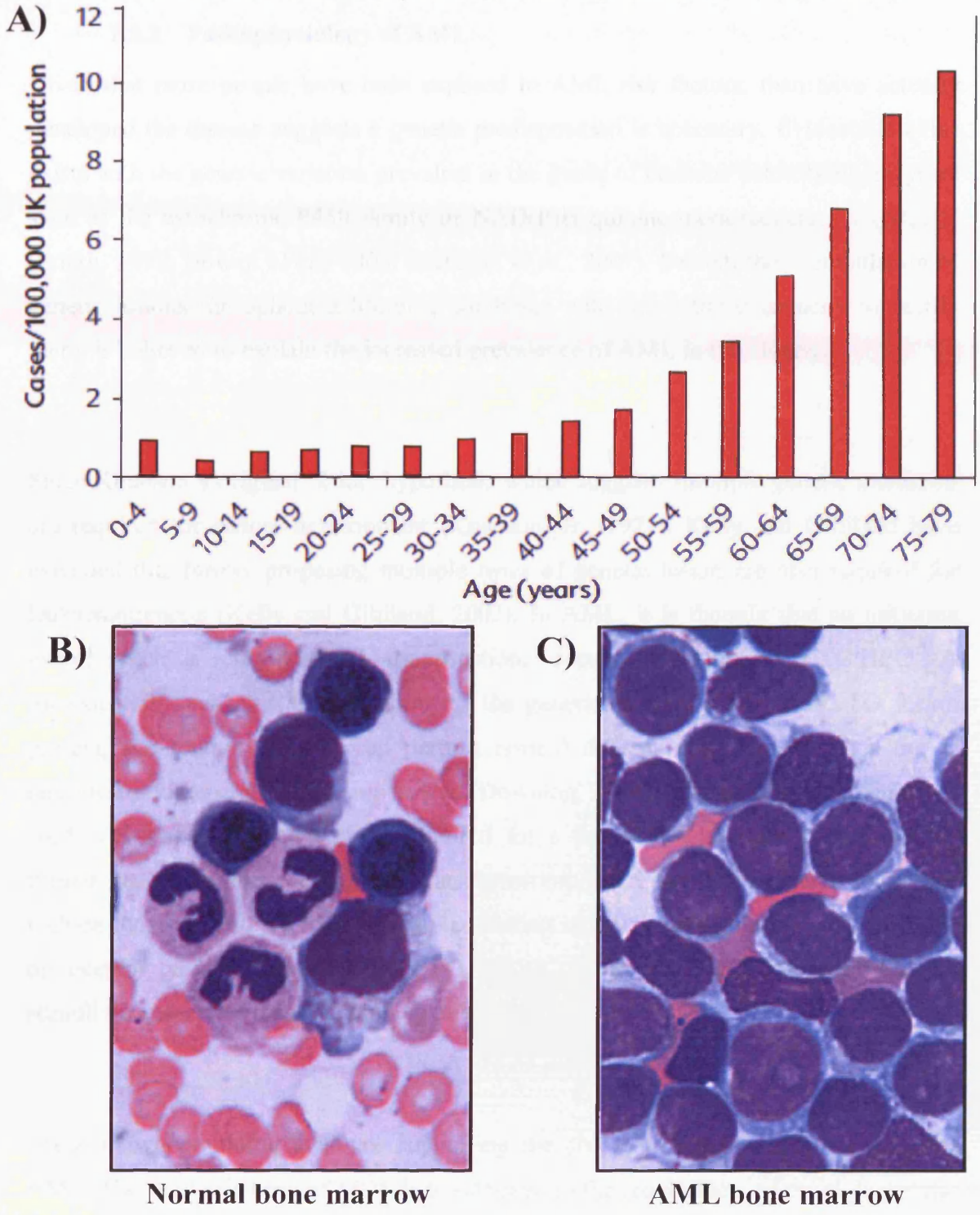


Figure 1.6 - The incidence and morphological appearance of AML.

A) The incidence of AML in the UK according to age group, illustrating this malignancy is primarily a disease of the elderly. Sourced from the Leukaemia and Lymphoma Research (LLR) website. **B)** The cellular morphology of normal BM by cytochemical staining shows a range of different blood cell lineages at varying stages of maturation. **C)** The BM of an AML patient exhibits the domination of dark staining, dysfunctional, developmentally perturbed blasts. Images obtained from http://pathology.wustl.edu/~yaseenlab/?page=180.

1.2.2 Pathophysiology of AML

Given that more people have been exposed to AML risk factors, than have actually developed the disease suggests a genetic predisposition is necessary. Evidence for this exists with the genetic variation prevalent in the genes of benzene detoxifying enzymes such as the cytochrome P450 family or NAD(P)H quinine oxidoreductase 1 (NQO1) (Smith, 1999; Bowen *et al.*, 2003; Barragan *et al.*, 2007). Indeed, the accumulation of genetic lesions throughout a lifetime, combined with the reduced capacity to rectify them, is believed to explain the increased prevalence of AML in the elderly.

Since Knudson's original '2-hit' hypothesis which suggests multiple genetic mutations are required for cancer development (Knudson, Jr., 1971), Kelly and Gilliland have extended this further proposing multiple *types* of genetic lesion are also required for leukaemogenesis (Kelly and Gilliland, 2002). In AML, it is thought that an initiating event, often a chromosomal translocation, occurs in primitive HSC/HPC. A translocation such as t(8;21) (leading to the generation of the RUNX-1/ETO fusion protein, see section *1.2.2.1*) can perturb normal differentiation programmes but is insufficient to generate leukaemia *in vivo* (Downing, 2003). Instead, this initiating event confers the necessary advantages required for a pre-malignant clone to acquire the further genetic lesions required for transformation. Such additional genetic mutations include those capable of constitutively activating intracellular signalling such as RAS, or receptor tyrosine kinases like FLT-3, which provide the survival or proliferative stimuli necessary for the malignant clone to dominate.

There is overwhelming evidence supporting the above proposed pathophysiology of AML. The very existence of MDS is a testament to the requirement of multiple genetic lesions in AML. MDS is generally regarded as a 'pre-leukaemic' syndrome, which displays many of the same haematopoietic abnormalities and symptoms, albeit with slower evolution. Should the patient survive long enough, this condition often develops into AML which is generally attributed to the acquisition of further transforming genetic mutations. The requirement for an early initiating event in AML is evidenced by the detection of chromosomal translocations in haematopoietic cells which remain stably retained no matter what the treatment outcome (remission or relapse). The RUNX-

1/ETO fusion transcript is one such abnormality that has been frequently detected in haematopoietic cells of patients even in long-term remission (Nucifora *et al.*, 1993; Miyamoto *et al.*, 2000). Further studies of clonal evolution in acute lymphoblastic leukaemia (ALL) by the Greaves lab have shown that whilst the intiating genetic lesion like ETS variant gene 6 (ETV-6)/RUNX-1 remains stably expressed in all malignant clones, the prevalence of additional gene mutations such as deletions of paired box protein 5 (Pax-5) and B-cell translocation gene 1 (BTG-1) remain variable between subpopulations of the malignant clone (Anderson *et al.*, 2011). Finally, the requirement for co-operating mutations in leukaemogenesis is clear from the observation that many AMLs harbouring a core binding factor[1] (CBF) abnormality also frequently co-express additional mutations such as c-kit (Care *et al.*, 2003; Valk *et al.*, 2004; Cammenga *et al.*, 2005; Cairoli *et al.*, 2006; Schnittger *et al.*, 2006). Despite the overwhelming evidence described above clearly this is not the limit of complexity in AML pathophysiology given that 50% of *de novo* AML patients present with an otherwise normal karyotype (Grimwade *et al.*, 1998).

1.2.2.1 *Frequent genetic aberrations in AML*

Frequent gene mutations involving transcriptional/signal transduction components have been identified in AML (reviewed by Ravandi *et al.*, 2007 and Dohner and Dohner, 2008), with the most frequent including *Nucleophosmin-1* (NPM1), *FLT-3*, *CEBPα*, *c-kit*, *neuroblastoma RAS viral oncogene homolog* (NRAS), *Wilms tumour 1* (WT1), *brain and acute leukaemia cytoplasmic* (BAALC), *mixed-lineage leukaemia* (MLL) and *ecotropic viral integration site 1* (EVI1). The mutational status of many of these genes is now routinely assessed due to the prognostic influence in normal karyotype AML (section *1.2.3.3*) and some have even become a therapeutic target given their pathological role in AML.

[1] CBFs are a group of heterodimeric transcription factors composed of a DNA-binding RUNX-1, RUNX-2 or RUNX-3 subunit (CBFα), and a non-DNA-binding CBFβ subunit. CBFs are required for both effective embryonic and adult haematopoiesis, and are frequently dysregulated in leukaemia (De Bruijn and Speck, 2004). Disruption of CBF genes as seen in the chromosomal translocations t(8;21)(q22;q22) or inv(16)(p13;q22) perturbs normal haematopoietic differentiation, and can co-operate with further mutations to promote proliferation.

Of particular note, NPM1 mutations represent the most frequent and well characterised molecular aberration in AML occurring in approximately 35% of AML cases but not reported in any other cancer (Falini *et al.*, 2005). NPM1 is a nuclear chaperone protein that resides in the nucleoli of cells and can regulate the ARF-p53 tumour suppressor pathway. However, a mutation in exon 12 of the gene leads to its cytoplasmic mislocalisation in AML blasts (Dohner and Dohner, 2008). Also of relevance in AML are the frequent internal tandem duplications (ITD) mutations (20-30%) observed in the *FLT-3* gene (Gilliland and Griffin, 2002). Such mutations lead to constitutive activation of tyrosine kinase activity and subsequent activation of downstream signalling pathways responsible for proliferation and survival.

Chromosomal abnormalities like inversion of chromosome 16 (inv(16)), or reciprocal translocations between chromosomes 15 and 17 (t(15;17)), and 8 and 21 (t(8;21)), have also been well characterised in AML. For example the t(8;21) CBF abnormality leads to fusion of the *RUNX-1* gene on chromosome 21, with the *eight twenty one* (ETO) gene on chromosome 8 generating the RUNX-1/ETO fusion protein. This abnormality is present in 10-15% of *de novo* AML and 40% of the FAB M2 subtype. The RUNX-1 portion of the protein can bind DNA and heterodimerise with CBFβ as normal, but lacks the transcription activation domains which are replaced with transcriptionally repressive sequences of ETO (Meyers *et al.*, 1993). This suppressive complex recruits additional nuclear receptor corepressors (N-CoR), paired amphipathic helix protein (Sin3A) and histone deacetylases (HDAC) which lead to the deacetylation of histones and chromatin remodelling (Wang *et al.*, 1998; Amann *et al.*, 2001). Therefore, normal transcriptional regulation by RUNX-1 in haematopoiesis is disturbed leading to reduced expression of some developmentally significant genes such as myeloperoxidase (MPO), the receptor for colony-stimulating factor 1 (CSF-1R), and the subunits of the T-cell antigen receptor (TCR) (Downing, 1999). Interestingly, this fusion protein has also been associated with transcriptional activation (Klampfer *et al.*, 1996).

It is becoming increasingly apparent that epigenetics also plays a role in AML pathology. Epigenetics is the alteration of gene expression unrelated to changes in the DNA sequence. One example of epigenetic change is DNA methylation which is

frequently dysregulated in tumourigenesis (Baylin and Herman, 2000). The addition of a methyl group to a cytosine-phosphate-guanosine (CpG island) stretch of DNA, often within the promoter region of a gene, renders that gene (typically tumour suppressors) permanently silenced. Methylated promoters of various Wnt signalling inhibitors (e.g. dickkopf-1; DKK1) have been discovered in AML no doubt contributing to the dysregulation of this pathway observed in this disease (Griffiths *et al.*, 2010).

Finally, the relatively recent and rapid emergence of microRNAs (miR) as important mediators in AML pathology must also be briefly considered. miRs are short (~22 nucleotides) regulatory non-coding RNAs that are abundantly expressed in the human genome and conserved across multiple species (reviewed by Zhao *et al.*, 2010). miRs are able to specifically repress gene expression through their incorporation into the RNA-induced silencing complex (RISC) and subsequent guidance to the target mRNA, which is degraded through mRNA cleavage or translational repression. Such regulation is vital to normal haematopoietic processes such as differentiation, proliferation and apoptosis, but deregulation is often observed in AML. For instance, Garzon *et al.* has reported the upregulation of miR-107 in acute promyelocytic leukaemia (APL), which specifically targets nuclear factor I/A (NFI-A), a gene important to normal CEBP-mediated granulocytic differentiation (Garzon *et al.*, 2007).

1.2.2.2 *Leukaemic stem cells*

Over the past two decades much excitement and controversy has surrounded the proposed existence of leukaemic stem cells (LSC) in AML. LSCs have historically been described as a rare subset of leukaemic cells which, through significant genetic damage, have acquired the sufficiently dysregulated self-renewal capacity necessary to both establish and maintain a malignant clone. They are typically defined by a primitive HSC-like phenotype (i.e. $CD34^+$) with the ability to initiate leukaemia when xenografted into immuno-suppressed mice. Despite the seemingly detailed characterisation of LSCs conflicting evidence exists over the origin, function and therapeutic potential of these cells in AML.

Evidence for the existence of an LSC population in AML was initially provided through the experiments of Lapidot and Dick in the 1990's which demonstrated that only specific subsets of the bulk AML clone ($Lin^-CD34^+CD38^-$ and *not* $Lin^-CD34^+CD38^+$) could successfully re-establish the disease when infused into sub-lethally irradiated mice (Lapidot *et al.*, 1994; Bonnet and Dick, 1997). However this work has since been challenged by the findings of Taussig and colleagues who showed that the CD38 antibodies used to purify such populations can in fact hinder the engraftment potential of these cells (Taussig *et al.*, 2008). Indeed, when such inhibitory factors were removed, the $CD34^+CD38^+$ fraction of AML samples was found to contain most, if not all, leukaemia intiating cells (LIC).

There are also limitations to the murine xenograft model as a system for assaying LSC activity. Indeed, rather than an assessment of LIC capability, this model could merely assess the ability of certain cells to engraft mice. In support of this it has been found that APL blasts, and indeed a significant proportion of other AML samples, fail to engraft at all, suggesting this system cannot efficiently model all LIC subsets (Bonnet and Dick, 1997). Interestingly, the vast majority of melanoma cells engraft this model (Quintana *et al.*, 2008). Whilst this could reflect a higher number of LSC/LIC in different tumours, this could also represent the variability in different cell types to engraft this model. Furthermore, the engraftment potential of certain cells can be influenced by the dose and site of inoculation (Yahata *et al.*, 2003; Wang *et al.*, 2003; Chabner *et al.*, 2004; Liu *et al.*, 2010).

Further work from the Dick lab has proposed that LSCs are analogous to normal $CD34^+$ HSCs (quiescence, high self-renewal potential, residence in the BM niche, phenotype) and hence are likely derived from them. Tracking of human LSCs in NOD-SCID mice showed heterogenous self-renewal potential leading the author to conclude that AML, similar to normal haematopoiesis, is organised hierarchically with self-renewing LT-LSCs with high LIC capacity, giving rise to leukaemic progeny with reduced self-renewal potential and LIC capability (Hope *et al.*, 2004). Although stem cell characteristics are indispensible for leukaemogenesis, it is not necessarily $CD34^+$ HSC themselves which represent the transformed LIC population. This is evidenced by the

fact intiating mutations such as the t(8;21) translocation have been observed in non-leukaemic cells (Miyamoto *et al.*, 2000; Downing, 2003). Also a substantial number of AML cases (including most NPM1 mutated AML) exhibit very little or no CD34 positivity suggesting the LSC/LIC population must contain considerably more heterogeneity than originally defined. Indeed much evidence now exists showing LIC capability at the level of committed myeloid progenitors (Jamieson *et al.*, 2004; Goardon *et al.* 2011). $CD34^+CD38^+$ granulocyte-macrophage progenitors in chronic myeloid leukaemia (CML) demonstrate greater self-renewal capacity than their normal counterparts and also expand more significantly than the $CD34^+CD38^-$ HSC pool during disease progression (Jamieson *et al.*, 2004). Furthermore the t(15;17) abnormality encoding the promyelocytic leukaemia/retinoic acid receptor alpha (PLM/RARα) fusion product in APL has been detected in $CD34^-CD38^+$ cell populations and not in the HSC enriched $CD34^+CD38^-$ subset (Turhan *et al.*, 1995) potentially explaining this subtype's failure to engraft.

The emergence of the LSC concept has provoked much optimism into the potential for therapeutic targeting in AML. Indeed the failure of current therapeutic regimens to fully eradicate LSC or LIC populations still represent the best explanation as to why so many patients relapse following CR or BM transplantation. However, clonal evolution experiments by Mel Greaves (see above *1.2.2*) have demonstrated that this strategy may be more difficult than first anticipated. These studies show that although the founding genetic mutation is stably retained throughout the malignant clone, differing selective (Darwinian) pressures within the leukaemic environment can lead to genetically and phenotypically distinct LIC subpopulations. Such clonal evolution means that if LSCs truly exist then they are likely to represent an elusive, continually moving target, rather than a fixed entity (Greaves, 2010).

1.2.3 Diagnosis, classification and prognosis in AML

1.2.3.1 *Diagnosis*

Traditionally, a diagnosis of AML was made upon presentation of BM containing 30% blasts in the BM or peripheral blood, using cytomorphological techniques of the FAB classification system (Estey and Dohner, 2006). The more recent World Health Organisation (WHO) classification lowers the threshold to 20% provided the AML blast population exhibits myeloid origin ($CD13^+CD33^+$) or is present with atleast one known cytogenetic abnormality (see *1.2.3.2* below).

1.2.3.2 *Classification*

Although AML is generally referenced as a single disease, it is heterogeneous and better resembles a collection of myeloid malignancies. In acknowledgement of this, two classification systems exist to address the distinct genetic, biochemical and morphological subsets of this disease.

The first and oldest FAB classification (*Figure 1.7A*) uses cytochemical techniques such as May-Grünwald-Giemsa staining to subtype AML according to blast morphology. AML is then classified by the extent of myeloid differentiation exhibited by the bulk blast population, with M0 representing the most undifferentiated blasts, through to the extensive megakaryocytic differentiation exhibited by the M7 variant. This system remains popular with developmental haematologists attempting to model the disease *in vitro*, since it provides information on the extent of differentiation prior to transformation.

This system has now been largely superseded by the recently updated 2008 WHO classification of AML which now incorporates all available clinical information including the cytogenetic, immunophenotypic, morphological and molecular properties of the disease (Vardiman *et al.*, 2009). From this it defines four major categories of AML as shown in *Figure 1.7B*. This allows a more accurate diagnosis of the subtype of

AML which has become increasingly vital given the emergence of genetically tailored therapeutics.

1.2.3.3 *Prognosis*

Major prognostic factors in AML include response to initial treatment, age, cytogenetic status, WBC count, secondary disease (e.g. from MDS, which results in more aggressive and resistant disease), and serum albumin, bilirubin and creatinine level (Estey and Dohner, 2006).

Performance status is a highly relevant predictor of therapy-related death. Using the Zubrod scale, the general patient health prior to induction is assessed on a 6-point scale from 0 (unaffected health), through to 4 (bed-bound) and 5 (death) (Estey and Dohner, 2006). Unsurprisingly, higher performance status scores are associated with a higher rate of treatment-related mortality.

Age is powerful predictor of resistant disease in AML. Despite significant progress in the survival of young adults (<60 years old) with AML, relatively little progress has been made in the last 40 years for elderly patients. Data from the UK Medical Research Council (MRC) AML trials (*Figure 1.8*), shows around 50% of young patients achieve a long-term 'cure' ($\geq$5 years), which halves for patients between 60-69 years and is less than 10% for patients 70 years or older. Major obstacles to CR in elderly patients include more aggressive and resistant disease (due to higher frequencies of acquired chromosomal aberrations and DNA mutations), and poor tolerance of intensive treatment regimes.

Cytogenetics provide a reliable predictor of treatment outcome in AML, such that established abnormalities can be categorised as adverse, intermediate or favourable for prognosis (*Figure 1.7C*). Approximately 60% of AML patients present with a karyotypic abnormality prior to treatment (Mrozek *et al.*, 2001), and large-scale AML patient studies by Grimwade *et al* have characterised the clinical relevance of such

abnormalities (Grimwade *et al.*, 1998; Grimwade *et al.*, 2001). For example, AML harbouring a CBF abnormality such as t(8;21) or inv(16) are highly responsive to chemotherapy and yield higher CR rates with longer overall survival. However patients with a complete deletion of chromosome 5 (-5) or chromosome 7 (-7), or complex karyotypes of multiple aberrations are linked with poor response to treatment, and often present in old age. Curiously, the largest cytogenetic group in AML is a normal karyotype (intermediate risk). In this instance, specific molecular mutations (such as those described in section *1.2.2.1*) are highly predictive of outcome. Indeed the NPM1 and CEPBα mutations carry such accurate prognostic power (higher CR rate, longer survival, and improved event-free survival; (Frohling *et al.*, 2004; Dohner *et al.*, 2005) that they have been recently included as a provisional entity in the WHO classification of AML (see *Figure 1.7B*). The presence of multiple molecular mutations can complicate the prognosis in AML. For example the otherwise favourable prognosis of an NPM1 mutation is all but abrogated when present with a FLT3-ITD (Dohner *et al.*, 2005; Thiede *et al.*, 2006). Furthermore, some of these individual gene mutations are capable of conferring a worse prognosis in otherwise favourable cytogenetic risk groups, as exemplified by the *c-kit* mutation in t(8;21) leukaemias (Schnittger *et al.*, 2006; Paschka *et al.*, 2006).

A

FAB subtype	Description
M0	Undifferentiated
M1	Myeloblastic without maturation
M2	Myeloblastic with maturation
M3	Promyelocytic
M4	Myelomonocytic
$M4_{Eo}$	Myelomonocytic with bone marrow eosinophilia
M5	Monocytic
M6	Erythroleukaemic
M7	Megakaryocytic

B)

Acute myeloid leukaemia and related neoplasms

Acute myeloid leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
APL with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
Provisional entity: AML with mutated NPM1
Provisional entity: AML with mutated CEBPA

Acute myeloid leukemia with myelodysplasia-related changes

Therapy-related myeloid neoplasms

Acute myeloid leukemia, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
  Pure erythroid leukemia
  Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis

C

Risk Group	Abnormality	Comment
Favourable	t(8;21) t(15;17) inv(16)	Alone or with other aberrations
Intermediate	Normal +8 +21 +22 del (7q) del (9q) Abnormal 11q23 All other structural and numerical abnormalities	Cytogenetic abnormlities not classified as favourable or adverse Lack of additional favourable or adverse cytogenetic changes
Adverse	-5 -7 del(5q) Abnormal 3q Complex	Alone or in conjunction with intermediate-risk or other adverse-risk

Figure 1.7 - The classification of AML subtype and prognostic cytogenetics.

A) The FAB classification of AML which defines eight subtypes of disease based on the blast morphology. Adapted from Tenen, 2003. **B)** The updated 2008 WHO classification of AML which defines four main categories of AML on the basis of morphological, immunophenotypic, molecular and clinical characteristics of the disease. Adapted from Vardiman *et al.*, 2009. **C)** The favourable, intermediate and adverse cytogenetic risk groups of AML. Adapted from Grimwade *et al.*, 1998.

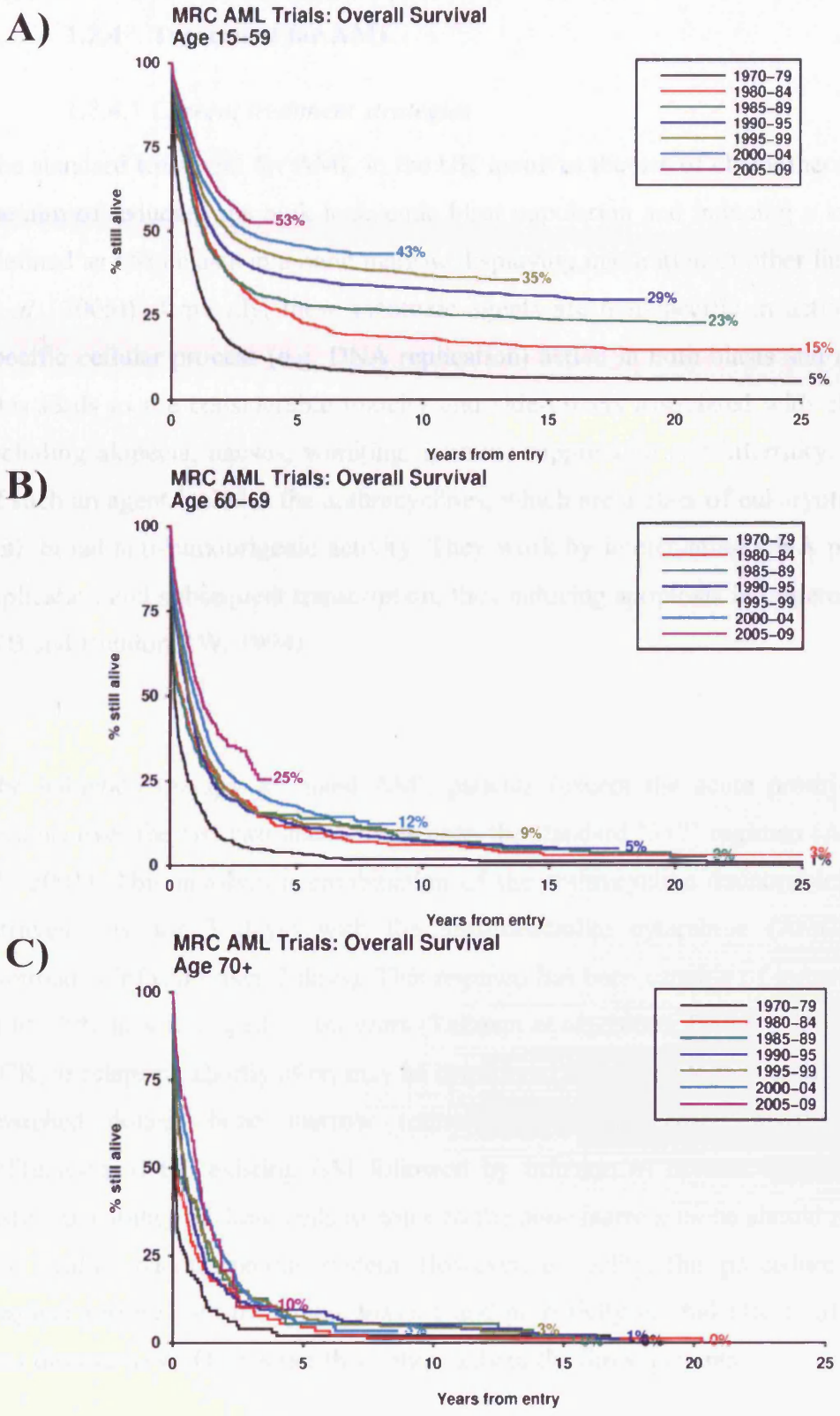


Figure 1.8 - The overall survival of different AML patient age groups.

Kaplan–Meier survival curves showing the overall survival rates of AML patients enrolled in the UK MRC AML trials since 1970 (courtesy of Professor Alan Burnett). Significant progress has been demonstrated in the **A)** younger patient cohort (15-59 years), however the prognosis remains poor for older patients of **B)** 60-69 years or **C)** 70 years plus.

1.2.4 Treatment for AML

1.2.4.1 *Current treatment strategies*

The standard treatment for AML in the UK involves the use of chemotherapeutics with the aim of reducing the bulk leukaemic blast population and inducing a long-term CR (defined as <5% blasts in a bone marrow displaying maturation of other lineages; (Gale *et al.*, 2005)). Typically, these cytotoxic agents are non-specific in action and target specific cellular process (e.g. DNA replication) active in both blasts and normal cells. This leads to the considerable toxicity and side-effects associated with chemotherapy including alopecia, nausea, vomiting, immune-suppression and infertility. An example of such an agent includes the anthracyclines, which are a class of eukaryotic antibiotics with broad anti-tumourigenic activity. They work by intercalating DNA preventing its replication and subsequent transcription, thus inducing apoptosis in targeted cells (Pratt WB and Ruddon RW, 1994).

The induction therapy for most AML patients (except the acute promyelocytic M3 variant) over the last two decades has been the standard '3+7' regimen (Appelbaum *et al.*, 2001). This involves a combination of the anthracycline daunorubicin (45mg/m^2 intravenously for 3 days) with the anti-metabolite cytarabine (AraC; 100mg/m^2 continuous infusion over 7 days). This regimen has been capable of inducing CR rates of 65-75% in adults aged 18-60 years (Tallman *et al.*, 2005). Patients failing to achieve a CR, or relapsing shortly after, may be considered for an autologous (self) or allogeneic (matched donor) bone marrow transplant. This procedure involves sub-lethal obliteration of the existing BM followed by infusion of normal CD34$^+$ HSCs. The instinctual ability of these cells to home to the bone marrow niche should reconstitute a new healthy haematopoietic system. However, in reality, this procedure is met with frequent relapse, and the severe toxicity and potentially morbid effects of graft versus host disease (GVHD), restrict this option only to the fittest patients.

The lack of progress in survival rates from elderly patients with AML over the last 40 years (*Figure 1.8C*), has led many experts to conclude that current chemotherapeutic regimens have reached 'the end of the road' in terms of treatment efficiency. The

genetic and molecular heterogeneity exhibited in AML means a more targeted approach to specific aberrations within the patient is now required. The Wnt signalling pathway is one such target that has rapidly accumulated interest of late because of its frequent dysregulation in AML.

1.3 Wnt signalling

1.3.1 Overview

Wnt signalling is an evolutionary conserved pathway critical for normal developmental processes in both the embryo and adult, including cell growth and differentiation. The human genome encodes for a total of 19 Wnt genes, which are all members of a lipid-modified family of secreted glycoproteins (Clevers, 2006). All are capable of activating the multiple pathways which comprise Wnt signal transduction when bound to their cognate receptor complex, with the net result of downstream Wnt target gene activation. At least three different intracellular signalling pathways are recognised to emanate from a Wnt stimulus. These include the well characterised canonical pathway, and the lesser understood planar cell polarity (PCP) and Wnt-Ca^{2+} non-canonical pathways (Staal *et al.*, 2008). Only the canonical pathway will be described further here given the central involvement of catenin molecules, which have particular relevance to this study.

In the absence of a Wnt signal, the canonical Wnt pathway is maintained in a state of suppression (*Figure 1.9A*) through continual degradation of the central mediator, β-catenin. In this state, β-catenin is bound by a catenin destruction complex (CDC) consisting of casein kinase 1 (CK-1), glycogen synthase kinase 3 beta (GSK-3β), axis inhibition protein 1 (Axin-1) and adenomatous polyposis coli (APC). This complex phosphorylates β-catenin on Serine 45 (Ser) by CK-1 and then on Ser33, Ser37 and Threonine 41 (Thr) by GSK-3β, generating recognition sites for the β-transducin-repeat-containing protein (β-TRCP) (Aberle *et al.*, 1997; Orford *et al.*, 1997). This protein tags β-catenin with ubiquitin molecules ultimately targeting it for proteasome-mediated degradation in the cytoplasm (Salomon *et al.*, 1997; Kitagawa *et al.*, 1999; Hart *et al.*, 1999). In the nucleus, the transcription factor TCF suppresses Wnt target gene activation by forming a repressor complex with members of the groucho (GRG)

family of transcriptional repressors, C-terminal binding protein (CtBP) and HDACs (Roose *et al.*, 1998).

The canonical pathway is activated (*Figure 1.9B*) upon binding of a Wnt ligand, such as Wnt3A, to a transmembrane receptor complex consisting of the frizzled (FZ) family of G protein-coupled receptors, and the low density lipoprotein receptor-related protein (LRP) family of multifunctional endocytic receptors. Activation of this complex leads to the recruitment of dishevelled (DVL) which in turn assists CK-1 and GSK-3β in the phosphorylation of LRP5/6 (Zeng *et al.*, 2005; Zeng *et al.*, 2008). The phosphorylated residues of LRP5/6 provide 'docking' points for Axin-1 which subsequently dissociates from the CDC, and re-locates to the plasma membrane. This ultimately causes the failure of all CDC components to assemble (through a mechanism that is incompletely understood) and so β-catenin is not phosphorylated or degraded. Instead the cytoplasmic pool of signalling competent β-catenin accumulates, culminating in its eventual translocation to the nucleus through a largely unknown mechanism. Here, β-catenin is able to activate Wnt target genes by displacing groucho repressors on DNA and binding the high-mobility group (HMG) family of nuclear proteins TCF and LEF in a complex also requiring B-cell CLL/lymphoma-9 protein (BCL-9) and pygopus (Kramps *et al.*, 2002; Thompson, 2004; Townsley *et al.*, 2004a; Townsley *et al.*, 2004b). The architectural TCF/LEF transcription factors serve to physically bend DNA and permit the association of the DNA-protein complexes necessary for gene transcription. Well characterised gene targets of TCF/LEF-mediated Wnt/β-catenin signalling include *c-myc* (He *et al.*, 1998), *cyclinD1* (Shtutman *et al.*, 1999), *survivin* (Zhang *et al.*, 2001; Kim *et al.*, 2003a), *CD44* (Wielenga *et al.*, 1999), and *TCF-1* (Roose *et al.*, 1999).

The canonical pathway is also negatively regulated at varying stages of the cascade. The binding of Wnt ligand to the FZ and LRP activation receptors is antagonised by the presence of soluble decoy proteins in the extracellular matrix (Kawano and Kypta, 2003). Inhibitory proteins such as DKK1, Wnt inhibitory factor (WIF), soluble frizzled-related protein (sFRP), and norrin are capable of binding, but not transducing, a Wnt stimulus. Within the nucleus, β-catenin mediated transcription is inhibited by sequestration of β-catenin from TCF/LEF complexes by the β-catenin-interacting

protein (ICAT) (Daniels and Weis, 2002). Finally, alternative splicing of the TCF gene, in particular TCF-1, can also regulate the sensitivity of the cell to Wnt signals. Longer forms of the transcription factor harbour the amino-terminal required to bind catenin, whilst shorter forms lack this domain and subsequently function as endogenous transcriptional repressors (Van de Wetering M. *et al.*, 1996).

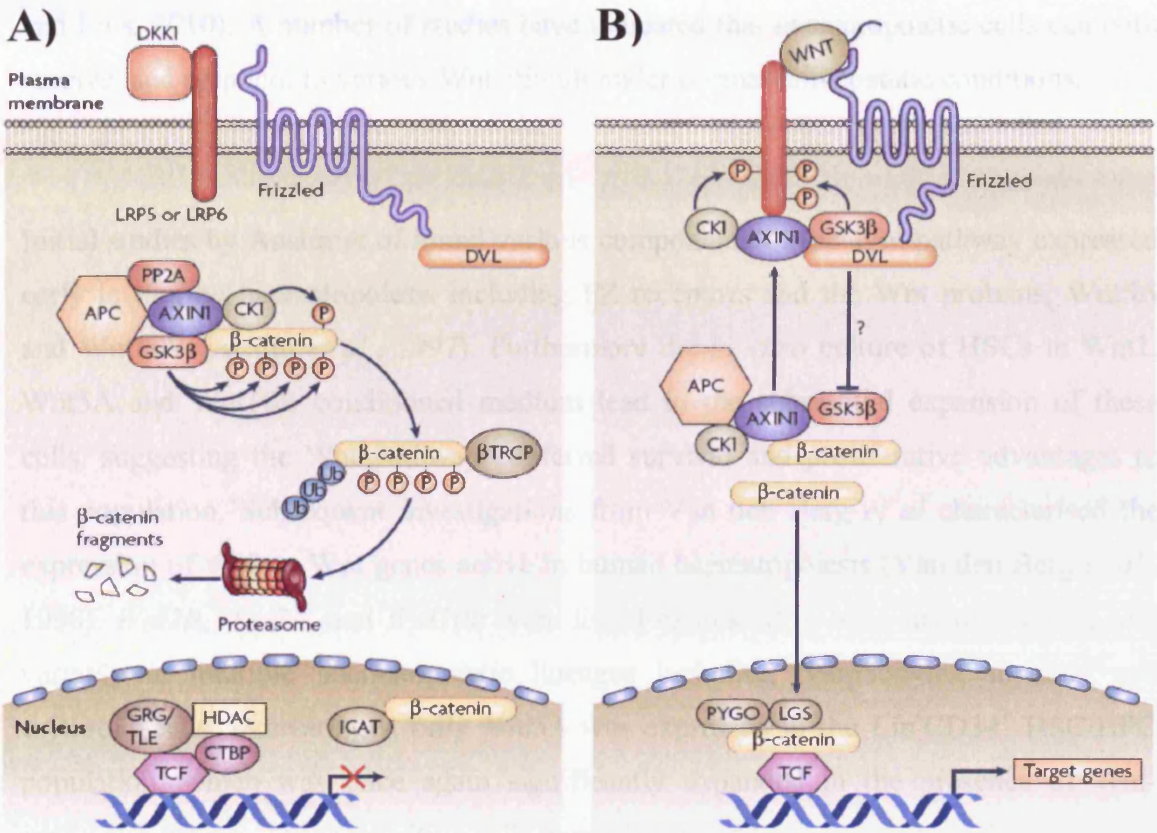


Figure 1.9 - Overview of the canonical Wnt signalling pathway.

A) In the absence of a Wnt ligand, β-catenin is bound by the CDC, phosphorylated, and targeted for degradation in a ubiquitin/proteasome-dependent manner. Wnt signalling is further inhibited by soluble antagonists such as DKK-1, or binding of nuclear β-catenin by ICAT. Wnt target gene expression is suppressed by recruitment of co-repressors such as GRG/TLE (Groucho/transducin-like enhancer), CTbP and HDACs. **B)** Wnt signalling is activated upon binding of a lipid-modified Wnt ligand FZ/LRP receptor complex. DVL initiates the sequestering of Axin-1 from the CDC by phosphorylation of LRP, leading to the dissolution of this complex. Failure of this complex to degrade β-catenin causes its accumulation and nuclear translocation where it binds TCF/LEF promoters on DNA to activate Wnt target genes. Additional co-factors such as legless (LGS; also known as BCL-9) and Pygopus (PYGO), CBP/p300, brahma and MED12 are recruited to facilitate transcription. Adapted from Staal *et al.*, 2008.

1.3.2 Wnt signalling in normal haematopoiesis

The Wnt pathway is known to regulate the fate and function of many different cell types (reviewed by Clevers, 2006 and Cadigan and Peifer, 2009), and evidence over the last 15 years has established that this pathway is also active in normal haematopoiesis (Staal and Luis, 2010). A number of studies have indicated that haematopoietic cells can both receive, and respond, to various Wnt stimuli under normal homeostatic conditions.

Initial studies by Austin *et al* found various components of the Wnt pathway expressed early in murine haematopoiesis including FZ receptors and the Wnt proteins, Wnt5A and Wnt10B (Austin *et al.*, 1997). Furthermore the *in vitro* culture of HSCs in Wnt1, Wnt5A and Wnt10B conditioned medium lead to the substantial expansion of these cells, suggesting the Wnt pathway conferred survival and proliferative advantages to this population. Subsequent investigations from Van den Berg *et al* characterised the expression of various Wnt genes active in human haematopoiesis (Van den Berg *et al.*, 1998). *Wnt2B*, *Wnt5A*, and *Wnt10B* were found expressed in bone marrow stroma and variably in multiple haematopoietic lineages including lymphocytes, myeloid and erythroid cells. Interestingly, only *Wnt5A* was expressed in the $Lin^{-}CD34^{+}$ HSC/HPC population, which was once again significantly expanded in the presence of Wnt-producing stroma. These primitive cells were also found to express atleast 6 members of the FZ family of Wnt receptors. Since these initial studies further reports have confirmed both the expression and influence of various Wnt components on normal haematopoietic cells (Reya *et al.*, 2000; Hackney *et al.*, 2002; Murdoch *et al.*, 2003; Willert *et al.*, 2003; Wagner *et al.*, 2005; Dosen *et al.*, 2006; Congdon *et al.*, 2008; Sercan *et al.*, 2010; Gallagher *et al.*, 2010). In particular, much interest has surrounded the influence of Wnt signalling within the HSC niche.

Although not completely characterised, there is much evidence to support active Wnt signalling within the BM niche. Indirectly, it is likely to affect HSC regulation through its role in maintaining mesenchymal tissue, such as osteoblasts (Guo *et al.*, 2004; Day *et al.*, 2005). Fleming and colleagues demonstrated a direct relationship *in vivo* through targeted expression of the Wnt pathway inhibitor dickkopf-1 (DKK1) in the niche (Fleming *et al.*, 2008). Loss of Wnt signalling arising from DKK1 overexpression

caused increased cell cycling and reduced regenerative capacity of HSC following transplant, leading the authors to conclude that Wnt signalling in the niche regulates HSC quiescence and reconstitution function. Kim *et al.* used a different approach to demonstrate Wnt importance in the niche (Kim *et al.*, 2009). Enforced expression of β-catenin in the surrounding stromal cells promoted HSC self-renewal in a contact-dependent manner, whilst direct stabilisation in HSC led to loss of this population. Indeed, both Wnt receptors and β-catenin were expressed more highly in the BM stroma than haematopoietic cells, and could be further induced through stimulation with Wnt3A. Notch ligands were also enriched in Wnt/β-catenin activated stroma leading to downstream activation of Notch signalling in HSCs suggesting cross-talk between these pathways within the niche.

Evidence for a direct role of Wnt signalling in haematopoietic cells is strengthened by recent functional studies into various components of the pathway (reviewed recently by (Staal and Luis, 2010)). For example, Wnt signalling is likely to be important for lymphopoiesis given thymic T-cell development is perturbed upon overexpression of the Wnt inhibitors ICAT (Pongracz *et al.*, 2006) and sFRP (Staal *et al.*, 2001). Conversely, the proliferation of B-cells appears to be negatively regulated by the Wnt signalling agonist, Wnt3A (Dosen *et al.*, 2006). Inhibition of the Wnt CDC constituent GSK-3β has been shown to promote the self-renewal and reconstitution capacity of HSCs *in vivo* indirectly through its influence on β-catenin level (Trowbridge *et al.*, 2006; Holmes *et al.*, 2008). The downstream transcriptional activators TCF/LEF have also demonstrated relevance in a normal haematopoietic setting. Repression of the downstream Wnt transcriptional activator, LEF-1, was shown to inhibit proliferation and induce apoptosis in $CD34^+$ progenitor cells (Skokowa *et al.*, 2006). Mice deficient in TCF-1 demonstrate severe thymocyte abnormalities with a reduction in overall thymocyte number, and a block in differentiation between transition from immature CD8+ precursors to more mature CD4+CD8+ T-cell lymphocytes (Verbeek *et al.*, 1995). Finally, the manipulation of differing exogenous Wnt proteins is capable of altering the course of haematopoietic development. Depletion of Wnt3A reduced the number and repopulation capacity of HSC/HPC, whilst further reducing the frequency of myeloid progenitors (Luis *et al.*, 2009). Furthermore, loss of Wnt11 caused the domination of vacuolated macrophages during *in vitro* culture of HPC, at the expense of

red cells and monocytes (Brandon *et al.*, 2000). These lineages were rescued by re-addition of Wnt11 and Wnt5A, and can actually stimulate the reverse phenotype when over-concentrated, implying that a fine balance of exogenous Wnt proteins is required for normal haematopoiesis.

Arguably the most disputed function of a Wnt signalling component in normal haematopoiesis is that of the central mediator β-catenin. A host of studies have demonstrated Wnt/β-catenin signalling is vital in mediating the survival and self-renewal of HSC (Reya *et al.*, 2003; Willert *et al.*, 2003; Jamieson *et al.*, 2004; Zhao *et al.*, 2007; Holmes *et al.*, 2008; Kim *et al.*, 2009; Nemeth *et al.*, 2009). However, other studies have contradicted these findings by demonstrating that constitutively activated β-catenin in HSC impairs multi-lineage differentiation and completely exhausts the HSC pool (Baba *et al.*, 2005; Baba *et al.*, 2006; Kirstetter *et al.*, 2006; Scheller *et al.*, 2006). To further complicate the picture, the constitutive deletion of β-catenin, and or the close homologue γ-catenin, in HSC did not affect the ability of these cells to fully reconstitute a normal haematopoietic system when transplanted into irradiated mice (Cobas *et al.*, 2004; Jeannet *et al.*, 2008; Koch *et al.*, 2008). Interestingly, TCF/LEF activation remained intact in these cells suggesting the existence of other catenin-like molecules that can also bind these transcription factors and even compensate in the absence of catenin. The disparate results arising from these many investigations has been suggested to arise from the considerable variation in the experimental approaches adopted to generate them (Staal and Luis, 2010). Although the precise role of individual Wnt signalling components requires further elucidation, it seems undeniable that Wnt signalling and its strict regulation are indispensible for normal, healthy haematopoiesis.

1.3.3 Dysregulated Wnt signalling in AML

The importance of Wnt signalling to normal development makes this pathway an ideal target during malignant transformation. Indeed, its dysregulation has been reported in many epithelial cancers (reviewed by others (Polakis, 2000; Giles *et al.*, 2003)) and there is much evidence, although some still controversial, pointing to its dysregulation in AML.

A landmark study by Majeti *et al* compared the gene expression profiles between normal BM HSCs and AML LSCs and identified the Wnt signalling pathway as aberrantly regulated (Majeti *et al.*, 2009). It must be noted however that very small patient numbers were used (<10 per comparison), and CD34 positivity was adopted as the only LSC marker which remains controversial (as previously discussed *1.2.2.2*). A recent study by Wang *et al* identified the Wnt/β-catenin axis to be vital in mediating the self-renewal and survival of murine LIC (Wang *et al.*, 2010). In particular, activation of Wnt/β-catenin signalling was suggested to represent the mechanism by which certain oncogenes can transform even committed progenitors. However, caution must be exercised when comparing this to human AML-initiating cells since the oncogenes used in this murine model (*Meis homeobox 1a* (*Meis1a*) and *homeobox A9* (*HoxA9*)) are rarely implicated in human AML.

Further dysregulation of Wnt signalling components has been reported in myeloid leukaemias including the soluble Wnt proteins Wnts1/2B/3A (Simon *et al.*, 2005; Kawaguchi-Ihara *et al.*, 2008), FZ-4 receptor (Tickenbrock *et al.*, 2008), GSK-3β (De Toni *et al.*, 2006; Abrahamsson *et al.*, 2009) LEF-1 (Li *et al.*, 2004; Petropoulos *et al.*, 2008) and TCF-4 (Siti Sarah Daud *et al.*, 2010). Using Western blotting or immunohistochemical techniques, many studies have demonstrated the variable expression of the central mediator β-catenin in primary AML blasts (Chung *et al.*, 2002; Serinsoz *et al.*, 2004; Simon *et al.*, 2005; Ysebaert *et al.*, 2006; Xu *et al.*, 2008; Chen *et al.*, 2009). The studies of Ysebaert, Xu and Chen *et al* were all able to further verify β-catenin expression as a prognostic indicator of poor survival in AML patients. Despite these findings, many of the above mentioned studies are hampered by relatively small cohort sizes (typically ≤ 80) which are too small to be definitive in such a heterogeneous disease as AML. The study of Xu *et al* investigated another possible level of β-catenin dysregulation in AML; namely the inappropriate nuclear localisation. The detection of non-phosphorylated nuclear β-catenin (the only form which should theoretically enter the nucleus) was detected immunohistochemically in nearly half of all AML samples examined, implying aberrant transcriptional activity in AML. However this study is once again hampered by small sample numbers and does not declare how nuclear localisation compares with that of normal HSC/HPC. Given the associated role of β-catenin with self-renewal in normal HSC, it is conceivable that β-

catenin would be expected in the nucleus of a HSC/HPC disorder. This concept was taken further by the research of Simon *et al*, who proposed aberrant β-catenin transcriptional activity in AML given the finding that leukaemic cells demonstrated higher outputs from the TOPFLASH reporter (internal measurement of TCF/LEF activity). However, the authors do not show how β-catenin correlates with TCF/LEF activity, which becomes important given that TOPFLASH is non-specific for catenin-TCF/LEF activation and other molecules are also capable of activating these transcription factors. Furthermore this paper compares β-catenin protein level in AML blasts with that of normal progenitors which have been cultured *in vitro* for 6 days. Such a comparison may be inappropriate given the lack of exogenous Wnt factors present in culture medium which may influence β-catenin level within a normal *in vivo* setting. Finally, methylation of Wnt negative regulators, such as WIF-1, DKK and sFRP have also been identified in AML which would be predicted to elicit uncontrolled Wnt signalling (Chim *et al.*, 2006; Valencia *et al.*, 2009; Griffiths *et al.*, 2010). Unfortunately these studies mainly focus on the gene status of these Wnt inhibitors in AML blasts and do not formally demonstrate inhibition at the protein level, or hyperactivity of the Wnt pathway through β-catenin or TCF/LEF measurement. All three studies are in agreement however, that methylation of Wnt negative regulators is associated with an adverse prognosis in AML.

Well established gene mutations in AML such as FLT3-ITD may also mediate their pathogenic effects through perturbation of Wnt signalling. Tickenbrock *et al* discovered the FLT3-ITD mutation can induce expression of the FZ-4 receptor and β-catenin, which could enhance the sensitivity of these cells to Wnt signal transduction (Tickenbrock *et al.*, 2005). The study by Simon *et al*, also noted high TCF/LEF output in FLT3-ITD$^+$ AML but this did not reach significance given the high levels observed in most patient samples regardless of FLT-3 status (Simon *et al.*, 2005).

Finally, an additional Wnt signalling component has recently been identified as dysregulated in AML (Zheng *et al.*, 2004; Muller-Tidow *et al.*, 2004; Tonks *et al.*, 2007). γ-Catenin, a close structural and functional homologue of β-catenin, was originally identified as dysregulated in AML by its overexpression (at mRNA and

protein level) in the presence of common AML translocation products such as RUNX-1/ETO (Zheng *et al.*, 2004; Muller-Tidow *et al.*, 2004). Indeed these fusion proteins were shown to be directly capable of activating the γ-catenin gene promoter, and γ-catenin mRNA levels were significantly higher in fusion protein-positive primary AML BM blasts. More extensive AML patient microarray studies from our laboratory identified γ-catenin mRNA expression as higher across the majority of AML FAB subtypes, regardless of fusion protein status, in comparison to normal $CD34^+$ progenitor cells (Tonks *et al.*, 2007). The highest levels of γ-catenin expression were observed in patients with a CBF abnormality (t(8;21) or inv(16)). Collectively, these data suggested that CBF abnormalities in AML mediate their pathogenic effects in part by disrupting Wnt signalling through γ-catenin activation. Such reports were the first to implicate γ-catenin within a haematopoietic context.

1.3.4 γ-Catenin

γ-Catenin (aka plakoglobin, junction plakoglobin (JUP)), is a close structural and functional homologue of β-catenin and is a member of the armadillo protein family, an evolutionary conserved group of proteins critical to normal physiological processes such as cell signalling, adhesion and motility. *Armadillo* was originally discovered as a segment-polarity gene vital for proper axis formation during *Drosophila* embryogenesis (Peifer and Wieschaus, 1990). The identification of human γ-catenin through immuno-precipitation with cadherin protein showed it shared close amino acid sequence homology with both *Drosophila* Armadillo (63%) and *Xenopus* β-catenin (68%) (McCrea and Gumbiner, 1991; Knudsen and Wheelock, 1992; Peifer *et al.*, 1992). Further work has shown that γ-catenin protein is highly conserved amongst mammalian species with mouse *(Mus musculus)*, rat *(Rattus norvegicus)*, cow *(Bos taurus)* and dog *(Canis familiaris)* all sharing ≥98% amino acid sequence homology with the human form[2]. Complete sequencing has shown it to span 745 amino acids, with a predicted molecular weight of 82kDa (Franke *et al.*, 1989). The γ-catenin gene (*CTNNG*) is located on chromosome 17q21 (Aberle *et al.*, 1995), and is subject to alternative splicing. Nine different transcripts have been identified to date (see Sanger Ensembl

[2] http://www.genecards.org/cgi-bin/carddisp.pl?gene=JUP

website[3]), nearly all of which are protein coding. Three of the transcripts encode the full-length 745 amino acid sequence, with the rest encoding short variants (<300 amino acids) with no known functional significance. Of note, an alternatively spliced form of γ-catenin has been identified harbouring a 120bp deletion leading to loss of the fourth armadillo repeat and prevents its binding with protein partners such as APC and E-cadherin (Ozawa *et al.*, 1995a; Ozawa *et al.*, 1995b). γ-Catenin protein is known to be regulated through the same post-translational modifications as are active for β-catenin protein (see below).

In addition, to the main constituents of the armadillo protein family (α-, β- and γ-catenin) a further subfamily of armadillo repeat containing proteins have been described including p120 catenin and δ-catenin. However, these proteins as yet have no known relevance to haematopoiesis and consequently will not be discussed further (for review see McCrea and Park, 2007).

Armadillo proteins share a common structure including a highly conserved central armadillo domain flanked by amino- (NH_2) and carboxy (COOH) -termini (Hatzfeld, 1999). The central domain consists of 12 armadillo repeat regions (consisting of 45 amino acids each) that exhibit considerable sequence homology (~85%) between γ- and β-catenin molecules (*Figure 1.10A*). Crystallographic analysis has revealed this 'arm' repeat motif can fold into a tertiary structure composed of densely packed α-helices that form a superhelix with a positively charged groove spanning the entire arm region (Huber *et al.*, 1997). This groove has been proposed to mediate the binding of many common protein partners including α-catenin, cadherin, APC, Axin and TCF/LEF transcription factors (Ben-Ze'ev and Geiger, 1998). The amino-termini of γ- and β-catenin share only modest similarity (*Figure 1.10A*), but both contain the crucial GSK-3β consensus site necessary for the phosphorylation and subsequent degradation of the molecules (Aberle *et al.*, 1997). Indeed, γ-catenin is also known to interact with many of the same degradation components also active in the maintenance of β-catenin protein stability (see *1.3.1*) including APC and Axin (Rubinfeld *et al.*, 1995; Miller and Moon,

[3] http://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000173801;r=17:39910856-39943183

1997; Kodama *et al.*, 1999; Sadot *et al.*, 2000). The NH_2-terminus of β-catenin has also been identified to carry functional significance by housing transcriptional activation domains. In particular these activation domains have been found important for binding and stimulating LEF-1 mediated transcription in epithelial cells, however no such function has yet been ascribed to the corresponding region of γ-catenin (Simcha *et al.*, 1998; Hsu *et al.*, 1998; Kolligs *et al.*, 1999). The COOH-termini of catenins (*Figure 1.10A*) share the least homology and the function of this domain is incompletely resolved. They are also believed to assist transcriptional function (Orsulic and Peifer, 1996; Simcha *et al.*, 1998; Hsu *et al.*, 1998; Hecht *et al.*, 1999), but substantial evidence also implicates this terminal with regulating the specificity of catenin binding with various adhesion and transcriptional partners including the desmosomal and classical cadherins (see *1.3.5.1* below) (Wahl *et al.*, 1996; Palka and Green, 1997; Wahl *et al.*, 2000), LEF-1 (Zhurinsky *et al.*, 2000a) and TCF-4 (Solanas *et al.*, 2004). Of particular note, the studies by Wahl *et al* confirmed the COOH-terminus of γ-catenin to be important for interactions with desmosomal cadherins, and furthermore, through chimaeric modelling, showed such COOH-terminal specificity can explain the exclusion of β-catenin from these adhesion structures. Also of interest, Zhurinsky *et al*, showed that the COOH-, and indeed NH_2-termini, of γ- and β-catenin are not required for actual binding to LEF-1-DNA complexes, but are required for subsequent transcriptional activation. The molecular similarity between γ- and β-catenin has led many to hypothesise, or demonstrate, that they share considerable functional overlap.

1.3.5 Functions of γ-catenin

γ-Catenin shares many common protein partners with β-catenin and, as a consequence, assumes many of the same functions within the cell, however, important differences also exist.

1.3.5.1 *Cell adhesion*

The best characterised function of γ-catenin is in cell adhesion. Like β-catenin, it is located within adherens junctions (AJ) between cells, where it anchors classical cadherins (i.e. N- or E-cadherin) in the membrane, to the actin cytoskeleton, via α-

catenin (*Figure 1.10B*). The presence of additional protein binding sites in the NH_2- and COOH-terminal domains, enable γ-catenin to function as a scaffold protein for these multi-protein assemblies (Aberle *et al.*, 1994; Nagafuchi *et al.*, 1994). Such types of adhesion are critical for maintaining tissue integrity within an epithelial context such as the skin or gut. Unlike β-catenin, it can also be found as a constituent of desmosomal plaques (*Figure 1.10B*), where it anchors the desmosomal cadherins desmocollin and desmoglein, to cytosolic intermediate filaments through desmoplakin and plakophilin (Schmidt *et al.*, 1994; Cowin and Burke, 1996). These strong adhesion structures are tissue-specific and limited to organs undergoing constant physical stress such as the heart. The is affirmed by the finding that γ-catenin-deficient murine embryos die early in development from severe cardiac defects resulting from improper formation of desmosomes (Bierkamp *et al.*, 1996; Ruiz *et al.*, 1996). Understandably, much knowledge of γ-catenin's adhesive function has arisen from experiments conducted within an epithelial context, given that adherence is not a feature of haematopoietic cells which exist in single-cell suspensions. Although homotypic interactions (between same cell types) are rare, heterotypic interactions (between different cell types) may be relevant within a haematopoietic context, particularly within the BM niche. The interaction between N-cad and β-catenin has been proposed to regulate the interaction of HSC with the local BM stroma (see *1.1.2.2*). However the questionable N-cad expression on HSC (see *3.5.2*), and the ability of double-catenin knockout (KO) HSC to fully reconstitute immunosuppressed mice (Cobas *et al.*, 2004; Jeannet *et al.*, 2008; Koch *et al.*, 2008), would challenge this concept.

1.3.5.2 *Transcription*

The functional role of γ-catenin as a transcription factor for the Wnt signalling pathway is more contentious. γ-Catenin is regulated in the same manner as β-catenin within the cell, being degraded by the same machinery (see above *1.3.1*) in the absence of a Wnt signal, and stabilised then translocated in the presence of Wnt ligand (*Figure 1.10B*) (Bradley *et al.*, 1993; Papkoff *et al.*, 1996; Kim *et al.*, 2011). Within an epithelial context, γ-catenin has previously been shown to effectively bind TCF/LEF transcription factors (Huber *et al.*, 1996; Simcha *et al.*, 1998; Hecht *et al.*, 1999; Kolligs *et al.*, 2000; Zhurinsky *et al.*, 2000a; Miravet *et al.*, 2002) and γ-catenin transcriptional activity has also been reported in β-catenin-null epithelial backgrounds (Conacci-Sorrell *et al.*,

2002; Maeda *et al.*, 2004; Kim *et al.*, 2011). γ-Catenin is further capable of transforming R3KE epithelial cells in a TCF/LEF dependent mechanism where it activates *c-myc* expression more extensively than β-catenin (Kolligs *et al.*, 2000). However, in general, its affinity for forming ternary complexes with DNA, and resulting transcriptional power, has been demonstrated as inferior to that of β-catenin (Simcha *et al.*, 1998; Zhurinsky *et al.*, 2000a; Maeda *et al.*, 2004). Furthermore γ-catenin is unable to compensate for the loss of β-catenin in KO mice (Haegel *et al.*, 1995; Huelsken *et al.*, 2000). The study by Huelsken *et al* shows the failure of γ-catenin to compensate the Wnt signal required for proper axis formation results in the death of β-catenin-deficient embryos.

There is a small quantity of evidence to suggest that γ-catenin could have transcriptional relevance within a haematopoietic context. Experiments by Müller-Tidow *et al*, have shown that induction of γ-catenin by AML fusion proteins, such as RUNX-1/ETO, in U937 cells lead to the increased frequency of γ-catenin/LEF-1 complexes (Muller-Tidow *et al.*, 2004). They were further able to show that γ-catenin was found bound directly to the promoter region of the well characterised Wnt target gene *c-myc*. It must be mentioned however that the frequency of β-catenin/LEF-1 complexes also increased with fusion protein induction in these cells making it more difficult to ascribe β-catenin independent TCF/LEF activation by γ-catenin. Work from our laboratory has also involved the analysis of gene expression profiles between normal human $CD34^+$ HSC/HPC and those overexpressing γ-catenin (Liddiard, unpublished data). Although global differences in gene expression were fairly unremarkable, a few noteworthy examples were observed including the positive induction of *myc-target protein 1* (*myc-T1*). Finally, following the demonstration of γ-catenin-mediated transcriptional control over *survivin* expression on a β-catenin deficient background (in epithelial NCI-H28 cells), Kim *et al* were further able to identify a correlation between γ-catenin and *survivin* expression in blast crisis CML patients (Kim *et al.*, 2011).

1.3.6 The functional relationship between γ- and β-catenin

The close structural and functional homology shared between γ- and β-catenin has provoked interest into the degree of competition, compensation, and synergy exhibited

between these two proteins. Salomon *et al* employed tumour cell lines (HT1080 and SVT2), which express β-catenin and N-cadherin but not γ-catenin, to demonstrate that exogenous γ-catenin was capable of displacing β-catenin from the AJ, augmenting its degradation by the proteasome (Salomon *et al.*, 1997). As well as promoting β-catenin degradation, this study also demonstrated how the ubiquitin-proteasome system can become overwhelmed by the extra-junctional β-catenin leading to nuclear translocation of β-catenin. Similar findings have been observed in *Xenopus* embryos where exogenous γ-catenin has been proposed to increase the cytoplasmic pool of signalling competent β-catenin, by saturating the degradation and CDC components (Miller and Moon, 1997; Klymkowsky *et al.*, 1999). Simcha *et al* also observed similar findings in an epithelial MDCK cell line, where β-catenin was identified as the primary driver of LEF-1 mediated transactivation (Simcha *et al.*, 1998). Despite the interdependence of γ- and β-catenin on the same regulatory components, the balance of protein expression/activity is likely to be dependent on the setting. For example in primary CML patient samples γ- and β-catenin level were found to inversely correlate, though it must be noted that only mRNA was assessed (Kim *et al.*, 2011). Lack of correlation between γ- and β-catenin protein levels has also been observed in primary tumour samples of brain, lung and breast (Toyoyama *et al.*, 1999; Bukholm *et al.*, 2000; Amitay *et al.*, 2001).

As well as competition for structural and regulatory constituents, γ- and β-catenin may also compete for the same nuclear partners with consequences for Wnt signal transduction. For example, Miravet *et al* have shown that TCF-4 contains distinct binding sites for both β- and γ-catenin and, although both proteins may bind simultaneously, interactions with the latter antagonises the transcriptional activity of the complex in epithelial cells (Miravet *et al.*, 2002). Further evidence from experiments in cardiac and lung cells have also highlighted γ-catenin as an inhibitor of β-catenin-TCF/LEF-mediated transcription (Winn *et al.*, 2002; Garcia-Gras *et al.*, 2006).

Compensatory mechanisms have also been described between γ-and β-catenin. Upregulation of γ-catenin has been found to partially compensate for the loss of β-catenin in adult cardiomyocytes (Zhou *et al.*, 2007), although it is unable to fully

compensate for the loss of β-catenin in the developing murine embryo (see *1.3.5.2* above). Similarly, β-catenin compensates for the loss of γ-catenin in early *Xenopus* development (Kofron *et al.*, 1997), yet cannot functionally compensate in the desmosomes of γ-catenin-null murine embryos (Bierkamp *et al.*, 1999). These results indicate that γ- and β-catenin probably serve distinct functions, and the nature of interplay between them is likely to be highly context-dependent.

The finding that both of these proteins are dysregulated in disease, and both specifically in AML, warrants further investigation into how the balance of expression can affect the pathology of the disease.

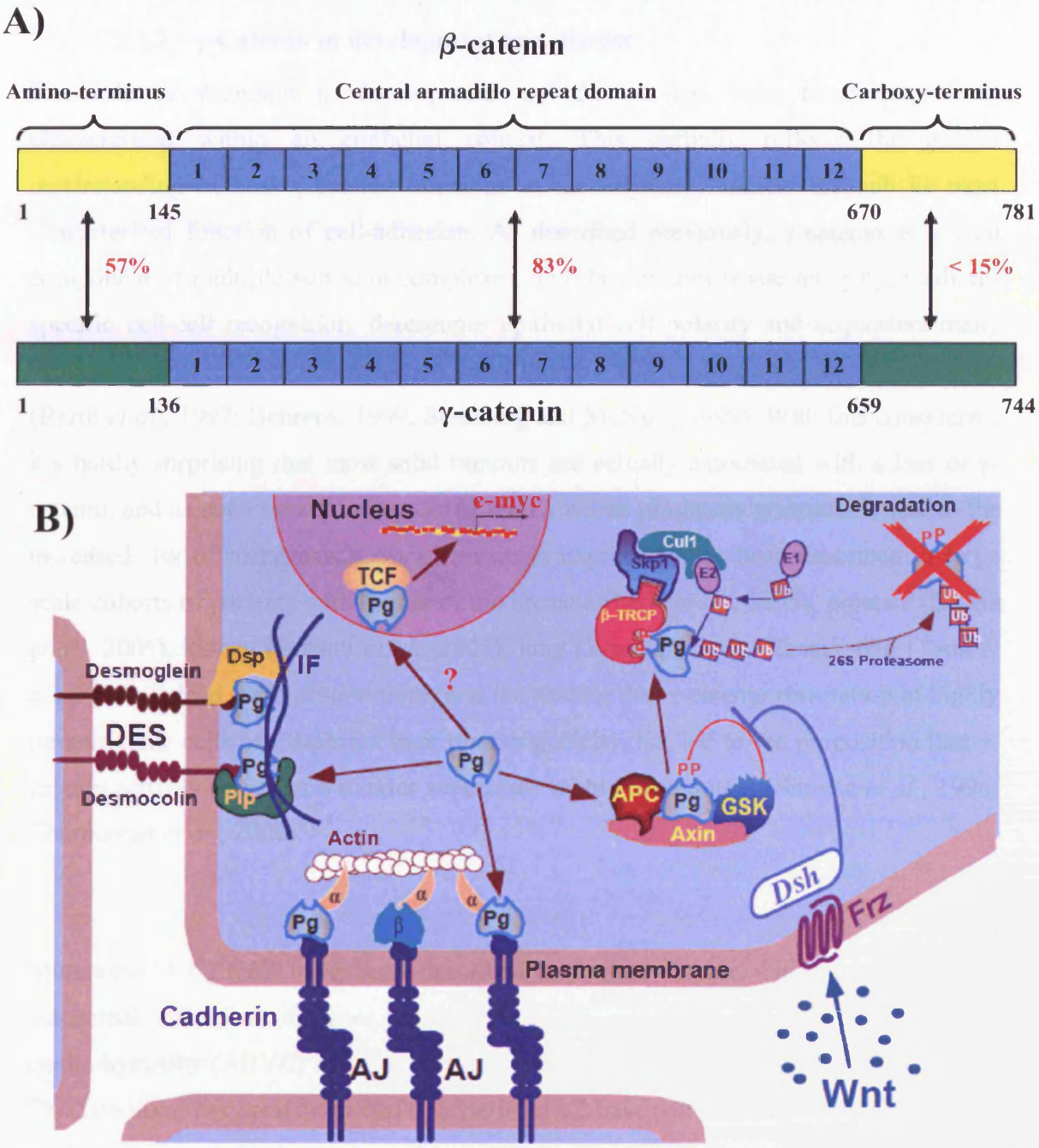


Figure 1.10 - The structural and functional homology between γ- and β-catenin.

A) γ-Catenin shares close molecular homology (red numbers) with β-catenin, with most identity shared between central armadillo domains and the least between the COOH-termini. Adapted from Solanas *et al.*, 2004. **B)** γ-Catenin (Pg) shares close functional homology with β-catenin (β). Both proteins are located in adherens junctions (AJ) where they mediate cadherin anchoring to the actin cytoskeleton via α-catenin (α). γ-Catenin (and β-catenin) can also translocate to the nucleus through an unknown (?) mechanism where it can interact with TCF/LEF complexes to activate Wnt target genes such as *c-myc*. Like β-catenin, γ-catenin is also subject to the same mechanism of proteasomal degradation as β-catenin. Additionally, a unique role for γ-catenin is observed in desmosomes (DES) where it anchors desmosomal cadherins to intermediate filaments (IF) via desmoplakin (Dsp) and plakophilin (Plp). Adapted from Zhurinsky *et al.*, 2000b.

1.3.7 γ-Catenin in development and disease

The role of γ-catenin in development and disease has been more extensively characterised within an epithelial context. This partially reflects the greater understanding of how γ-catenin functions in these tissues, namely through its most characterised function of cell-adhesion. As described previously, γ-catenin is a vital component of multiple adhesion complexes, and thus defines tissue integrity, mediates specific cell-cell recognition, determines epithelial cell polarity and sequesters many signalling molecules to cell adhesion sites, thereby also regulating signal transduction (Barth *et al.*, 1997; Behrens, 1999; Steinberg and McNutt, 1999). With this considered, it's hardly surprising that most solid tumours are actually associated with a loss of γ-catenin, and as such are often associated with a worse prognosis presumably due to the increased risk of metastasis. Loss of γ-catenin expression has been described in large scale cohorts of patients with cancer of the breast (Aberle *et al.*, 1995), prostate (Shiina *et al.*, 2005), kidney (Breault *et al.*, 2005), lung (Winn *et al.*, 2002) and skin (Tada *et al.*, 2000). Indeed these observations, and the finding that γ-catenin restoration in highly tumourigenic cells can suppress their tumourigenicity, has led to the proposition that γ-catenin actually serves as a tumour suppressor within this context (Simcha *et al.*, 1996; Charpentier *et al.*, 2000).

Mutations of *CTTNG* have been described in human disease. Naxos disease is an autosomal recessive disorder characterised by arrhythmogenic right ventricular cardiomyopathy (ARVC) and abnormalities of hair and skin (Protonotarios *et al.*, 1986). This condition has been described to arise from a 2 base pair deletion of *CTTNG*, which renders the γ-catenin protein unstable and thus disturbs the integrity of cardiac tissue (McKoy *et al.*, 2000). A slightly different dominant mutation of *CTNNG* has been identified in familial ARVC. This mutation inserts an extra Ser residue at position 39 in the NH_2-terminus of γ-catenin resulting in the ubiquitination of the protein and its preferential translocation from membrane to cytoplasm (Asimaki *et al.*, 2007). This ultimately reduces the frequency of desmosomes within cardiac tissue leading to its overall weakening. Interestingly, only one mutation of γ-catenin has ever been identified in human cancer (Caca *et al.*, 1999). This was a missense mutation of Ser 28 found in gastric cancer, depriving the molecule of a potential phosphorylation site. Lack of phosphorylation can protect the molecule from degradation and this study further

identified increased TCF transcription when this mutation was modelled *in vitro*. Similar mutations have been described for the β-catenin gene (*CTTNB*) in human epithelial cancers, albeit with much higher frequency (reviewed by Polakis, 2000). Missense mutations or in-frame deletions of the NH_2-terminus deprive the molecule of its GSK3-β consensus site and render it resistant to degradation. These types of mutation have been shown to constitutively activate LEF/TCF-dependent transcription in cancer of the colon (Korinek *et al.*, 1997; Morin *et al.*, 1997), skin (Rubinfeld *et al.*, 1997), uterus (Fukuchi *et al.*, 1998), liver (Miyoshi *et al.*, 1998), and brain (Zurawel *et al.*, 1998).

No literature exists regarding the function, or even expression, of γ-catenin in normal human haematopoietic cells. An *in vivo* study of murine haematopoiesis using Western blotting showed γ-catenin (and β-catenin) was ubiquitously expressed across all murine haematopoietic tissues analysed, including bone marrow, thymocytes and splenocytes (Koch *et al.*, 2008). However this study, and others, have shown that the deletion of γ-catenin (and/or β-catenin) in HSC/HPC did not effect the long term reconstitution ability of these cells *in vivo* (Cobas *et al.*, 2004; Jeannet *et al.*, 2008). Although these studies would initially suggest a redundant role for catenins in haematopoiesis, a number of issues must first be considered (discussed in more detail in *5.5.1*). Briefly, these studies were tailored to assess a small aspect of haematopoiesis; namely the repopulating capacity of γ/β-catenin KO HSC/HPC in mice *only*. Consequently, the endpoints focus on absolute numbers of cell lineages, rather than the intricate morphological, functional and immunophenotypic development of specific haematopoietic lineages. The lack of knowledge regarding γ-catenin expression and function in normal human haematopoiesis will continue to hinder any understanding of a pathological role in haematological malignancy.

In AML, and indeed all haematological malignancy, only a few studies exist examining the pathological role of γ-catenin (mentioned previously in section *1.3.3*). On a functional level Müller-Tidow *et al*, showed that overexpression of γ-catenin in murine HPC and myeloid 32D cells promoted their growth and proliferation (Muller-Tidow *et al.*, 2004). This phenotype was however relatively weak and was only demonstrated in

murine cells. Injection of the γ-catenin-induced 32D cells into irradiated mice accelerated the development of leukaemia *in vivo*, albeit with long latency (~60days) considering this was a cell line. A study by Zheng *et al* showed similar findings whereby γ-catenin induction accelerated the cell cycle progression of murine HSCs and preserved an immature phenotype (Zheng *et al.*, 2004). These cells were also capable of accelerating myeloid leukaemia development when transplanted into irradiated mice. However, these phenotypes were again relatively weak with only 2 out of 4 mice developing leukaemia after very long latency periods of 9 and 12 months. Unpublished data from our laboratory also indicates a very modest drive of self-renewal in γ-catenin transduced human $CD34^+$ primary cells. However this phenotype was considerably weaker than the self-renewal driven by the RUNX-1/ETO fusion protein (Tonks, unpublished data). It should be noted that none of aforementioned studies assessed the co-expression of β-catenin level. Previous evidence has shown γ-catenin induction to be capable of co-stabilising β-catenin and thus the self-renewal phenotypes observed may simply arise from indirect stabilisation of β-catenin. Indeed, such a phenotype has commonly been associated with β-catenin in these cell types (see *1.3.2*). Finally *γ-catenin* expression has been found elevated in blast crisis CML patients, which further correlated with *survivin* expression implying a transcriptional role in CML (Kim *et al.*, 2011). However, this correlation was only identified at the mRNA level and cannot be assumed to reflect the protein level given the post-translational modifications active in γ-catenin regulation (see *4.5.1*).

Collectively, these findings justify further investigation into the potential pathological roles and relationship, of γ- and β-catenin, in AML.

1.4 Aims of the study

The main objective of this study is to gain an understanding of the role of γ-catenin in normal haematopoiesis and in AML. This will be achieved through the following aims:-

1) **To determine the expression and cellular localisation of γ-and β- catenin during normal human haematopoiesis.**

Any pathological role for γ-catenin in AML cannot be established without first understanding its role in normal haematopoiesis. Analysis of γ-catenin expression level in different human haematopoietic developmental subsets will allow identification of stages of normal haematopoiesis where γ-catenin potentially has a role. These studies will be supported by subcellular localisation assays which will provide correlative evidence of function within these cell lineages. A comparison with the close homologue β-catenin will be necessary to identify whether there is the possibility for interaction/interdependence of function between these catenins

2) **To determine if γ-catenin expression is dysregulated in AML.**

Analysis of γ-catenin protein level and localisation in primary AML patient blasts will allow the identification of any aberrant expression, when compared to normal primary haematopoietic cells. As for aim 1, comparison with β-catenin will be made to assess whether interplay between these catenins is altered in the malignant context compared with that established in normal haematopoietic cells.

3) **To determine the functional significance of γ-catenin dysregulation on haematopoeitic development.**

The role of γ-catenin in normal haematopoiesis and AML will be investigated through genetic manipulation (overexpression and silencing) of γ-catenin using primary human progenitor cells and myeloid leukaemia cell lines to respectively model the consequences of change in γ-catenin expression level in normal and malignant haematopoiesis.

2 - General Materials and Methods

2.1 General chemicals and reagents

Unless otherwise stated all chemicals and reagents were obtained from Sigma-Aldrich (Dorset, UK). Isopropanol, ethanol (EtOH), methanol (MeOH), tris(hydroxymethyl)aminomethane (Tris), sodium chloride (NaCl), sucrose, potassium hydroxide (KOH), magnesium acetate, ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), sodium orthovanadate (NaV), triton detergent (TX-100) and sodium azide (NaAz) were obtained from Fisher Scientific (Loughborough, UK). Hanks balanced salt solution (HBSS), sodium bicarbonate ($NaHCO_3$) and phosphate buffered saline (PBS) were purchased from Invitrogen (Paisley, UK). Foetal bovine serum (FBS) and horse serum were obtained from Biosera (Sussex, UK). Human Transferrin was supplied by Roche Diagnostics (Burgess Hill, UK).

Gentamicin (Amdipharm, Essex, UK), heparin (Wockhardt, Wrexham, UK) and sterile water (Fresenius Kabi Ltd, Cheshire, UK) were supplied from pharmacy, University Hospital of Wales, Cardiff, UK.

2.2 Tissue and cell culture

2.2.1 General cell culture conditions

All tissue culture work was performed in a Microflow Class II biological safety cabinet (Bioquell, Andover, UK), where all surfaces had been pre-sterilised with 70% (*v/v*) EtOH. Cultures were incubated in a Hera Cell humidified incubator (DJB Labcare) at 37° Celsius (C) supplied with 5% carbon dioxide (CO_2) in air.

Dulbecco's Modified Eagle's Medium (DMEM), Iscove's Modified Dulbecco's Medium (IMDM), Roswell Park Memorial Institute - 1640 (RPMI-1640) were obtained from Sigma-Aldrich. L-Glutamine, human interleukin-3 (IL-3), human interleukin-6 (IL-6), granulocyte macrophage-colony stimulating factor (GM-CSF) and FMS-like tyrosine kinase 3 (FLT-3) were supplied by Invitrogen. Granulocyte-colony stimulating factor (G-CSF) and stem cell factor (SCF) were supplied by Amgen (Cambridge, UK).

All plasticware including tips, serological pipettes (Greiner Bio-One, Stonehouse, UK), culture flasks (Nunc, Rochester, USA), multi-well culture dishes, Falcon tubes (Scientific Laboratory Supplies, Nottingham, UK) and universal containers (UC), Sterilin, Caerphilly, UK) were purchased in a pre-sterilised condition. All contaminated waste was soaked in Precept Mini Haz-Tabs (Guest Medical, Kent, UK) for 24 hours prior to disposal, or disregarded into sharps bins or autoclavable waste bins if appropriate. If the culture work involved contact with retrovirus, then the strength of the Precept was doubled accordingly.

2.2.2 Culture of transformed cell lines

The commonly used cell lines and their culture requirements are summarised in *Table 2.1* (Drexler, 2001). Unless otherwise stated, all cell lines were maintained at a density of $1x10^5$-2 $x10^6$/ml by serial passage every 48 hours.

Table 2.1 - Commonly used cell lines and their specific growth requirements.

Cell line	Cell type	Source	Culture medium	Doubling time (hours)
K562	Human Chronic Myeloid Leukaemia	European Collection of Cell Cultures (ECACC) (Ref. 89121407)	RPMI-1640, 10% (*v/v*) FBS, 2mM (*v/v*) L-Glutamine, 20μg/ml (*v/v*) Gentamicin	24-30
THP-1	Human Monocytic Leukaemia	ECACC (Ref. 88081201)	RPMI-1640, 10% (*v/v*) FBS, 2mM (*v/v*) L-Glutamine, 20μg/ml (*v/v*) Gentamicin	34-40
HEL	Human Erythroleukaemia	ECACC (Ref. 92111706)	RPMI-1640, 10% (*v/v*) FBS, 2mM (*v/v*) L-Glutamine, 20μg/ml (*v/v*) Gentamicin	24-36
U937	Human histiocytic lymphoma	American Type Culture Collection (ATCC) (Ref. CRL-1593.2)	RPMI-1640, 10% (*v/v*) FBS, 2mM (*v/v*) L-Glutamine, 20μg/ml (*v/v*) Gentamicin	24-30
FDCP mix	Murine haematopoietic progenitor cell line	Prof. Tony Whetton, University of Manchester, UK.	IMDM, 20% (*v/v*) horse serum, 10% (*v/v*) WEHI conditioned medium (IL-3 source), 4mM (*v/v*) L-Glutamine, 20μg/ml (*v/v*) Gentamicin.	24-30
Phoenix - Ampho	Murine Retrovirus producing cell line	Prof. Gary Nolan, Stanford University, California, USA.	DMEM, 10% FBS (*v/v*), 4mM (*v/v*) L-Glutamine, 20μg/ml (*v/v*) Gentamicin.	24-36

2.2.3 Primary material

Neonatal cord blood (CB) was obtained from healthy full-term pregnancies at the Maternity Unit of University Hospital of Wales (Cardiff) following informed consent. Samples were taken into a 50ml falcon tube containing 500 international units (IU) (*v/v*) of bacteriocide-free heparin to prevent blood clot formation. Normal bone marrow (BM) was obtained from the Welsh Bone Marrow Donor Registry, Spire Private Hospital

(Cardiff) following informed consent. Bone Marrow was sampled into EDTA coated Vacutainer® tubes (Becton Dickinson, Oxford, UK).

2.2.4 Estimation of cell number

Cell enumeration was performed using an improved Neubauer haemocytometer counting chamber (Hawksley, Sussex, UK), and an Eclipse TS100 light transmission microscope (Nikon, Surrey, UK). An 8μl aliquot of cells to be counted were removed from culture aseptically and pipetted directly under the cover slip on the counting chamber. For enumeration of primary cell material, (CB or BM cells), ZAP-OGLOBIN™ Lytic Reagent (Beckman Coulter, Buckinghamshire, UK) was added to the cell aliquot (2μl per 200μl) to lyse contaminating erythrocytes. Cellularity per ml was ascertained by counting the cells residing in each of the four corners of the counting square (which has a known volume of 0.9μl) and multiplying the average by $1x10^4$.

2.2.5 Cryopreservation and thawing of cell lines and primary cells

For freezing, $1x10^6$-10^8 cells were collected by centrifugation at 200 x *g* for 5 minutes and resuspended in the relevant growth medium (see section *2.2.2*) before being added drop-wise to an equal volume of freezing medium (50% IMDM, 30% FBS (*v/v*), 20% (*v/v*) dimethylsulphoxide (DMSO)). Cells were aliquoted into 1.8ml cryopreservation vials (Nunc) and immediately placed in a controlled refrigeration container (using 100% isopropanol as the thermal interface). After 24 hours at -80°C the tubes were transferred to cryovats in liquid nitrogen (LN_2) for long-term storage.

Cryopreserved cells were recovered from LN_2 by rapidly thawing in a 37°C water bath in the presence of 900μl 0.45μm-filtered FBS and 200μg sterile DNase I. Freezing medium was slowly diluted and osmotic balance restored by the drop-wise addition of an equal volume of magnetic-activated cell sorting (MACS) buffer (1 x PBS, 0.5% (*v/v*) bovine serum albumin (BSA), 5mM (*v/v*) $MgCl_2$) over 3 minutes. This dilution by dropwise doubling of volume was repeated twice more followed by centrifugation at 200 x *g* for 10 minutes. Cells were resuspended in the relevant growth medium (see

section *2.2.2*) and seeded in tissue culture flasks for 24 hour recovery at 37°C gassed with 5% (*v/v*) CO_2 in air.

2.3 Subcloning of Plasmid DNA

2.3.1 General subcloning

All restriction endonucleases, reaction buffers, BSA and DNA ladders were supplied by New England Biolabs (Massachusetts, USA). Molecular grade water was obtained from Roche. QIAquick™ Gel Extraction, QIAprep® Mini and Maxi-prep kits were obtained from Qiagen (Sussex, UK) and used according to manufacturer's instructions (see *Appendices*). All DNA was quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Delaware, USA) as per manufacturer's guidelines and stored at -20°C.

2.3.2 Assessment of restriction digested DNA by agarose gel electrophoresis

To analyse DNA fragments generated from restriction digests, a 0.8% agarose gel was prepared in Tris-Borate-EDTA (TBE) buffer with 0.5μg/ml (*w/v*) Ethidium Bromide (EtBr, Promega, Hampshire, UK). DNA was prepared for electrophoresis by resuspending up to 250ng of restriction digested DNA in 10μl of deionised water (dH_20), and making up to a 12μl volume using loading buffer (0.25% Bromophenol Blue, 0.25% Xylene Cyanol, 30% Glycerol). A 1 kilobase (kb) DNA ladder was prepared in a similar fashion by ensuring a 1:10 dilution. The prepared samples were loaded onto the wells of the gel (submerged in TBE buffer containing 0.5μg/ml EtBr) and electrophoresed at 80V for 45-60 minutes followed by digital image acquisition using the LAS-3000 digital scanner. A raw FujiFilm data file was acquired after the gel was exposed to the digital sensor for 0.03-0.5 seconds.

2.3.3 Composition of medium for growing *E.coli* cells

Luria Bertani (LB) medium, required for the culture of competent *E.coli* cells, was made up to 1 litre from 950ml deionised water, 10g bacto-yeast extract, 5g tryptone peptone (BD) and 10g sodium chloride. To ensure sterility, the medium was autoclaved immediately using standard laboratory procedure. LB agar was made with the addition of 15g Bacto-agar (BD) for every litre of LB medium. The LB agar was heated to 100°C before adding ampicillin (University Hospital of Wales Pharmacy) to the concentration of 50μg/ml. To pour a plate, 30ml of ampicillin supplemented agar medium was used per 90mm Petri dish plate. The plates were cooled (avoiding condensation and dust to ensure sterility) and used immediately.

2.3.4 Transformation of competent *E.coli* cells with plasmid DNA

In order to identify, isolate and amplify plasmid DNA of interest transformation of bacteria was necessary. As well as containing essential coding elements for gene expression in mammalian cells, retroviral plasmids also contain the necessary components for plasmid replication in prokaryotic (bacterial) cells. One such vital component is that which encodes ampicillin resistance, allowing the identification of bacteria transformed with, and expressing, the vector of interest. Ampicillin resistance is encoded by the *bla* gene, which enables the bacterium to translate a protein (β-lactamase) capable of hydrolysing the β-lactam ring structure (critical for ampicillin stability) rendering the antibiotic ineffective and the bacteria resistant.

Each DNA to be amplified by transformation was thawed on ice alongside one 50μl vial of One Shot® TOP10 Chemically Competent *E.coli* (Invitrogen). For each DNA sample 6μl was aseptically transferred directly into the respective vial of competent cells and tapped gently to mix, followed by 30 minutes incubation on ice. Following this, the cells were placed at 42°C for 30 seconds, without agitation, before replacing on ice and adding 250μl of pre-warmed S.O.C medium (Invitrogen) to each vial to maximise transformation efficiency (Hanahan, 1983). All vials were secured in a shaking incubator at 37°C for exactly 1 hour at 225 revolutions per minute (rpm). Once the incubation had elapsed, potentially transformed bacteria were spread onto pre-warmed

ampicillin-containing LB agar plates using plastic spreaders to generate a film of bacterial growth, and left at 37°C overnight. The process of identifying bacterial colonies harbouring the DNA plasmid of interest, and the expansion of these relevant colones, is described in sections *3.3.1.6-3.3.1.9*.

2.4 Purification of human CD34$^+$ cells

2.4.1 Isolation of mononuclear cells from whole human cord blood or bone marrow

Whole human CB/BM was diluted 1:1 with HBSS containing 25mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 20μg/ml gentamicin and 10IU/ml bacteriocide-free heparin. This preparation was then layered over Ficoll-Paque™ (GE Healthcare, Hemel Hempstead, UK) in 50ml falcon tubes at a ratio of 8:5 (Blood:Ficoll) before centrifuging at 400 x *g* for 40 minutes with slow acceleration and the brake disengaged. Following centrifugation the mononuclear cells formed a visible interface between plasma and Ficoll, and were subsequently aspirated into a UC containing 15ml of wash medium (RPMI-1640, 5% FBS, 20μg/ml Gentamicin, 10IU/ml). The cells were centrifuged at 200 x *g* for 10 minutes and further washed in the same way until the supernatant was free of platelet contamination as determined by visual inspection of the supernatant clarity. Mononuclear cell number was estimated as described in section *2.2.4* before resuspending in RPMI-1640 with 10% FBS (*v/v*), aliquoting at 5x10^7 mononuclear cells/vial and cryopreserving in LN_2 (section *2.2.5*).

2.4.2 Isolation of human CD34$^+$ haematopoeitic progenitor cells from isolated mononuclear cells

CD34$^+$ haematopoietic progenitor cells were purified (from previously isolated mononuclear cells, see section *2.4.1*) using a magnetic-activated cell separation (miniMACS™) kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, following recovery from LN_2 (section *2.2.5*), 1x10^8 mononuclear cells were resuspended in 150μl MACS buffer before incubation at 4°C for 15 minutes with 50μl hapten-conjugated monoclonal CD34 antibody (clone QBEND/10) per 10^8 cells, in the presence of FcR

blocking agent. Next, 5ml of MACS buffer was added to stop the reaction and the cells pelleted by centrifugation at 200 x *g* for 5 minutes. Washed cells were resuspended in MACS buffer and incubated at 4°C with the addition of 50μl of anti-hapten microbeads (per 10^8 cells) for a further 15 minutes. After repeating the previously described wash step, mononuclear cells were resuspended in MACS buffer and applied to a magnetised (MS) column. Once the flow through had ceased, the column was removed from the magnet and the magnetically-labelled $CD34^+$ cells were eluted with 1ml MACS buffer. This fraction was next passed through a second column to maximise purity. Once flow through had stopped, $CD34^+$ cells were eluted as before and cell number estimated (section *2.2.4*). For larger or smaller starting densities of mononuclear cells the relative volumes of the protocol were adjusted accordingly.

To assess the $CD34^+$ purity of the eluent, $1x10^4$ cells were resuspended in staining buffer (SB) (1 x PBS, 0.5% (*v/v*) BSA, 0.02% (*v/v*) NaAz) and combined with 2.5μg/ml R-PE-conjugated anti-human CD34 monoclonal antibody (Clone 8G12, BD). After 30 minutes at 4°C, cells were washed in 1ml of staining buffer and pelleted by centrifugation at 200 x *g* for 5 minutes. Cells were then analysed by flow cytometry as described in section *2.7*.

2.5 Retroviral transduction

2.5.1 Principles of retroviral transduction

Retroviral transduction represents a highly efficient system for transferring genes of interest into haematopoietic cells. This may be for the purpose of overexpressing or silencing (through shRNA mechanisms described further in section *5.3.1.3*) a particular protein within cells. Most transforming retroviruses used to transduce haematopoietic cells are derived from the Moloney Murine Leukaemia Virus (MMLV), except they are replication deficient; that is the coding regions of their *gag*, *pol* and *env* genes have been replaced with genes of interest (Morgenstern and Land, 1991). The absence of such critical genes means a packaging cell line such as Phoenix (see proceeding section *2.5.2*) is necessary to provide the full spectrum of retroviral proteins required for proper virus formation. Retroviral vectors are typically designed to express two exogenous

genes, one being the gene of interest and the other being a selectable marker such as drug resistance or green fluorescent protein (GFP). Once the infected cells have been 'selected' using the appropriate marker, one can be sure any resulting phenotype is almost exclusively due to the introduced gene. Genetically engineered retroviruses exploit many of the features that allow the wild type (wt) virus to efficiently infect host cells. The viral envelope recognises and interacts with the specific host cell surface receptors permitting cellular entry of the virion. The viral core, including the reverse transcriptase protein, is then essential for the conversion of single-stranded viral RNA (including the engineered gene of interest) into double-stranded DNA, which is randomly integrated into the host genome with the assistance of viral integrase (see Stanford university website[4]). This unique property allows stable, long-term expression (from powerful retroviral promoters) of desired genes within target cells and all their subsequent progeny.

2.5.2 Generation of Amphotropic retrovirus

Phoenix amphotropic packaging cell lines are adapted to yield high titre retroviral virions by providing the full complement of viral polypeptides necessary for RNA packaging including the gag (viral core), pol (enzymes required for integration of RNA into host genome) and env proteins (protein capsid). Phoenix cells were seeded at 6.8 x 10^6 cells per $80cm^2$ tissue culture flask using the conditions specified in *Table 2.1.* The following day, the medium was aspirated and replaced with 15ml of fresh medium, after which calcium-phosphate mediated transfection of plasmid DNA was performed. Firstly, 45μg of the relevant plasmid DNA was combined with 125mM (*v/v*) Calcium Chloride ($CaCl_2$) and made up to a total volume of 450μl in sterile water. This mixture was then added drop-wise to 450μl HEPES-buffered saline, under gentle 'bubbling' using a 1ml serological pipette. The mixture was then briefly vortexed and allowed to incubate undisturbed at room temperature (RT) for 20 minutes. When $CaCl_2$, DNA and a phosphate-containing buffer are combined at a neutral pH, a visible precipitate is generated consisting of calcium-phosphate-DNA complexes (Okayama H and Chen C, 1991). This sediment can then be actively incorporated from the surface of the cell by endocytosis. Approximately 5 minutes before completion of the incubation, 25μM

[4] http://www.stanford.edu/group/nolan/tutorials/tutorials.html

chloroquine was applied to all phoenix cultures to be transformed. Chloroquine promotes the uptake of the foreign DNA by neutralizing the pH within lysosomal vesicles which prevents DNases destroying plasmid DNA. Once the incubation had elapsed, the full 900μl of calcium-phosphate-DNA precipitate was pipetted gently onto the cells, gassed with CO_2, and returned to 37°C. The following morning, the medium was aspirated from every culture and discarded (to minimize the toxic side effects of chloroquine exposure) before replacing with 8ml of fresh medium, gassing with CO_2, and incubating at 33°C for optimal retrovirus production. On the final day, the retrovirus-containing medium was harvested by pipetting and centrifuged at 200 x *g* for 10 minutes. The retroviral-containing supernatant was then distributed into 1ml cryovials aliquots, snap frozen in LN_2, and stored at -80°C until further use. DNA transfections with the PINCO retroviral vector permitted a second retroviral harvest the following day given the presence of the Epstein Barr Virus (EBV) origin of replication and *EBV nuclear antigen 1* (EBNA-1) gene. These components allow the construct to be episomally replicated within mammalian cells which maintains retroviral titre (Grignani *et al.*, 1998).

2.5.3 Retroviral transduction procedure

The required number of wells of a sterile, untreated 24 multi-well plate were coated with 25μg of retronectin (Takara-Bio, Shiga, Japan) and incubated at RT for 2 hours. The retronectin was then aspirated and replaced with 250μl of PBS containing 1% BSA (*w/v*) for 30 minutes at RT. Meanwhile, the relevant viruses were prepared by rapidly thawing at 37°C, adding 320μg of polybrene, and incubating at 37°C for 20 minutes. Polybrene is a positively charged molecule that increases the efficiency of retroviral transduction by neutralising the negative charge of cell surfaces. This allows the viral glycoproteins to more efficiently bind their receptors on the target cell surface, by reducing the repulsion between sialic acid-containing molecules (Davis *et al.*, 2002). Once the incubation with 1% BSA (*w/v*) had elapsed it was removed from each well in turn and immediately replaced with 1ml of the relevant retrovirus, before centrifugation of the multi-well plate for 90 minutes at 3,200 x *g* in a double sealed carrier. High speed centrifugation is required to rapidly purify and concentrate the retroviral particles to the plate surface thus allowing the increased frequency of retrovirus-target cell interactions.

Incidentally, the use of polybrene also assists this process by providing a vehicle with which retroviral particles may sediment (Landazuri *et al.*, 2006).

During the centrifugation, cell lines or primary haematopoietic progenitor cells (isolated as described in section *2.4*) were prepared by counting (section *2.2.4*) and resuspending at a density of 1 x 10^5/ml in the relevant continuation cell culture medium (refer to section *2.2.2*). Upon completion of retroviral sedimentation, the supernatant was removed from all wells and replaced with 1ml of non-specific, serum-containing medium to wash off any residual polybrene. This medium was aspirated after 5 minutes and substituted with 1ml (i.e. 1 x 10^5) of the relevant cells, followed by an overnight incubation at 37°C in 5% (*v/v*) CO_2 in air. The following day, a second round of infection was carried out. The cells of each well were carefully harvested to a separate UC and maintained at 37°C in the same conditions, whilst the plate was centrifugally loaded with a second round of virus (as above). At the end of retroviral sedimentation, the cells were returned to the appropriate well for the second round of retroviral transduction overnight.

2.6 Determination of protein expression by Western blot

2.6.1 Generation of protein homogenate from whole cells

For extraction of protein from whole cells, approximately 1x10^6 cells were collected and washed twice in Tris-buffered Saline (TBS) (H_2O, 20mM Tris, 135mM NaCl) by centrifugation for 10 minutes at 200 x *g*. Following the removal of supernatant, cell pellets were snap-frozen by placing directly into LN_2 and stored at -80°C until required. Cell pellets were recovered from storage by thawing on ice, in the presence of 1mg/ml DNase I. Once thawed, 50μl of homogenisation buffer (0.25M sucrose, 10mM HEPES-KOH pH7.2, 1mM Magnesium acetate, 0.5mM EDTA, 0.5mM EGTA, 10mM beta-mercaptoethanol (BME)) containing one complete mini EDTA-free protease inhibitor tablet (Roche), 1mM activated sodium orthovanadate (pH10) and 1% TX-100 (*v/v*), was added per 1x10^6 cells and incubated on ice for 30 minutes with occasional vortexing to promote cell lysis. Following incubation, the cell homogenate was transferred to a pre-chilled eppendorf tube, before centrifuging at 16,000 x *g* for 5 minutes in a Biofuge (Heraeus) pre-cooled to 4°C to pellet insoluble cellular components. The supernatant,

containing the isolated cellular proteins, was transferred to a fresh eppendorf tube and immediately assessed for protein concentration.

2.6.2 Generation of fractionated cytosolic and nuclear homogenate

Either $2x10^6$ transformed cells or $1x10^7$ primary cells were fractionated using a nuclear/cytosol fractionation kit (Biovision, California, USA). Briefly, cells were first washed twice with 20ml washes of TBS followed by centrifugation at 200 x *g* for 10 minutes. Following washing, cells were fully resuspended in 200μl CEB-A (Cytosol extraction buffer) containing 1mM dithiothreitol (DTT) and 1 X protease inhibitor cocktail (PIC). This suspension was then transferred to a clean, pre-chilled eppendorf, vortexed for 15 seconds and returned to ice for 10 minutes. Once the incubation had elapsed, 11μl of CEB-B was added to the cells followed by a brief 5 second vortex before resuming incubation on ice for 1 further minute. Cells were again vortexed for 5 seconds before undergoing centrifugation at 16,000 x *g* for 8 minutes in a pre-chilled (4°C) microcentrifuge. The supernatant, representing the cytoplasmic extract, was carefully removed to a fresh mini-eppendorf tube and held on ice for the remainder of the protocol. The residual pellet (now containing mostly nuclei) was washed free of cytosolic contamination by carefully applying 500μl of pellet wash (1 x PBS, 5mM $MgCl_2$) and centrifuging as above for 3 minutes. After removal of supernatant, nuclear cell pellets were submerged in LN_2 to begin the process of nuclear destruction. Semi-lysed nuclear pellets were then rapidly thawed on ice in the presence of 1mg/ml DNase, before being resuspended in 100μl of NEB (Nuclear Extraction Buffer) also containing 1mM DTT and 1 X PIC. This suspension was then vortexed maximally for 15 seconds before being returned to ice, a process repeated every 10 minutes during a 40 minute incubation. After the final vortex, eppendorfs were centrifuged for 15 minutes at 16,000 x *g* to pellet insoluble material, before finally isolating the supernatant (nuclear extract) to a separate tube. The cytosolic and nuclear homogenate was then either immediately measured for protein concentration (see section *2.6.3* below) or stored at -80°C.

2.6.3 Quantification of protein in cell homogenate

Protein concentration of cell lysates was determined using the Bradford protein assay (Bradford, 1976). Firstly, protein calibration standards ranging from 0, 10, 40, 70 and 100µg/ml BSA were diluted in lysis buffer. 10μl of each protein standard, and 10μl of

each cell lysate was placed into the well of a 96-well microtitre plate (all samples were run in duplicate) and 190μl of Bradfords working solution (Bradford's stock 1:1 with ultra pure water) applied to the samples. The absorbances of the samples were read at 590nm using an ASYS Hitech Expert plus spectrophotometer (Biochrom, Cambridge, UK). For estimation of protein concentration see *Equation 2.1* below:

$$\textbf{Protein (μg/ml)} = \frac{A_{590} - c}{m}$$

Where;

A_{590} = the absorbance of the sample at 590nm
c = the value at which the standard curve intersects the y-axis
m = the gradient of the standard curve

Equation 2.1 - Linear equation for a straight line used to calculate the protein concentration within cell homogenate

2.6.4 Resolution of proteins by SDS-PAGE electrophoresis

Western blotting was completed using the NuPAGE® Pre-Cast Gel System (Invitrogen). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) involves the separation of proteins within a gel matrix under the influence of an electric field. Denaturisation and reduction of sample proteins is necessary to linearise proteins and dissociate polypeptides from each other. Protein lysates were removed from -80°C, thawed on ice, and then a sufficient volume of lysate was used so that all samples were normalised in terms of cell equivalents. In general, 50μl of cell homogenate was prepared with 50nM NuPAGE® sample reducing agent (DTT), 1 X NuPAGE® lithium dodecyl sulphate (LDS) sample buffer and made to volume with dH_20. Once well mixed, these samples were denatured by incubation at 70°C for 10 minutes, and then returned to ice.

Pre-cast NuPAGE® Novex 4-12% bis-Tris gels were rinsed with water, and the wells washed with 1 x NuPAGE® MOPS-sodium dodecyl sulphate (MOPS-SDS) running buffer, before being secured into an XCell *SureLock*™ Mini Cell electrophoresis tank. The inner chamber of the tank was filled with 200ml of running buffer containing 500μl

NuPAGE® anti-oxidant, and the reduced samples loaded onto lanes of the gel alongside a MagicMark™ XP Western Standard protein ladder (Invitrogen). The outer chamber of the tank was filled with running buffer, and electrophoresis was performed at 200V (400mA) over 50 minutes. Resolved proteins within the gel were next transferred onto a nitrocellulose membrane.

2.6.5 Transfer of separated proteins onto a nitrocellulose membrane

Following electrophoresis the gel was removed from the tank and released from its plastic holding. One surface of the gel was moistened with transfer buffer (dH_20, 10% MeOH, 1 X NuPAGE® Transfer Buffer, 1ml antioxidant) before layering a piece of pre-soaked Whatman 3M filter paper on the gel. Any air bubbles were removed by rolling out trapped air. The other gel side was also wetted with transfer buffer, before layering a pre-soaked nitrocellulose membrane (0.45 μm pore size) complete with an additional piece of filter paper on top of the gel surface ensuring all trapped air bubbles were removed. The gel/membrane was sandwiched between pre-soaked blotting pads as shown in

Figure 2.1 and inserted into an XCell II™ Blot Module. The blot module was filled to the top of the pads with transfer buffer and the outside of the tank filled with distilled water, before completing the transfer over 1 hour at 30V (400mA).

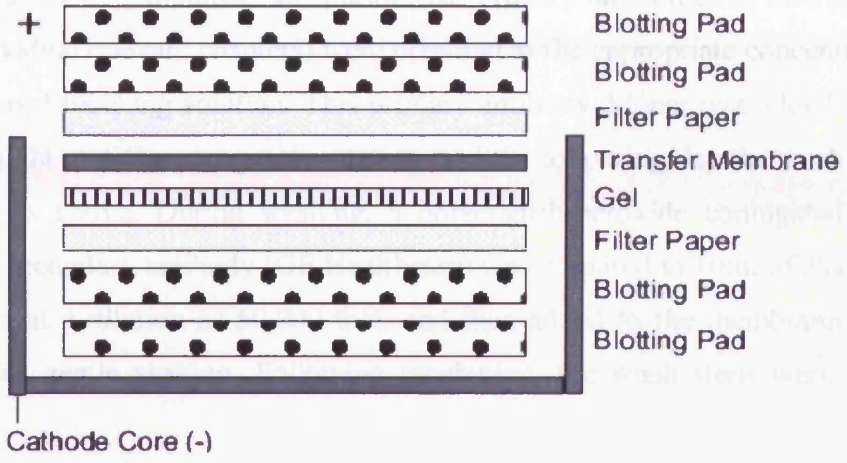


Figure 2.1 - Assembly of components during Western blot transfer.

The pre-soaked nitrocellulose membrane was placed immediately against the gel, and this arrangement sandwiched between two soaked pieces of filter paper with all trapped air bubbles removed. This assembly was then laid on top of 2 saturated blotting pads located closest to the cathode core within the blot module. A further 2 saturated blotting pads were aligned on top of the membrane sandwich, or enough so that adequate tension was placed on the assembly when then module was sealed together.

Upon completion of electroblotting the membrane sandwich was removed from the blot module and the nitrocellulose membrane rinsed twice with ultra-pure water, followed by two further water washes of 5 minutes on a rotary shaker. To check for efficient transfer of proteins, 30ml of Ponceau S solution was incubated with the membrane for 30 seconds with gentle agitation. Inaccurate loading and transfer errors could be noted at this point. A further two washes with water of 5 minutes were necessary to remove excess Ponceau S stain. The membrane was then ready blocking and detection stages.

2.6.6 Detection of transferred proteins by chemiluminescence

The visualization of transferred proteins present on the membrane was assisted using the Amersham ECL™ Advance Western Blotting Detection Kit (GE Healthcare). The

membrane was placed in 10ml of blocking solution (5% ECL advance blocking reagent in TBS supplemented with 1% (*v/v*) Tween20 (Invitrogen) (TBS-T)) on the shaker for 1 hour at RT. Following blocking, a fixed wash method of 15 minutes in TBS-T followed by three short washes of 5 minutes, was performed. Primary antibodies to the relevant antigen (see individual relevant chapters) were prepared to the appropriate concentration in 10ml of 5% (*w/v*) blocking solution. This primary antibody diluent was added to the membrane overnight at 4°C under gentle rotation, and the following day the wash steps were performed as above. During washing, a horseradish-peroxide conjugated anti-mouse or -rabbit secondary antibody (GE Healthcare) was prepared in 10ml of 2% (*w/v*) blocking solution at a dilution of 50,000-fold, and then added to the membrane for 1 hour at RT under gentle shaking. Following incubation, the wash steps were again performed.

Chemiluminescent detection was performed according to the manufacturer's instruction. Briefly, 'solution A' and 'solution B' were combined at RT in a 1:1 working solution (10ml per full size membrane 10 x 10 cm) and protected from the light. The membrane surface was blotted dry using filter paper and flooded with 4ml of the combined chemiluminescent substrate. The reaction developed over 5 minutes, after which the excess substrate was blotted away, and a piece of clean acetate placed over the membrane with all trapped air pockets smoothened out. A digital image of the chemiluminescent reaction was captured over a 1-30 minute exposure using a LAS-3000 digital scanner (FUJIFILM UK Ltd, Bedfordshire, UK).

2.6.7 Analysis of protein using densitometry

Captured images were analysed semi-quantitatively using post-acquisition software (Advanced Image Data Analyzer (AIDA) software version 4.26.038, Raytek Scientific, Sheffield, U.K)) and protein intensity measured using pixel-based densitometric analysis. This software utilises the intensity of pixels to quantitate the amount of protein present on the blot. Using the raw data files, a region of interest (ROI) was constructed around the desired band(s) with which a histogram of peak intensity was produced. From this histogram, a baseline of background (noise) pixel intensity was set which was

established from the surrounding area of the protein band within the ROI. Following the establishment of the background baseline, the area under the curve was calculated to give an arbitrary intensity value, from which estimates of fold-overexpression and under-expression of protein were calculated.

2.7 Flow cytometry

2.7.1 Principles of flow cytometry

Flow cytometry is capable of analysing multiple characteristics of a single cell through light scattering or fluorescence emission. This makes flow cytometry particularly useful for analysing heterogenous mixtures of cells such as blood within a short time-frame. The general configuration of a standard flow cytometer is illustrated in *Figure 2.2A*. Hydrodynamic focusing is pivotal to the technology, since this generates a laminar flow of single-cells in sheath fluid that passes through a flow cell allowing multi-parameter analysis of individual cells. Cells are typically exposed to a light source (usually a laser) at an interrogation point within the flow cell. The ability of a cell to scatter light when hit directly with a laser allows the measuring of physical properties such as size or internal complexity (see *Figure 2.2B* below). The ability of fluorescent dyes or compounds (section *2.7.2*) to emit light when excited by varying wavelengths of laser light provides a further parameter for measuring distinct biochemical features such as DNA content or antigen expression (Brown and Wittwer, 2000). Scattered or emitted light from analysed cells is collected by optical detectors and directed through a series of filters and dichroic mirrors, to isolate light of a particular wavelength. Photomultiplier tubes (PMT) are then responsible for converting the collected light from an analogue to a digital signal that is usually displayed as 2D plots or histograms on computer software.

2.7.2 Concept of fluorescence

A fluorescent molecule (or fluorophore) is any which becomes excited to a higher energy state upon light absorption and subsequently emits a higher wavelength of light upon returning to resting (ground) state (*Figure 2.3A*). The fluorophore only emits light of a given wavelength (or colour) when absorbing light of a particular optimal

wavelength for its initial excitation. This property has resulted in a large range of fluorophores (or fluorochromes) being utilised in flow cytometry (and other fluorescence technologies such as confocal microscopy) to provide additional parameters for detecting biochemical features of cells. Commonly, these fluorochromes are conjugated to an antibody targeted to a particular antigen of interest allowing its specific detection upon exposure to the correct light wavelength. Some frequently used fluorochromes include synthetic compounds such as fluorescein isothiocyanate (FITC), or naturally occurring fluorogenic molecules such as R-Phycoerythrin (R-PE) or allophycocyanin (APC). Alternatively, fluorescent dyes exist which only emit when bound in a particular confirmation such as TO-PRO-3 which only fluoresces when bound to nucleic acid. All such fluorochromes have distinct excitation and emission spectra (*Figure 2.3B*), which can be satisfied by the many configurations of lasers and filters present in the cytometer. A precise knowledge of the excitation/emission spectra of experimental fluorochromes is necessary so that; A) the correct laser line is employed for excitation, B) the correct filters are applied for collection of a specific wavelength of emitted light, and C) additional fluorochromes present in the sample sharing spectral overlap may be appropriately 'compensated' for.

The heterogeneity of flow cytometry experiments undertaken in this project means no-one single protocol is appropriate to cover all, so please consult individual chapters for detailed outlines of the flow cytometric methods and analyses performed.

2.8 Statistics

Significance of difference was tested using the Student's t-test. For additional specific statistical tests performed see individual chapters. GraphPad Prism version 5.01 (GraphPad Software, California, USA) was used for all analyses.

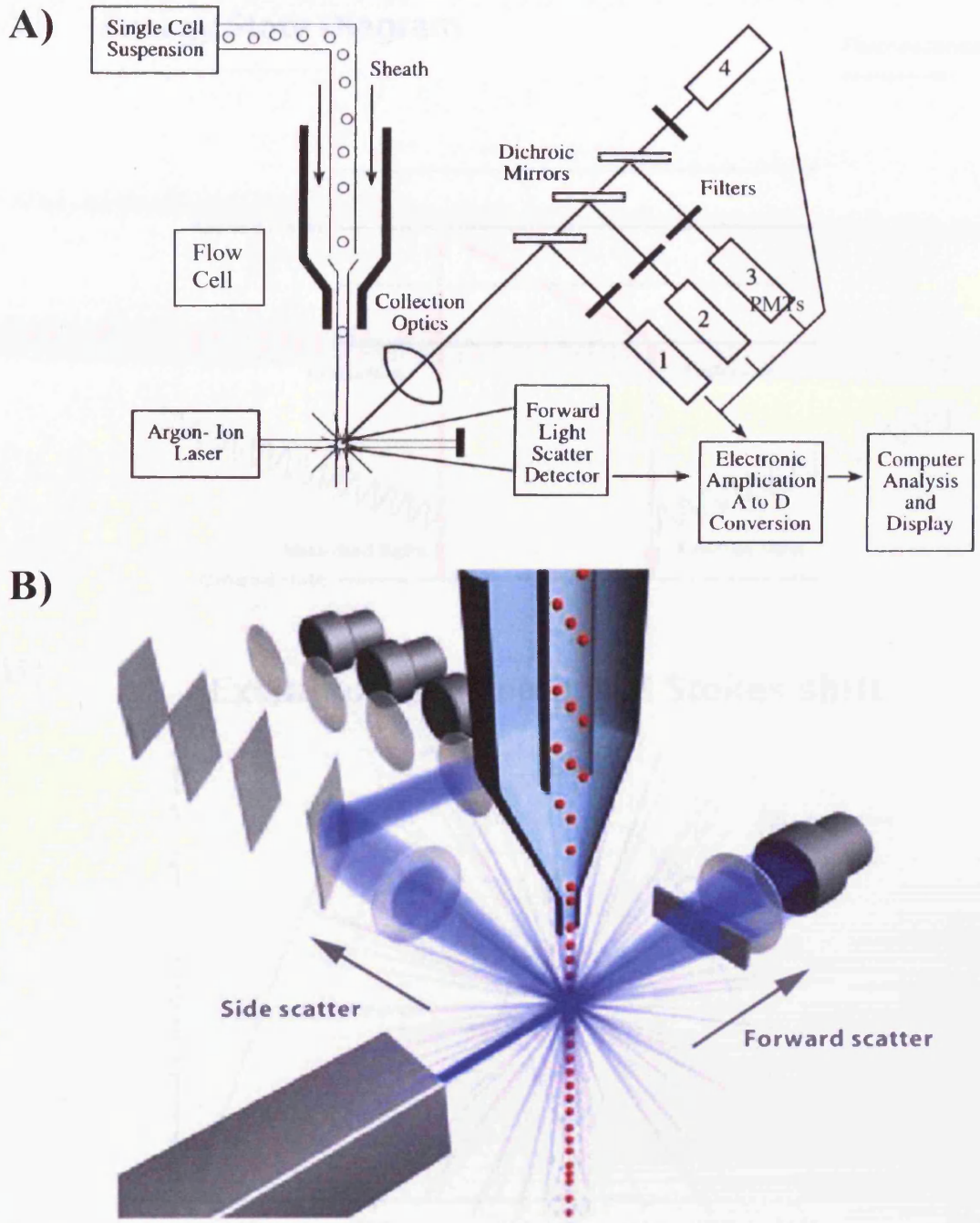


Figure 2.2 - The principles of flow cytometry.

A) The configuration of a standard benchtop flow cytometer. Cells are hydrodynamically focussed in sheath fluid to a single-cell suspension which are analysed individually at an interrogation point within the flow cell. Resulting scattered or emitted light is directed by optics and dichroic mirrors, through specific filters, onto PMTs (1-4). The collected analogue signal is digitised and displayed on a connected computer. Adapted from Brown and Wittwer, 2000. **B)** The measuring of physical properties of cells through the ability of individual cells to scatter direct laser light. The amount of forward scatter generated is proportional to the cell size, whilst the amount of side scatter is relative to the internal complexity of the cell (e.g. granularity). Adapted from http://probes.invitrogen.com/resources/education/tutorials/4Intro_Flow/player.html.

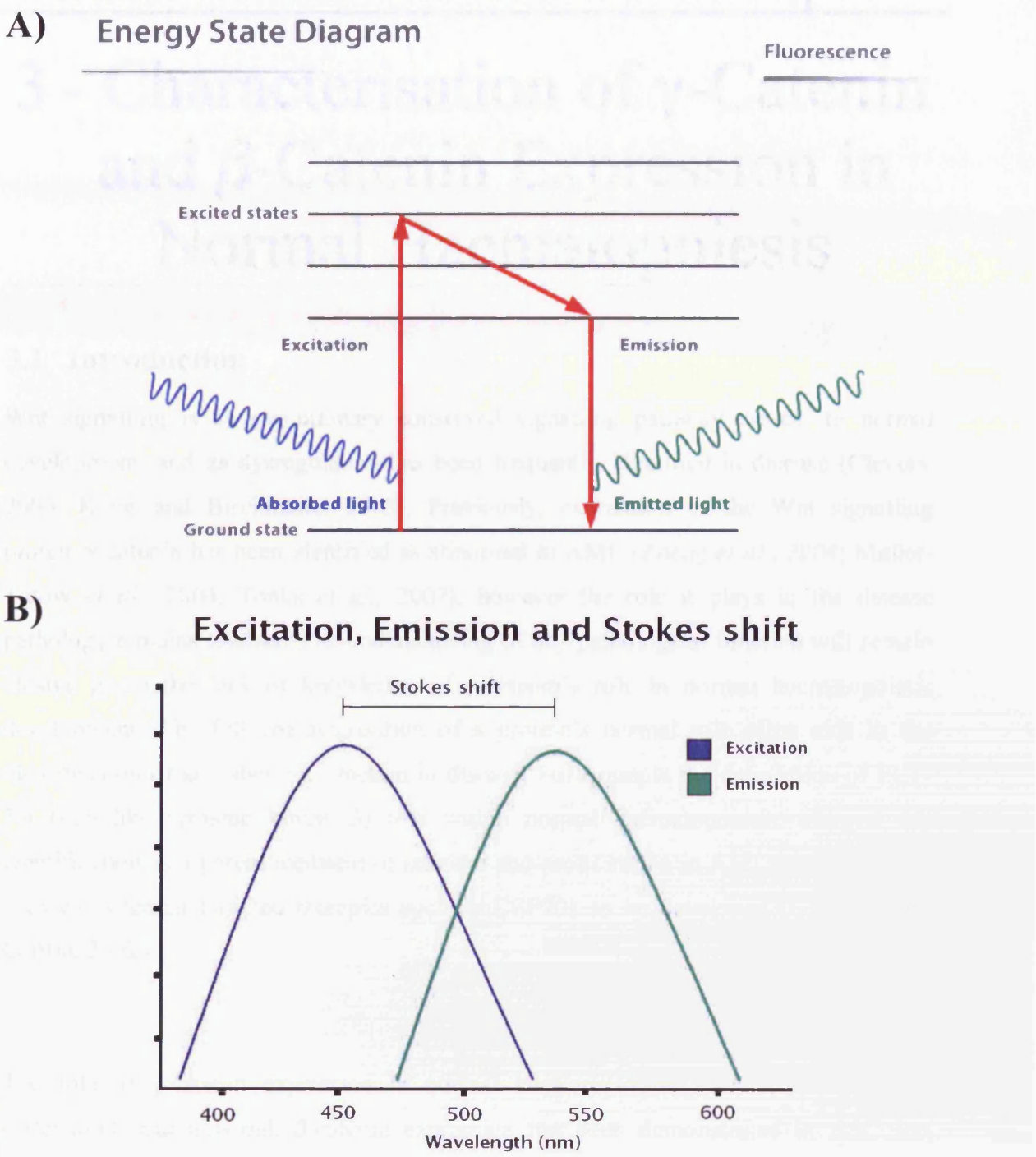


Figure 2.3 - The concept of fluorescence.

A) Schematic showing the definition of fluorescence. A fluorescent molecule becomes excited to a higher energy state upon absorption of a particular light wavelength and can consequently emit light of a higher wavelength when it returns to ground state. Adapted from http://probes.invitrogen.com/resources/education/tutorials/4Intro_Flow/player.html. **B)** Example of the excitation/emission spectra of a fluorophore. This particular molecule absorbs optimally at ~450nm and emits maximally at ~550nm. Stokes shift represents the difference in wavelength (nm) between the peak excitation and emission wavelengths. Adapted from http://www.advancedaquarist.com/2006/9/aafeature.

3 - Characterisation of γ-Catenin and β-Catenin Expression in Normal Haematopoiesis

3.1 Introduction

Wnt signalling is an evolutionary conserved signalling pathway critical to normal development, and its dysregulation has been frequently identified in disease (Clevers, 2006; Klaus and Birchmeier, 2008). Previously, expression of the Wnt signalling protein γ-catenin has been identified as abnormal in AML (Zheng *et al.*, 2004; Muller-Tidow *et al.*, 2004; Tonks *et al.*, 2007), however the role it plays in the disease pathology remains unclear. The understanding of any pathological function will remain elusive given the lack of knowledge of γ-catenin's role in normal haematopoietic development. The full characterisation of a protein's normal role often aids in the identification of any aberrant function in disease. For example the delineation of FLT-3's (Fms-like tyrosine kinase 3) role within normal haematopoiesis, allowed it's identification as a potent mediator of survival and proliferation in AML blasts, and has allowed potential targeted therapies such as CEP701 to be developed (Gilliland and Griffin, 2002).

The role of γ-catenin expression in normal haematopoiesis is currently not well understood, and although β-catenin expression has been demonstrated in HSC and progenitor cells (Reya *et al.*, 2003; Jamieson *et al.*, 2004; Simon *et al.*, 2005; Holmes *et al.*, 2008), it's involvement in latter stages of haematopoiesis remains largely unknown. Studies have been published attempting to decipher the roles of γ- and β-catenin in normal haematopoiesis; however, the variety of experimental models and techniques used has produced contrasting and contradictory results, as summarised recently (Staal and Luis, 2010). The fact that these catenins have dual roles in cell adhesion and transduction of Wnt signalling (see section *1.3.5*) suggests a multitude of potential functions within haematopoietic cells. This chapter sought to fully characterise γ- and β-

catenin expression in haematopoietic development. To this end, assays were developed that could accurately detect intra-cellular γ- and β-catenin expression in discrete developmental subsets of haematopoiesis at a single-cell level using flow cytometric and confocal microscopy. Such methods for γ-catenin measurement have not been previously reported and therefore required optimisation.

3.2 Aims

In order to establish a better understanding of the role of γ- and β-catenin in normal haematopoiesis the aims of this chapter were:

1) To optimise and validate a flow cytometric protocol capable of accurate and specific detection of intracellular γ- and β-catenin protein.

2) To use the above assay to determine the γ- and β-catenin expression level in discrete developmental subsets of human cord blood (CB) and bone marrow (BM).

3) To optimise and validate a confocal immunofluorescence protocol that could accurately and specifically identify the sub-cellular localisation of γ- and β-catenin protein in haematopoietic cells.

4) To use the above assay (point 3) to identify the sub-cellular localisation of γ- and β-catenin protein within specific haematopoietic lineages of human CB and BM.

3.3 Materials and Methods

3.3.1 Establishment of γ-catenin overexpression system

In order to establish the expression level and subcellular localisation of both γ- and β-catenin in developmental subsets of normal human haematopoiesis, a specific flow cytometric assay was optimised using K562 cells overexpressing both these catenins (*3.3.4*). A K562 cell line overexpressing β-catenin was already available (see *3.3.4.2*). For γ-catenin it was necessary to create an expression vector based on the retroviral vector pBabe-Puro (Morgenstern and Land, 1990) which gave stable expression of γ-catenin and conferred resistance to the antibiotic puromycin (the mechanism of which is described in detail in section *3.3.2*). The following section describes the creation of this vector.

3.3.1.1 *Excision of human γ-catenin cDNA from PINCO plasmid*

The PINCO retroviral vector (illustrated in *Figure 3.1*) was used as a source of human γ-catenin cDNA and was excised using a *Bam*H1/*Eco*R1 double restriction digest (*Figure 3.1B*). PINCO-γ-catenin DNA (10μg) was mixed with 1X EcoR1 buffer (recommended buffer for optimal enzymatic activity of both restriction endonucleases), 100U each of *Bam*H1 and *Eco*R1, 100μg/ml BSA, and the reaction volume made up to 100μl with molecular grade water. A control tube was also set up containing the above mixture minus the restriction endonucleases to ensure the PINCO-γ-catenin vector was free of contaminating nucleases. The reaction mixture was incubated at 37°C for 60 minutes.

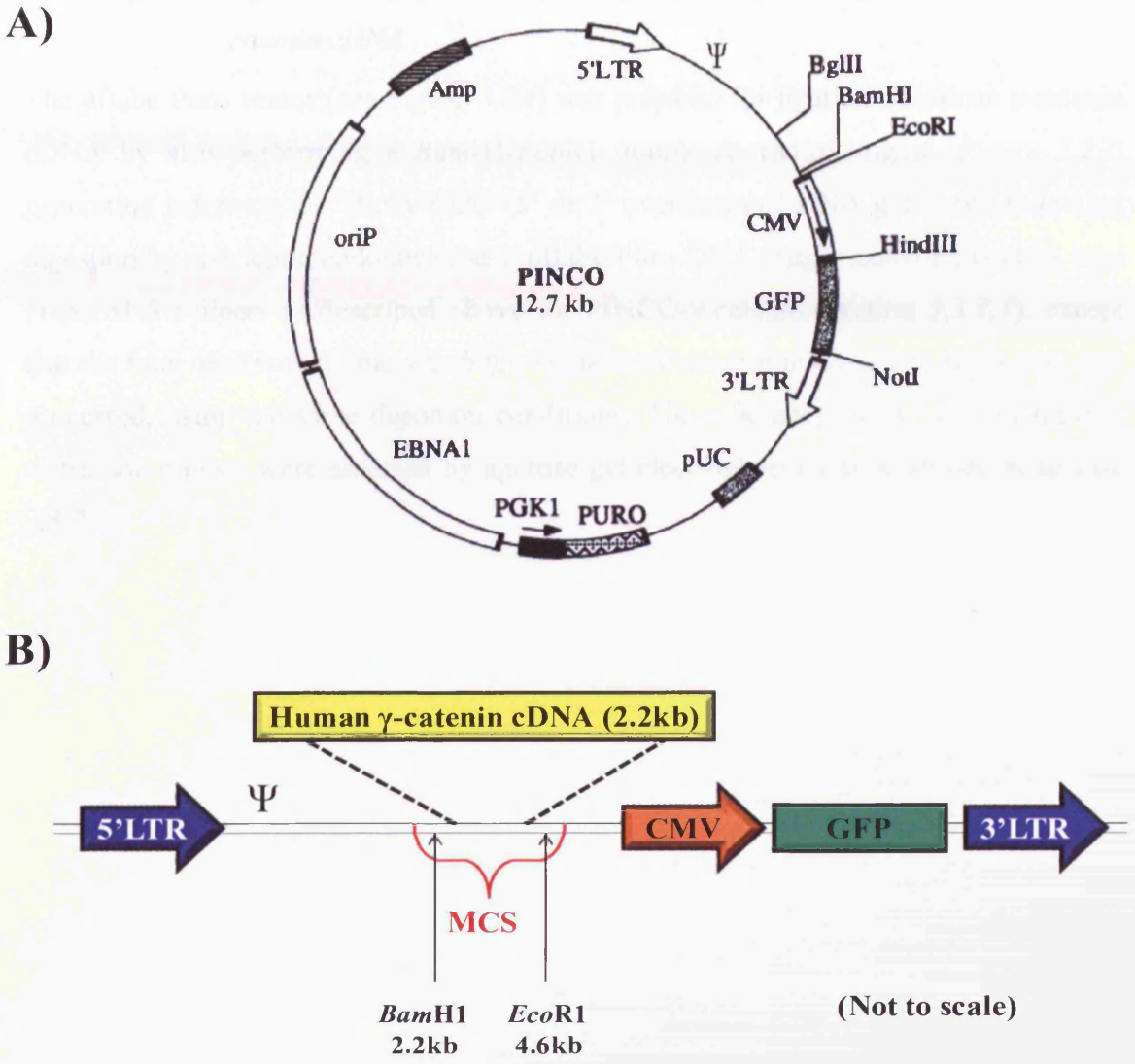


Figure 3.1 - An endonuclease enzyme restriction map of the PINCO vector from which γ-catenin was excised.

A) Key restriction enzyme recognition sites of the PINCO retroviral expression vector (adapted from Grignani *et al.*, 1998). **B)** Human γ-catenin cDNA was originally subcloned into the multiple cloning site (MCS) of the PINCO expression vector (Zheng *et al.*, 2004). The strategy used to excise γ-catenin cDNA from PINCO plasmid is illustrated. Indicated are the restriction enzymes used and the distance of the recognition sites from the start of the 5'LTR sequence expressed in kb. Amp: Ampicillin resistance gene; OriP: origin of replication; EBNA1= EBV nuclear antigen 1 gene which promotes episomal replication of DNA; PURO= Puromycin resistance gene driven by PGK1 promoter; pUC= prokaryotic control sequence; LTR= Moloney Murine Leukaemia Virus (MMLV) Long Terminal Repeats; Ψ= Retroviral RNA packaging signal sequence; CMV= Cytomegalovirus promoter; GFP= Enhanced Green Fluorescent Protein gene.

3.3.1.2 *Preparation of pBabe-Puro vector for ligation of excised human γ-catenin cDNA*

The pBabe-Puro vector (see *Figure 3.2A*) was prepared for ligation of human γ-catenin cDNA by also performing a *Bam*H1/*Eco*R1 double restriction digest (*Figure 3.2B*) generating heterologous 'sticky ends' (5' or 3' overhangs of DNA generated following digestion by restriction endonucleases). pBabe-Puro DNA (5μg), including control, was prepared for digest as described above for PINCO-γ-catenin (section *3.3.1.1*), except that the total reaction volume was 50μl. Undigested control pBabe-Puro DNA was also processed, using the same digestion conditions. The efficiency of the aforementioned restriction digests were assessed by agarose gel electrophoresis as described in section *2.3.2.*

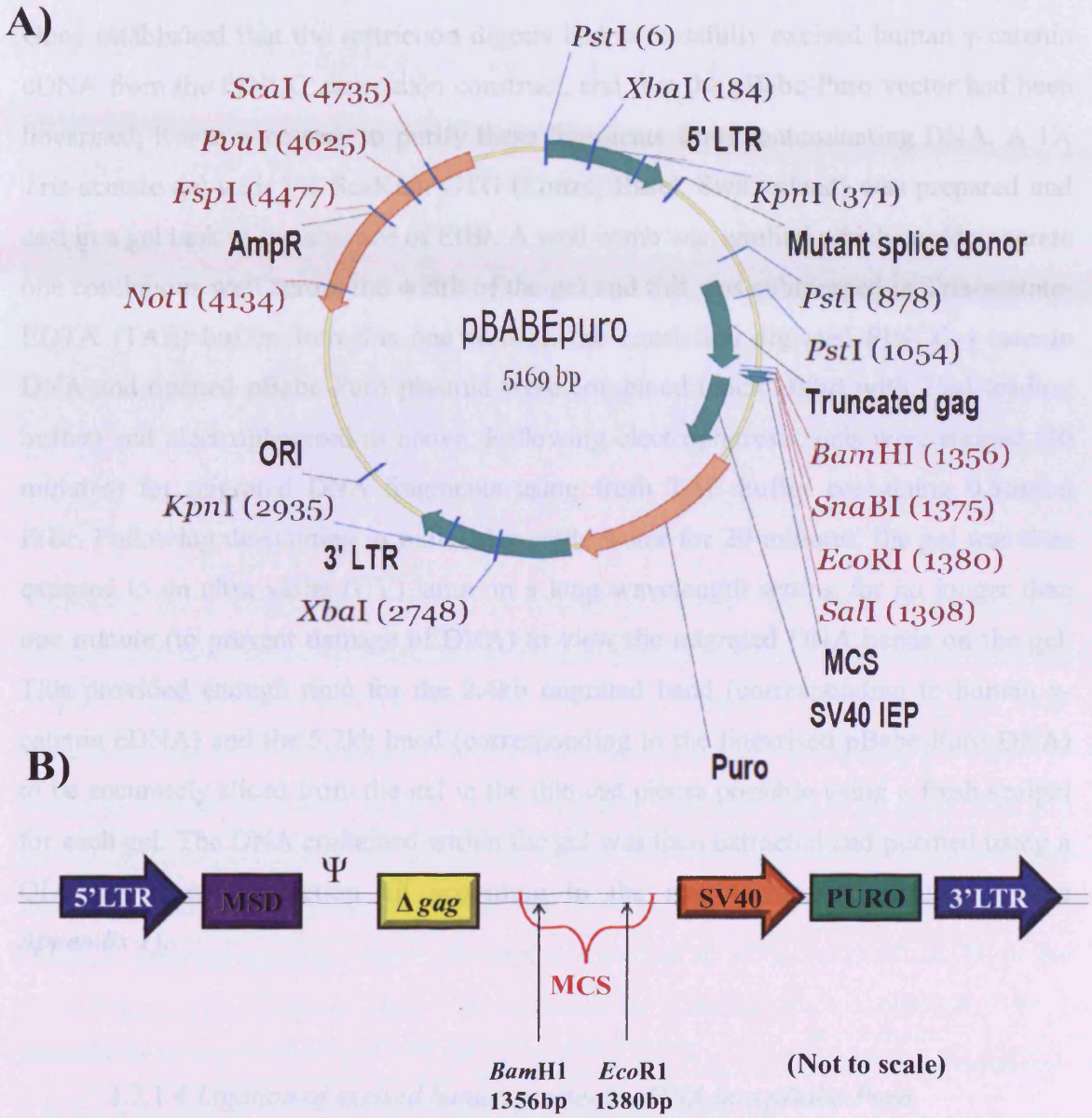


Figure 3.2 - An endonuclease enzyme restriction map of the pBabe-Puro retroviral vector into which γ-catenin was subcloned.

A) The enzyme restriction map shows key restriction enzyme recognition sites and essential coding sequences. Adapted from www.addgene.org. **B)** Illustration of the *Bam*H1 and *Eco*R1double restriction digest used to linearise the pBabe-Puro vector. The restriction enzyme recognition sites, within the MCS, are shown as a distance in base pairs (bp), from the start of the 5'LTR. Like PINCO, the LTR are based on that of MMLV. The retroviral RNA packaging signal sequence (Ψ) is flanked by a mutant splice donor (MSD) and a truncated *gag* (Δ *gag*) sequence to boost the retroviral titre. Transduced cells are selected by drug resistance encoded by the puromycin gene (PURO) driven by the Simian Virus 40 (SV40) promoter.

3.3.1.3 *Purification of γ-catenin and pBabe-Puro DNA*

Once established that the restriction digests had successfully excised human γ-catenin cDNA from the PINCO expression construct, and that the pBabe-Puro vector had been linearised, it was necessary to purify these fragments from contaminating DNA. A 1X Tris-acetate gel with 1% SeaKem GTG (Lonza, Basel, Switzerland) was prepared and cast in a gel tank in the absence of EtBr. A well comb was applied which could generate one continuous well across the width of the gel and this was submerged in Tris-acetate-EDTA (TAE) buffer. Into this one well all the restriction digested PINCO-γ-catenin DNA and opened pBabe-Puro plasmid were combined (each 100μl with 25μl loading buffer) and electrophoresed as above. Following electrophoresis, gels were stained (20 minutes) for migrated DNA fragments using fresh TAE buffer containing 0.5μg/ml EtBr. Following de-staining in molecular grade water for 20 minutes, the gel was then exposed to an ultra violet (UV) lamp on a long wavelength setting for no longer than one minute (to prevent damage of DNA) to view the migrated DNA bands on the gel. This provided enough time for the 2.4kb migrated band (corresponding to human γ-catenin cDNA) and the 5.2kb band (corresponding to the linearised pBabe-Puro DNA) to be accurately sliced from the gel in the thinnest pieces possible using a fresh scalpel for each gel. The DNA contained within the gel was then extracted and purified using a QIAquick™ gel extraction kit according to the manufacturer's instructions (see *Appendix 1*).

3.3.1.4 *Ligation of excised human γ-catenin cDNA into pBabe-Puro*

To maximize the efficiency of human γ-catenin cDNA (insert) ligation into the pBabe-Puro vector, an insert to vector molecular ratio of 10:1 was used. Given that the molecular weights of the γ-catenin and pBabe-Puro DNA were 2.35kb and 5.17kb respectively, a molecular ratio of 10:1 actually corresponds to a weight ratio of 4.5:1. Thus, for a 10μl ligation reaction, 113ng of γ-catenin DNA was combined with 25ng of pBabe-Puro DNA, 200 cohesive end units/μl T4 DNA ligase and 1X T4 DNA ligase reaction buffer (New England Biolabs). To estimate the level of background ligation (mainly due to self-ligation of the linearised pBabe-Puro plasmid) a 'background' reaction tube was also set up as above, but lacking γ-catenin DNA. A 'transformation control' was also used containing 25ng of undigested pBabe-Puro DNA. All reaction

volumes were incubated over 1 hour at 16°C then stored at -20°C until transformation of competent *E.coli.*

3.3.1.5 *Transformation of competent E.coli with ligated DNA*

Each vial of ligation reaction (3 in total as outlined in section *3.3.1.4*) was thawed on ice and used for bacterial transformation as described in *2.3.4* .Following transformation 75-225μl of competent cells were spread onto pre-warmed LB agar plates using plastic spreaders to generate a film of bacteria. Plates were incubated at 37°C overnight.

3.3.1.6 *Amplification of E.coli colony DNA*

Following an overnight incubation, 12 ampicillin-resistant colonies were randomly picked from the plate containing pBabe-Puro-γ-catenin transformants. Single, isolated bacterial colonies were isolated from the agar surface using a sterile loop, and each transferred into a UC containing 5ml of pre-warmed ampicillin-containing LB medium. These UCs were incubated at 37°C under vigorous agitation (approximately 300rpm) for 16 hours. At this point, an aliquot from each bacterial culture was taken for long-term freezer storage by preparing 800μl of confluent, transformed, ampicillin-resistant, bacterial culture into 200μl sterile glycerol and storing at -80°C in cryovials. From the remaining culture, bacterial cells were collected by centrifugation at 6,800 x *g* for 3 minutes in a microcentrifuge (Biofuge), and the DNA extracted using a QIAprep® Miniprep kit and the manufacturer's protocol (see *Appendix 2*).

3.3.1.7 *Identification of E.coli colony harbouring pBabe-Puro-γ-catenin DNA*

To identify colonies transformed with pBabe-Puro-γ-catenin, *Bam*H1/*Eco*R1 double restriction digests were performed, as described previously (section *3.3.1.1*), on all DNA isolated from colonies transformed in *3.3.1.5*. *Figure 3.3* shows the expected restriction sites and resulting fragment sizes generated should the colony contain pBabe-Puro-γ-catenin DNA. Restriction digested DNA was visualised as described in section *2.3.2.*

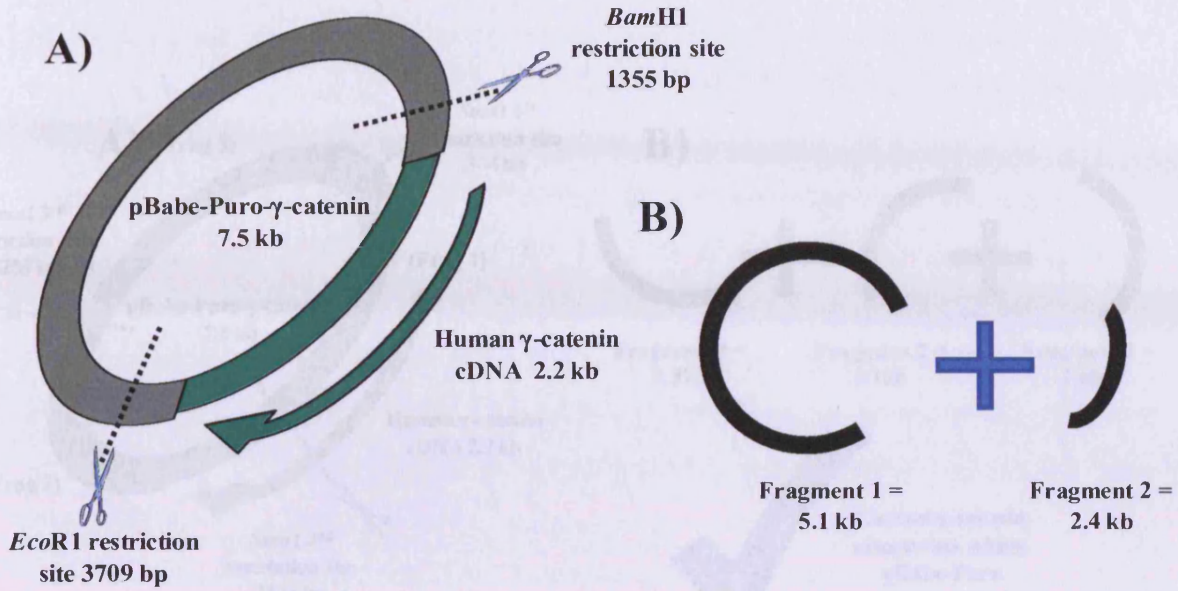


Figure 3.3 - Strategy for identification of colonies containing pBabe-Puro-γ-catenin vector.

A) Cartoon of *Bam*H1 and *Eco*R1 restriction sites of newly formed pBabe-Puro-γ-catenin vector with the location of restriction enzyme recognition sequences. Values represent the distance from the 5'LTR in bp. **B)** The predicted fragment sizes obtained from pBabe-Puro-γ-catenin following a *Bam*H1/*Eco*R1 double restriction digest.

3.3.1.8 *Confirmation of correct orientation of human γ-catenin cDNA within the pBabe-Puro vector*

The correct orientation of insert within the pBabe-Puro vector was confirmed using a *Sma*1 endonuclease digest which should generate the fragments illustrated in *Figure 3.4*. The fragments obtained from a potentially incorrect insertion are also shown in this diagram. A 10µl reaction included 350ng of colony DNA, 100U *Sma*1, NEB buffer 4 and the remainder molecular grade water. Digested fragments were assessed as described previously (section *2.3.2*).

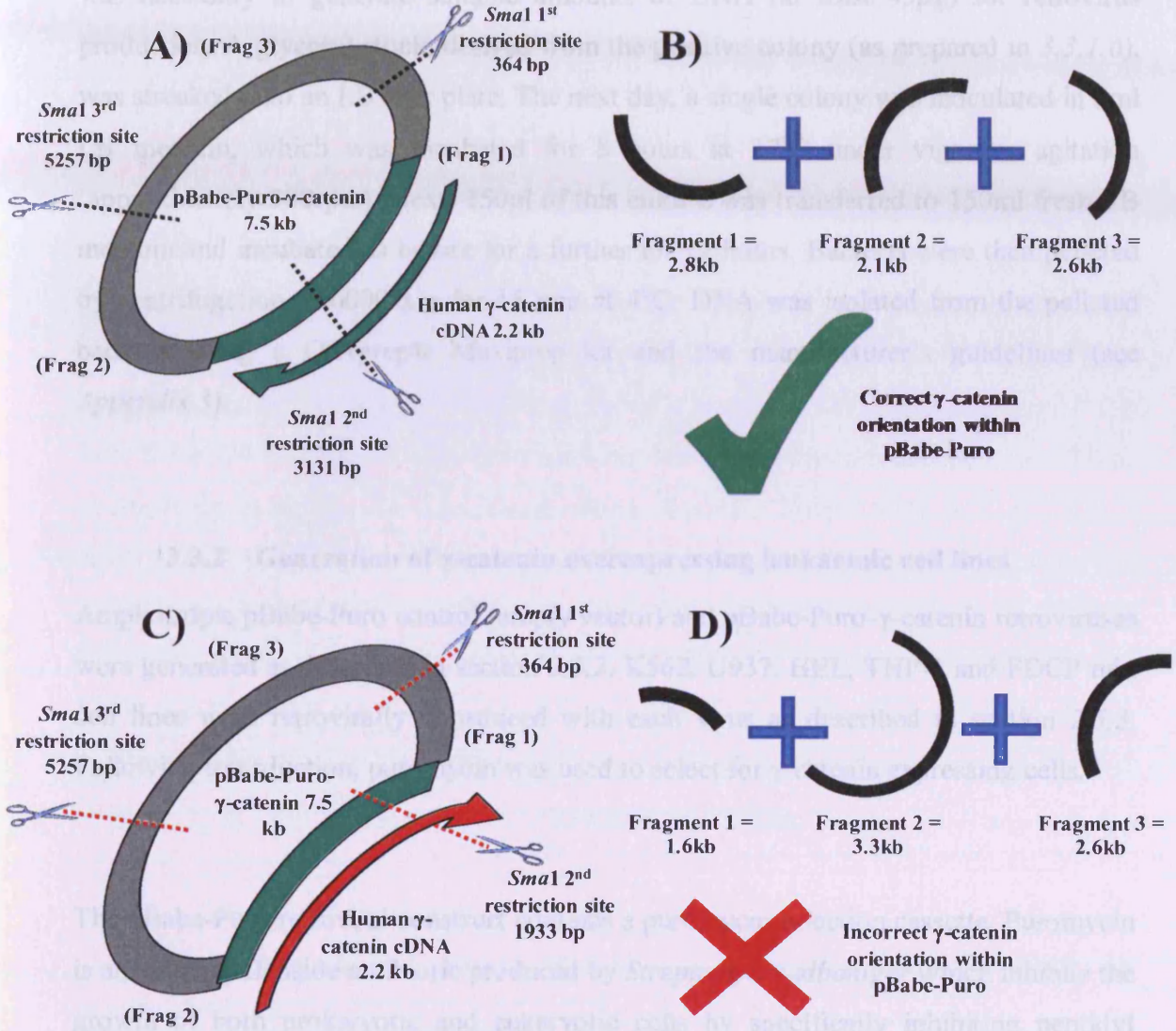


Figure 3.4 - Strategy to confirm correct orientation of γ-catenin cDNA within pBabe-Puro vector.

A) Plasmid map of pBabe-Puro-γ-catenin with the newly formed *Sma*1 recognition sites and **B)** the 2.8, 2.1 and 2.6kb fragment sizes expected upon correct γ-catenin orientation within pBabe-Puro. **C)** The predicted *Sma*1 recognition sites with an incorrectly orientated γ-catenin within pBabe-Puro and **D)** the 1.6, 3.3 and 2.6kb fragments expected following a single *Sma*1 restriction digest.

3.3.1.9 *Plasmid preparation of pBabe-Puro-γ-catenin*

Upon identification of a correctly orientated pBabe-Puro-γ-catenin positive colony it was necessary to generate suitable amounts of DNA (at least 45μg) for retrovirus production. A glycerol stock, derived from the positive colony (as prepared in *3.3.1.6*), was streaked onto an LB agar plate. The next day, a single colony was inoculated in 5ml LB medium, which was incubated for 8 hours at 37°C under vigorous agitation (approximately 300rpm). Next, 150μl of this culture was transferred to 150ml fresh LB medium and incubated as before for a further for 12 hours. Bacteria were then pelleted by centrifugation at 6000 x *g* for 15 min at 4°C. DNA was isolated from the pelleted bacteria using a QIAprep® Maxiprep kit and the manufacturer's guidelines (see *Appendix 3*).

3.3.2 Generation of γ-catenin overexpressing leukaemic cell lines

Amphotropic pBabe-Puro control (empty vector) and pBabe-Puro-γ-catenin retroviruses were generated as described in section *2.5.2*. K562, U937, HEL, THP-1 and FDCP mix cell lines were retrovirally transduced with each virus as described in section *2.5.3*. Following transduction, puromycin was used to select for γ-catenin expressing cells.

The pBabe-Puro retroviral construct contains a puromycin selection cassette. Puromycin is an aminonucleoside antibiotic produced by *Streptomyces alboniger* which inhibits the growth of both prokaryotic and eukaryotic cells by specifically inhibiting peptidyl transfer on ribosomes (Vara *et al.*, 1985). Transfection with the pBabe-Puro vector leads to expression of the *pac* gene which in turn encodes the enzyme Puromycin N-acetyl-tranferase (PAC) which inactivates puromycin by N-acetylation (Lacalle *et al.*, 1989; de la Luna S. and Ortin, 1992).

The day immediately following round two of retroviral transduction, puromycin (2μg/ml) was added to the medium of cells lines transfected with pBabe-Puro, and pBabe-Puro-γ-catenin. A 'mock transduced' (i.e. cultured in the presence of conditioned medium of phoenix cells and thus no contact with any retrovirus) control was included.

Selection was adjudged to be finished when over 99% of this culture was dead, as approximated by light microscopy. Viable cells were then washed free of puromycin by centrifugation at 200 x *g* for 5 minutes and resuspended in fresh medium. Finally, cells were assessed for γ-catenin expression (below in section *3.3.3*).

3.3.3 Analysis of γ- and β-catenin protein expression in transfected cell lines

In order to assess the level of endogenous and ectopic γ- and β-catenin expression driven by the respective retroviral vectors, protein levels were assessed from control and catenin transfected cells by Western blotting. Whole cell protein lysates of control and γ- or β-catenin transfected cells from each cell line were prepared and immuno-blotted as described in section *2.6*. The normalisation of protein lysates from each cell line to equal weight allowed any differences in catenin expression to be identified. A mouse monoclonal antibody was used for the detection of γ-catenin (Clone 15, BD) at 250ng/ml, whilst a rabbit monoclonal (Clone 6B3, Cell Signalling, Massachusetts, USA) was deployed for β-catenin detection at 1ng/ml. The detection of β-actin (Clone mAbcam 8226, Abcam, Cambridge, UK), was achieved using a mouse monoclonal at 20ng/ml, to provide an assessment for equal protein loading.

3.3.4 Optimisation of intracellular catenin staining

3.3.4.1 *Optimisation of γ-catenin staining*

For the optimisation of intracellular γ-catenin detection by flow cytometry, the K562 pBabe-Puro control and γ-catenin overexpressing cell lines created in section *3.3.2* were utilised since these showed both high endogenous and exogenous γ-catenin expression.

Detection of intracellular expression of γ-catenin was optimised using 1 x 10^6 each of the K562 pBabe-Puro control and γ-catenin cells. These cells were washed in 20ml of serum-free (SF) RPMI-1640 and centrifuged at 200 x *g* for 10 minutes. Cells were resuspended in 200μl SF RPMI-1640 and fixed in an equal volume of 4%

paraformaldehyde for 20 minutes under gentle agitation from a rotary shaker. Cells were subsequently washed with 1ml SF RPMI-1640 and centrifuged at 200 x *g* for 5 minutes. Cell membranes were permeabilised by resuspending the cells in 1ml 0.1% TX-100 detergent for 5 minutes with occasional agitation. The TX-100 was completely removed from the cells by 2 consecutive washes of 5ml staining buffer (SB, see section *2.4.2* for composition) each followed by centrifugation at 200 x *g* for 5 minutes. Cells were subsequently resuspended in 200μl SB and half were incubated with the primary anti-γ-catenin antibody at concentrations of 1, 3, 10, 30 or 60μg/ml, whilst the other half was incubated with the corresponding isotype-matched ($IgG2_a$ or $IgG2_b$) antibody at the same concentrations. Three different clones of γ-catenin primary mouse monoclonal antibody were tested; Clone 15 ($IgG2_a$, BD), PG 5.1 ($IgG2_b$, AbD Serotec, Kidlington, UK) and M111 ($IgG2_a$, AbCam). Primary antibody incubations of 30, 45 and 60 minutes at RT were tested, after which, the cells were washed in 1ml SB by centrifugation as above. Stained cells were resuspended in SB, and detected using secondary labelling step, where 1.5μg/ml R-PE conjugated rat-anti-mouse (RAM) $IgG2_{a+b}$ antibody (Clone X57, BD) as incubated for 30 minutes in the dark at RT. The cells were washed as above and resuspended in 100μl FACSFlow™ (BD) prior to flow cytometric acquisition (see *3.3.6*).

3.3.4.2 *Optimisation of β-catenin staining*

Detection of intracellular expression of β-catenin was optimised using the K562 cell line expressing a mutant form of β-catenin. This amino (NH_2)-terminally truncated form of β-catenin, encoded within the pPOLYPOZ retroviral plasmid (β-catΔN89, kind gift by Prof. Dale, originally constructed by Polakis *et al.* (Munemitsu *et al.*, 1996)), has a 89 amino acid sequence missing from the NH_2-terminus, thus depriving the molecule of its Casein Kinase α (CKα) and Glycogen Synthase Kinase 3β (GSK3β) phosphorylation sites (serine 45, and threonine 41, serines 33 and 37, respectively (Liu *et al.*, 2002)). The absence of phosphorylation means the protein is not ubiquitinated and targeted for degradation by the proteasome leading to constitutive accumulation of β-catenin in the cytosol. The K562 pBabe-Puro transduced cells were used again as the control line.

Cells were processed as described above (section *3.3.4.1*) using a primary mouse monoclonal β-catenin antibody (Clone 14, IgG1, BD) conjugated directly to FITC. The immuno-reactivity of this antibody was also raised to an epitope in the COOH-terminus of β-catenin so was not affected by the NH_2-terminal truncation. Primary antibody concentrations were optimised by incubation of 1, 3, 10 and 30μg/ml as above for 30 minutes in the dark. Detection of the primary labelling stage was achieved by flow cytometry (see *3.3.6*).

3.3.4.3 *Cross reactivity of γ- and β-catenin antibodies*

Given the high degree of molecular homology shared between these armadillo proteins (Hatzfeld, 1999), it was necessary to ensure that the respective antibodies did not cross-react under assay conditions. Therefore, K562-ΔN89-β-catenin cells were stained for γ-catenin (using optimised staining conditions), whilst the K562 pBabe-Puro-γ-catenin cell line was immuno-stained with the optimal conditions for the β-catenin primary antibody in each case employing K562 pBabe-Puro cells as controls. Assessment of cross-reactivity was measured using flow cytometry (*3.3.6*). It was also necessary to determine whether there was cross-reaction as determined by Western blotting (see below section *3.3.3*).

3.3.5 Detection of intracellular catenin expression in normal human haematopoietic cells

3.3.5.1 *Isolation of normal human haematopoietic cells from cord blood/bone marrow*

In order to assess the expression of γ- and β-catenin in haematopoietic lineages (including developmental subsets) human CB or BM was obtained as described in section *2.2.3*. A buffy coat containing total leukocytes, as shown in *Figure 3.5*, was isolated by centrifuging 15ml of whole CB or BM under high speed centrifugation at 2,500 x *g* for 15 minutes. Under these conditions a pale film of white blood cells forms between the red cell and serum layers, which was carefully removed using a Pasteur pipette. A degree of red cell contamination was inevitable with this aspiration, but also

desirable (provided it was not excessive) given these cells were also relevant for the analysis. Leukocytes were washed in 15ml of SF RPMI-1640 and centrifuged at 200 x *g* for 10 minutes, after which total leukocyte number was estimated as described in section *2.2.4*.

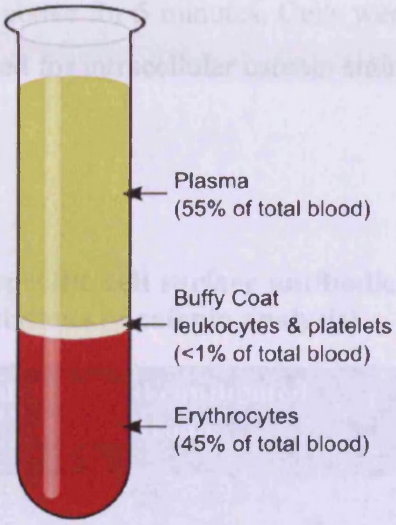


Figure 3.5 - The fractionation of leukocytes by centrifugation of whole cord blood or bone marrow.

The percentages stated for each fraction represent those obtained from centrifugation of peripheral blood. The method was the same for human CB or BM although the relative percentage of leukocytes was higher in BM.

3.3.5.2 *Preparation and pre-labelling of haematopoietic cells prior to staining*

Prior to intracellular staining of catenin, pre-labelling of cells with cell-surface markers was required in order to allow haematopoietic cell subset analysis by flow cytometry. A panel of antibodies specific for myeloid, lymphoid, erythroid or haematopoietic stem cell/progenitor populations were applied.

Total leukocytes ($1x10^7$) were resuspended in 100μl of staining buffer (SB) and subjected to the relevant panel of cell surface anti-human antibodies (each at 10μg/ml) for 30 minutes at 4°C. The threshold for cell surface staining was ascertained by also incubating cells with the isotype- and manufacturer-matched irrelevant control

antibodies conjugated to the same fluorochromes. The panel of mouse monoclonal antibodies (including clone and manufacturer) used in conjunction with γ-catenin and β-catenin immune-stained cells are shown in *Table 3.1* and *Table 3.2*, respectively. These differed primarily because of the different fluorochromes used to label γ- and β-catenins. Following the incubation, 1ml of SB was added to all tubes which were subsequently centrifuged as above for 5 minutes. Cells were next resuspended in 200μl of SF-RPMI-1640 and assayed for intracellular catenin staining as below.

Table 3.1 - The panels of specific cell surface antibodies used in the identification of haematopoietic subpopulations (γ-catenin analysis).

Lineage Panel	Antibodies and conjugated fluorochrome	Clone	Manufacturer
Haematopoietic Stem/Progenitor Cell	CD34-PerCP/Cy5.5	4H11	Biolegend (California, USA)
	CD38-APC	HIT2	Biolegend
	CD45-FITC	HI30	Biolegend
Myeloid lineages	CD15-FITC	W6D3	Biolegend
	CD14-APC	HCD14	Biolegend
	CD45-PerCP	MEM-28	AbCam
Lymphoid lineages	CD3-FITC	UCHT1	Biolegend
	CD19-APC	HIB19	Biolegend
	CD45-PerCP	MEM-28	AbCam
Erythroid lineage	GlyA-FITC	YTH89.1	AbD Serotec
	CD45-PerCP	MEM-28	AbCam

Table 3.2 - The panels of specific cell surface antibodies used in the identification of haematopoietic subpopulations (β-catenin analysis).

Lineage Panel	Antibodies and conjugated fluorochrome	Clone	Manufacturer
Haematopoietic Stem/Progenitor Cell	CD34-PE	8G12	BD
	CD38-APC	HIT2	Biolegend
	CD45-PerCP	MEM-28	AbCam
Myeloid lineages	CD15-PE	W6D3	Biolegend
	CD14-APC	HCD14	Biolegend
	CD45-PerCP	MEM-28	AbCam
Lymphoid lineages	CD3-PE	SK7	BD
	CD19-APC	HIB19	Biolegend
	CD45-PerCP	MEM-28	AbCam
Erythroid lineages	GlyA-PE	JC159	DAKO (Cambridgeshire, UK)
	CD45-PerCP	MEM-28	AbCam

3.3.5.3 *Intracellular staining of γ- and β-catenin*

Pre-labelled haematopoietic cells already suspended in SF-RPMI-1640 were stained for intracellular γ- and β-catenin using the protocol optimised in section *3.3.4.1.* These optimised conditions involved using the primary catenin, and appropriate isotype, antibodies at a concentration of 10μg/ml for 30 minutes.

3.3.5.4 *Intracellular staining of γ- and β-catenin in erythrocytes*

Owing to the fragile structure of mature erythrocytes, the staining method outlined above was modified to ensure enough red cells survived the lysis stage and were present in the final analysis. Cells pre-stained with the erythroid panel of antibodies received a longer (30 minute) fixation to increase cross-linking of proteins. For permeabilisation,

timings and washings remained the same, however erythroid labelled cells were permeabilised with 100% methanol, a gentler permeabilising agent than the triton TX-100 used in the above section. Finally, upon resuspension in 100μl FACSFlow™ just prior to flow cytometric analysis (below *3.3.6*), erythroid cells were stained with 165nM TO-PRO®-3 iodide (Invitrogen). This nuclear stain was an important parameter in discriminating mature anucleate red cells from nucleated cells.

3.3.6 Analysis of total γ- and β-catenin expression by flow cytometry

Six-parameter flow cytometric analyses of total catenin expression in haematopoeitic cells were performed using a FACSCalibur™ cytometer (BD) with CellQuest™ software version 3.3 (BD). Cells to be analysed were resuspended in 100μl FACSFlow™ in mini-flow tubes (Greiner Bio-one) and kept on ice in the dark until flow cytometric analysis. Given the presence of low-frequency developmental sub-populations within CB/BM, such as $CD34^+$ cells (0.05-3%), a minimum of $5x10^5$ events were collected for each panel of cells stained. The homogenous nature of cell lines meant acquisition of only 1×10^4 events was necessary. Debris was excluded from the analyses on the basis of forward- and side-scatter characteristics, whilst specific individual sub-populations were identified using the gating strategies outlined in results section *3.4.2.1.* Flow cytometric data were analysed using WinMDI version 2.8 (Joe Trotter, Pharmingen, California, USA) and the mean fluorescence intensity (MFI) of γ- and β-catenin within discrete developmental subsets and cell lines were calculated using *Equation 3.1* below:

Mean fluorescence intensity (logged arbitrary units) = CFI – BFI

Where;

CFI = Intensity of catenin fluorescence (given by γ- or β-catenin antibody)
BFI = Intensity of background staining (given by isotype matched control antibody on same population)

Equation 3.1 - Calculation used to generate the mean fluorescence intensity of catenin within subpopulations of cord blood.

3.3.7 Analysis of subcellular localisation of γ- and β-catenin by CLSM

3.3.7.1 *Principles of CLSM*

The ability of Confocal Laser Scanning Microscopy (CLSM) to accurately resolve different compartments of a single cell make it ideal for analysing the subcellular distribution of intracellular proteins such as catenin. CLSM is able to deliver such high resolution images, unlike conventional wide-field fluorescence microscopes, through its unique configuration (see *Figure 3.6*). A laser light source is passed through a narrow aperture and focussed, by an objective lens, onto a small focal volume within a tissue specimen. Fluorophores (see *2.7.2*) targeted to specific proteins and DNA within the pre-treated sample become excited and emit fluorescence of a higher wavelength that is re-collected by the objective lens and directed towards a beam splitter. This beam splitter is capable of separating the re-collected light by allowing reflected laser light to pass through whilst directing collected fluorescent light wavelength of interest through a small pinhole onto a PMT detector. The PMT converts light signals into an electrical one as described previously for flow cytometry (*2.7.1*) allowing a digital read-out (imaging) on a computer. The pinhole aperture thus excludes all out-of-focus light not originating from the focal point allowing a very sharp, resolved, thin area of focussed tissue to be examined as the laser scans over the plane of interest (Z-plane). Furthermore, the specimen stage can be adjusted vertically allowing fluorescence data to be collected from multiple focal planes (Z-stacks). These Z-stacks can eventually be compiled by intricate CLSM software into 3D information on protein expression throughout a single cell.

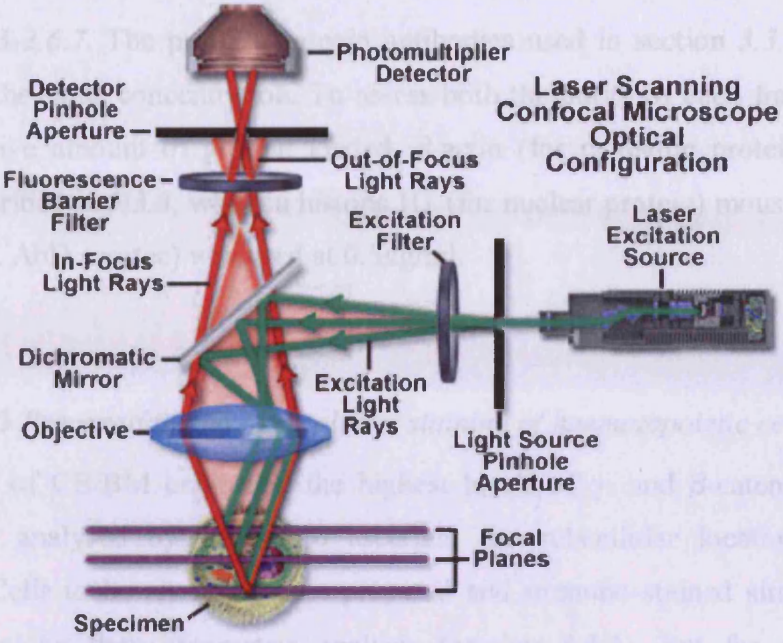


Figure 3.6 - The standard configuration of CLSM.

Schematic of a CLSM optical set-up showing the light laser source which is directed through pinhole apertures and the objective lens onto a focal plane within the specimen. Emitted fluorescence from within the sample is subsequently redirected back through the lens, and reflected by a dichromatic mirror through a pinhole aperture onto a PMT. Adapted from www.olympusfluoview.com/theory/confocalintro.html.

3.3.7.2 *Validation of CLSM as a technique for subcellular localisation analysis*

Before this technique could be routinely used for the analysis of primary haematopoietic cells, it was first validated by Western blotting. It was necessary to establish whether the intracellular staining protocol previously described could be used in conjunction with CLSM to detect shifts in the localisation of catenin between the cytosol and nucleus of individual cells.

To this end, the K562 pBabe-Puro control and γ-catenin overexpressing lines previously established (see section *3.3.2*) were intracellularly stained as outlined in section *3.3.4.1* but for the exceptions listed in points 4) and 5) of section *3.3.7.3*. CLSM image acquisition and post-acquisition analysis were completed as described in sections *3.3.7.4* and *3.3.7.5*, respectively. To validate the distribution of γ-catenin obtained from CLSM, cells from the respective K562 cultures were fractionated into cytosolic and nuclear homogenate using the method in section *2.6.2*, and Western blotted as in

sections *2.6.3-2.6.7.* The primary catenin antibodies used in section *3.3.3* were again deployed at the same concentration. To assess both the purity of each fraction and the amount relative amount of protein loaded, β-actin (for cytosolic protein) was used, again as described in *3.3.3*, whilst a histone H1 (for nuclear protein) mouse monoclonal (Clone AE-4, AbD serotec) was used at 0.5μg/ml.

3.3.7.3 *Preparation and intracellular staining of haematopoietic cells*

The lineages of CB/BM exhibiting the highest levels of γ- and β-catenin expression were further analysed by CLSM to ascertain the subcellular location of catenin expression. Cells to be analysed were prepared and immuno-stained similarly to that described for the flow cytometric analysis (section *3.3.5*), but for a number of fundamental differences;

1) The starting number of cells to be analysed was never more than $2x10^6$ since fewer cells were required for confocal analysis.

2) Due to the relative infrequency of $CD34^+$ HSC/progenitor cells within CM/BM, their prior purification before intracellular staining was required using the MACS technique outlined in section *2.4.2.*

3) The limited fluorescence channels for CLSM analysis meant that just one fluorochrome (R-PE) was available for the pre-labelling of target subsets described in section *3.3.5.2.* Thus, for HSC/progenitor cells and granulocytes, the CD34 and CD15 antibodies, respectively, described in *Table 3.2* were used. An R-PE conjugated CD14 monoclonal antibody (Clone HCD14, Biolegend) was used for the detection of monocytes.

4) Given the relative lack of efficiency of R-PE as a confocal fluorochrome, both γ- and β-catenin were labelled with FITC for optimal detection. The immuno-staining of β-catenin (section *3.3.4.2*) was thus unaffected (due to direct FITC conjugation), whilst γ-catenin immuno-detection required a different RAM secondary antibody (Clone LO-MG2a-9, AbD Serotec) used exactly as above (section *3.3.4.1).*

5) All cells to be analysed by confocal microscopy were stained just prior to analysis with 165nM TO-PRO®-3 iodide upon final re-suspension in 100μl FACSFlow™. This allowed the nuclei of cells to be confidently discriminated.

3.3.7.4 *CLSM data acquisition*

Cells to be analysed by CLSM were resuspended in 100μl FACSFlow™ containing 165nM TO-PRO®-3 iodide and kept in the dark on ice until analysis (<1 hour). At the time of analysis, a 30μl aliquot of the cells of interest was slowly and evenly pipetted onto a clean 22 x 22mm coverslip (Fisher) which was secured onto the adjustable Z-stage of the DMIRBE2 upright light microscope (Leica, Buckinghamshire, UK) and allowed to settle for approximately 5 minutes prior to focussing.

Confocal immunofluorescence was achieved using the resonant scanning head of a Leica TCS SP2 confocal laser microscope with a 63x objective NA 1.32 (HCX-PL-APO) assisted by Leica™ Confocal Software (LCS) version 2.61 (Leica Microsystems, Heidelberg, Germany). For each lineage a minimum of 50 cells were imaged using the strategy outlined in *Figure 3.7*. The threshold for catenin fluorescence was ascertained by imaging approximately 20 cells per lineage immuno-stained with the isotype-matched irrelevant control antibodies. Throughout image acquisition only whole nucleated cells were captured as determined by TO-PRO®-3 iodide staining. A fixed path of field navigation was adopted (*Figure 3.7A*), whilst a concerted effort was made to include as many cells of interest per field as possible. All cells scanned with a single Z-plane were imaged with the focal plane dissecting as close to the centre of the cells as possible (shown in *Figure 3.7B*), ensuring representative areas of membrane, cytosol and nucleus were observed for each cell. For each field, a light transmission image was captured, along with membrane fluorescence, nuclear fluorescence (TO-PRO®-3), and catenin fluorescence (γ or β) in the order exemplified in *Figure 3.7C*. This ensured that the necessary post-acquisition analysis could be conducted as described in the immediately proceeding section. For each lineage, representative Z-stacks (image series) were constructed of single cells which demonstrated exemplary catenin localisation, the principle of which is illustrated in *Figure 3.7D*.

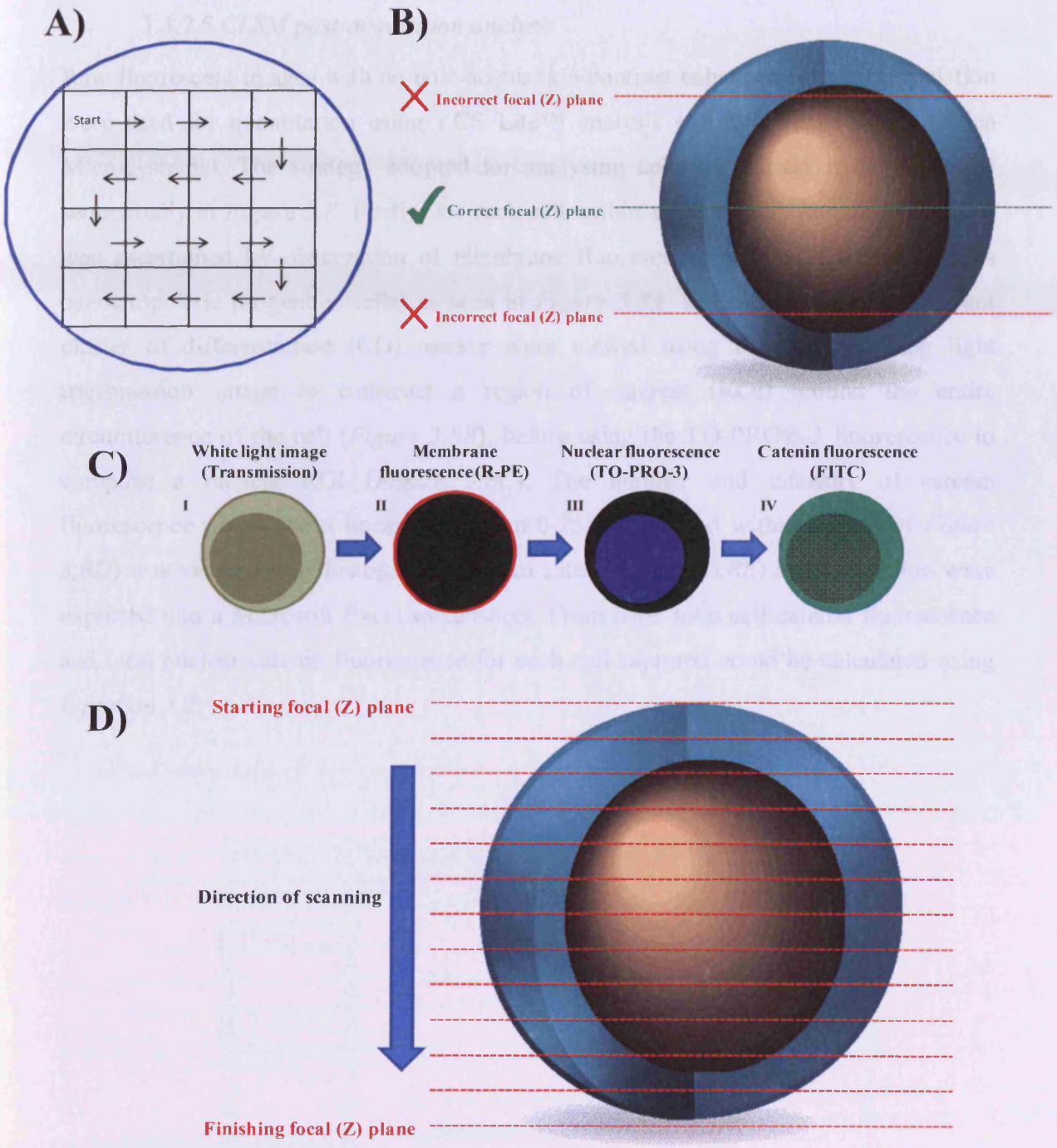


Figure 3.7 - Strategy used to acquire images by CLSM.

A) The fixed direction of movement adopted to navigate through the multiple fields of immunofluorescence to avoid selection bias. **B)** 3D cross section through a cell demonstrating the correct (green) and incorrect (red) focal planes used for imaging. Targeting the correct focal plane ensures a single image is captured which fairly represents the actual nuclear to cytoplasmic ratio of the target cell. **C)** Demonstration of sequential fluorescence images captured for each cell and **D)** visual illustration of how a confocal image series (Z-stack) is compiled by the CLSM.

3.3.7.5 *CLSM post-acquisition analysis*

Raw fluorescent images with no post-acquisition contrast enhancement or manipulation were used for quantitation using LCS Lite™ analysis software version 2.61 (Leica Microsystems). The strategy adopted for analysing cells of interest is demonstrated sequentially in *Figure 3.8*. Firstly, for each cell within a given field, immunophenotype was ascertained by observation of membrane fluorescence (e.g. CD34-PE identifies haematopoietic progenitor cells) as seen in *Figure 3.8A*. Cells expressing the relevant cluster of differentiation (CD) marker were viewed using the corresponding light transmission image to construct a region of interest (ROI) around the entire circumference of the cell (*Figure 3.8B*), before using the TO-PRO®-3 fluorescence to compose a nuclear ROI (*Figure 3.8C*). The number and intensity of catenin fluorescence pixels (on a linear scale from 0-255) contained within each ROI *Figure 3.8D*) was visualised by histograms in Leica Lite™ (*Figure 3.8E*) and raw values were exported into a Microsoft Excel spreadsheet. From here, total cell catenin fluorescence and total nuclear catenin fluorescence for each cell captured could be calculated using *Equation 3.2*.

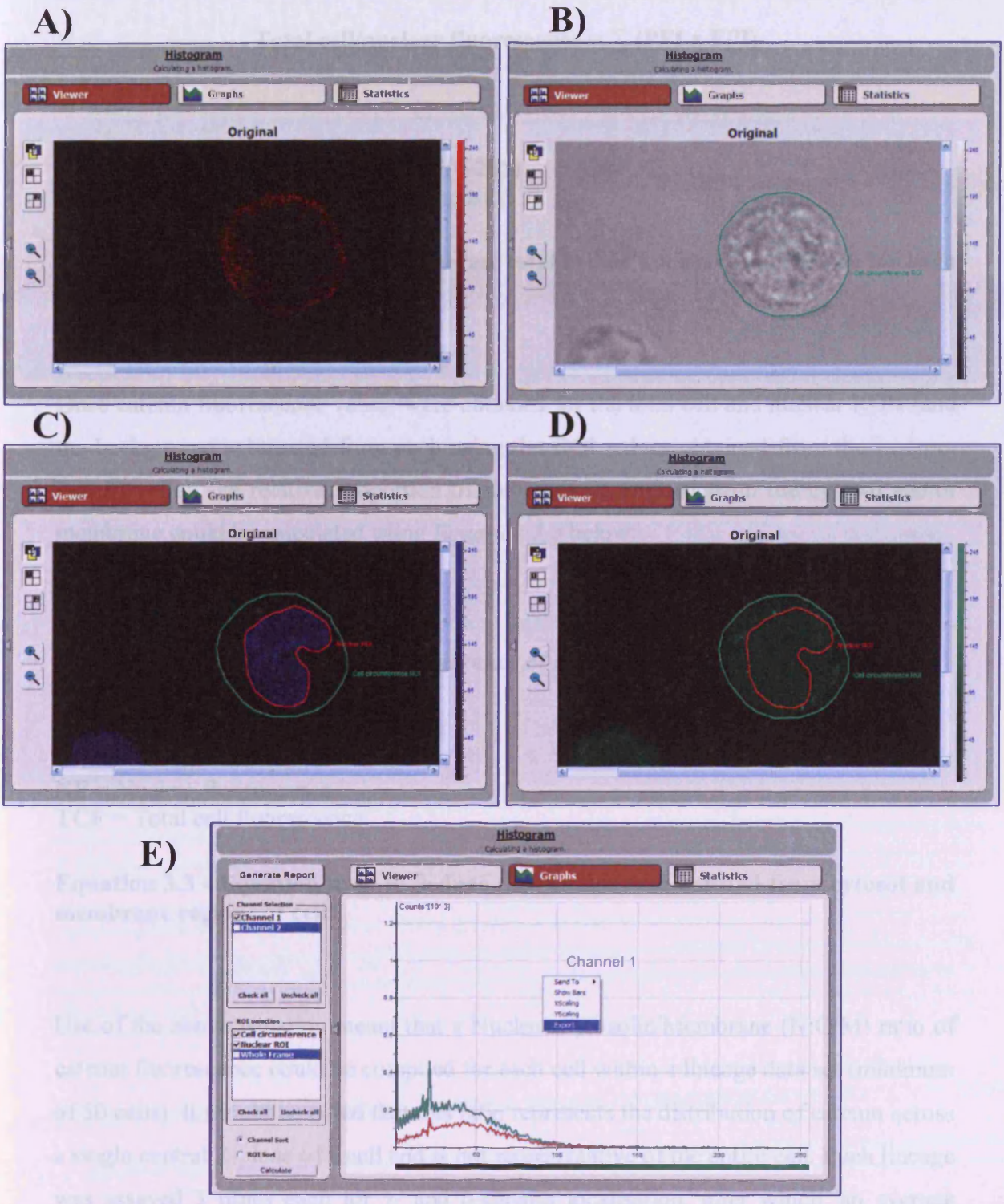


Figure 3.8 - Analytical technique used to quantify fluorescence emitted from whole cell and nuclear regions of individual haematopoietic cells.

A) Lineages of interest were first identified by immunophenotyping given by membrane fluorescence (red) before using the light transmission image **(B)** to draw an ROI around the entire cell circumference. **C)** TO-PRO-3 staining (blue) assisted in composing a nuclear ROI. **D)** Catenin fluorescence (green) was quantified within each ROI and used to generate histograms **(E)** of fluorescence intensity.

$$\textbf{Total cell/nuclear fluorescence} = \sum \textbf{(PFI x FPI)}$$

Where;

PFI = Fluorescence Intensity of Pixel (0-255)
FPI = Frequency of Pixels at given Intensity

Equation 3.2 - Equation used to generate total cell or nuclear fluorescence for each cell of given lineage.

Once catenin fluorescence values were obtained for the total cell and nuclear ROIs (and the background subtracted from each using the ROI values obtained from the isotype-stained cells), the relative proportion of fluorescence emitted from the cytosol and/or membrane could be calculated using *Equation 3.3* below:

$$\textbf{\% Cytosol/Membrane fluorescence of cell} = 100 - ([NF/TCF] \times 100)$$

Where;

NF = Nuclear fluorescence
TCF = Total cell fluorescence

Equation 3.3 - Equation used to deduce the fluorescence emitted from cytosol and membrane regions of cell.

Use of the above equation meant that a Nuclear:Cytosolic/Membrane (N:C/M) ratio of catenin fluorescence could be compiled for each cell within a lineage data set (minimum of 50 cells). It should be noted that this ratio represents the distribution of catenin across a single central Z-plane of a cell and is not representative of the entire cell. Each lineage was assayed 3 times each for γ- and β-catenin localisation, after which, an average N:C/M ratio, (with standard deviation (SD)), of γ- or β-catenin per cell was calculated.

3.4 Results

3.4.1 Optimisation of intracellular γ-catenin detection assay

3.4.1.1 *The generation of a γ-catenin retroviral expression vector*

To overexpress human γ-catenin, cDNA was subcloned from the PINCO construct into the pBabe-Puro retroviral vector. Using a double restriction digest (see *3.3.1.1*) the expected fragments sizes of 2.4kb (corresponding to γ-catenin sequence) and 12.7kb (corresponding to PINCO sequence) were generated (*Figure 3.9B*).

The recipient pBabe-Puro vector was linearised using the same restriction digest as mentioned above and *Figure 3.9C* confirms the efficiency of digestion by the generation of a 5.2Kb DNA fragment that corresponds to the full length pBabe-Puro sequence. After transformation of *E. coli* cells with the newly ligated pBabe-Puro-γ-catenin it was necessary to identify potential colonies expressing the plasmid (*Figure 3.9D*). Of 12 ampicillin-resistant colonies (c) isolated, 3 were identified (c2, c5 and c8) as potentially expressing the relevant DNA. This is shown in *Figure 3.9D* whereby c2 and c5 exhibit the DNA fragment sizes predicted in *Figure 3.3*.

Correct insert orientation was confirmed using a *Sma*1 restriction digest which produced the predicted fragment sizes (*Figure 3.4*) that corresponded to a correct orientation as illustrated in *Figure 3.9E*.

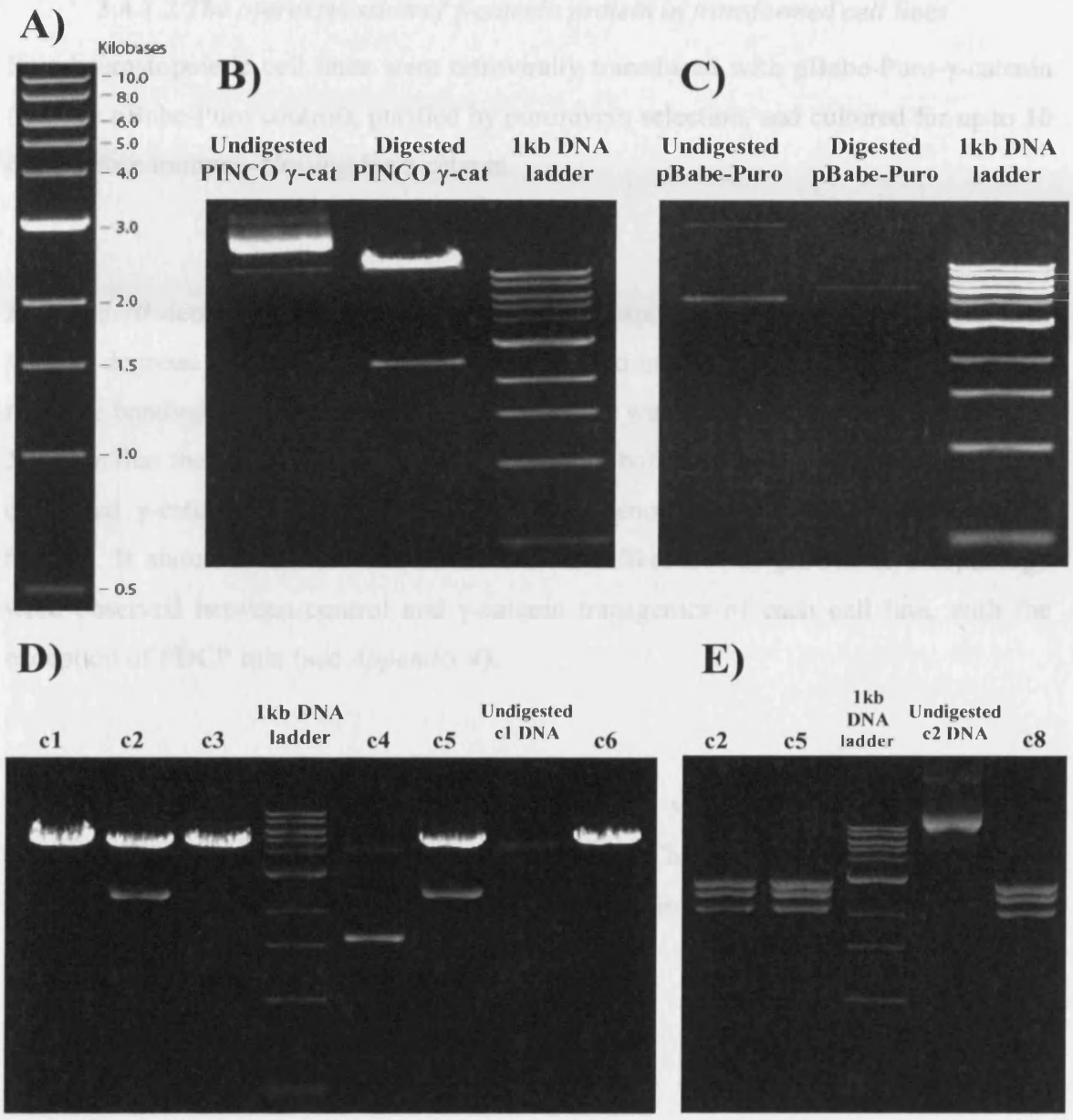


Figure 3.9 - Subcloning of human γ-catenin cDNA from PINCO into pBabe-Puro.

A) Range of molecular weights indicated by the 1kb DNA ladder used on all agarose gels (adapted from NEB website). **B)** Successful excision of γ-catenin from PINCO using *BamH*1/*EcoR*1 double digest as shown by generation of 12.7kb and 2.4kb DNA fragments. **C)** Successful linearization of pBabe-Puro using the same double digest as given by 5.1kb and otherwise undetectable 24bp DNA fragment. **D)** Representative agarose gel showing the range of ampicillin-resistant colonies (c) *BamH*1/*EcoR*1 restriction digested to test for the presence of pBabe-Puro-γ-catenin DNA. Presence of 5.2kb and 2.4kb DNA fragments in c2 and c5 (c8 not shown) identified them as potential colonies harbouring the plasmid of interest. **E)** Correct orientation of γ-catenin cDNA within pBabe-Puro as shown in c2, 5 and 8 by generation of 2.1, 2.6 and 2.8kb DNA fragments from a single *Sma*1 restriction digest.

3.4.1.2 *The overexpression of γ-catenin protein in transformed cell lines*

Five haematopoietic cell lines were retrovirally transduced with pBabe-Puro-γ-catenin (and the pBabe-Puro control), purified by puromycin selection, and cultured for up to 10 days before immuno-blotting for γ-catenin.

Figure 3.10 demonstrates how all cell lines overexpressed γ-catenin protein albeit to varying degrees. Interestingly, the γ-catenin detected in most of the cell lines exhibited a multiple banding pattern, the significance of which was further investigated in *Chapter 5.* Given that the multiple banding was a feature of both the endogenous and ectopically expressed γ-catenin made it unlikely that this phenomenon was due to non-specific binding. It should be noted that no discernible differences in growth or morphology were observed between control and γ-catenin transgenics of each cell line, with the exception of FDCP mix (see *Appendix 4*).

These data confirmed that the pBabe-Puro-γ-catenin vector was capable of ectopic expression of γ-catenin in all cell lines tested. The transgenic K562 line was subsequently employed for optimisation of γ-catenin staining because of a high induction of γ-catenin relative to endogenous levels.

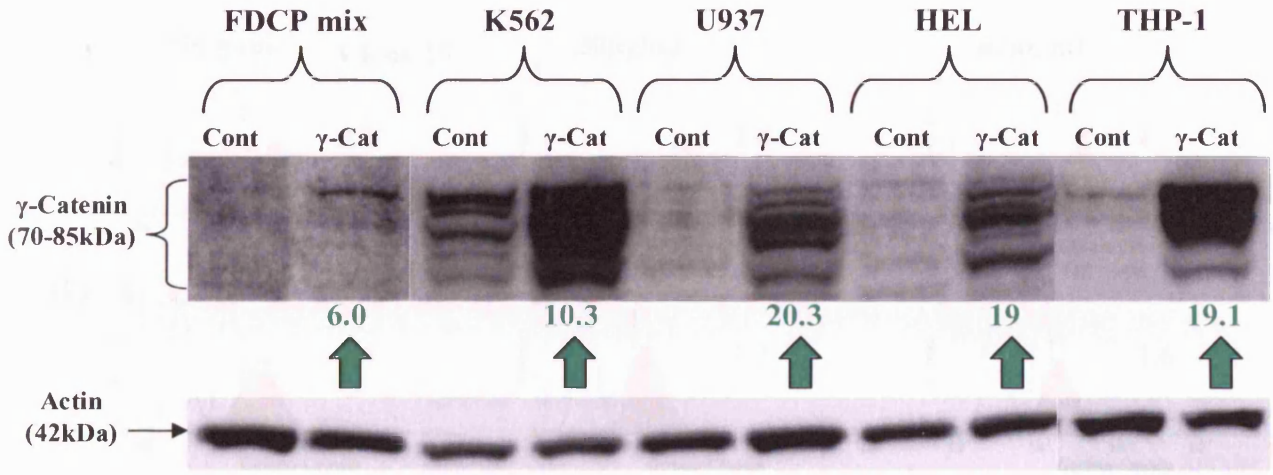


Figure 3.10 - Overexpression of γ-catenin protein in transformed cell lines.

Western blotting reveals the pBabe-Puro-γ-catenin retroviral vector ('γ-Cat') is capable of stable ectopic expression of γ-catenin in comparison to pBabe-Puro controls ('Cont'). Green numerals indicate the fold overexpression of γ-catenin versus controls that was achieved in each cell line as determined by densitometric analysis. Immunoblotting for β-actin indicates relative protein loading.

3.4.1.3 *The optimisation of a primary γ-catenin antibody*

As can be observed in *Figure 3.11A, B and C*, all monoclonals tested in a flow cytometric assay were able to detect the overexpression of γ-catenin in K562 cells to varying degrees. Optimal working concentration for all clones examined appeared to be 10μg/ml. However, clone M111 (*Figure 3.11C*) demonstrated the greatest efficiency of staining detecting a 9.7 fold overexpression of intracellular γ-catenin from control level. The specificity of this detection is validated by the fact it closely matched the 10.3 fold overexpression of γ-catenin present in the K562 cells as shown by Western blotting in *Figure 3.11D*. Furthermore, the broad range of fluorescence detected by the M111 clone (as demonstrated in the histograms) most accurately reflects the heterogeneity of protein overexpression often generated by the random process of retroviral-mediated gene integration. To ensure that incubation time was not a limiting factor in γ-catenin detection a range of primary antibody incubation times were attempted (30, 45 and 60 minutes all at RT) with the M111 clone. *Figure 3.11E* illustrates that the 30 minute incubation was optimal.

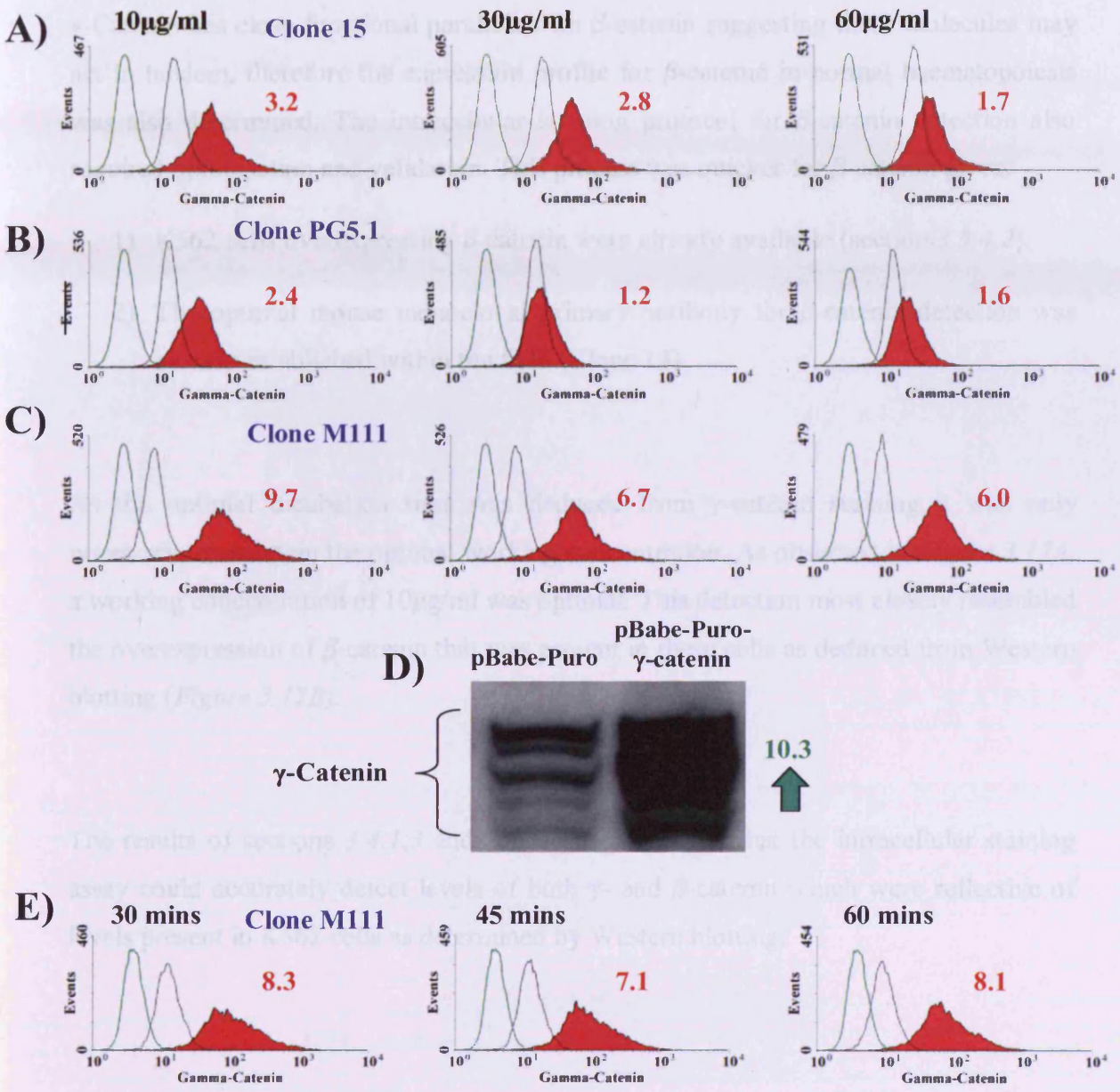


Figure 3.11 - Optimisation of γ-catenin intracellular staining in K562 cells.

Histograms of fluorescence obtained from cells stained with γ-catenin primary antibodies. The primary clones **A)** 15, **B)** PG5.1 **C)** M111, at the concentrations indicated, were used to stain: K562 pBabe-Puro (open black histograms); K562 pBabe-Puro-γ-catenin (red filled); or isotype-control cells (green). Numerals (red) represent the fold overexpression of γ-catenin detected in overexpressing versus control K562 cells as given by cytometric analysis. **D)** The fold overexpression of γ-catenin protein (in green) present in the K526 pBabe-Puro-γ-catenin cell line at the time of intracellular staining optimisation as deduced by western blotting. **E)** Optimisation of antibody incubation time using the optimal primary γ-catenin antibody clone (M111) at times of 30, 45 and 60 minutes.

3.4.1.4 *The optimisation of a primary β-catenin antibody*

γ-Catenin has close functional parallels with β-catenin suggesting these molecules may act in tandem, therefore the expression profile for β-catenin in normal haematopoiesis was also determined. The intracellular staining protocol for β-catenin detection also required optimisation and validation. This process was quicker for β-catenin given:

1) K562 cells overexpressing β-catenin were already available (section *3.3.4.2*).

2) The optimal mouse monoclonal primary antibody for β-catenin detection was already established within the field (Clone 14).

As the optimal incubation time was deduced from γ-catenin staining it was only necessary to ascertain the optimal working concentration. As observed in *Figure 3.12A*. a working concentration of 10μg/ml was optimal. This detection most closely resembled the overexpression of β-catenin that was present in these cells as deduced from Western blotting (*Figure 3.12B).*

The results of sections *3.4.1.3* and *3.4.1.4* demonstrated that the intracellular staining assay could accurately detect levels of both γ- and β-catenin which were reflective of levels present in K562 cells as determined by Western blotting.

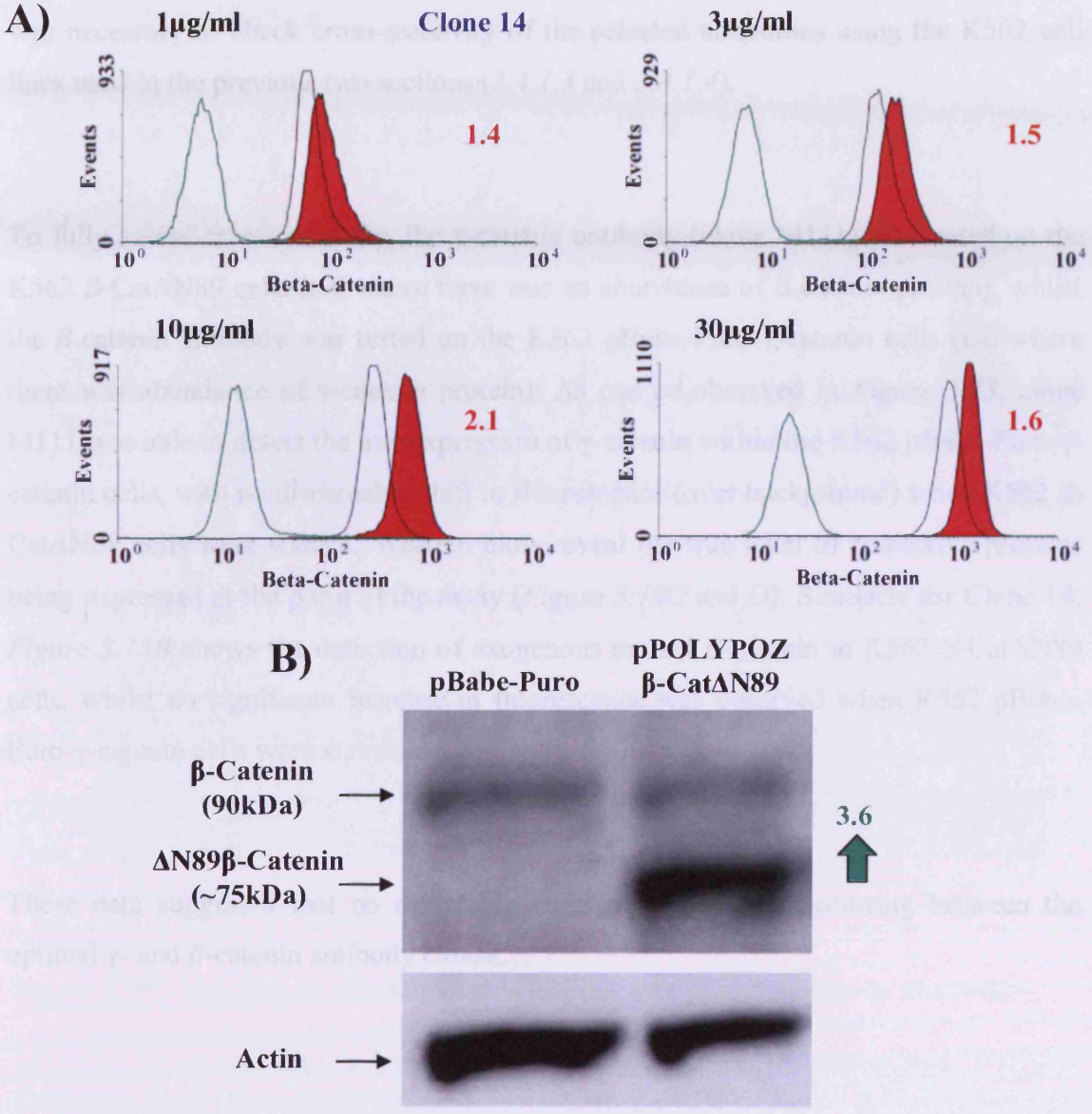


Figure 3.12 - The optimisation of β-catenin intracellular staining in K562 cells.

A) Histograms of fluorescence obtained from staining with primary β-catenin antibody (clone 14) on: K562 pBabe-Puro cells (open black histograms); K562 β-CatΔN89 cells (red filled); and isotype-control stained cells (green) at the indicated concentrations. Numerals (red) represent the fold overexpression of β-catenin detected in overexpressing versus control K562 cells as given by cytometric analysis. **B)** Western blot shows the fold overexpression of mutant β-catenin protein present in pPOLYPOZ β-CatΔN89, versus *wt* protein in control cells, at the time of intracellular staining optimisation. Note the mutant form migrates at a molecular weight of 75kDa whilst the *wt* migrates to around 90kDa.

3.4.1.5 *Lack of cross-reactivity between primary γ- and β-catenin antibodies*

Given the degree of homology between γ- and β-catenin molecules (see *Figure 1.10A*) it was necessary to check cross-reactivity of the selected antibodies using the K562 cell lines used in the previous two sections (*3.4.1.3* and *3.4.1.4*).

To fully assess cross-reactivity, the γ-catenin antibody (clone M111) was tested on the K562 β-CatΔN89 cells (i.e. where there was an abundance of β-catenin protein), whilst the β-catenin antibody was tested on the K562 pBabe-Puro-γ-catenin cells (i.e. where there was abundance of γ-catenin protein). As can be observed in *Figure 3.13*, clone M111 was able to detect the overexpression of γ-catenin within the K562 pBabe-Puro-γ-catenin cells, with no discernable shift in fluorescence (over background) when K562 β-CatΔN89 cells were stained. Western blots reveal the true level of respective proteins being expressed at the point of the assay (*Figure 3.13C* and *D*). Similarly for Clone 14, *Figure 3.13B* shows the detection of exogenous mutant β-catenin in K562 β-CatΔN89 cells, whilst no significant increase in fluorescence was observed when K562 pBabe-Puro-γ-catenin cells were stained.

These data suggested that no detectable cross reactivity was occurring between the optimal γ- and β-catenin antibody clones.

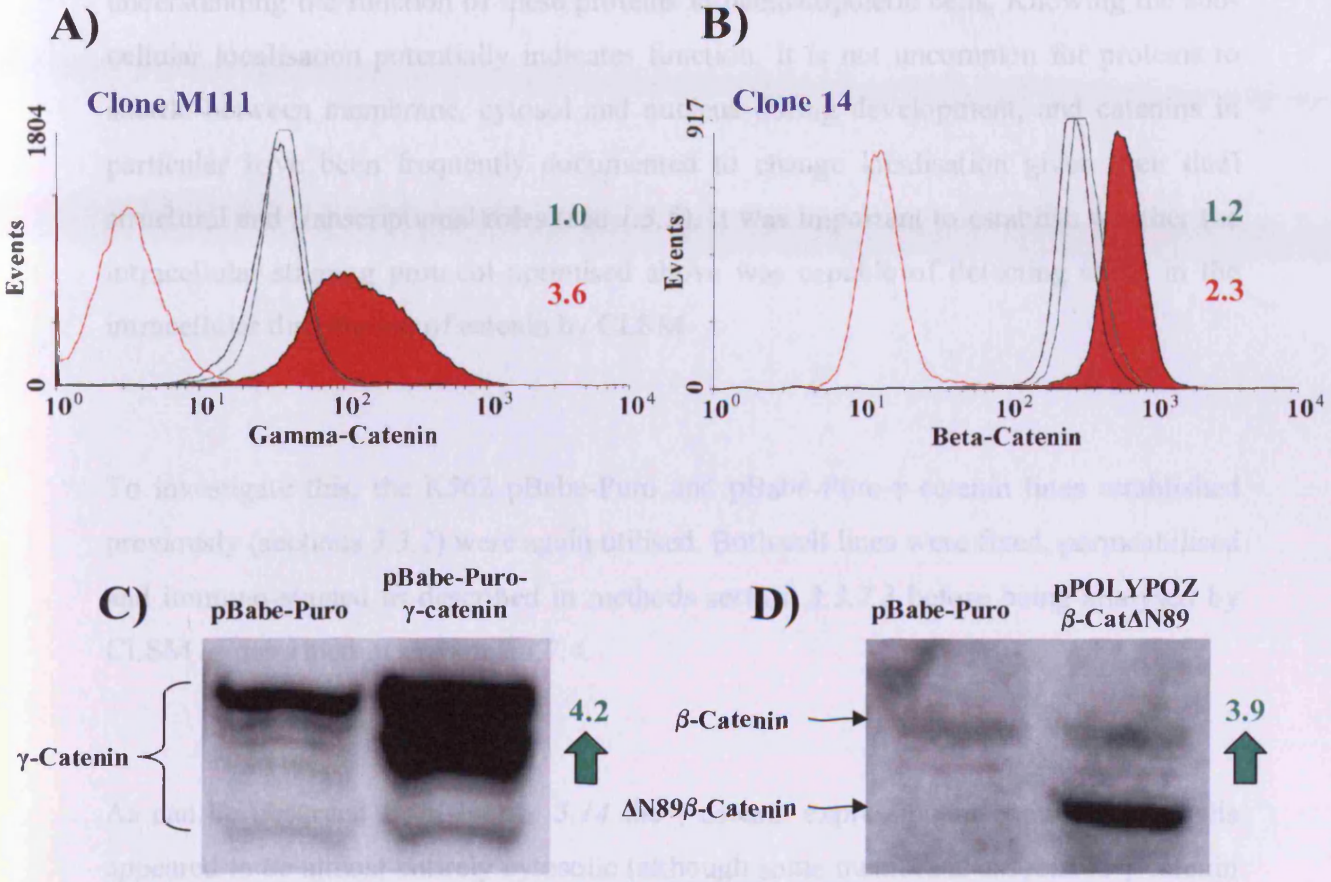


Figure 3.13 - The degree of cross-reactivity between γ- and β-catenin antibodies.

A) γ-catenin primary antibody (Clone M111) staining of: K562 pBabe-Puro-γ-catenin cells (red filled histogram); pBabe-Puro controls (black); K562 β-CatΔN89β cells (green); isotype-control stained cells (open red histogram). **B)** Reciprocal reactivity of the β-catenin primary antibody (Clone 14): K562 β-CatΔN89 cells (red filled histogram); K562 pBabe-Puro cells (black) K562 pBabe-Puro-γ-catenin cells (green); isotype-control stained cells (open red histogram). Red and green numerals represent the fold overexpression of the respective proteins in each K562 cell lines as given by cytometric analysis. **C)** The degree of γ-catenin and **D)** β-catenin overexpression present in respective K562 cell lines at the time of flow cytometric assessment as determined by Western blotting. Green numerals represent the fold overexpression of catenin relative to the pBabe-Puro control line.

3.4.1.6 *CLSM can detect nuclear translocation of catenin*

The total expression level of γ- and β-catenin would only provide limited scope for understanding the function of these proteins in haematopoietic cells. Knowing the sub-cellular localisation potentially indicates function. It is not uncommon for proteins to shuttle between membrane, cytosol and nucleus during development, and catenins in particular have been frequently documented to change localisation given their dual structural and transcriptional roles (see *1.3.5*). It was important to establish whether the intracellular staining protocol optimised above was capable of detecting shifts in the intracellular distribution of catenin by CLSM.

To investigate this, the K562 pBabe-Puro and pBabe-Puro-γ-catenin lines established previously (sections *3.3.2*) were again utilised. Both cell lines were fixed, permeabilised and immuno-stained as described in methods section *3.3.7.3* before being analysed by CLSM as described in section *3.3.7.4*.

As can be observed from *Figure 3.14* the γ-catenin expression in control K562 cells appeared to be almost entirely cytosolic (although some membrane associated γ-catenin cannot be ruled out). This figure and further examples of γ-catenin localisation in K562 pBabe-Puro controls can be viewed within the ***Chapter 3*** section on the ***supplementary disc*** within ***Folder 1, Fields 1.1-1.3***. Also present in this folder (***Field 1.4***) is a representative image of the level of background fluorescence obtained from K562 cells as determined by the isotype matched control antibody. This image demonstrates that non-specific fluorescence was virtually undetectable.

The analysis of K562 pBabe-Puro-γ-catenin cells, exemplified in *Figure 3.15*, demonstrated a clear increase in fluorescence intensity indicating the expected higher expression of γ-catenin. As observed for control cells, γ-catenin expression was primarily cytoplasmic, however, a greatly increased nuclear signal was frequently observed, an observation not made in control K562 cells. This nuclear fluorescence was confirmed by the fact that TO-PRO-3 (nucleic acid stain) signal was also present in the

same region. This figure and further examples of γ-catenin localisation in K562 pBabe-Puro-γ-catenin cells can be observed on the supplementary disc within ***Fields 2.1-2.5*** located in ***Folder 2***.

These data indicate that CLSM was capable of detecting changes in the level of γ-catenin in distinct subcellular locations.

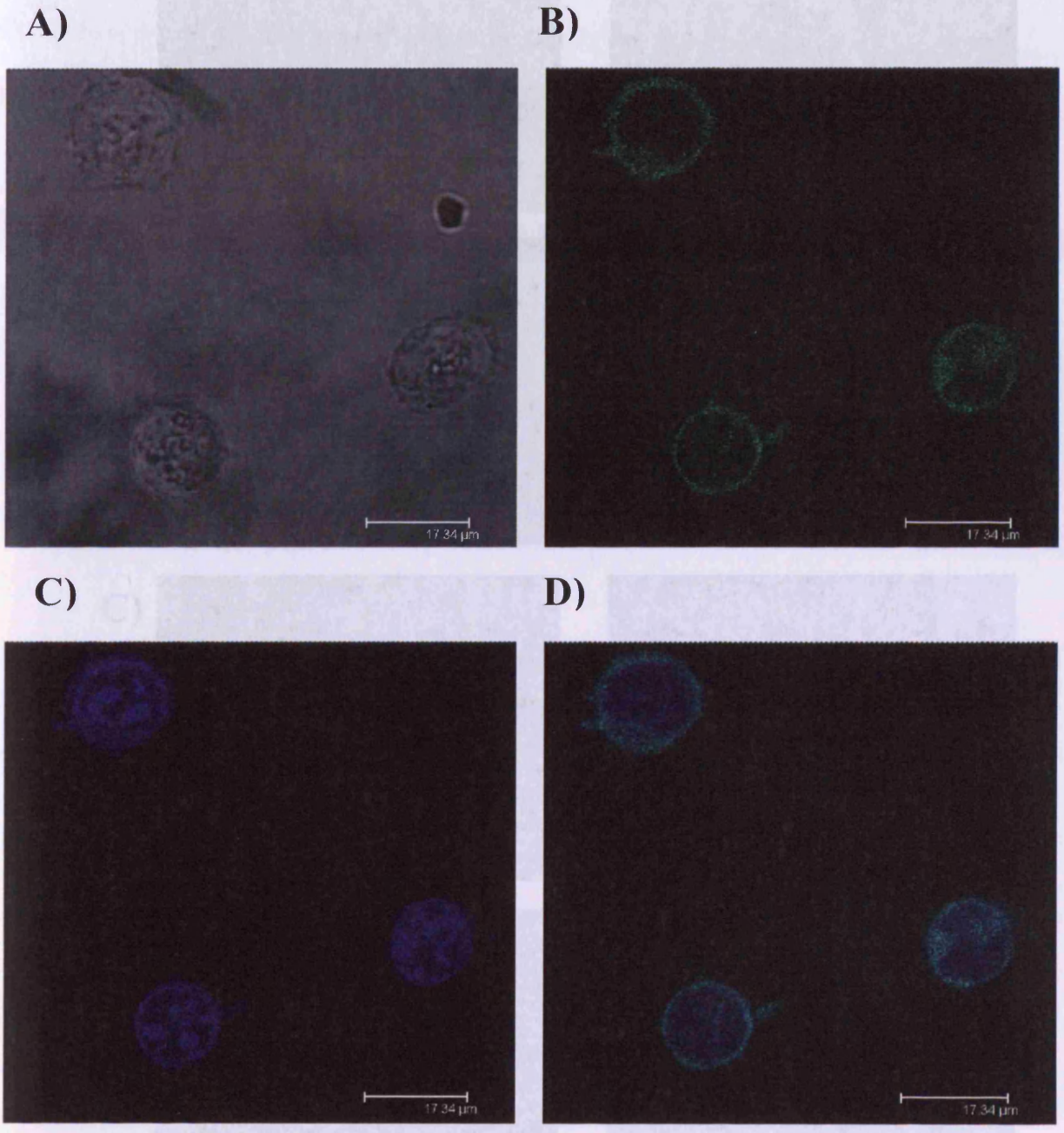


Figure 3.14 - Subcellular localisation of γ-catenin in K562 control cells by CLSM.

Representative confocal Z-sections of K562 pBabe-Puro control cells showing γ-catenin subcellular localisation with **A)** phase contrast, **B)** γ-catenin (green), **C)** nuclear TO-PRO-3 (blue) and **D)** merged images.

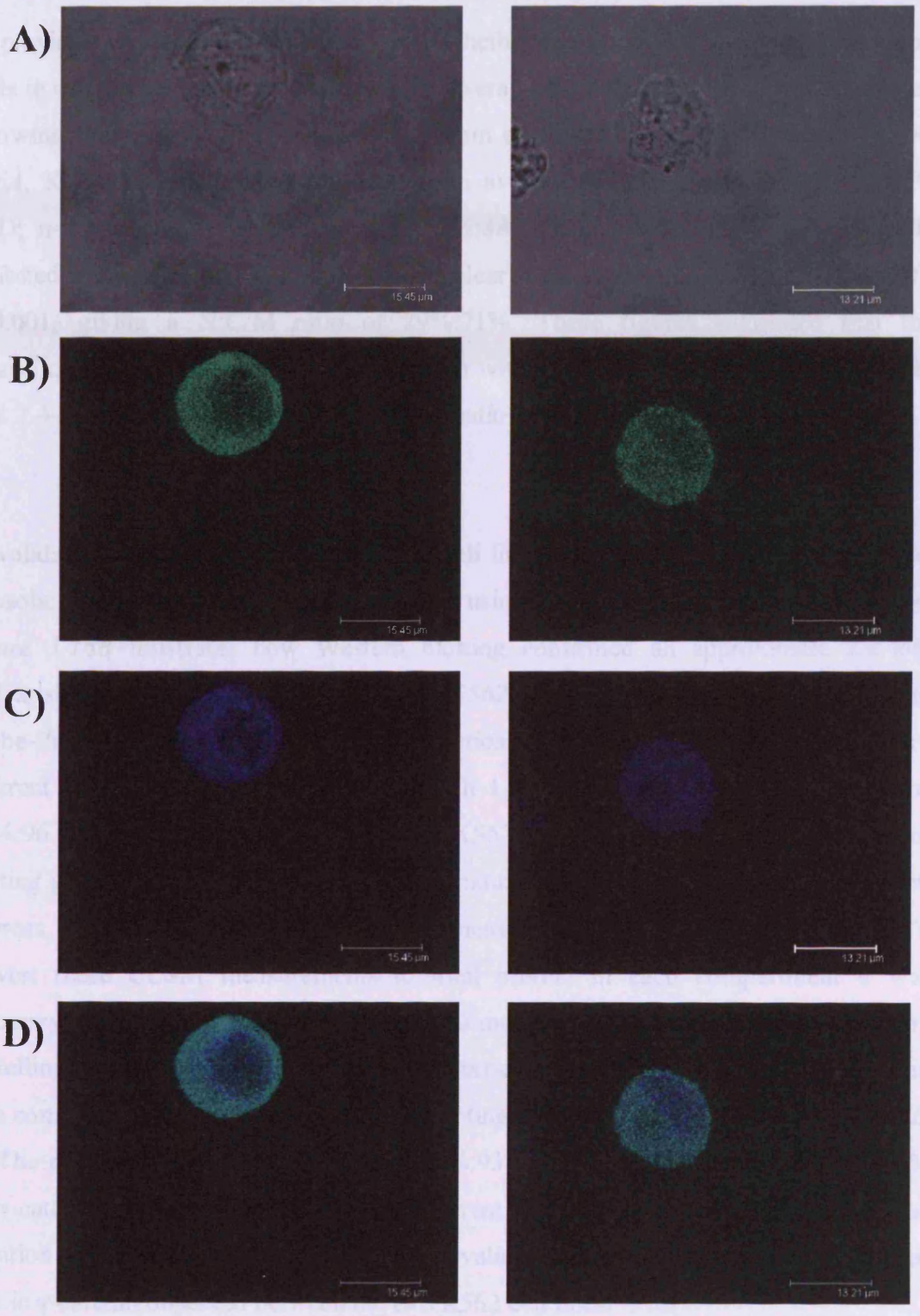


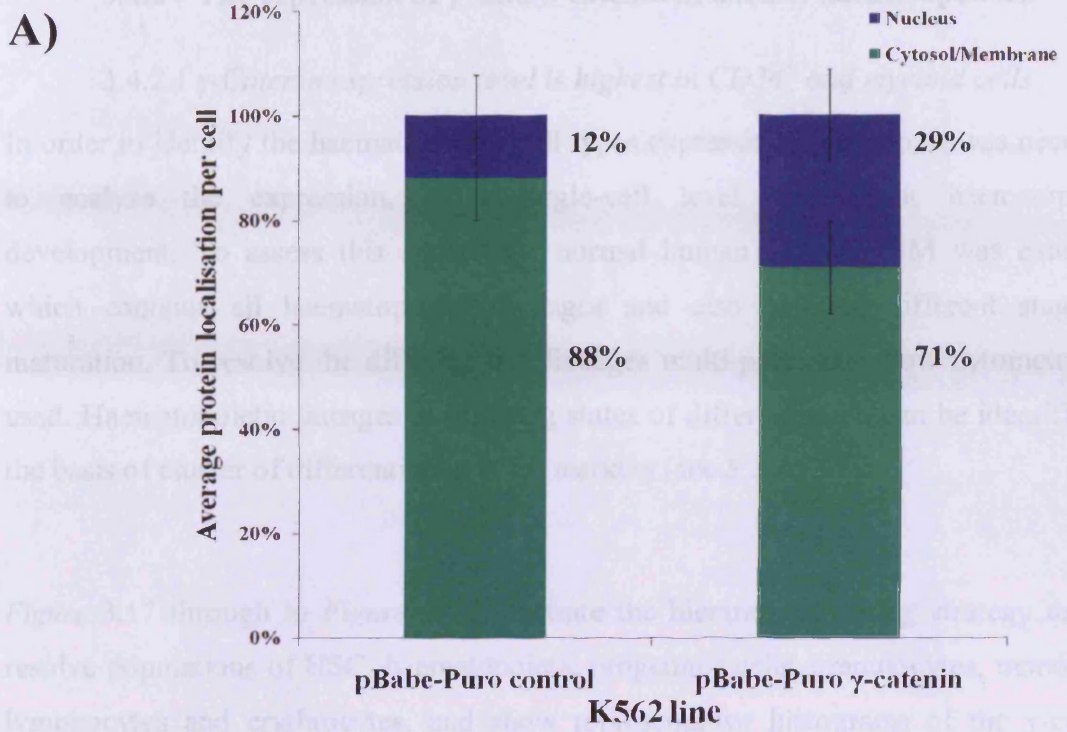
Figure 3.15 - Subcellular localisation of γ-catenin in overexpressing K562 control cells by CLSM.

Representative confocal Z-sections of K562 pBabe-Puro-γ-catenin cells showing γ-catenin subcellular localisation with **A)** phase contrast, **B)** γ-catenin (green), **C)** nuclear TO-PRO-3 (blue) and **D)** merged images.

To provide more objective evidence as to whether the assay was capable of detecting shifts in catenin subcellular localisation, the average N:C/M ratio per cell was calculated following the analysis of individual cells from each K562 line. As viewed in *Figure 3.16A*, K562 pBabe-Puro cells exhibited an average nuclear localisation of 12±8% (1SD; n=50) giving a N:C/M ratio of 12%:88%. K562 pBabe-Puro-γ-catenin cells exhibited a significantly higher average nuclear localisation of 29±9% (1SD; n=50), $P<0.001$, giving a N:C/M ratio of 29%:71%. These figures suggested that the intracellular staining protocol in combination with CLSM was capable of detecting at least 2.4-fold shifts in nuclear catenin translocation.

To validate the above findings, both K562 cell lines were fractionated into nuclear and cytosolic homogenate and Western blotted using methods described in section *2.6*. *Figure 3.16B* illustrates how Western blotting confirmed an approximate 2.2-fold nuclear shift in γ-catenin localisation within K562 pBabe-Puro-γ-catenin cells relative to pBabe-Puro controls. The apparent N:C/M ratios obtained from this method were very different to that obtained from CLSM, with 1.6%:98.4% for control K562s versus 3.5%:96.5% for γ-catenin overexpressing K562 cells. This arose because Western blotting gave rise to a ratio based on total amounts of γ-catenin in each compartment, whereas CLSM compared area-intensity measurements in each compartment. To convert these CLSM measurements to total protein in each compartment it was necessary to take into account the relative volumes of each compartment. Therefore, by modelling individual K562 cells as two concentric (or eccentric) spheres, total protein in each compartment could be estimated by adapting the volume of a sphere (see *Appendix 5*). The resulting CLSM N:C/M ratios (6.5%:93.5% for controls versus 15.0%:85.0% for γ-catenin overexpressing) remained different to Western blot values (although the variation had approximately halved), but still validated the approximate 2.2-fold nuclear shift in γ-catenin observed between the two K562 cell lines.

Taken together, these data confirm that the intracellular catenin detection assay could be used in conjunction with CLSM to detect shifts in catenin subcellular localisation within haematopoietic cells.



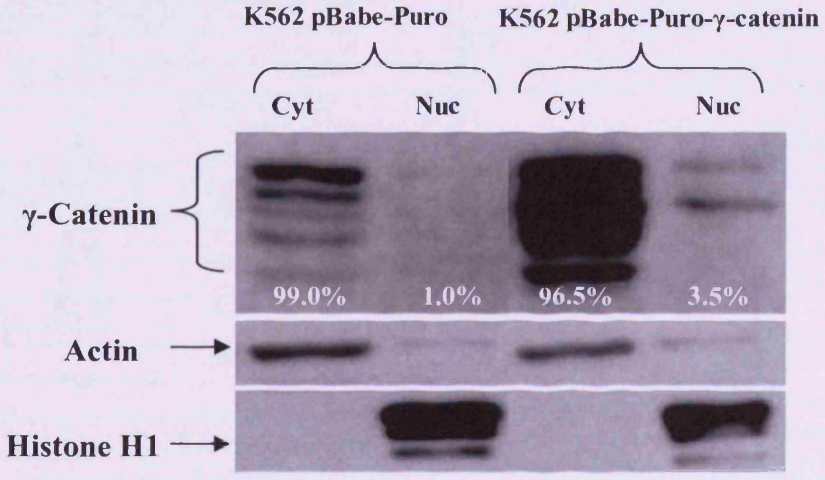


Figure 3.16 - Direct comparison of CLSM and Western blotting techniques in the detection of subcellular γ-catenin.

A) The average localisation of γ-catenin protein per K562 cell summarised from the analysis of cells (n=50) from control and γ-catenin overexpressing K562 cells, $*P<0.001$. Black numerals give the percentage localisation of γ-catenin within that fraction relative to the total γ-catenin present. Error bars represent SD from each data set. **B)** Western blot demonstrates γ-catenin protein present in nuclear and cytosolic lysates from control and γ-catenin overexpressing K562 cells. White numerals represent the percentage of γ-catenin contained within each fraction relative to the total amount of γ-catenin detected. Probing for actin and histone H1 confirmed the purity of each fraction.

3.4.2 The expression of γ- and β-catenin in normal haematopoiesis

3.4.2.1 *γ-Catenin expression level is highest in CD34$^+$ and myeloid cells*

In order to identify the haematopoietic cell types expressing γ-catenin, it was necessary to analyse the expression, at a single-cell level, throughout haematopoietic development. To assess this accurately, normal human CB and BM was examined which contains all haematopoietic lineages and also cells at different stages of maturation. To resolve the different cell lineages multi-parameter flow cytometry was used. Haematopoietic lineages at differing states of differentiation can be identified on the basis of cluster of differentiation (CD) markers (see *5.3.1.1*).

Figure 3.17 through to *Figure 3.20* illustrate the hierarchical gating strategy used to resolve populations of HSC, haematopoietic progenitor cells, granulocytes, monocytes, lymphocytes and erythrocytes, and show representative histograms of the γ-catenin expression detected from each lineage.

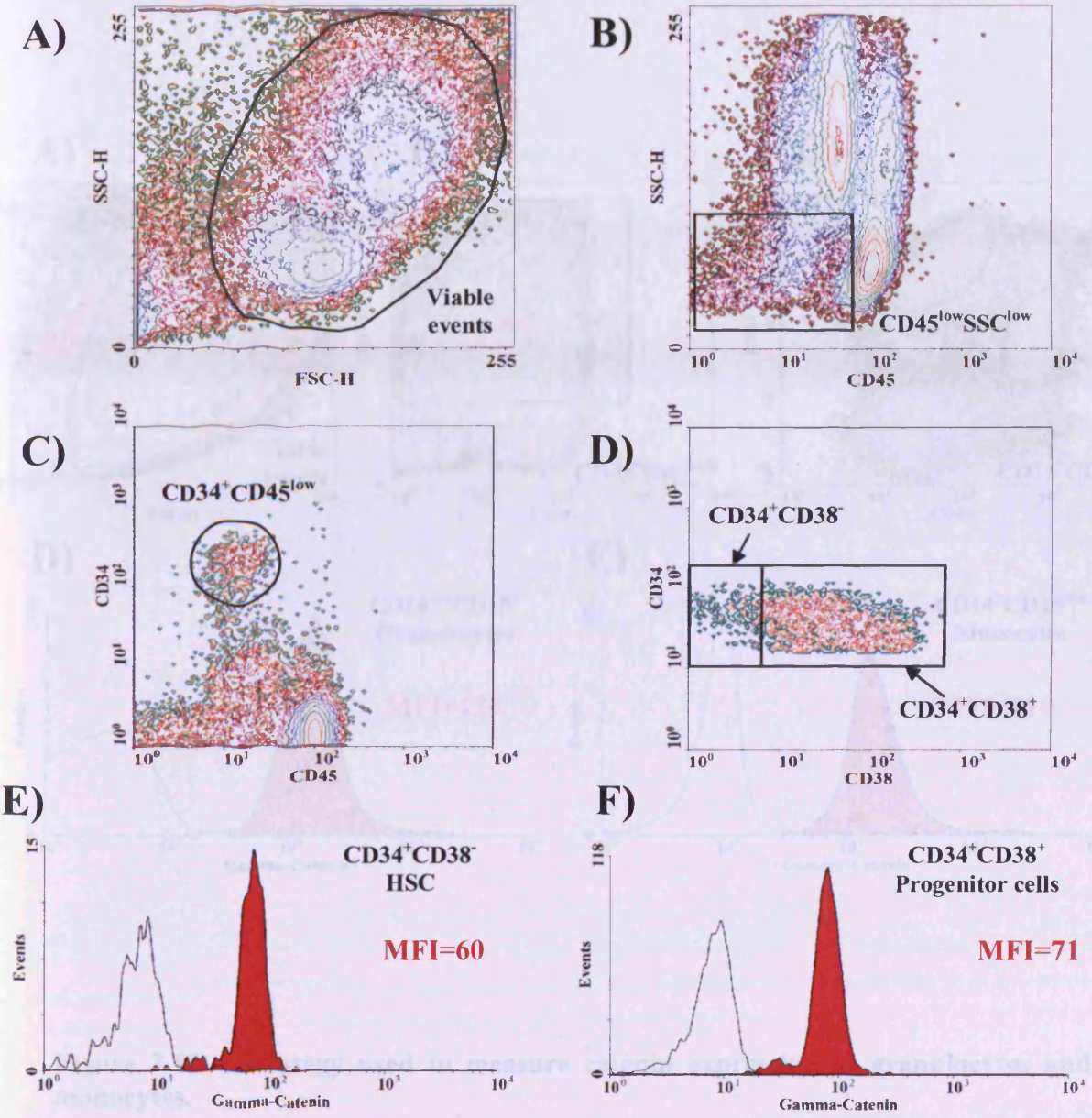


Figure 3.17 - Strategy used to measure catenin expression in haematopoietic stem cells and progenitor cells.

Representative flow cytometric plots showing the sequential strategy used to gate and measure catenin in haematopoietic stem cells and progenitor cells. **A)** Firstly, red cells, debris and cell aggregates were excluded using FSC versus SSC parameters. **B)** High SSC and $CD45^{bright}$ cells were excluded from analysis followed by **C)** gating on $CD34^{+}CD45^{low}$ events. These events were further re-gated on FSC and SSC parameters to exclude remaining debris and doublets (not shown). **D)** $CD34^{+}$ events were divided into $CD38^{+/-}$ using a threshold obtained from isotype staining for CD38 on the $CD34^{+}$ population. Representative histograms showing γ-catenin (red filled) and isotype (open black) staining obtained from **E)** $CD34^{+}CD38^{-}$ HSCs and **F)** $CD34^{+}CD38^{+}$ progenitor cells. Red numerals indicate the representative MFI of γ-catenin staining obtained for the indicated subset.

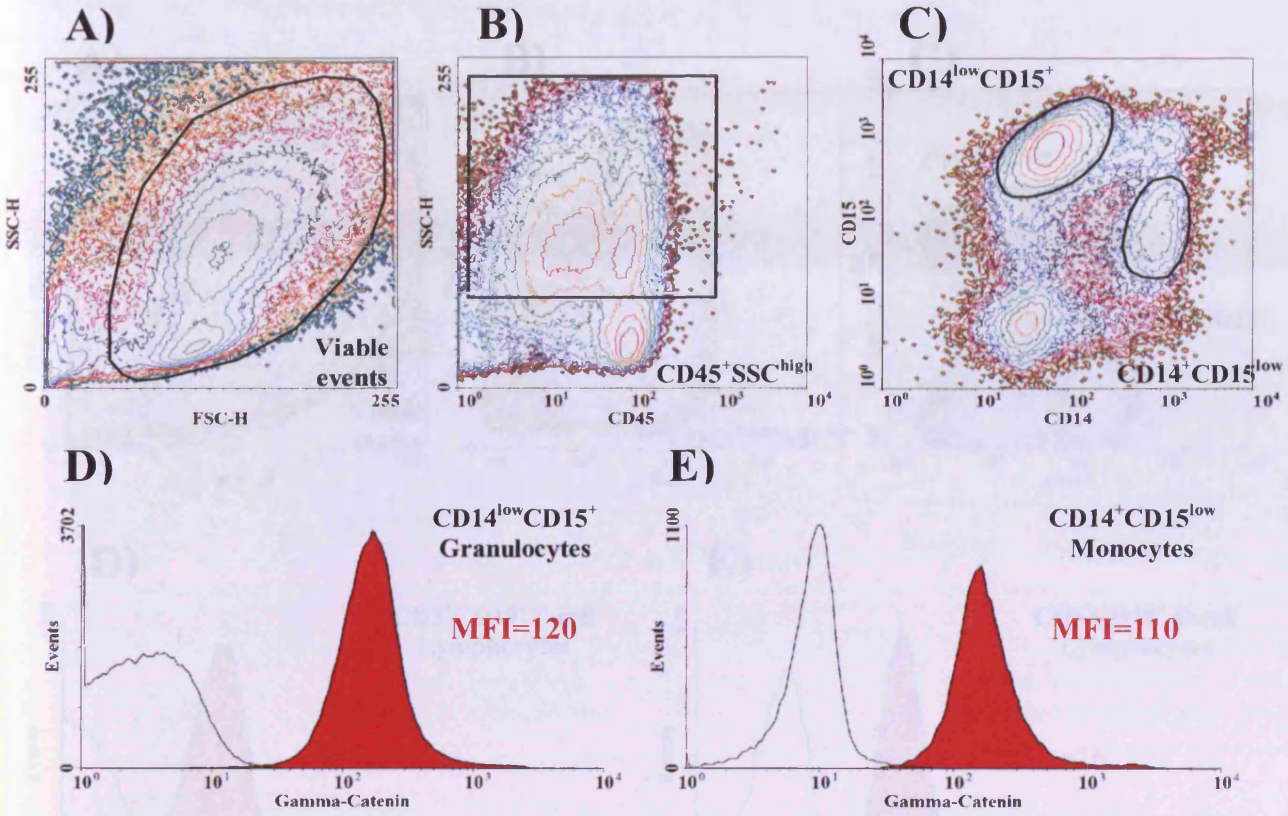


Figure 3.18 - Strategy used to measure catenin expression in granulocytes and monocytes.

Representative flow cytometric plots showing the sequential strategy used to measure catenin in granulocytic and monocytic cells. **A)** Firstly, red cells, debris and cell aggregates were excluded using FSC versus SSC parameters. **B)** Gating of $CD45^+$ cells with high side scatter allowed identification of granular myeloid cells, which were re-gated on a **C)** CD14 versus CD15 plot to allow the resolution of granulocytes ($CD14^{low}CD15^+$) and monocytes ($CD14^+CD15^{low}$). Representative histograms showing γ-catenin and isotype staining obtained from **D)** granulocytes and **E)** monocytes. Red numerals indicate the representative MFI of γ-catenin staining obtained for each subset.

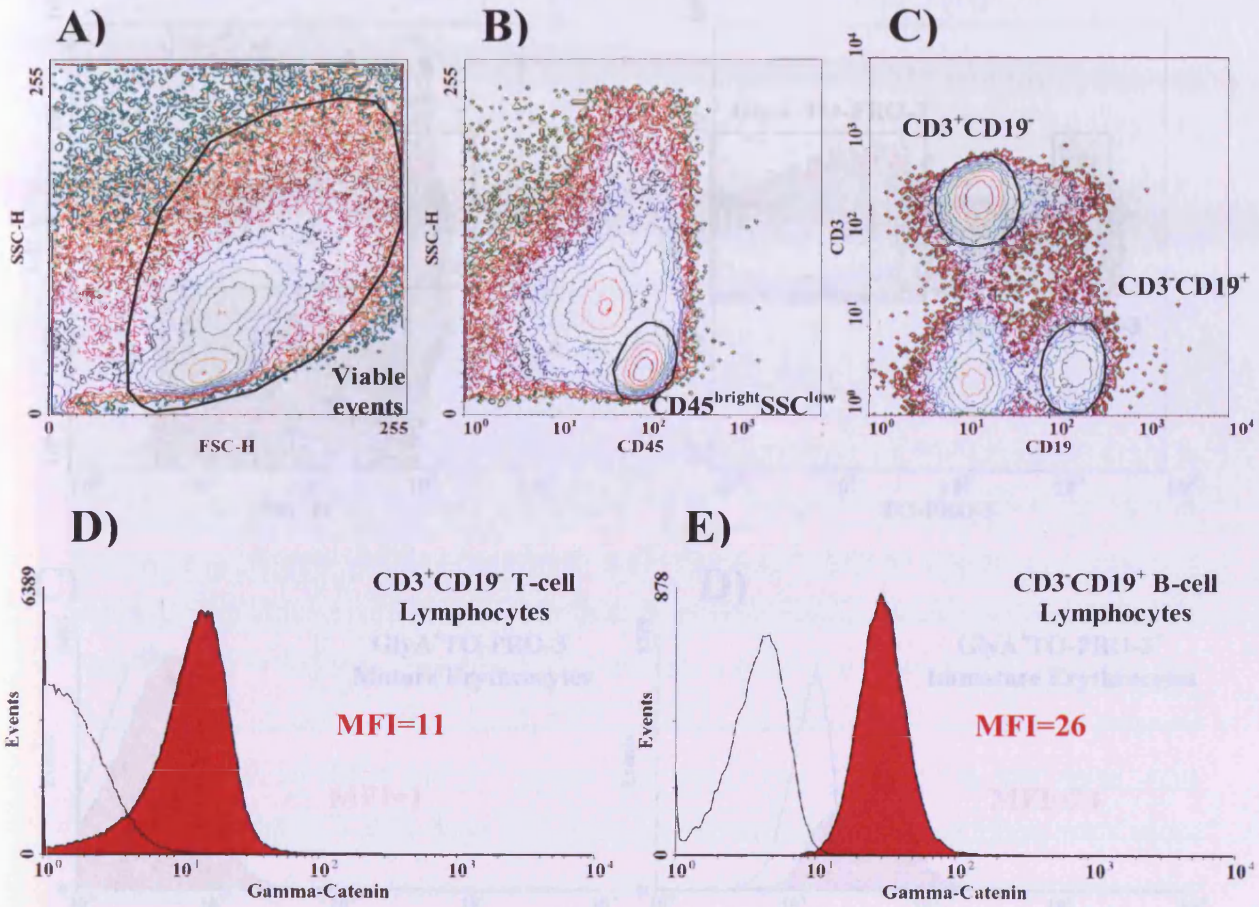


Figure 3.19 - Strategy used to measure catenin expression in lymphocytes.

Representative flow cytometric plots showing the sequential strategy used to measure catenin in lymphoid cells. **A)** Firstly, red cells, debris and cell aggregates were excluded using FSC versus SSC parameters. **B)** Gating on low side scatter $CD45^{bright}$ cells allowed identification of the total lymphocyte pool. **C)** CD3 versus CD19 plot allowed the separation of T-cells ($CD3^{+}CD19^{-}$) and B-cells ($CD3^{-}CD19^{+}$). Representative histograms showing γ-catenin and isotype staining obtained from **D)** T-cells and **E)** B-cells. Red numerals indicate the representative MFI of γ-catenin staining obtained for each subset.

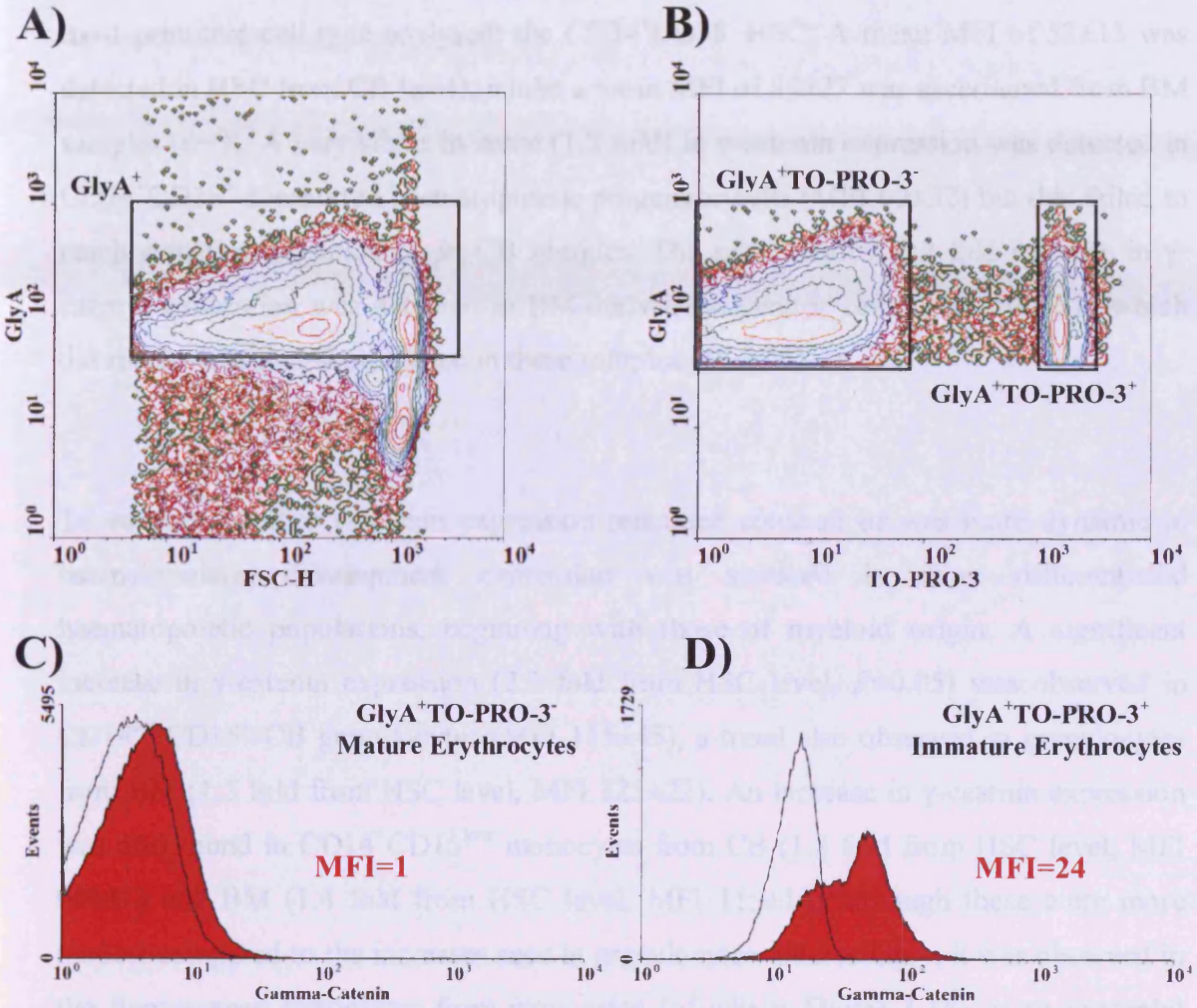


Figure 3.20 - Strategy used to measure catenin expression in erythrocytes.

Representative flow cytometric plots showing the sequential strategy used to measure catenin in erythroid cells. **A)** Erythroid cells were identified, and debris excluded, by gating on $GlyA^+$ events. **B)** Erythroid cells were further resolved into nucleated red cells ($GlyA^+TO\text{-}PRO\text{-}3^+$) and enuclueated red cells ($GlyA^+TO\text{-}PRO\text{-}3^-$). Representative histograms showing γ-catenin and isotype staining obtained from **C)** enucleated erythrocytes and **D)** nucleated erythrocytes. Red numerals indicate the representative MFI of γ-catenin staining obtained for each subset.

The relative expression level of γ-catenin detected in haematopoietic cells of normal human CB and BM is summarised in *Figure 3.21*. γ-Catenin expression was high in the most primitive cell type analysed; the $CD34^+CD38^-$ HSC. A mean MFI of 52±13 was detected in HSC from CB (n=4), whilst a mean MFI of 84±27 was ascertained from BM samples (n=3). A very slight increase (1.2 fold) in γ-catenin expression was detected in $CD34^+CD38^+$ committed haematopoietic progenitor cells (MFI 60±32) but this failed to reach statistical significance in CB samples. The same modest 1.2 fold increase in γ-catenin expression was detected in BM-derived progenitor cells (MFI 100±31) which did reach statistical significance in these samples ($P<0.05$).

To address whether γ-catenin expression remained constant or was more dynamic in haematopoietic development expression was assessed in more differentiated haematopoietic populations, beginning with those of myeloid origin. A significant increase in γ-catenin expression (2.2 fold from HSC level, $P<0.05$) was observed in $CD14^{low}CD15^+$ CB granulocytes (MFI 115±45), a trend also observed in granulocytes from BM (1.5 fold from HSC level, MFI 125±22). An increase in γ-catenin expression was also found in $CD14^+CD15^{low}$ monocytes from CB (1.8 fold from HSC level, MFI 90±51) and BM (1.4 fold from HSC level, MFI 115±12) although these were more modest compared to the increases seen in granulocytes. Interestingly, it was observed in the fluorescence histograms from monocytes (of which *Figure 3.18E* is an example) that a distinct 'shoulder' of higher γ-catenin expression was frequently present, the significance of which is raised later in this chapter.

Cells of lymphoid origin were also included in the analysis and generally demonstrated a low expression of γ-catenin. $CD3^+CD19^-$ T-cell lymphocytes exhibited a significant down-regulation of γ-catenin from HSCs both in CB (0.5 fold, MFI 27±18, $P<0.05$) and BM samples (0.4 fold, MFI 38±14, $P<0.05$). $CD3^-CD19^+$ B-cell lymphocytes also demonstrated lower levels of γ-catenin expression compared to HSCs, with a 0.8 fold reduction in CB B-cells (MFI 44±21) and significantly lower 0.5 fold reduction in BM B-cells (MFI 43±14, $P<0.05$). Interestingly also, B-cells had consistently higher levels of γ-catenin expression than T-cells in both CB ($P<0.01$) and BM ($P<0.05$).

Finally, erythroid cells were also analysed using a modified permeabilisation method as described in section *3.3.5.4*. Using this technique, mature erythrocytes ($GlyA^+TO\text{-}PRO\text{-}3^-$) demonstrated virtually no γ-catenin expression from CB (MFI 1±0.6, $P<0.01$) or BM (MFI 1±0.6, $P<0.05$). Unfortunately, even this adapted protocol was unable to consistently preserve the immature red cell population ($GlyA^+TO\text{-}PRO\text{-}3^+$) as illustrated in *Figure 3.20A* and *B*. From a few examples where analyses were possible, it appeared that γ-catenin expression was present within this immature subset *al*beit at low levels (MFI=30±8, n=2 for CB, MFI=28±6, n=2 for BM).

Taken together, these data present a clear expression profile for γ-catenin throughout haematopoietic development. Expression starts relatively high in primitive HSC and committed progenitor cells, before increasing for subsequent granulocytic and monocytic differentiation. Expression is lower in both types of lymphocytes, with B-cells harbouring slightly higher levels than T-cells, and expression is altogether lost in mature red cells.

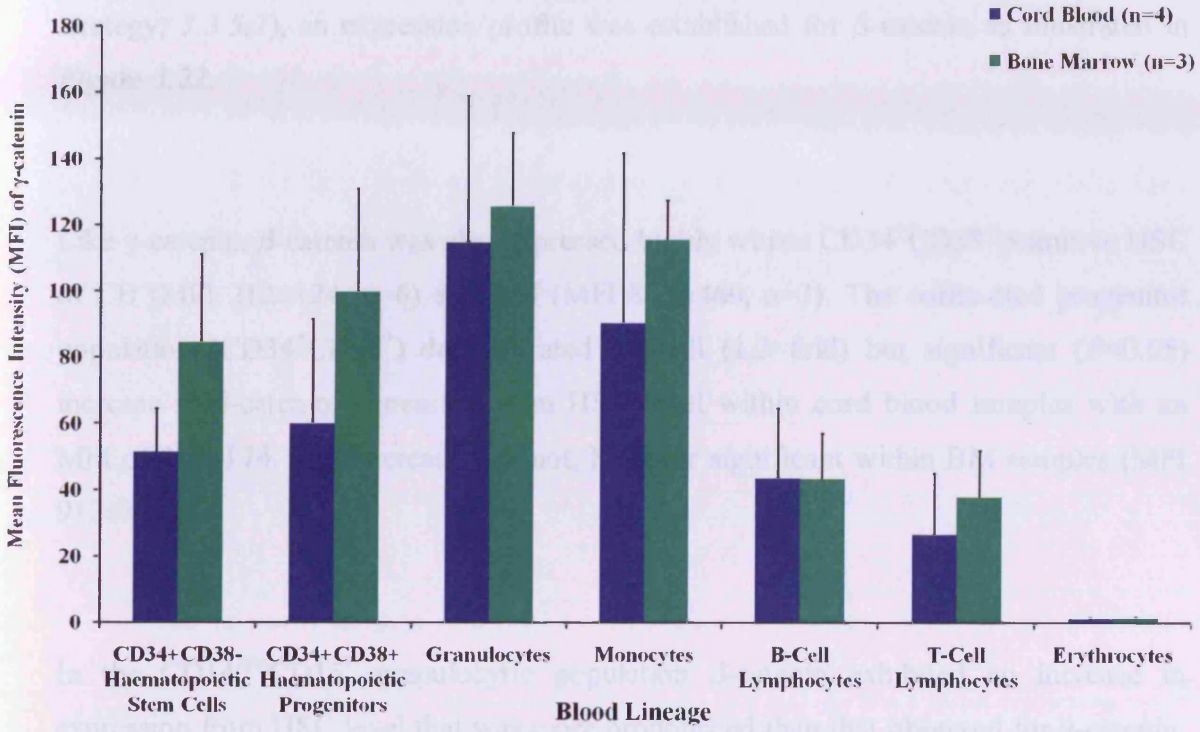


Figure 3.21 - The relative expression level of γ-catenin throughout normal haematopoiesis.

The expression level of γ-catenin within discrete developmental subsets of normal haematopoietic development as determined by the average MFI obtained from normal cord blood (blue bars, n=4) and bone marrow (green bars, n=3) samples. Data represents mean ± 1SD obtained from each data set.

3.4.2.2 *β-Catenin expression level is similar to that of γ-catenin in normal haematopoiesis*

To establish whether γ-catenin has distinct or similar expression in haematopoiesis to its close homologue β-catenin, a direct comparison was performed. Using the same protocol as for γ-catenin analysis, (but incorporating a slightly different pre-labelling strategy; *3.3.5.2*), an expression profile was established for β-catenin as illustrated in *Figure 3.22*.

Like γ-catenin, β-catenin was also expressed highly within $CD34^+CD38^-$ primitive HSC of CB (MFI 212±124, n=6) and BM (MFI 873±460, n=3). The committed progenitor population ($CD34^+CD38^+$) demonstrated a small (1.3 fold) but significant ($P<0.05$) increase in β-catenin expression from HSC level within cord blood samples with an MFI of 279±174. This increase was not, however significant within BM samples (MFI 912±327).

In the $CD14^{low}CD15^+$ granulocytic population β-catenin exhibited an increase in expression from HSC level that was more pronounced than that observed for γ-catenin. In CB granulocytes a 4.2 fold increase in expression was observed relative to HSC level (MFI 1182±568, $P<0.05$), whilst BM derived granulocytes exhibited a 3.3 fold up-regulation of β-catenin (MFI 3042±635, $P<0.05$). Unlike γ-catenin, β-catenin did not demonstrate a significantly increased expression level within the $CD14^+CD15^{low}$ monocyte population compared with $CD34^+$ progenitor cells.

A significant down-regulation of β-catenin from HSC level was present in $CD3^+CD19^-$ T-cell lymphocytes from both CB (0.3 fold, MFI 56±34, $P<0.05$) and BM (0.3 fold MFI 297±74, $P<0.05$). B-cells also consistently harboured a significantly higher level of β-catenin expression than T-cells in both CB ($P<0.05$) and BM ($P<0.05$), an observation also previously made for γ-catenin.

Finally, analysis of mature erythrocytes ($GlyA^+TO\text{-}PRO\text{-}3^-$) confirmed that, like γ-catenin, β-catenin expression was completely absent in this terminally differentiated subset (MFI 9±5, $P<0.05$ in CB and MFI 25±19, $P<0.05$ for BM). As mentioned previously, analysis of immature ($GlyA^+TO\text{-}PRO\text{-}3^+$) red cell populations was problematic, however a few analyses suggested that β-catenin expression was present within this subset, although at low levels (MFI 47±15, n=3 for CB).

In summary, these data imply that β-catenin assumes a very similar expression profile to that of γ-catenin throughout haematopoietic development. The only differences being a more marked increase in expression within the granulocytic population and the lack of any increased in expression associated with monocyte maturation.

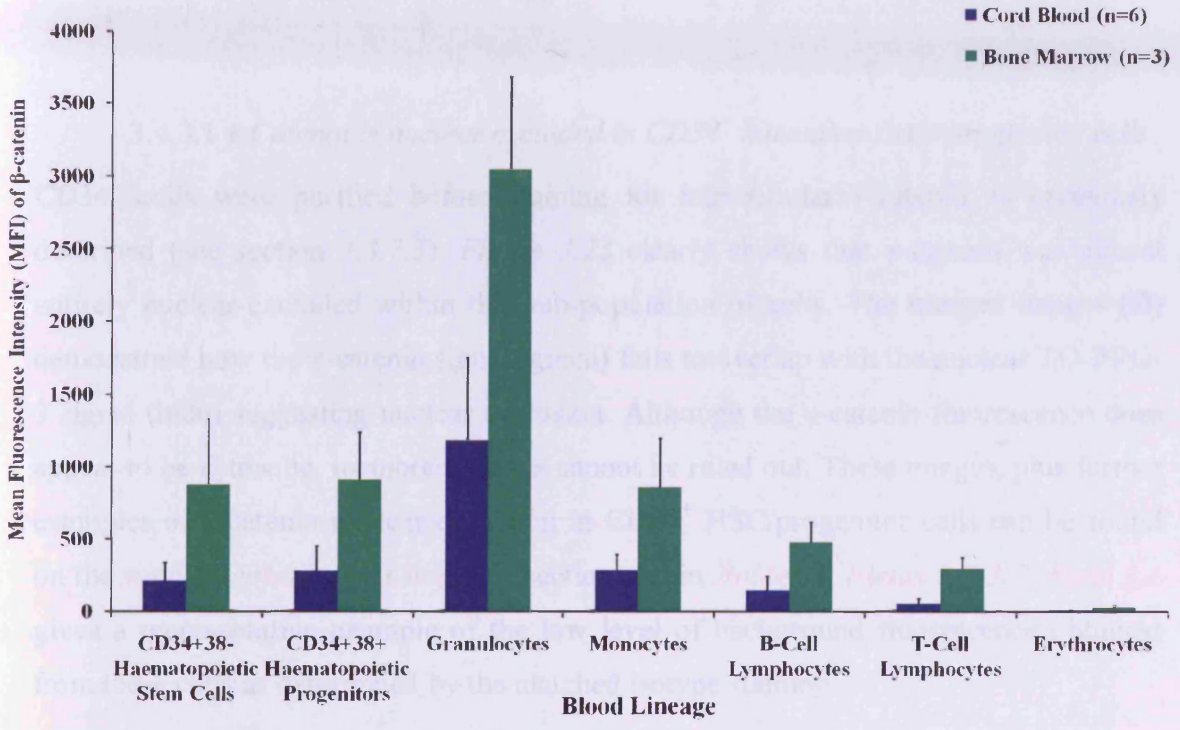


Figure 3.22 - The relative expression level of β-catenin throughout normal haematopoiesis.

The expression level of β-catenin in discrete developmental subsets of normal haematopoietic development as determined by the average MFI obtained from normal CB (blue bars, n=6) and bone marrow (green bars, n=3) samples. Data represents mean ± 1SD obtained from each data set.

3.4.3 The subcellular localisation of γ- and β-catenin in haematopoietic cells

The expression profile of γ- and β-catenin in haematopoietic development has shown expression of both proteins to be highest in HSC/progenitor, granulocyte and monocyte populations. Therefore these subsets were analysed for the subcellular localisation of expression using CLSM.

3.4.3.1 *γ-Catenin is nuclear excluded in CD34$^+$ haematopoietic progenitor cells*

CD34$^+$ cells were purified before staining for intracellular γ-catenin as previously described (see section *3.3.7.3*). *Figure 3.23* clearly shows that γ-catenin was almost entirely nuclear-excluded within this sub-population of cells. The merged images (**D**) demonstrate how the γ-catenin signal (green) fails to overlap with the nuclear TO-PRO-3 signal (blue) suggesting nuclear exclusion. Although the γ-catenin fluorescence does appear to be cytosolic, membrane forms cannot be ruled out. These images, plus further examples of γ-catenin nuclear exclusion in CD34$^+$ HSC/progenitor cells can be found on the ***supplementary disc Chapter 3*** section within ***Folder 3***, ***Fields 3.2-3.7***. ***Field 3.1*** gives a representative example of the low level of background fluorescence obtained from these cells as determined by the matched isotype staining.

Figure 3.24 consists of a Z-stack series whereby multiple Z-planes of a CD34$^+$ cell captured by CLSM are compiled to provide a cross-section through a single cell. This figure can be found in movie form on the ***supplementary disc*** as ***Z-Stack 3.1*** within ***Folder 3***. It illustrates clearly how γ-catenin is nuclear excluded throughout the entirety of a CD34$^+$ cell and not just on a given single plane.

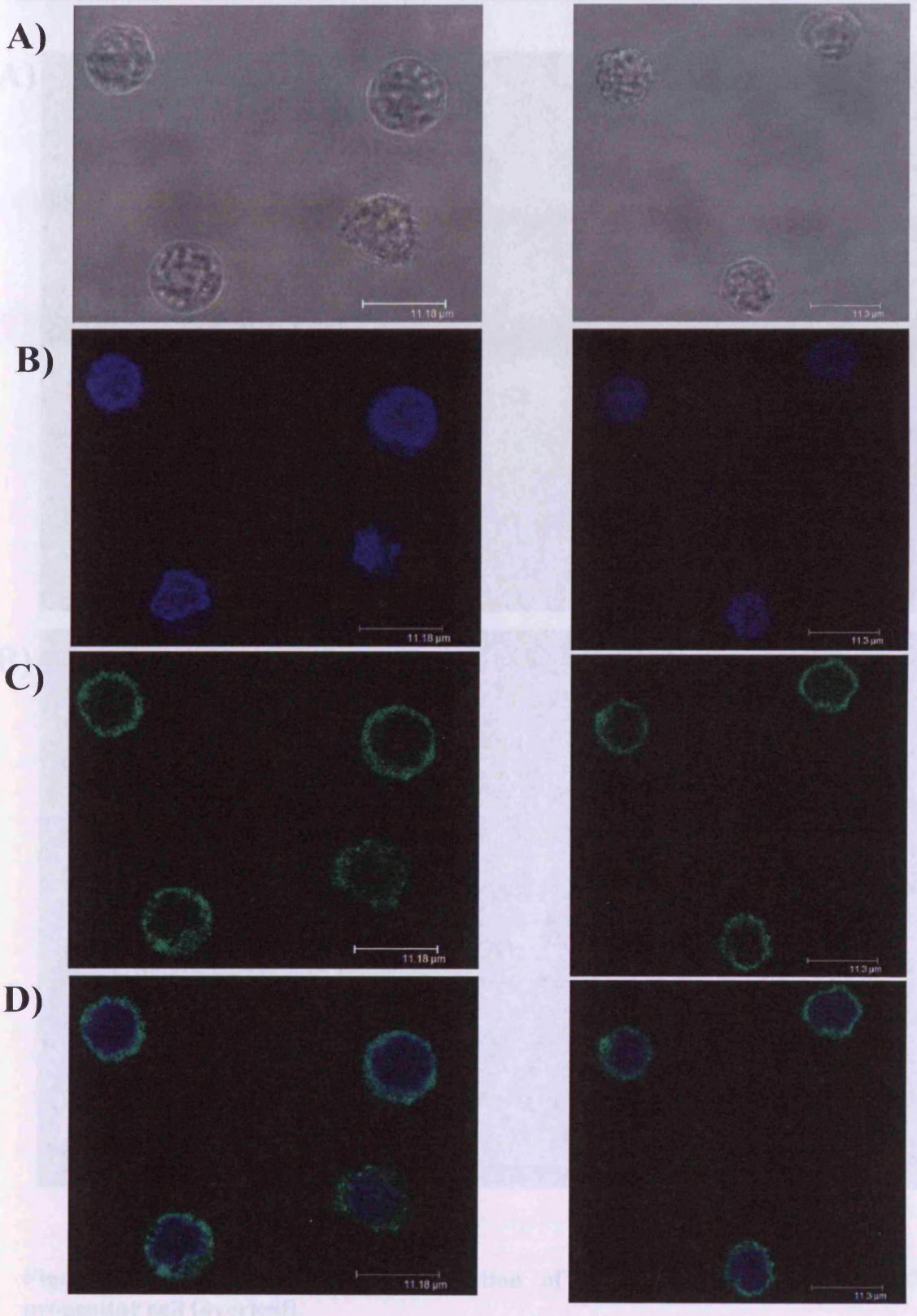


Figure 3.23 - The subcellular localisation of γ-catenin expression in CD34$^+$ progenitor cells.

Pair of representative CLSM images of γ-catenin localisation in CD34$^+$ haematopoietic progenitor cells with **A)** phase contrast, **B)** nuclear (blue), **C)** γ-catenin (green), and **D)** merged images.

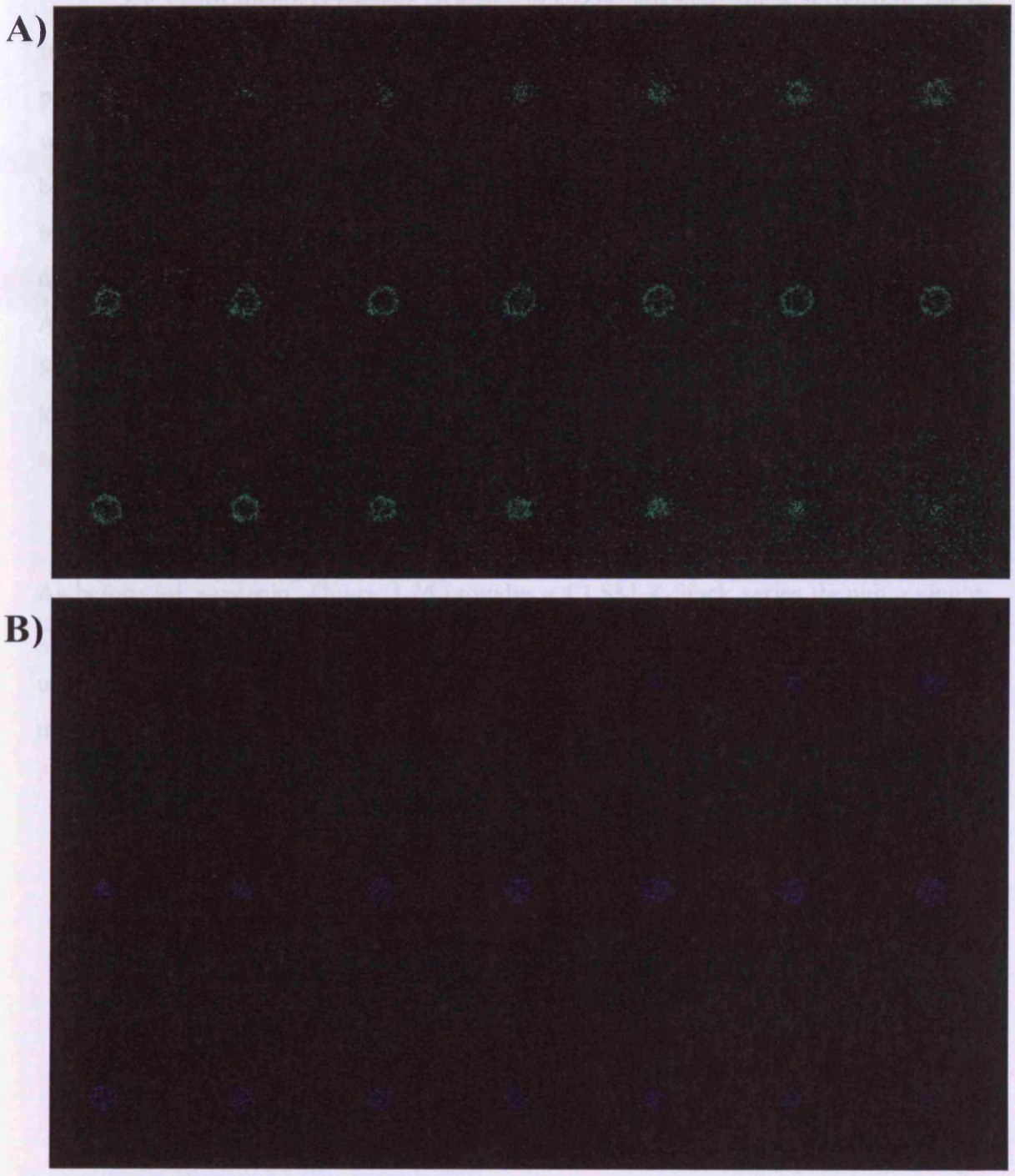


Figure 3.24 - The subcellular localisation of γ-catenin throughout a CD34$^+$ progenitor cell (overleaf).

Representative CLSM Z-stack series showing **A)** γ-catenin localisation throughout an entire CD34$^+$ progenitor cell, and the **B)** identified nuclear region as given by TO-PRO-3 staining.

3.4.3.2 *β-Catenin is nuclear localised in CD34$^+$ haematopoietic progenitor cells*

In contrast to γ-catenin, *Figure 3.25* illustrates how β-catenin expression was predominantly nuclear localised within CD34$^+$ cells. This is particularly emphasised when viewing the merged images (**D**) where there is a considerable degree of overlap between the (yellow) β-catenin signal and (blue) nuclear region. Interestingly, mid-field in *Figure 3.25*, there appeared to be a CD34$^+$ cell in the final stages of cell division (two nuclei sharing a single cytoplasm) where the β-catenin remained mostly nuclear. Another interesting observation seen also in *Figure 3.25* was the presence of multiple foci of intense β-catenin staining. These images, plus further examples of β-catenin localisation in CD34$^+$ cells can be browsed in ***Fields 4.2-4.5*** of ***Folder 4*** on the ***supplementary disc*** within the ***Chapter 3*** section.

As before for γ-catenin, *Figure 3.26* provides a CLSM Z-Stack series though a single CD34$^+$ HSC/progenitor, which clearly demonstrates that β-catenin distribution is ubiquitous through the nucleus of the cell and not just localised on a single Z-plane. A movie form of this Z-Stack series and others can be viewed in ***Z-Stacks 4.1-4.3*** of ***Folder 4*** on the ***supplementary disc***.

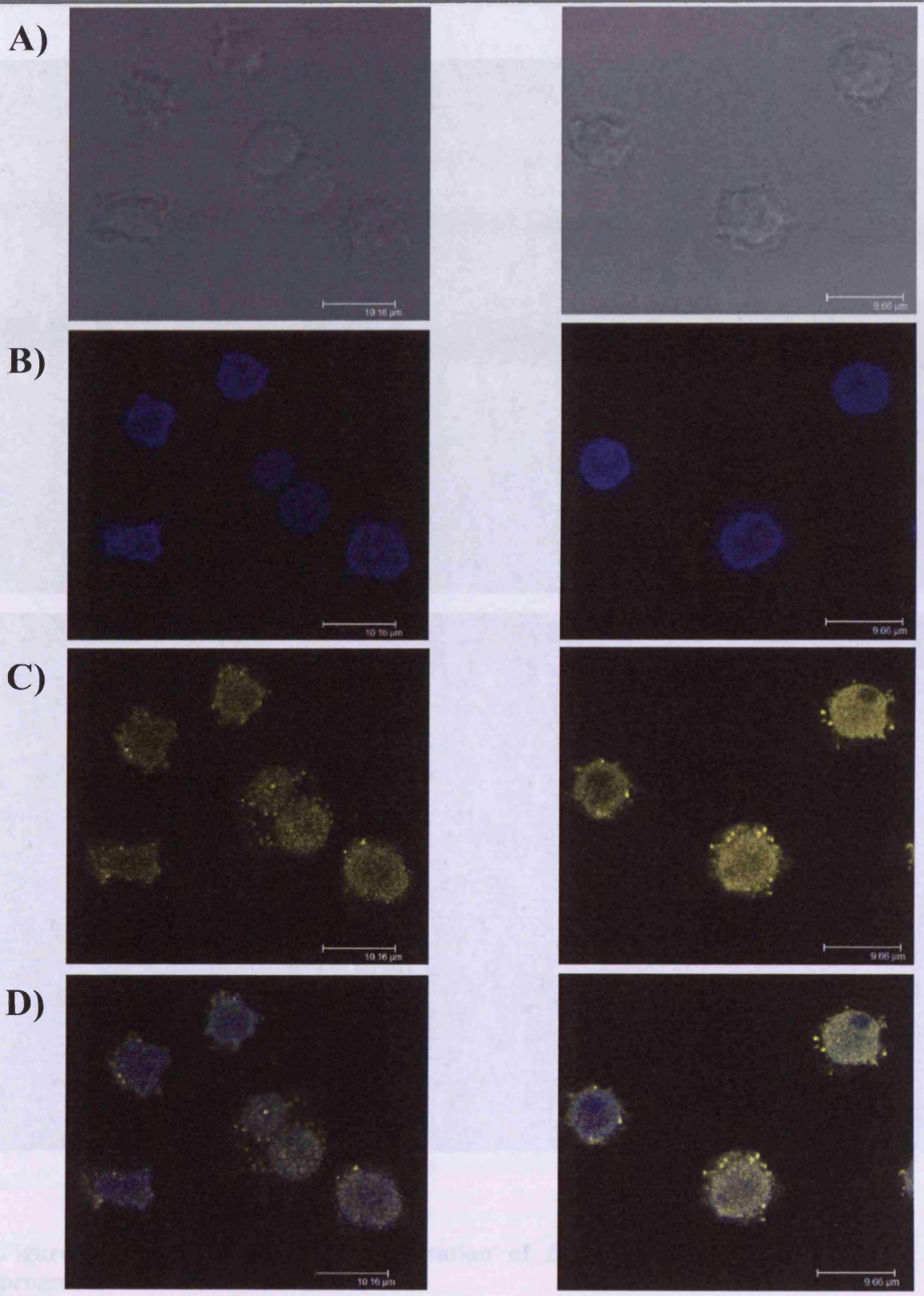


Figure 3.25 - The subcellular localisation of β-catenin expression in $CD34^+$ progenitor cells.

Pairs of representative CLSM images of β-catenin localisation in $CD34^+$ haematopoietic progenitor cells with **A)** phase contrast, **B)** nuclear (blue), **C)** β-catenin (yellow), and **D)** merged images.

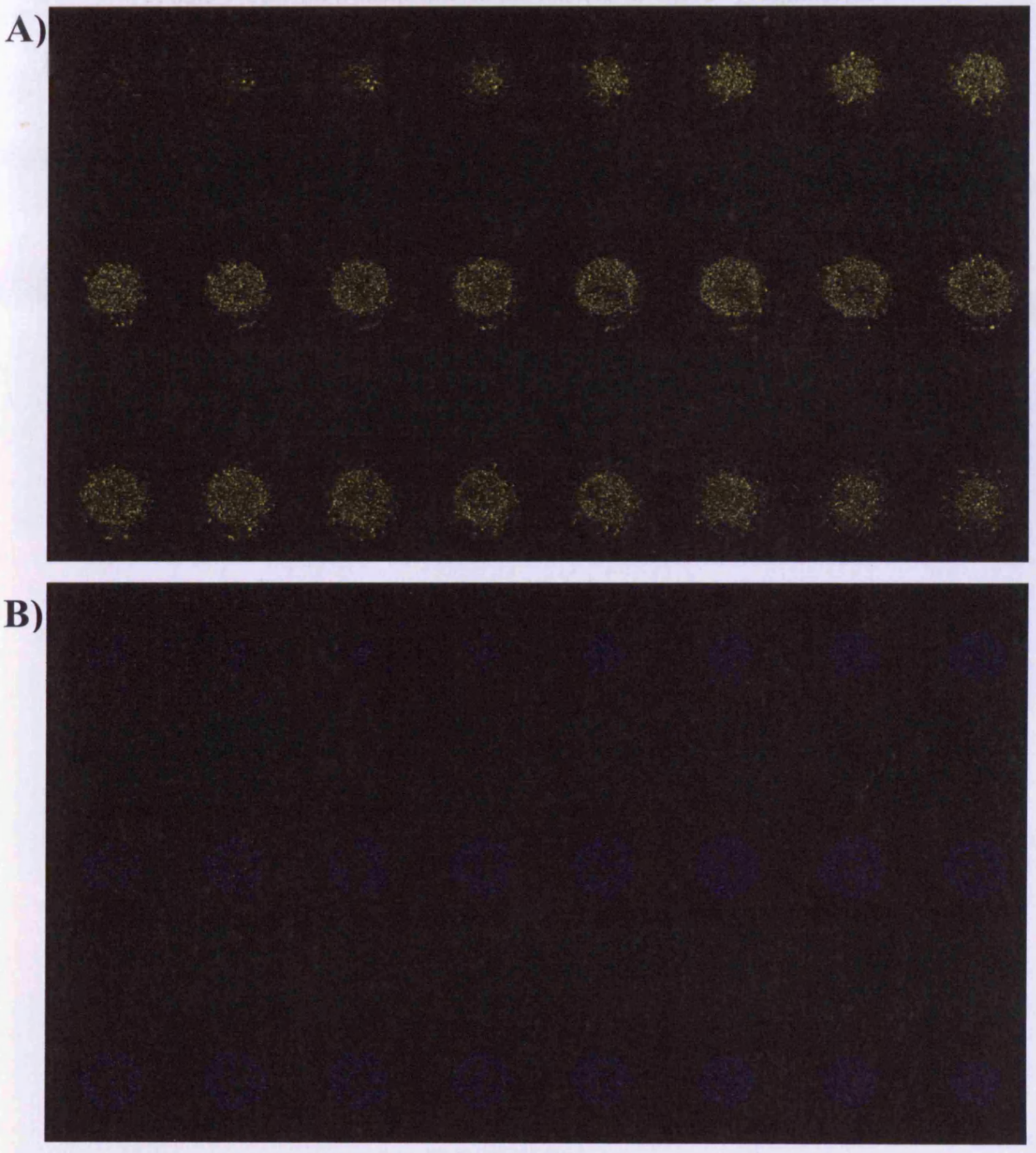


Figure 3.26 - The subcellular localisation of β-catenin throughout a CD34$^+$ progenitor cell.

Representative CLSM z-stack series showing **A)** β-catenin localisation throughout an entire CD34$^+$ progenitor cell. and the **B)** identified nuclear region as given by TO-PRO-3 staining.

3.4.3.3 *γ-Catenin translocates to the nucleus of CD15$^+$ granulocytes*

Granulocytes exhibited the highest levels of γ-catenin observed in haematopoietic development and interestingly, although γ-catenin remained for the most part cytosolic, increased levels of nuclear γ-catenin were observed. It should be noted that there are 3 types of granulocyte, and CD15 surface expression mainly identifies neutrophils and eosinophils, but not basophils. Also, of the total granulocyte pool present in normal peripheral blood, neutrophils make up over 90% (Hoffbrand *et al.*, 2005b), making it likely that the wide range of maturity and morphology observed using this stain belonged to the neutrophil pool. However to be technically correct the term granulocyte will still be used for the remainder of this chapter. *Figure 3.27* provides a representative picture of the varying states of granulocytic maturation encountered in the analysis. The immature form represented in *Figure 3.27A*, demonstrated a strong cytosolic expression of γ-catenin but also showed an increased level of nuclear γ-catenin compared to that observed in CD34$^+$ cells. The part-differentiated (bi-lobed nucleus) granulocytic form in *Figure 3.27B* also harboured higher levels of nuclear γ-catenin that are exemplified in the merged image (**IV**). Even in the terminally differentiated, hyper-segmented granulocytes seen in *Figure 3.27C* there remained a nuclear presence of γ-catenin, as seen by the 'grainy' γ-catenin signal observed in the TO-PRO-3$^+$ nuclear regions.

Further examples of γ-catenin localisation in granulocytic cells can be viewed in ***Fields 5.2-5.7*** of ***disc Folder 5*** in the ***Chapter 3*** section. Also within this folder a number of Z-Stack series (***5.1-5.3***) can be viewed in movie form, which demonstrate that γ-catenin is ubiquitous throughout the granulocyte nuclei and not just localised on a single Z-plane.

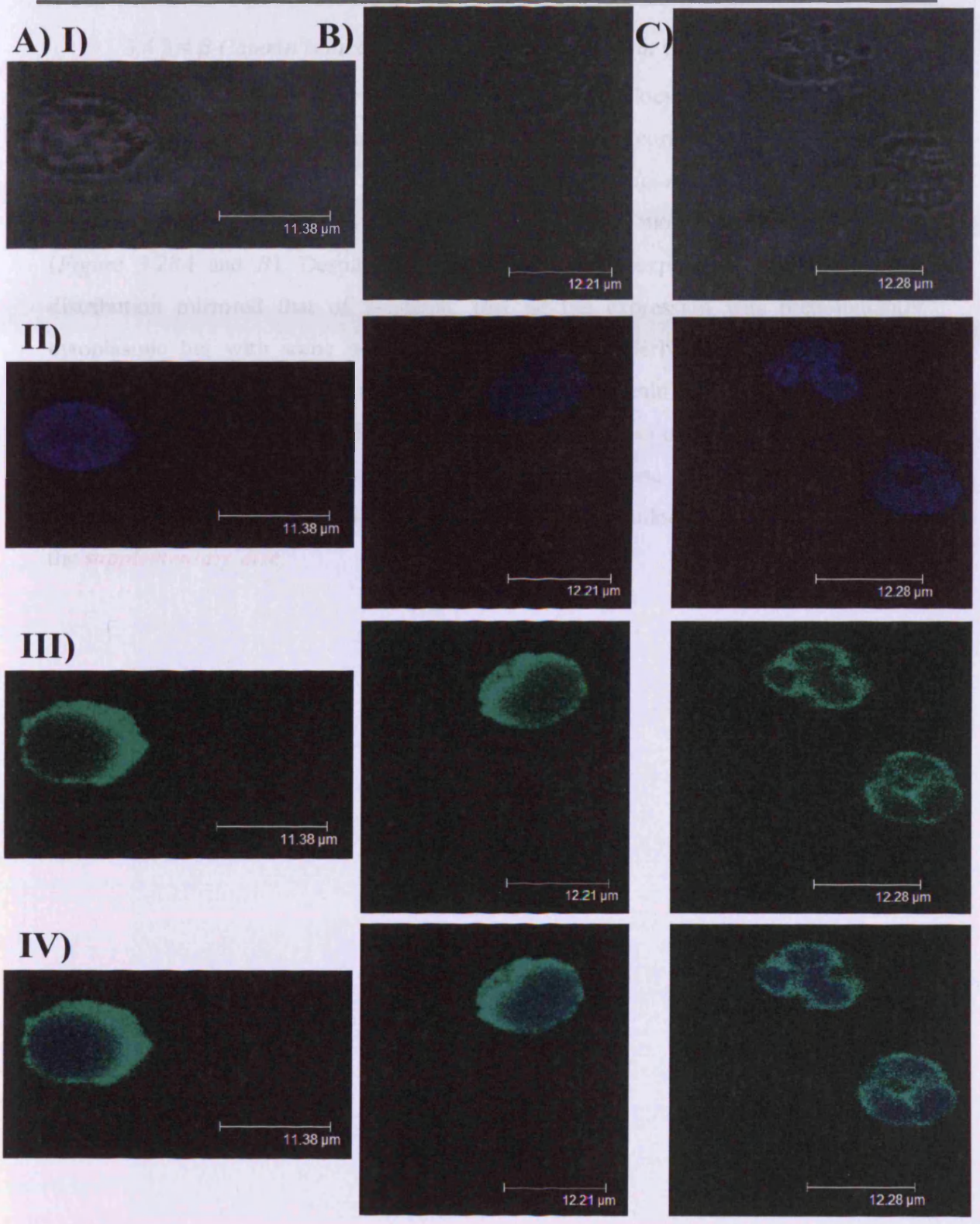


Figure 3.27 - The subcellular localisation of γ-catenin expression in CD15$^+$ granulocytic cells.

Representative CLSM images of γ-catenin localisation in granulocytes with I) phase contrast, II) nuclear (blue), III) γ-catenin (green), and IV) merged images. **A)** Example of undifferentiated, **B)** partially differentiated with bi-lobed nucleus and **C)** terminally differentiated granulocytes.

3.4.3.4 *β-Catenin is localised similarly to γ-catenin in CD15$^+$ granulocytes*

The levels of β-catenin were at their highest in granulocytic cells with enhanced intensity of β-catenin staining in this subset of cells which correlated well with the high fluorescence levels detected from flow cytometry (*Figure 3.22*). As illustrated previously for γ-catenin, a wide range of granulocyte morphology was examined (*Figure 3.28A* and *B*). Despite the increase in overall expression, the intracellular distribution mirrored that of γ-catenin, that is; the expression was predominantly cytoplasmic but with some nuclear localisation, particularly in the undifferentiated granulocytes. As with γ-catenin however, nuclear β-catenin was still present in the terminally differentiated granulocytes. Interestingly, the foci of β-catenin staining seen in CD34$^+$ cells were no longer frequently observed in these mature cells. For further images and Z-Stack series of β-catenin localisation in granulocytes consult ***Folder 6*** of the ***supplementary disc***.

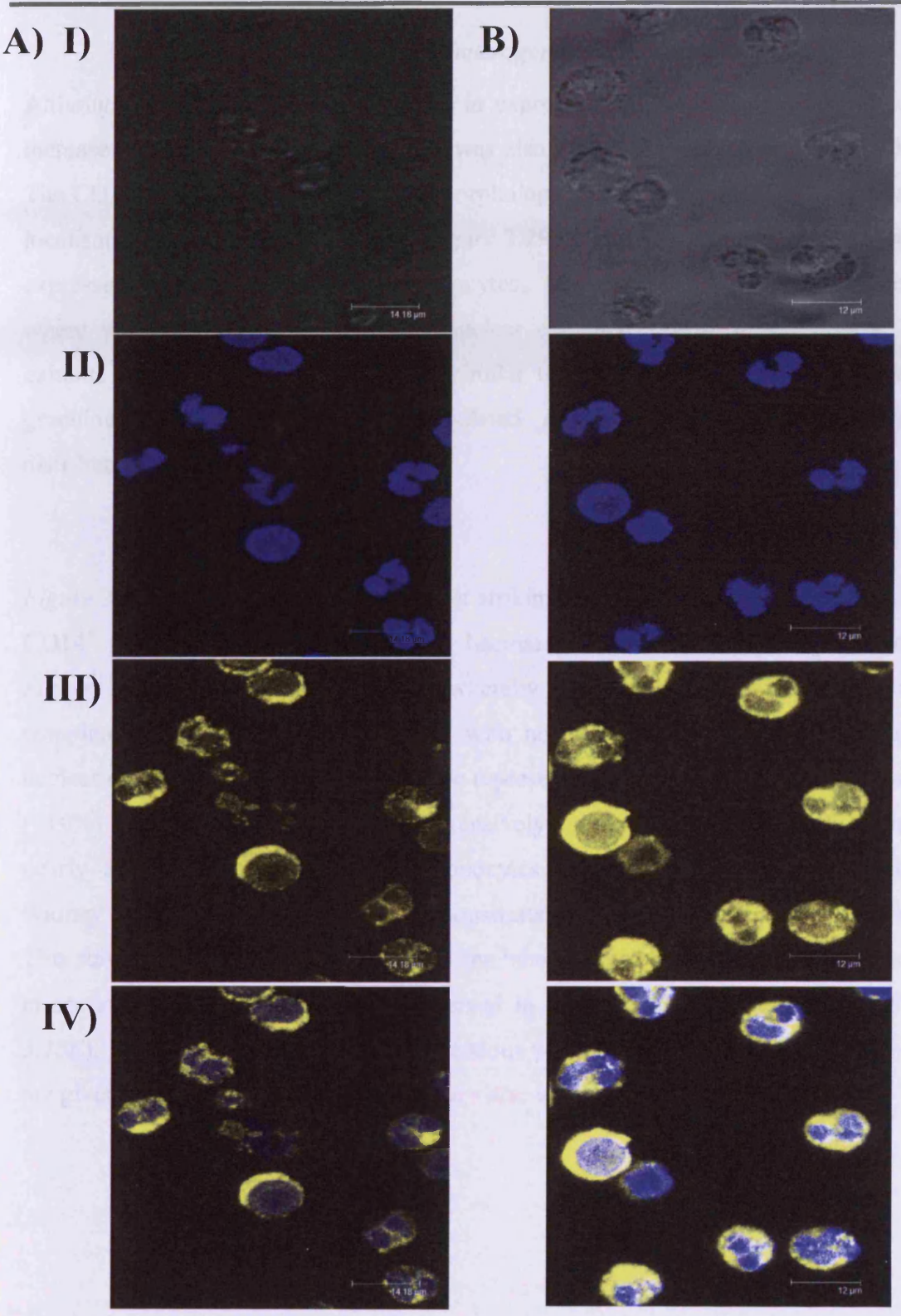


Figure 3.28 - The subcellular localisation of β-catenin expression in CD15$^+$ granulocytic cells.

A) and **B)** Representative CLSM images of β-catenin localisation in granulocytes with I) phase contrast, II) nuclear (blue), III) β-catenin (yellow), and IV) merged images. Mixed fields containing examples of undifferentiated, partially differentiated and terminally differentiated granulocytes are shown.

3.4.3.5 *γ-Catenin localisation is heterogenous in CD14$^+$ monocytes*

Although not as pronounced as the rise in expression observed from granulocytes, an increase in overall γ-catenin expression was also observed in the monocyte population. The CD14$^+$ immuno-stain identified a morphologically diverse subset and the γ-catenin localisation was also heterogeneous. *Figure* 3.29*A* shows an example of low γ-catenin expression (frequently observed in monocytes). Image *Figure* 3.29*B* shows monocytes where γ-catenin was almost entirely nuclear excluded, whilst image *Figure* 3.29*C* exhibits a partial nuclear localisation (similar to the distribution observed in mature granulocytes). Interestingly, hyper-lobulated monocytes frequently displayed the distribution observed in images *B* and *C*.

Figure 3.29*D*, *E* and *F* represent the most striking examples of γ-catenin localisation in CD14$^+$ monocytes (and indeed of any haematopoeitic cell observed in this study). *Figure* 3.29*D* represents an example whereby γ-catenin expression appeared to be completely ubiquitous through the cell with no discrimination between cytosolic or nuclear regions. *Figure* 3.29*E* and *F* were representative of a small subset of monocytes (<10%) whereby the γ-catenin was intensively nuclear localised. Curiously, it was nearly always the undifferentiated monocytes or those with a semi-differentiated 'kidney bean' shaped nucleus that demonstrated this degree of nuclear translocation. This subset of cells is likely to represent the 'shoulder' of high fluorescence referred to in section *3.4.2.1* and consistently observed in the monocyte histograms (see *Figure 3.18E*). Further examples of the heterogeneous γ-catenin localisation within monocytes are given in ***Folder 7*** of the ***supplementary disc*** within the ***Chapter 3*** section.

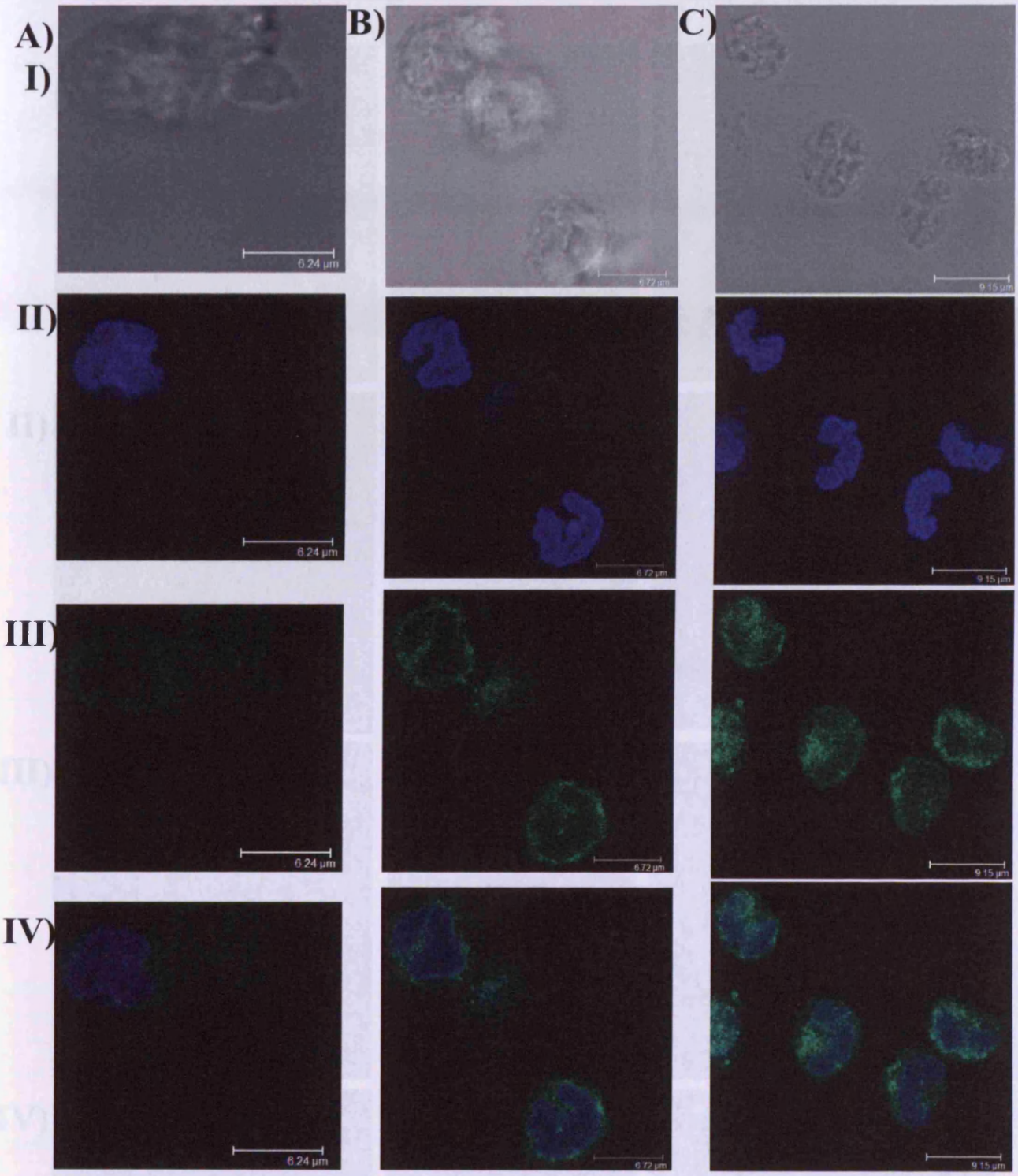
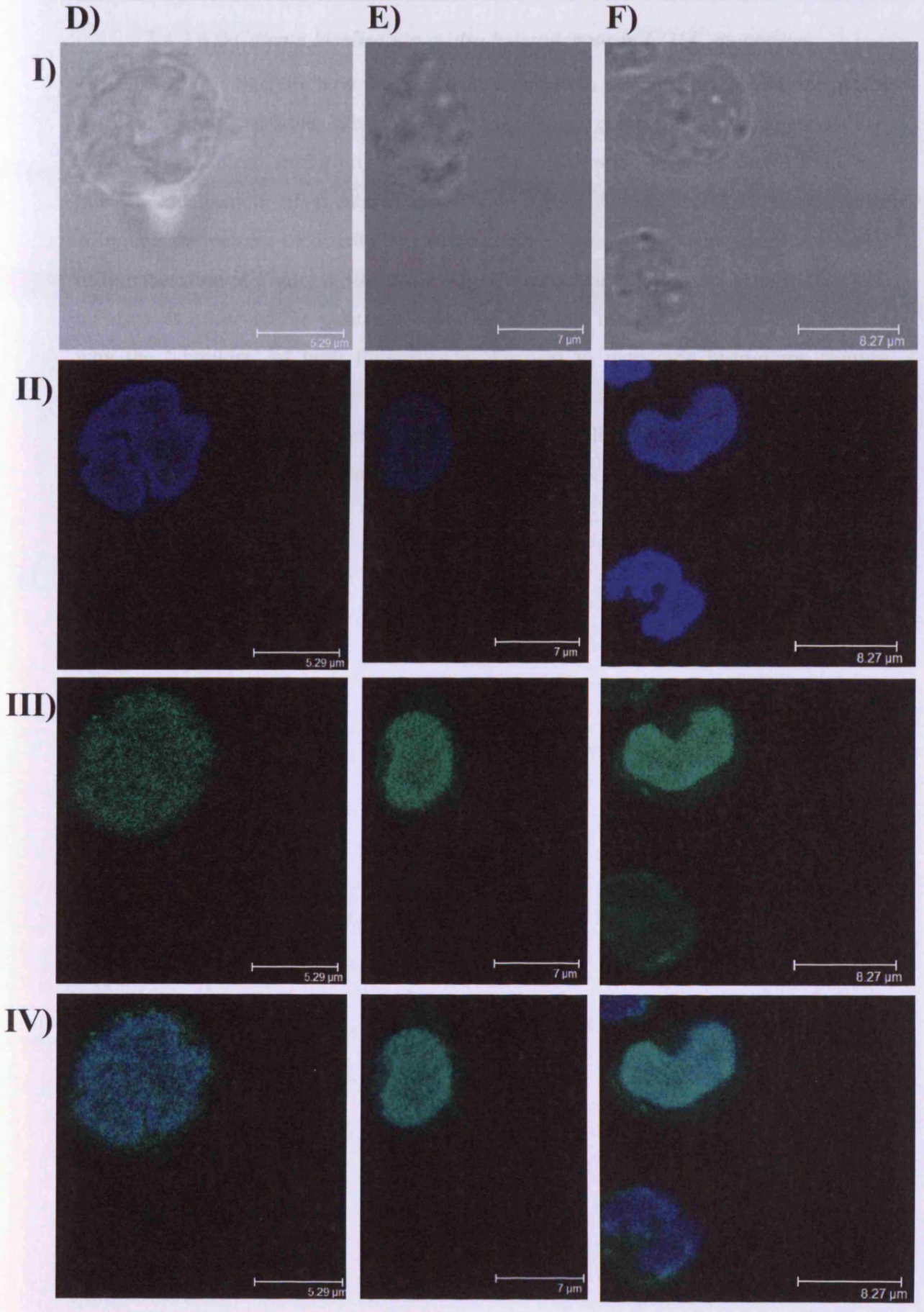


Figure 3.29 - The subcellular localisation of γ-catenin expression in CD14$^+$ monocytic cells.

Representative CLSM images of γ-catenin localisation in monocytes with I) phase contrast, II) nuclear (blue), III) γ-catenin (green), and IV) merged images. A range of monocyte morphology and γ-catenin expression is indicated. Examples of **A)** low expression, **B)** nuclear exclusion, **C)** partial nuclear localisation, **D overleaf)** diffuse distribution and **E + F)** strong nuclear localisation of γ-catenin are shown.


D)
E)
F)
I)
II)
III)
IV)
5.29 µm
7 µm
8.27 µm

3.4.3.6 *β-Catenin localisation is also heterogenous in CD14$^+$ monocytes*

Figure 3.30A-F illustrate how the β-catenin distribution was also heterogeneous in this morphologically variable subset. As for γ-catenin, representative images of low expression, nuclear exclusion, partial nuclear localisation, diffuse distribution and nuclear localisation of β-catenin are shown from *Figure 3.30A-F*, respectively. Although the pattern of distribution corresponds to that of γ-catenin there are subtle differences seen in *Figure 3.30E* and *F* where the nuclear localisation is not to the same intensity as observed for γ-catenin within cells of this morphology. This may explain why the 'shoulder' of high fluorescence observed in monocyte histograms (*Figure 3.18E*) was only present for γ-catenin staining and not for β-catenin (data not shown). Further images and Z-Stack series of β-catenin intracellular distribution in monocytes are present in ***Folder 8*** of the ***supplementary disc***.

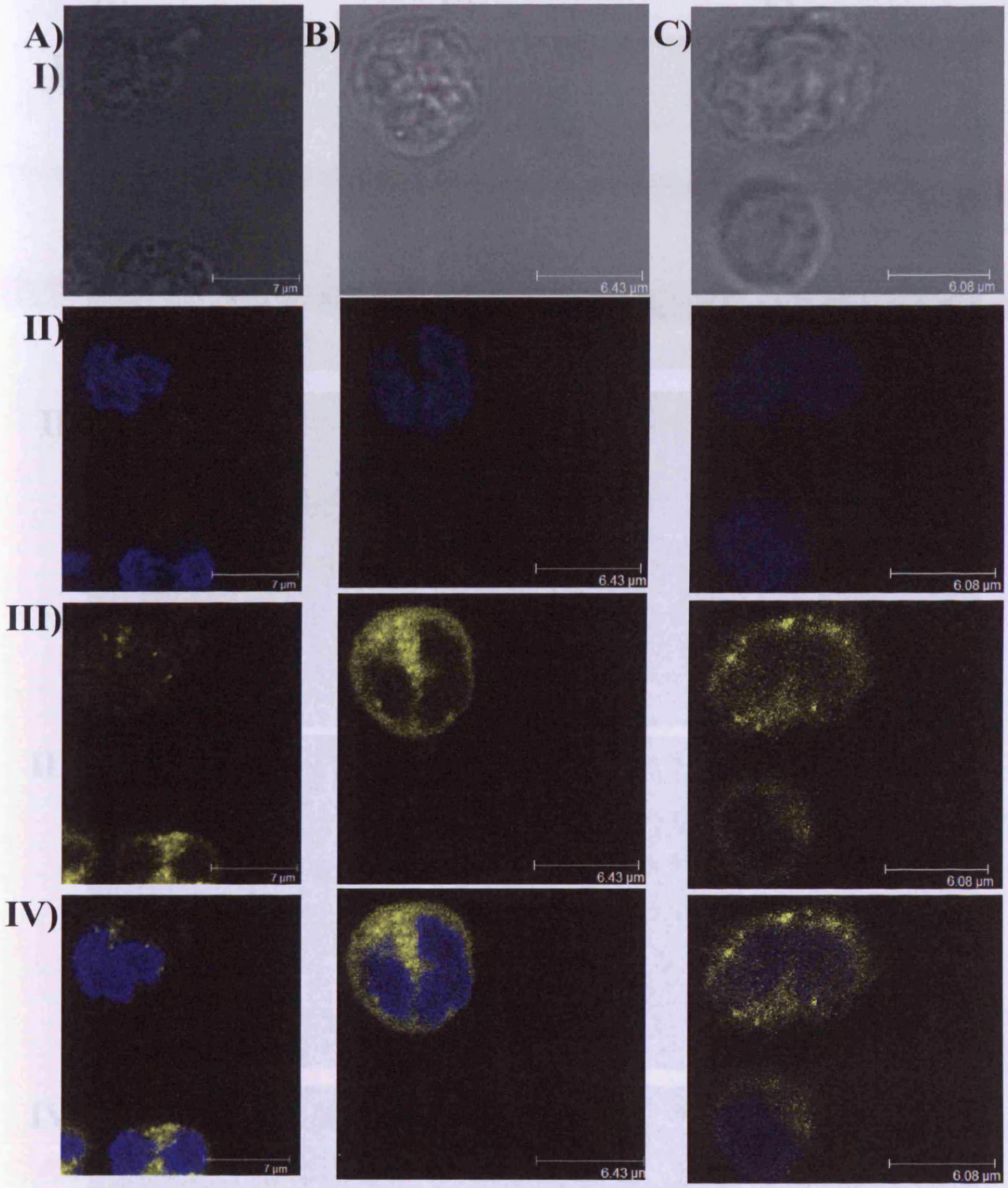
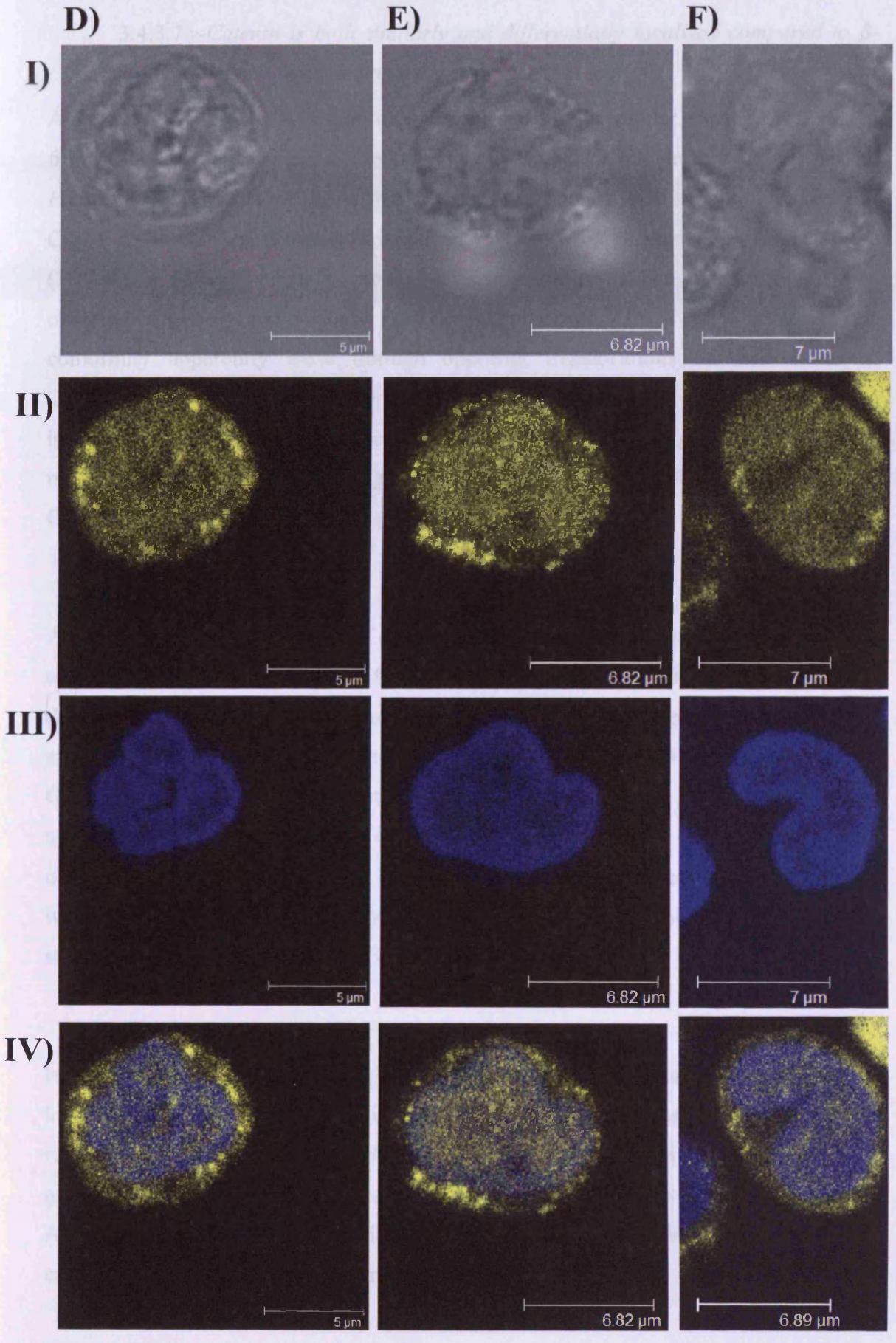


Figure 3.30 - The subcellular localisation of β-catenin expression in CD14$^+$ monocytic cells (overleaf).

Representative CLSM images of β-catenin localisation in monocytes with I) phase contrast, II) nuclear (blue), III) β-catenin (yellow), and IV) merged images. A range of monocyte morphology and β-catenin expression is indicated. Examples of **A)** low expression, **B)** nuclear exclusion, **C)** partial nuclear localisation, **D overleaf)** diffuse distribution and **E + F)** strong nuclear localisation of β-catenin are shown.


D)
E)
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I)
II)
III)
IV)
5 µm
6.82 µm
7 µm
5 µm
6.82 µm
7 µm
5 µm
6.82 µm
7 µm
5 µm
6.82 µm
6.89 µm

3.4.3.7 *γ-Catenin is both similarly and differentially localised compared to β-catenin in haematopoietic cells*

A summary of the N:C/M ratios of γ- and β-catenin subcellular expression obtained from haematopoietic progenitor cells, granulocytes and monocytes are summarised in *Figure 3.31A* and *B*. This shows that the degree of nuclear localisation of γ-catenin in $CD34^+$ cells was significantly lower than observed for β-catenin in this cell type (10%±4 vs 58%±12, $P<0.01$, n=3). No significant difference in localisation was observed between γ- and β-catenin in $CD15^+$ granulocytes (23%±8 vs 27%±9, n=3), this conformity apparently arose through opposing translocations of these catenins. Compared with $CD34^+$ cells γ-catenin demonstrated an increase in nuclear translocation in $CD15^+$ cells (10%±4 in $CD34^+$ cells vs 23%±8, $P<0.05$) whilst β-catenin exhibited a marked exit from the nucleus in granulocytes (58%±12 in $CD34^+$ cells vs 27%±9 in $CD15^+$ cells, $P<0.01$).

An average nuclear localisation of 44%±16 (n=3) was observed for γ-catenin in $CD14^+$ monocytes was not significantly different from the localisation of β-catenin, 38%±12, in this cell type. As observed for granulocytic development, this apparently arose from a marked translocation of γ-catenin to the nucleus (10%±4 in $CD34^+$ cells vs 44%±16 in $CD14^+$ cells, $P<0.001$) whilst β-catenin shifted distribution to the cytosol/membrane in monocytes (58%±12 in $CD34^+$ cells vs 38%±12 in $CD14^+$ cells, $P<0.05$). The observation that γ-catenin nuclear localisation was more heterogeneous than β-catenin within this cell type is borne out by the larger standard deviation observed for γ-catenin stained cells (16 vs 12, $P<0.01$, n=3).

In summary, this section of data shows that γ-catenin and β-catenin are oppositely localised in early haematopoiesis suggesting distinct functions and independent regulation of translocation in $CD34^+$ cells. The distribution of both proteins is similar in granulocytic and monocytic differentiation despite reciprocal directions of translocation. A small subset of monocytes exhibited a unique and strong nuclear localisation of γ-catenin suggestive of an exclusive role within this context.

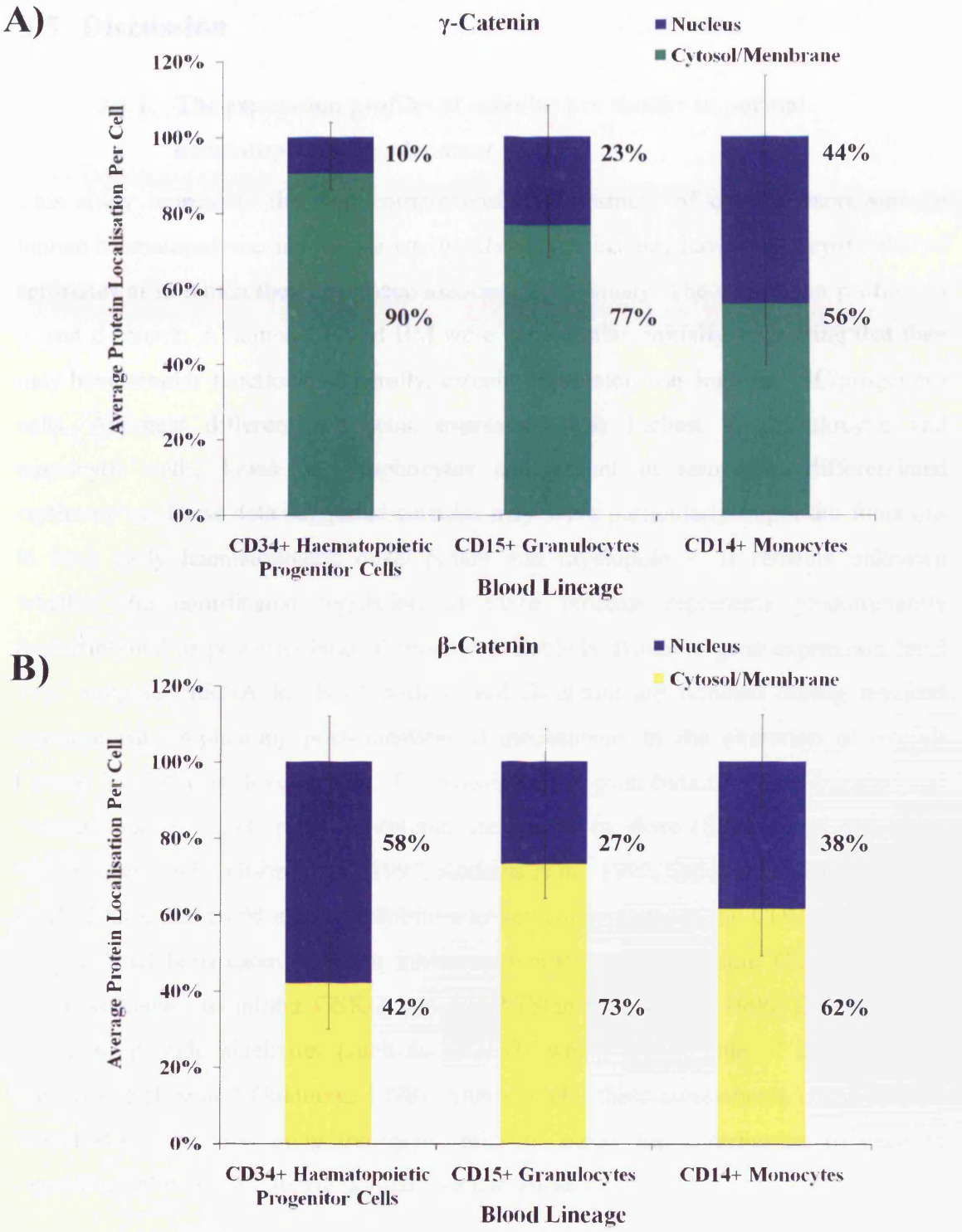


Figure 3.31 - Direct comparison of γ-catenin and β-catenin subcellular localisation in haematopoietic cells.

Summary bar graphs of **A)** γ-catenin and **B)** β-catenin subcellular localisation in haematopoietic cells (n=3 for each cell type). Percentage represents average protein localisation in that compartment per cell. Data represents mean ± 1SD obtained from each data set.

3.5 Discussion

3.5.1 The expression profiles of catenins are similar in normal haematopoietic development

This study represents the first comprehensive assessment of catenin expression in human haematopoiesis, and has shown that these proteins may have roles beyond that of self-renewal in which they have been associated previously. The expression profiles of γ- and β-catenin in human CB and BM were very similar, initially suggesting that they may have similar functions. Generally, catenin expression was high in HSC/progenitor cells. Amongst differentiated cells, expression was highest in granulocytic and monocytic cells, lower in lymphocytes and absent in terminally differentiated erythrocytes. These data suggested catenins may serve particularly important functions in both early haematopoietic development and myelopoiesis. It remains unknown whether the coordinated regulation of these proteins represents predominantly transcriptional or post-translational processes. Publicly available gene expression level data[5] suggests mRNA levels of both γ- and β-catenin are retained during myeloid development; implicating post-translational mechanisms in the alteration of overall catenin level during development. To investigate the contribution of post-translational mechanisms, of which γ- and β-catenin are known to share (Salomon *et al.*, 1997; Aberle *et al.*, 1997; Orford *et al.*, 1997; Kodama *et al.*, 1999; Sadot *et al.*, 2000; Kolligs *et al.*, 2000), one could employ inhibitors to key components of the CDC and monitor the levels of both catenins. Such inhibitors would include Lithium Chloride (LiCl) which is known to inhibit GSK-3 and CK-2 (Stambolic *et al.*, 1996; Davies *et al.*, 2000), or peptide aldehydes (such as MG132) which are capable of inhibiting the proteasome (Lee and Goldberg, 1998). Alternatively, these components could also be 'knocked-out' *in vivo* using transgenic mice to assess the contribution of each in regulating catenin level during normal haematopoiesis.

Studies using murine models null for γ- and/or β-catenin suggest that these proteins have redundant roles in both short- and long-term haematopoiesis (Cobas *et al.*, 2004;

[5] http://www.ncbi.nlm.nih.gov/geoprofiles

Jeannet *et al.*, 2008; Koch *et al.*, 2008). In contrast, current data of this study would argue for important roles in more than one stage of haematopoietic development. The shortcomings of these papers are critically appraised in the more relevant discussion of *Chapter 5*, however, these experiments focused on the general reconstitution capacity of catenin-deleted haematopoietic progenitor cells, rather than any specific developmental abnormalities. This investigation was tailored to analyse catenin expression within specific developmental subsets, which was found to be dynamic, rather than uniform/absent (which could have been expected from functionally redundant proteins).

Evidence from this study conflicts with studies showing that β-catenin protein expression decreases with development (Simon *et al.*, 2005; Yeung *et al.*, 2010). These studies analysed the expression of endogenous β-catenin protein using *in vitro* cultured $CD34^+$ human haematopoietic progenitor cells. They showed, by time-course Western blotting, that although β-catenin expression was readily detectable in early undifferentiated progenitor cells (in agreement with us), this expression was down-regulated and virtually absent in later differentiated cultures (in contrast to the upregulation observed in this study). Reasons for this apparent discrepancy may be related to sample preparation and the inclusion of bulk haematopoietic cell populations, whereas this analysis was tailored to a specific lineage by flow cytometry. Further, similar experiments conducted in this study (featured later in *Figure 5.12* of *Chapter 5*) have shown (at worst) maintenance of β-catenin (and γ-catenin) expression in later differentiated haematopoietic cultures from progenitor cell level.

The finding that β-catenin expression is high in primitive haematopoietic progenitor cells is in keeping with β-catenin's well documented role as a transcriptional mediator of self-renewal within this cell type (Reya *et al.*, 2003; Zhao *et al.*, 2007; Holmes *et al.*, 2008; Congdon *et al.*, 2008). Such a role in these cells has not been documented previously for γ-catenin even though results from this study and others suggest γ-catenin may have transcriptional activity (Simcha *et al.*, 1998; Kolligs *et al.*, 2000; Williams *et al.*, 2000; Zhurinsky *et al.*, 2000a; Muller-Tidow *et al.*, 2004; Maeda *et al.*, 2004; Fukunaga *et al.*, 2005). No significant differences in expression of γ- or β-catenin were

observed between the two respective types of primitive $CD34^+$ haematopoietic cell ($CD34^+38^-$ LT-HSC and $CD34^+38^+$ committed progenitor cells). If anything a slight rise in expression was associated with $CD34^+CD38^+$ progenitor cells which would fit with the apparent rise in expression associated with myeloid differentiation. These findings are in agreement with Jamieson *et al.* (Jamieson *et al.*, 2004) who, using a similar method, detected no discernable differences in β-catenin expression between HSC and committed progenitor cells.

The finding that γ- and β-catenin expression actually increases with haematopoietic differentiation is perhaps in contrast to the pattern of expression observed in other normal tissues. In the gut, a small pool of stem cells (termed Paneth cells) reside in the intestinal crypts with high self-renewal potential and thus high β-catenin expression (Batlle *et al.*, 2002; Van de Wetering *et al.*, 2002). These cells continually replenish the high-turnover of differentiated villus epithelium which have abrogated self-renewal capability and thus low β-catenin expression (Clevers, 2006).

Lower levels of catenin protein were identified in lymphocytes, a finding partly backed up by Chung *et al.* (Chung *et al.*, 2002) who noted a low level of β-catenin protein in normal peripheral blood T-cells. Furthermore removal of γ-catenin seems to have no consequences for thymocyte development (Goux *et al.*, 2005). A number of studies have implicated canonical Wnt signalling in effective lymphopoiesis (Reya *et al.*, 2000; Pongracz *et al.*, 2006; Dosen *et al.*, 2006; reviewed by Staal and Sen, 2008). The finding that expression of either catenin was completely absent in terminally differentiated erythrocytes is in agreement with a study of the erythrocyte proteome that found no catenins present (Pasini *et al.*, 2006). Indeed, there is little evidence that Wnt signalling can influence erythrocytes.

3.5.2 Catenins are both similarly and reciprocally localised in normal haematopoietic development

The investigations into catenin subcellular localisation by CLSM were crucial in attempting to link function to the overall levels observed in the initial part of the study. Intriguingly, γ- and β-catenin exhibit reciprocal localisation in $CD34^+$ cells suggesting distinct functions and independent regulation of translocation in early haematopoiesis. Nuclear exclusion of γ-catenin at this early stage of haematopoiesis would imply a structural role in these cells. A function with which γ-catenin is known to participate is in adhesion both within adherens junctions (reviewed by Takeichi, 1990) and desmosomes (Koch and Franke, 1994; Lewis *et al.*, 1997). However, to our knowledge such homotypic (same cell to cell) adhesive junctional structures are not present or relevant for haematopoietic cells. Heterotypic (joining of two different cell types) adhesion has previously been reported for haematopoietic cells (Allport *et al.*, 2000) and is known to anchor HSC/progenitor cells within BM stroma. However whether this interaction is governed by cadherin-catenin complexes is unresolved and there remains considerable debate as to whether cadherins (e.g. N-cadherin) are actually expressed on HSC (Kiel *et al.*, 2007; Hooper *et al.*, 2007; Haug *et al.*, 2008; Kiel *et al.*, 2009; Hosokawa *et al.*, 2010; Li and Zon, 2010). Irrespective of this the γ-catenin signal detected in $CD34^+$ cells by CLSM did not appear to be exclusively membranous. It is plausible that this cytosolic localisation may represent a primed reservoir of γ-catenin that is nuclear translocated upon myeloid differentiation.

That β-catenin is predominantly nuclear localised in $CD34^+$ cells strongly suggests a transcriptional function in early haematopoiesis. This is in keeping with the wealth of documented evidence implicating β-catenin with a role in self-renewal in these cells (Reya *et al.*, 2003; Willert *et al.*, 2003; Jamieson *et al.*, 2004; Zhao *et al.*, 2007; Holmes *et al.*, 2008; Kim *et al.*, 2009; Nemeth *et al.*, 2009), and this study is not the first to image β-catenin in the nuclei of HSC by CLSM (Jamieson *et al.*, 2004; Congdon *et al.*, 2008). An interesting observation made (but not so much for γ-catenin) was the extra-nuclear presence of intense foci of β-catenin staining (as seen in *Figure 3.25A* and *B*). It is unknown whether these punctate aggregates represent genuine biological phenomenon or staining artefact. Such aggregates have been observed in other studies

utilising β-catenin immunofluorescence by CLSM and have been suggested to be indicative of genuine biological events. A study by Kim *et al.* (Kim *et al.*, 2003b) concluded that the foci represent cytoplasmic vesicles containing β-catenin and other Wnt signalling components such as Axin and DVL. Alternatively, a study by Dashwood *et al.* (Dashwood *et al.*, 2005) demonstrated that the aggregates contained β-catenin localised within lysosomal vesicles.

The presence of γ- and β-catenin in the nuclei of differentiated granulocytes in similar nuclear/cytosolic (N/C) ratios is particularly interesting for two reasons. Firstly, the localisation of both catenins suggests reciprocal directions of translocation from HSC/progenitor level. That is, γ-catenin translocated from cytoplasm to nucleus, whilst β-catenin appeared to have shifted from nucleus to cytoplasm. This implies that a tightly regulated mechanism exists governing the nuclear entry or exit of both catenins in normal haematopoiesis, and that this system can act independently for each catenin (discussed further below). Secondly, the increased presence of γ-catenin, and retained expression of β-catenin, in the nucleus would insinuate a potential transcriptional role within granulocytes. Although infrequently reported, translocation of γ-catenin has been observed elsewhere in normal development whereby nuclear localisation of γ-catenin was found to be important for anterior axis duplication within developing *Xenopus* embryos (Karnovsky and Klymkowsky, 1995; Rubenstein *et al.*, 1997). In granulocytes, the highest level of nuclear catenin was evident in relatively immature $CD15^+$ subsets which had just began, but not finished, the process of nuclear segmentation (see *Figure 3.27B*). A study by Serinsöz *et al.* (Serinsoz *et al.*, 2004) appeared to partly corroborate these observations by also noting high β-catenin expression in immature granulocytic cells, albeit by immunohistochemistry rather than CLSM. This finding would suggest that any potential transcriptional activity of γ- and β-catenin would be more relevant to early granulopoietic development. Such a hypothesis is interesting given that severely reduced LEF-1 (of which both catenins are known to bind in order to transduce a Wnt stimulus) in granulopoiesis leads to congenital neutropenia (CN; (Skokowa *et al.*, 2006)). In this study the highest level of LEF-1 in healthy individuals was present in promyelocytes (similar to catenins) and CN arrested promyelocytes exhibited defective expression of the well established catenin target genes *cyclinD1* (Shtutman *et al.*, 1999),

myelocytomatosis oncogene (*c-myc* or *myc*; (He *et al.*, 1998)) and *survivin* (Kim *et al.*, 2003a). However to infer such a connection would be highly speculative, especially given that LEF-1 has been shown to have β-catenin independent activity through the transforming growth factor β (TGF-β) (Nawshad and Hay, 2003) and Notch (Ross and Kadesch, 2001) pathways, and may be activated by other unknown catenin-like molecules (reviewed by Staal and Luis, 2010).

The $CD14^+$ monocytes represented the most morphologically diverse subset analysed and the distribution of catenin was also heterogeneous. Of particular interest were the large degrees of nuclear translocation exhibited by γ-catenin within these cells, indeed the largest observed of all the analysed subsets. Specifically, a rare subgroup (<10%) of the $CD14^+$ monocyte pool exhibited intense and heavy nuclear localisation of γ-catenin (featured in *Figure* 3.29*E* and *F*). This subgroup is highly likely to represent cells responsible for generating the 'shoulder' of intense fluorescence observed consistently in monocyte MFI histograms (example in *Figure 3.18E*). Although distribution of β-catenin was similar in monocytes, nuclear localisation to the same intensity was not observed suggesting an exclusive transcriptional role for γ-catenin within these cells. Incidentally, a study by Tickenbrock *et al.* (Tickenbrock *et al.*, 2006) would agree with the distribution of β-catenin observed in these cells, with their investigations also detecting cytosolic and nuclear forms by CLSM.

Initially, it was queried whether this unique monocytic sub-group could represent an 'activated' population following exposure to a stimulus such as bacterial lipopolysaccharide (LPS), interferon-γ (IFN-γ) or tumour necrosis factor-α (TNF-α), thus implicating γ-catenin in a potential immune function. Such a role has been implicated for β-catenin and Wnt signalling previously in monocytes (Monick *et al.*, 2001; Staal *et al.*, 2008; George, 2008; Otero *et al.*, 2009). However, an overnight incubation with IFN-γ appeared to not significantly promote the nuclear translocation of γ-catenin in isolated human monocytes (data not shown). Alternatively, given the consistent nuclear morphology (smooth oval or often kidney-bean shaped) of this subgroup, these monocytes could represent a developmental intermediary (as previously

characterised by (Meuret *et al.*, 1974)), requiring the transcriptional capabilities of γ-catenin at this stage of monocyte/macrophage differentiation. However, recent studies have suggested that the make-up of the monocyte population is actually far more complex than initially presumed (reviewed by Gordon and Taylor, 2005 and Robbins and Swirski, 2010) and therefore these cells could represent a developmental subset (though this would have to be substantiated through use of additional markers). There seems to be a degree of consensus amongst a number of studies that catenins, and more general Wnt signalling, contribute to the trans-endothelial migration of monocytes (Sandig *et al.*, 1997; Allport *et al.*, 2000; Thiele *et al.*, 2001; Tickenbrock *et al.*, 2006; Lee *et al.*, 2006). Indeed the level of γ-catenin was actually shown to fluctuate within a VE-cadherin adhesion complex during this physiological process (Allport *et al.*, 2000). One study showed frizzled receptors (which bind a Wnt ligand) expressed on monocytes, which no doubt contributed to the Wnt-3A mediated increase of β-catenin observed in these cells (Tickenbrock *et al.*, 2006). This reaction was shown to increase the adhesive properties of monocytes to endothelial layers, although a lack of Wnt target gene expression (*c-myc* and *connexin43*) implied this was not a transcriptional-mediated effect of β-catenin. In a similar investigation by Lee *et al.* (Lee *et al.*, 2006) lithium chloride treatment of THP-1 cells (monocytic cell line) enhanced adhesion to endothelium, but again this could not be attributed to any transcriptional upregulation of established adhesion molecules. Conversely, Thiele *et al.* (Thiele *et al.*, 2001) proposed that β-catenin could not be involved with cadherin-mediated adhesion because they failed to detect any such molecules on human monocytes, and furthermore failed to observe any activation of the TCF/LEF promoter upon induction of β-catenin. An adhesive function for γ-catenin in this unique subgroup of monocytes does not seem likely from findings of this study given that γ-catenin is intensely nuclear localised. Such a function would require γ-catenin to be distributed to the membrane as seen for α- and β-catenin during this process (Sandig *et al.*, 1997), though the monocytes in these experiments were not in contact with endothelium.

3.5.3 Multiple mechanisms could regulate the nuclear localisation of catenin in haematopoietic development

The movement of proteins (especially those as large as catenin ~85-90kDa) in and out of the nucleus is a tightly regulated process. In the case of the classical import pathway, a protein's nuclear localisation sequence (NLS) binds an NLS receptor comprising two cytosolic proteins: importin-α and importin-β, which subsequently docks the complex to a nuclear pore by binding nucleoporins (reviewed by Nigg, 1997). The protein is then translocated through the nuclear pore utilising the small soluble GTPaseRan/TC4 (Melchior *et al.*, 1993; Moore and Blobel, 1993) and p10 (Moore and Blobel, 1994; Paschal *et al.*, 1996), in an energy-dependent process. Studies have suggested that the central armadillo domains of β-catenin (and hence γ-catenin) are sufficiently similar to the HEAT[6] repeats of the importin-β central domain to allow nuclear translocation of these proteins through direct interaction with nuclear pore proteins (Fagotto *et al.*, 1998; Yokoya *et al.*, 1999). However, this has been challenged more recently by a study showing β-catenin cannot associate with nuclear pore proteins (Suh and Gumbiner, 2003), and it seems unlikely that unregulated entry of β-catenin would be desirable in normal cells. Indeed this study and others (Cong and Varmus, 2004; Krieghoff *et al.*, 2006), are incompatible with unregulated entry of β and γ-catenin and it is doubtful that NLS or even nuclear export sequences (NES) exist for either catenin.

Nuclear chaperone proteins have been documented to assist nuclear translocation or export of proteins. A wealth of candidates have been proposed to regulate the nuclear localisation of catenin, including APC (Henderson, 2000; Henderson and Fagotto, 2002), Axin (Cong and Varmus, 2004; Wiechens *et al.*, 2004), Chibby (Li *et al.*, 2010), mothers against decapentaplegic homolog 3 (Smad-3) (Zhang *et al.*, 2010), menin (Cao *et al.*, 2009), zinc finger protein 639 (ZASC-1) (Bogaerts *et al.*, 2005), insulin-like growth factor-1 (IGF-1) (Chen *et al.*, 2005), Presenilin-1 (Raurell *et al.*, 2006), pygopus (Townsley *et al.*, 2004a) and B-cell CLL/lymphoma-9 protein (BCL-9) (Krieghoff *et al.*, 2006). Interestingly, TCF-4 (Maeda *et al.*, 2004; Krieghoff *et al.*, 2006) and LEF-1

[6] HEAT repeats are tandemly repeated, 37-47 amino acid long modules occurring in a number of cytoplasmic proteins, including the four name-giving proteins huntingtin, elongation factor 3 (EF3), the 65 Kd alpha regulatory subunit of protein phosphatase 2A (PP2A) and the yeast PI3-kinase TOR1. HEAT repeats form a rod-like helical structure and appear to function as protein-protein interaction surfaces.

(Behrens *et al.*, 1996; Huber *et al.*, 1996; Simcha *et al.*, 1998) have also been shown to translocate γ-catenin and β-catenin to the nucleus. Given that these molecules are the target complex for Wnt signalling, and the fact they carry an NLS, raises the intriguing possibility of a 'piggy back' mechanism for catenins *en route* to transducing a Wnt signal. The finding that some of the aforementioned chaperones interact with β-catenin, but not γ-catenin, would fit with the apparent reciprocal localisation of β-catenin and γ-catenin observed during haematopoietic development. Since none of the above candidate shuttling mechanisms have been examined within a haematopoietic context it is difficult to identify which of these potential mechanisms are operating within this context.

Post-translation modification of β-catenin is also known to affect its translocation. Whilst serine/threonine phosphorylation of the catenin NH_2-terminus targets the molecule for degradation, phosphorylation (especially of tyrosine residues) has been reported to promote it's nuclear translocation and enhance transcription (Bonvini *et al.*, 2001; Piedra *et al.*, 2001; Ilan *et al.*, 2003; Bourguignon *et al.*, 2007; Wu *et al.*, 2008). This has also been demonstrated in a haematopoietic context with FLT-3 (Kajiguchi *et al.*, 2007). In AML, FLT-3 internal tandem duplication and tyrosine kinase domain mutations (FLT3-ITD and FLT3-TKD, respectively) were shown to increase tyrosine phosphorylation of β-catenin, relative to *wt* FLT-3 receptor, leading to nuclear accumulation.

As well as nuclear import mechanisms, it is likely that nuclear export mechanisms may be just as relevant for regulating nuclear levels of γ- and β-catenin in normal haematopoietic subsets. Many of the candidate chaperone proteins described in the above section have also been linked with transporting catenin out of the nucleus including APC (Henderson, 2000), Axin (Wiechens *et al.*, 2004), and menin (Cao *et al.*, 2009). Additionally, a novel nuclear β-catenin binding protein, Ran binding protein 3 (RanBP-3), was discovered by Hendriksen *et al.* to antagonise TCF-4 mediated transactivation in human cell lines by re-localising active β-catenin from the nucleus to the cytoplasm (Hendriksen *et al.*, 2005). Interestingly, one report has shown that whilst

APC and Axin enrich β-catenin in the cytoplasm, and TCF and β-catenin co-activators (BCL9 and Pygopus) increase nuclear β-catenin, they do not accelerate the export or import rate of β-catenin (Krieghoff *et al.*, 2006). This would suggest that regulation of β-catenin (and indeed γ-catenin) subcellular localisation could represent a dynamic balance between shuttling and retention. Thus, it is tempting to speculate that many of the haematopoietic cells exhibiting high nuclear catenin may also harbour high levels of the nuclear binding partners such as TCF4, pygopus and BCL-9.

Finally, in a related theme, junctional proteins (such as cadherin and α-catenin) have been frequently shown to negatively regulate the nuclear level of catenin (and hence Wnt signal transduction), by sequestering it from the nucleus and to adhesion complexes at the cell membrane instead (Fagotto *et al.*, 1996; Simcha *et al.*, 1998; Orsulic *et al.*, 1999; van Hengel *et al.*, 1999; Giannini *et al.*, 2000; Gottardi *et al.*, 2001; Stockinger *et al.*, 2001; Nelson and Nusse, 2004; Gottardi and Gumbiner, 2004). Establishment of the cadherin expression profile (type and level) for haematopoietic cells (of which there is much debate) would first have to be performed before such a mechanism could be proposed for the regulation of catenin localisation observed in developmental subsets.

In summary, this chapter has provided the first comprehensive assessment of the expression of γ- and β-catenin in normal haematopoiesis. Most notably, it seems neither of the proteins has HSC/progenitor restricted expression and are thus likely to serve roles in more developed haematopoietic cells. This study has also raised important questions of the precise role γ-catenin in myeloid differentiation, and how subcellular localisation of catenin is regulated, issues of which are addressed in *Chapter 5*. However, it is next important to examine if, and how, normal expression of these proteins is dysregulated in acute myeloid leukaemia which is investigated in the following chapter.

4 - Characterisation of γ-Catenin Expression in Acute Myeloid Leukaemia and Implications for Patient Survival

4.1 Introduction

The previous chapter examined the developmental expression of γ-catenin and determined the subcellular localisation of expression. Such information, however, is not available for primary AML blasts. Evidence from this laboratory and others suggests γ-catenin is dysregulated in AML (Zheng *et al.*, 2004; Muller-Tidow *et al.*, 2004; Tonks *et al.*, 2007), particularly in the presence of aberrant translocation products (see introductory section *1.3.3*), but little is known of its pathological relevance.

It remains to be determined if γ-catenin *protein* is dysregulated in primary AML blasts and how this compares with normal levels in haematopoietic progenitor cells. If protein expression is abnormally high (which the mRNA levels would suggest (Tonks *et al.*, 2007)) then it is necessary to characterise the subcellular location of this protein within these malignant cells. Knowledge of the intracellular distribution provides an indication of the functional significance of overexpression with a nuclear presence implying a transcriptional function. The previous chapter has suggested not only that γ-catenin (and β-catenin) is capable of shifting localisation with development, but that this is a tightly regulated process in normal haematopoiesis. How this system is maintained, if at all, in AML cells remains unknown.

The inappropriate nuclear translocation of β-catenin in colon cancer, arising from mutations in the adenomatous polyposis coli (APC) protein (Korinek *et al.*, 1997; Morin *et al.*, 1997), is a well characterised pathological mechanism, although there appears to be no clear consensus on the prognostic influence (Hugh *et al.*, 1999; Chung *et al.*,

2001; Baldus *et al.*, 2004; Wong *et al.*, 2004; Wong *et al.*, 2005; Martensson *et al.*, 2007; Elzagheid *et al.*, 2008; Horst *et al.*, 2009). Whether γ- or β-catenin have a pathological role in AML is not well established, although dysregulated β-catenin expression has previously been identified in AML blasts and linked with an adverse prognosis (Ysebaert *et al.*, 2006; Xu *et al.*, 2008). However, for such a heterogeneous disease, these studies were very small and thus unlikely to be definitive.

How γ-catenin expression (mRNA or protein) contributes to the pathology of AML is thus far unanswered and by no means (despite homology with β-catenin) predictable. Although one study has suggested γ-catenin is a mediator of self-renewal in leukaemogenesis (Zheng *et al.*, 2004) (supported by unpublished data from this laboratory, see *Appendix 6*), other studies in different disease settings have associated γ-catenin with a tumour suppressor role (Aberle *et al.*, 1995; Simcha *et al.*, 1996; Charpentier *et al.*, 2000; Polychronopoulou *et al.*, 2002; Breault *et al.*, 2005; Misaki *et al.*, 2005; Shiina *et al.*, 2005). The large number of patient samples and clinical information available through the AML trials will allow this question to be addressed.

Finally, evidence exists (in other contexts) that these proteins are capable of influencing not only total levels of one other, but also their respective subcellular locations (Salomon *et al.*, 1997; Miller and Moon, 1997; Simcha *et al.*, 1998; Sadot *et al.*, 2000; Zhou *et al.*, 2007; Li *et al.*, 2007). Therefore if the stringent regulation of β and γ-catenin observed in normal haematopoiesis is lost in AML this could give rise to cooperating or even opposing functions in AML. For this reason it is important to analyse the expression and location of both these catenins.

4.2 Aims

In order to establish whether γ-catenin expression is dysregulated in AML, and whether this has prognostic implications for the patient, the aims of this chapter were:

1) To validate a large cohort of AML patient γ-catenin mRNA values, and determine the effect of mRNA expression on patient survival by correlation with AML clinical parameters.

2) To establish how γ-catenin *protein* is expressed in AML blasts relative to normal haematopoietic progenitor cells.

3) To examine the subcellular localisation of γ-catenin expression in AML blasts and compare this with β-catenin, and then to observations made for normal haematopoiesis.

4) To investigate the significance of the subcellular localisation of γ- and β-catenin protein on AML patient survival using a similar analysis to above (point 1).

4.3 Materials and Methods

4.3.1 The statistical analysis of the effect of γ-catenin mRNA expression on AML patient survival

4.3.1.1 *The validation of Affymetrix microarray mRNA values by qRT-PCR*

Before a large cohort of patient γ-catenin mRNA expression values could be used for patient survival analysis it was first necessary to validate these values using an alternative mRNA assay. Affymetrix (California, USA) MicroArray Suite version 5 (MAS5.0)-normalised (as described in Liddiard *et al.*, 2010) γ-catenin mRNA values from Affymetrix HGU133A GeneChip® hybridization were validated using quantitative Reverse Transcriptase - Polymerase Chain Reaction (qRT-PCR).

Patient RNA (of high quality, as assessed by Agilents Bioanalyser 2000 as in (Haferlach *et al.*, 2010)) isolated by Trizol lysis and reverse transcribed into cDNA as described

elsewhere (Tonks *et al.*, 2007; Haferlach *et al.*, 2010), was already available from a random selection of 19 AML patient samples (from the main cohort of 243 patients in *Table 4.2* below). For each AML sample, a 10μl qRT–PCR reaction was prepared containing 50ng cDNA, 1μl FastStart DNA SYBR Green I master mix (Roche), 3mM $MgCl_2$, 500nM forward and reverse γ-catenin or *ABL* (Abelson murine leukaemia viral oncogene homolog 1) primers (Eurofins MWG Operon, Ebersberg, Germany, see *Table 4.1*) and made to volume with water. A 40-cycle qRT-PCR was performed on a LightCycler 2.0 (Roche) with an annealing temperature of 60°C. The relative level of γ-catenin mRNA in each AML patient sample was assessed based on the $2^{-\Delta\Delta C_T}$ method (Schmittgen and Livak, 2008), summarised in *Equation 4.1* below. Briefly, the level of γ-catenin mRNA was normalised between samples by taking the C_T (cycle threshold) value (the cycle number whereby the fluorescence detection becomes greater than background and exponential) and subtracting the C_T level obtained from a housekeeping internal control gene, in this case *ABL*. The degree of correlation between the two techniques was assessed using Pearson's correlation coefficient (R) with the software of section *2.8*.

$$\textbf{Relative mRNA level GOI} = C_{TGOI} - C_{TICG}$$

Where;

C_T = Cycle threshold
GOI = Gene of interest
ICG = Internal control gene

Equation 4.1 – Equation based on $2^{-\Delta\Delta C_T}$ method for calculating relative mRNA level for a gene of interest

Table 4.1 - Primers used for qRT-PCR validation of Affymetrix microarray.

Gene	Forward primer (5'to 3')	Reverse primer (3' to 5')
γ-catenin	5'-ACTGAACTCCACCGACCAAC-3'	3'-CACCCTGGAGAGAGAAGCTG-5'
ABL	5'-CCCAACCTTTTCGTTGCACTGT-3'	3'-CGGCTCTCGGAGGAGACGTAGA-5'

4.3.1.2 *The clinical endpoints used in statistical analysis of AML patient survival*

To determine the relevance of γ-catenin mRNA dysregulation on AML patient prognosis, merged MAS5.0 normalised RNA values previously obtained from Affymetrix HGU133A and HGPlus 2.0 GeneChip® hybridisations (Tonks *et al.*, 2007) were correlated with available patients survival data for 243 AML patients enlisted in the MRC/NCRI AML trials 10 to 15. Demographic and clinical details of the AML patient cohort, such as age, sex and French-American-British (FAB) type are featured in *Table 4.2* below.

Table 4.2 - Clinical data for 243 AML patients included in γ-catenin mRNA survival analysis.

Parameter	No. of patients
Trial	
AML10	6
AML11	8
AML12	45
AML14 (Intensively treated)	50
AML14 (Non-intensively treated)	6
AML15	128
AML16 (Intensively treated)	0
AML16 (Non-intensively treated)	0
Age	
<15	0
15-29	34
30-39	30
40-49	41
50-59	65
60-69	47
70+	26
Median (range)	53 (15-87)
Sex	
Female	118
Male	125
WHO performance status	
0	140
1	84
2	10
3	7
4	2
White blood cell count ($x10^9/l$)	
<10	70
10-49.9	83
50-99.9	43
100+	46
Unknown	1
Median (range)	27.9 (1-316)
Cytogenetics	
Favourable	64
Intermediate	129
Adverse	29
Unknown	21
Secondary Disease	
No	225
Yes	18
FAB Type	
M0	12
M1	43
M2	59
M3	25
M4	46
M5	32
M6	3
M7	2
ALL	0
Bilineage	2
RAEB/RAEB-t	2
Unknown/Other	17

4.3.1.3 *Statistical techniques used to assess AML patient survival*

Statistical analyses were kindly assisted by Dr. Robert Hills using a previously described statistical model (Gale *et al.*, 2005) and SAS version 9.1.3 software (SAS Institute Inc., Buckinghamshire, UK). The Wilcoxon 2-sample test (for continuous data), Mantel-Haenszel test for trend (for ordinal data), and the chi-square test (for heterogeneity) were used to test for differences in clinical and demographic data by γ-catenin mRNA level. Kaplan-Meier life tables were constructed for survival data and were compared by means of the log-rank test. Follow-up was up available for the vast majority of patients, and the small number of patients lost to follow-up were censored at the date they were last known to be alive. Analysis of time-to-event data was performed using standard log-rank methods and odds ratio (OR) plots. Univariate analysis was used to examine the association between γ-catenin mRNA level and CR rate, and univariate Cox models were used to analyze OS, DFS, and RR. Univariate models were adjusted for known baseline diagnostic variables including age, white blood cell count, sex, WHO performance status, de novo/secondary disease and cytogenetic risk group, with interaction for each with γ-catenin mRNA level tested using Pearson's correlation coefficient (R). Because of multiple testing, the level of significance was set at $P<0.05$ for all tests. All P values are 2 tailed. Odds Ratio (OR) and 95% confidence intervals (CI) are quoted where relevant. In all cases an OR <1 indicates a benefit for patient prognoses, whilst OR >1 are indicative of adverse patient prognoses.

4.3.2 Source of primary AML blasts and normal $CD34^+$ haematopoietic cells

Peripheral blood (PB) or bone marrow (BM) was obtained from AML patients enrolled in the MRC-NCRI AML clinical trials following informed consent. Mononuclear cells (containing AML blasts) were isolated and cryopreserved in the tissue bank according to clinical trial protocol as previously described (Pallis *et al.*, 2001). AML samples were rapidly thawed (*2.2.5*) and assessed for a minimum of 75% viability as judged by light microscopy prior to use in experiments. Normal human neonatal cord blood (CB) was obtained as described in section *2.2.3* and the $CD34^+$ haematopoietic progenitor cell population enriched to over 85%, as described in section *2.4*.

4.3.3 The determination of γ-catenin protein level in AML blasts versus normal CD34$^+$ haematopoietic progenitor cells

To establish whether γ-catenin protein expression (like mRNA) was dysregulated in primary AML blasts, total levels were compared to that from normal human CD34$^+$ haematopoietic progenitor cells. Whole cell protein homogenate was isolated (*2.6.1*) from 54 individual AML blast samples (for patient demographic and clinical details see *Table 4.3*) and CD34$^+$ cells (from 3 separate CB samples) and Western blotted as described in sections *2.6.3-2.6.7*. To compare γ-catenin protein expression (as determined by densitometry) from AML blasts and CD34$^+$ cells, all samples were first normalised to β-actin to control for minor differences in protein loading. To control for variation in transfer and detection sensitivity between gels, actin-normalised values were further normalised to the signal obtained from the 20kDa band on the protein ladder. This marker was always derived from the same batch and loaded in the same way (20μl) on each gel, thus acting as a marker of consistency on each gel immuno-blotted. A K562 lysate served as a positive control for the primary γ-catenin antibody, as the previous chapter has shown these cells contain high levels of endogenous γ-catenin.

Table 4.3 - Clinical data for the 54 AML patients included in whole blast γ-catenin protein analysis.

Parameter	No. of patients
Trial	
AML10	0
AML11	0
AML12	0
AML14 (Intensively treated)	1
AML14 (Non-intensively treated)	0
AML15	49
AML16 (Intensively treated)	1
AML16 (Non-intensively treated)	3
Age	
<15	0
15-29	3
30-39	10
40-49	14
50-59	18
60-69	6
70+	3
Median (range)	50.5 (23-77)
Sex	
Female	25
Male	29
WHO performance status	
0	27
1	20
2	3
3	4
4	0
White blood cell count ($x10^9/l$)	
<10	10
10-49.9	19
50-99.9	10
100+	15
Unknown	0
Median (range)	38.7 (1.2-244.0)
Cytogenetics	
Favourable	9
Intermediate	31
Adverse	3
Unknown	11
Secondary Disease	
No	48
Yes	6
FAB Type	
M0	4
M1	17
M2	9
M3	0
M4	10
M5	5
M6	1
M7	0
ALL	0
Bilineage	0
RAEB/RAEB-t	0
Unknown/Other	8

4.3.4 The determination of γ- and β-catenin subcellular location in AML blasts

The subcellular localisation of β and γ-catenin in AML blasts were assessed by CLSM (γ-catenin only) and Nuclear/Cytosol (N/C) Western blotting as follows below.

4.3.4.1 *The determination of γ-catenin subcellular expression in AML blasts by CLSM*

For subcellular expression analysis by CLSM, cells were pre-labelled with the CD34-PE mouse monoclonal antibody featured in *Table 3.2* using the associated method of section *3.3.5.2.* AML cells were then intracellularly stained for γ-catenin using the optimal conditions highlighted in section *3.3.5.3* with the necessary modifications required for CLSM analysis as described in *3.3.7.3.* CLSM images were acquired and analysed as before in sections *3.3.7.4* and *3.3.7.5*, respectively. The blasts of five AML patient samples were analysed in total all of which were FAB type M0 or M1.

4.3.4.2 *The determination of γ- and β-catenin subcellular localisation in AML blasts by Western blotting*

For subcellular analysis of catenin by N/C Western blotting, 59 AML samples were processed as described in methods section *2.6.2.* Demographic and clinical details were available for 49 patients of this cohort and are featured in *Table 4.4.* Nuclear and cytosolic homogenate were Western blotted and analysed as in sections *2.6.3-2.6.7.* The mouse monoclonal antibodies used to detect γ- and β-catenin by immuno-blotting are described in section *3.3.3.* The normalisation technique described above (*4.3.3*) was again applied to allow γ- and β-catenin localisation comparisons between AML samples blotted on different gels. The correlations between cytosolic/nuclear γ-catenin and or cytosolic/nuclear β-catenin, was assessed using Spearman's rank correlation coefficient (R) test and the degree of significance obtained via the Student's t-test.

Table 4.4 - Clinical data for 49 AML patients included in N/C fractionated catenin protein analysis

Parameter	No. of patients
Trial	
AML10	0
AML11	1
AML12	0
AML14 (Intensively treated)	7
AML14 (Non-intensively treated)	2
AML15	39
AML16 (Intensively treated)	0
AML16 (Non-intensively treated)	0
Age	
<15	0
15-29	8
30-39	5
40-49	5
50-59	18
60-69	7
70+	6
Median (range)	55 (17-78)
Sex	
Female	27
Male	22
WHO performance status	
0	27
1	20
2	2
3	0
4	0
White blood cell count ($x10^9/l$)	
<10	6
10-49.9	16
50-99.9	16
100+	11
Unknown	0
Median (range)	64.1 (3.8-257.0)
Cytogenetics	
Favourable	4
Intermediate	31
Adverse	6
Unknown	8
Secondary Disease	
No	46
Yes	3
FAB Type	
M0	6
M1	19
M2	10
M3	0
M4	8
M5	4
M6	0
M7	0
ALL	0
Bilineage	0
RAEB/RAEB-t	0
Unknown/Other	2

4.3.5 The correlation of Affymetrix microarray mRNA values with γ-catenin protein from Western blotting

It was necessary to determine any correlation between γ-catenin mRNA and γ-catenin protein level within AML blasts. To examine this, cytosolic protein values from the fractionated AML samples of section *4.3.4* (*Table 4.4*), were compared with corresponding microarray values (obtained from section *4.3.1*), where available. The degree of correlation between normalised γ-catenin mRNA and normalised cytosolic protein expression was assessed by means of a Spearman's rank correlation coefficient (R) test and the degree of significance obtained via the Student's t-test.

4.3.6 The effect of γ- and β-catenin localisation on AML patient prognosis

To investigate whether the specific localisation of γ- or β-catenin protein to the cytoplasm or nucleus had any implications for AML prognosis these values were used for survival analyses. Respective, normalised cytosolic and nuclear protein values for γ- and β-catenin, were generated from the 49 patients (featured in *Table 4.4*) of section *4.3.4*, were each correlated with survival parameters measured within the MRC/NCRI AML trials, exactly as outlined above (*4.3.1.2* and *4.3.1.3*).

4.4 Results

4.4.1 The validation of microarray derived γ-catenin mRNA levels by qRT-PCR

In order to utilise γ-catenin mRNA values from a cohort of 243 AML trial patients for survival analyses it was first necessary to validate these values using qRT-PCR. A random selection of 19 patients from within the bigger cohort featured in *Table 4.2* were compared for γ-catenin expression between microarray and qRT-PCR. A highly significant correlation ($R=0.85$, $P<0.001$) was found between MAS5.0 normalised RNA values from the Affymetrix HGU133A GeneChip® hybridization and the relative γ-catenin mRNA level obtained from qRT-PCR cycle threshold analysis (*Figure 4.1*). This finding would suggest that the level of γ-catenin mRNA detected by microarray closely reflects the actual level present within primary AML patient blasts.

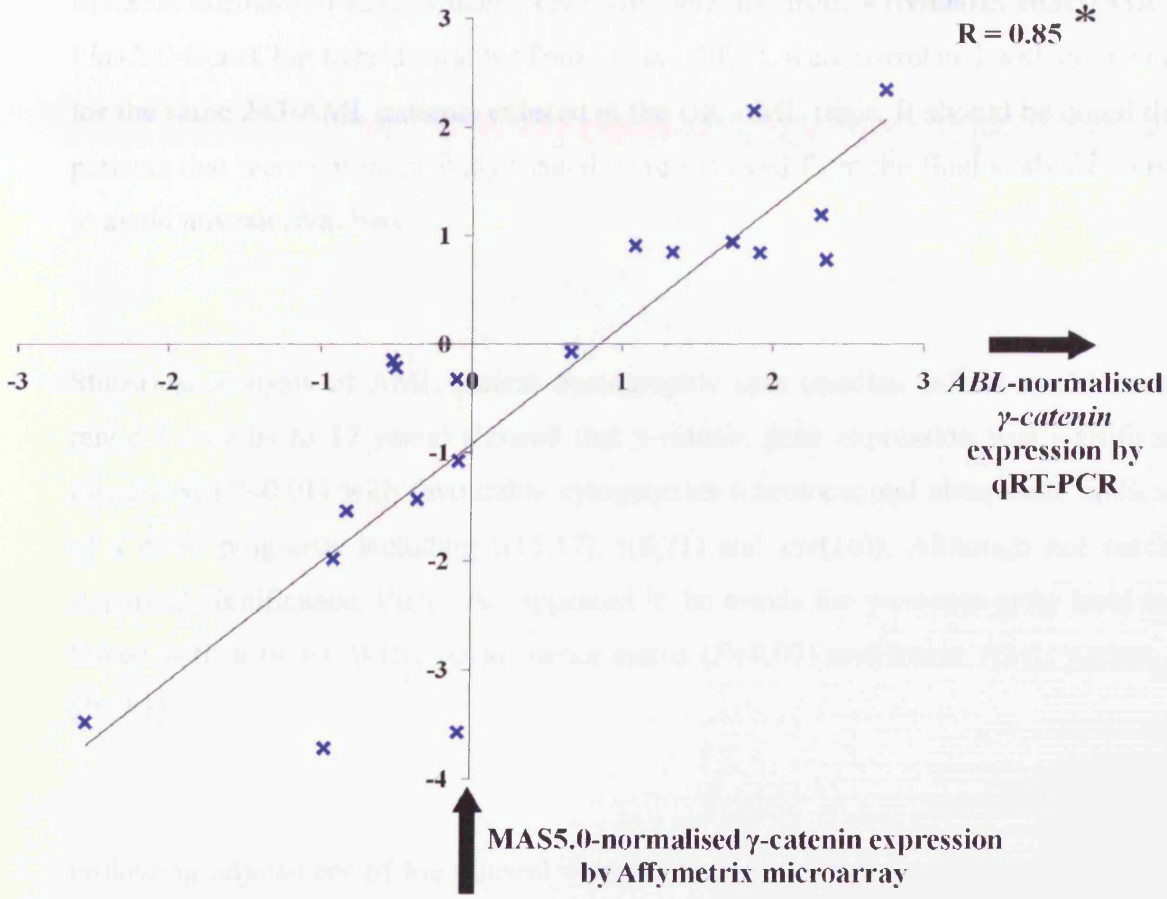


Figure 4.1 - The validation of γ-catenin mRNA detection by microarray using qRT-PCR.

The correlation between Affymetrix microarray and qRT-PCR in the detection of γ-catenin mRNA in AML blasts of 19 patients. MAS5.0 normalised γ-catenin values from each patient by microarray are compared to *ABL* normalised *γ-catenin* values for the corresponding sample obtained from qRT-PCR. Pearson's correlation coefficient (R) gives the degree of correlation between the two techniques,* $P<0.001$.

4.4.2 γ-Catenin mRNA expression is associated with poor prognosis in AML

To examine the consequences of γ-catenin mRNA expression on AML patient survival, MAS5.0 normalised RNA values previously obtained from Affymetrix HGU133A and Plus 2.0 GeneChip hybridisations (Tonks *et al.*, 2007), were correlated with clinical data for the same 243 AML patients enlisted in the UK AML trials. It should be noted that 6 patients that were not intensively treated were removed from the final analysis, in order to avoid any survival bias.

Statistical analysis of AML patient demographic data (median follow up 64 months, range 7 months to 17 years) showed that γ-catenin gene expression was significantly associated ($P<0.01$) with favourable cytogenetics (chromosomal aberrations indicative of a good prognosis including t(15;17), t(8;21) and inv(16)). Although not reaching statistical significance, there also appeared to be trends for γ-catenin gene level to be linked with a better WHO performance status ($P=0.07$) and lower AML patient age ($P=0.1$).

Following adjustment of the clinical data for the known baseline diagnostic variables outlined in section *4.3.1.3*, statistical analysis of AML survival data revealed a significant reduction in CR rate in association with higher γ-catenin mRNA levels (OR 1.25 per log increase, $P<0.05$, CI 1.03-1.51). Importantly, the analysis further demonstrated that this lower CR rate appeared to arise from more resistant disease (OR 1.60 per log increase, $P<0.01$, CI 1.18-2.18). No discernible effects on RR, DFS or OS for AML patients were observed.

This data imply that although γ-catenin mRNA expression is associated with favourable prognostic indicators, as a single entity it is associated with an adverse prognosis for the AML patient.

4.4.3 γ-Catenin protein level is dysregulated in AML blasts versus normal CD34$^+$ haematopoietic cells

To gauge the overall level of γ-catenin protein dysregulation in AML blasts, relative levels from whole cell extracts of AML patient samples were compared to the levels present in normal human cord blood derived CD34$^+$ haematopoietic progenitor cells. Owing to the low frequency of CD34$^+$ cells within cord blood and the limited availability of sufficiently large samples, just 3 samples were available from which to generate protein lysates. *Figure 4.2A* shows that γ-catenin was abundant within these cells (in accord with *3.4.2.1*). Further, a similar multiple-banding pattern as previously observed for the protein lysates of AML cell lines was also observed (*Figure 3.10*). These samples generated a mean normalised γ-catenin protein expression value of 10.3±4.9.

Of the 54 AML samples Western blotted 10 were removed from analysis because the protein level was too low in concentration to reliably quantitate (γ-catenin and β-actin) leaving 44 samples in total. As can be seen from the representative Western blot featured in *Figure 4.2B*, the range of protein expression and banding observed for γ-catenin within the AML samples was heterogeneous and because of this the overall γ-catenin expression from the AML cohort (mean 32.8, med 2.2, range 0.2-279.0, n=44) was not significantly different from values obtained from normal CD34$^+$ progenitor cells (mean 10.3, med 11.4, range 5.0-14.6, n=3, P=0.2). However, taking a threshold of 2 standard deviations above the normal mean ($\bar{X}$ + 2SD) as a marker for γ-catenin overexpression, it can be observed from *Figure 4.3* that 9 of 44 AML samples fall into this overexpression category. Using the same method but subtracting 2SD from mean ($\bar{X}$ - 2SD) a similar proportion (8/44) of blasts were found to underexpess γ-catenin relative to normal CD34$^+$ progenitor cells.

These data show that although the majority of AML samples express γ-catenin within a normal range, around 20% of AML patients demonstrate over- and underexpression of the protein.

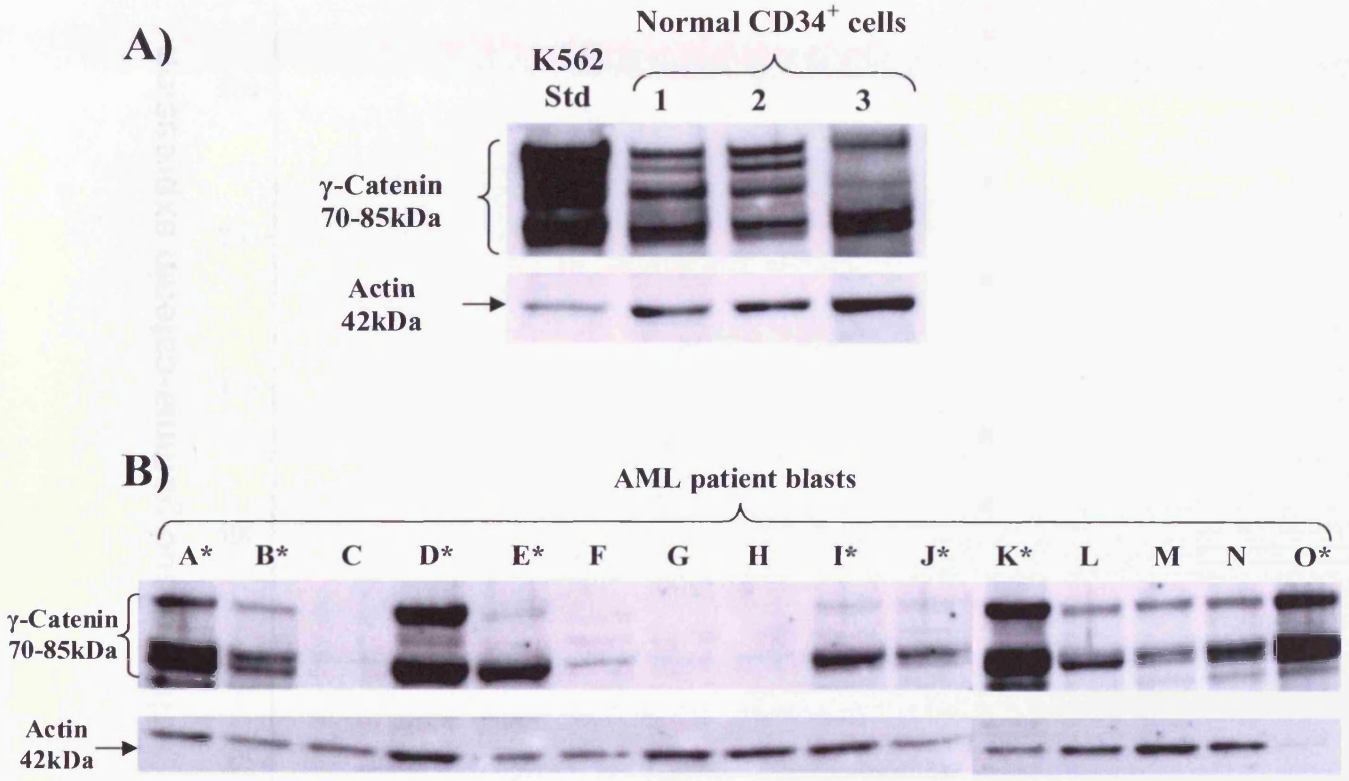


Figure 4.2 - The expression of γ-catenin protein in normal CD34$^+$ cells and AML blasts.

A) Representative Western blot showing γ-catenin protein expression obtained from normal haematopoietic progenitor cells (n=3) and **B)** from AML blasts (n=15). Patient samples marked with * indicate an overexpression of γ-catenin protein relative to the normal CD34$^+$ cells. Actin detection shows relative protein loading between samples.

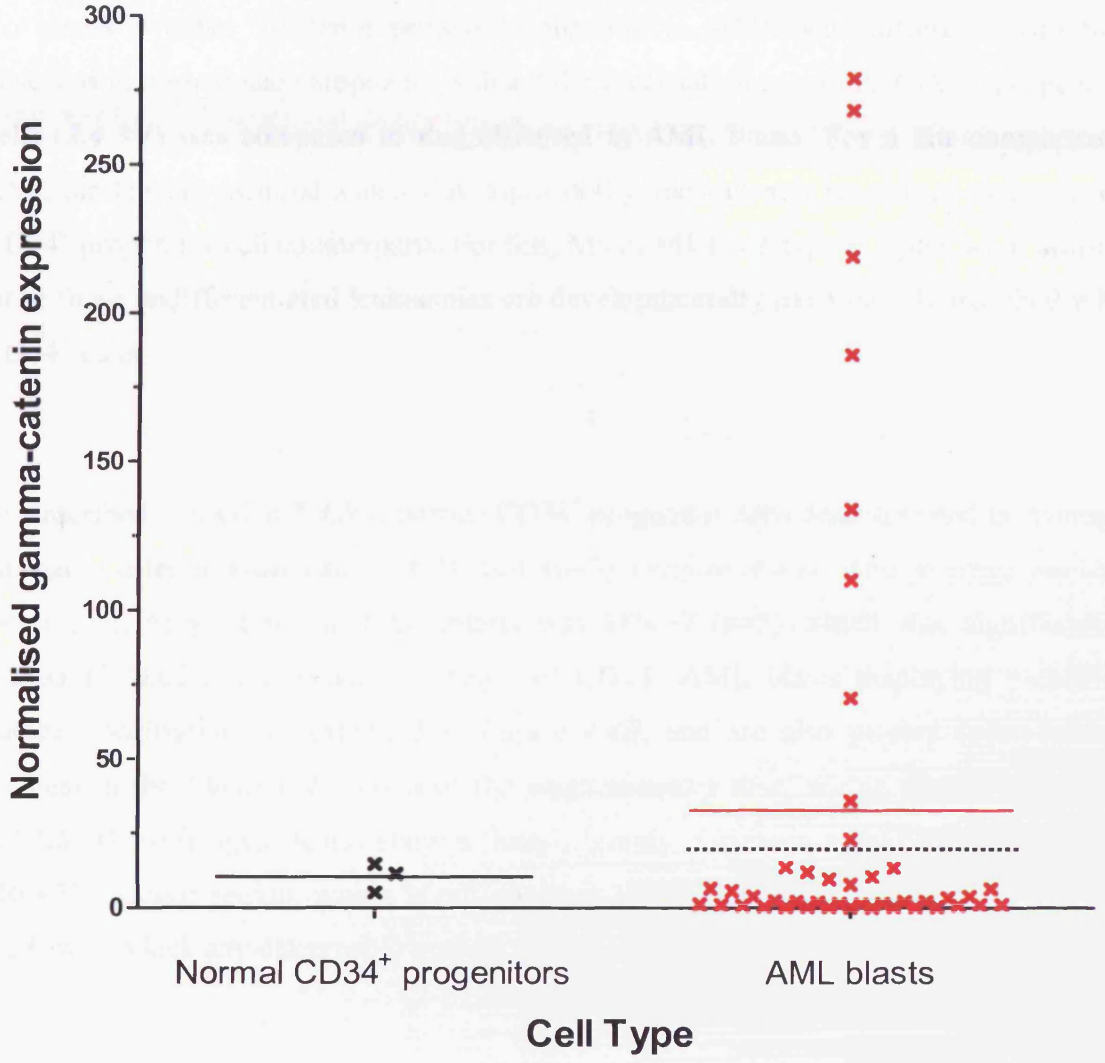


Figure 4.3 - Comparison of γ-catenin protein expression between normal CD34$^+$ cells and AML blasts.

Normalised protein values obtained from normal human cord blood derived CD34$^+$ progenitor cells (n=3) and AML blasts (n=44) as determined from densitometric analysis. Black and red solid lines represent respective means from each cohort, whilst black dashed line amongst AML values represents the threshold of $\bar{x}$ + 2SD obtained from normal cells, used to define γ-catenin overexpression.

4.4.4 The subcellular localisation of γ- and β-catenin expression in AML blasts

4.4.4.1 *γ-Catenin is aberrantly localised to the nucleus of M0/1 AML blasts*

To assess whether γ-catenin protein localisation in AML was different from that observed in normal haematopoiesis, subcellular localisation in normal $CD34^+$ progenitor cells (*3.4.3.1*) was compared to that observed in AML blasts. For a fair comparison, AML blasts were required which, developmentally, most closely resembled their normal $CD34^+$ progenitor cell counterparts. For this, M0 or M1 FAB-type samples were utilised since these undifferentiated leukaemias are developmentally most closely matched with $CD34^+$ cells.

As described in section *3.4.3.1*, normal $CD34^+$ progenitor cells demonstrated an average nuclear γ-catenin localisation of 10%±4 (n=3) (*Figure 4.4A)*. The average nuclear localisation of γ-catenin in M0/1 blasts was 18%±7 (n=5) which was significantly greater ($P<0.05$). Representative images of $CD34^+$ AML blasts displaying γ-catenin nuclear localisation are exhibited in *Figure 4.4B*, and are also present (plus further images) in the ***Chapter 4*** section of the ***supplementary disc***, within ***Folder 1, Fields 1.2-1.8***. These images clearly show a 'hazy', 'grainy' γ-catenin signal within the (TO-PRO-3^+) nuclear region, which is not apparent in the $CD34^+$ progenitor cells (*Figure 3.23)* which lack any discernable nuclear γ-catenin signal over background.

These data imply that γ-catenin is aberrantly nuclear translocated in M0/1 undifferentiated AML.

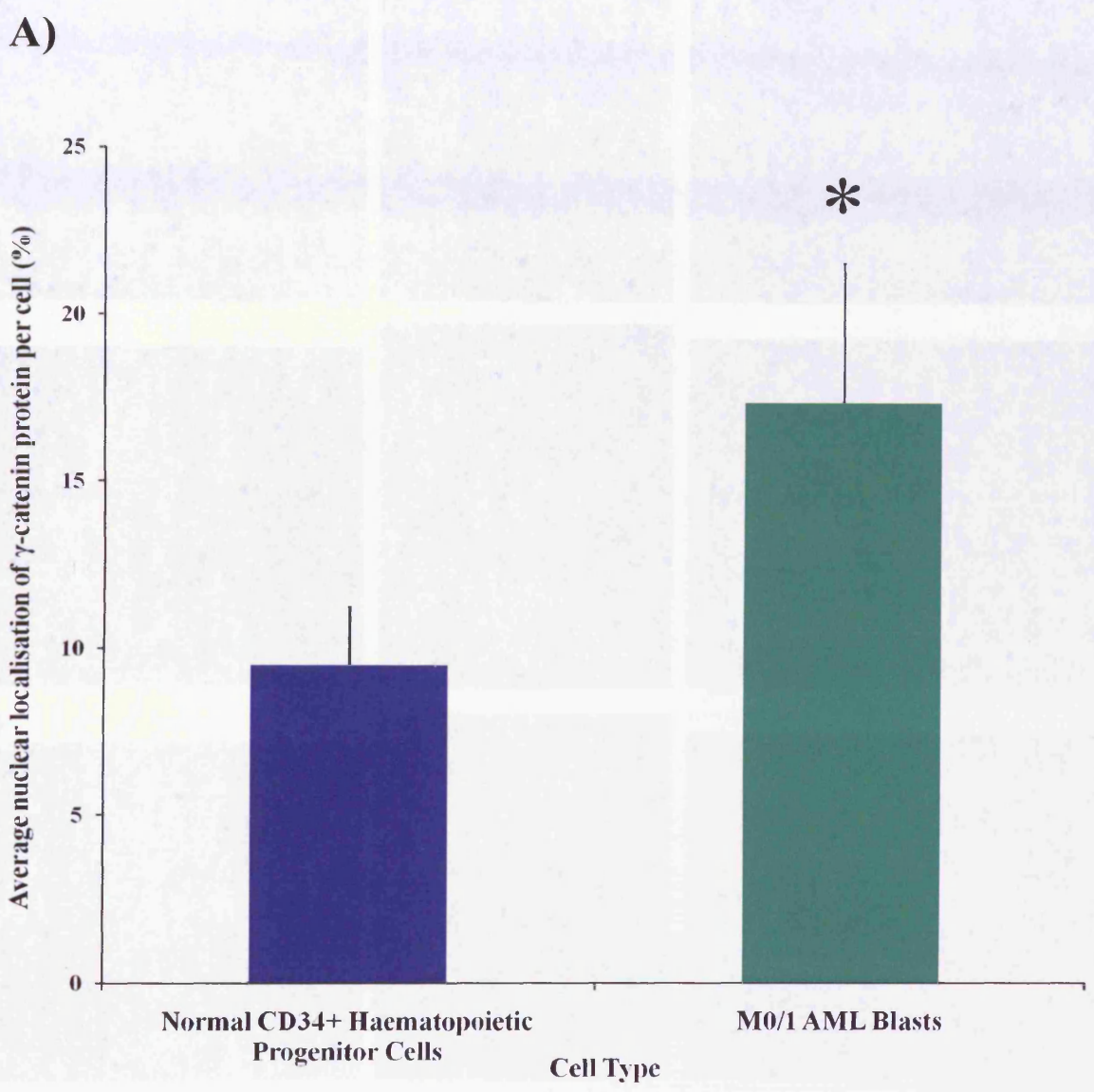
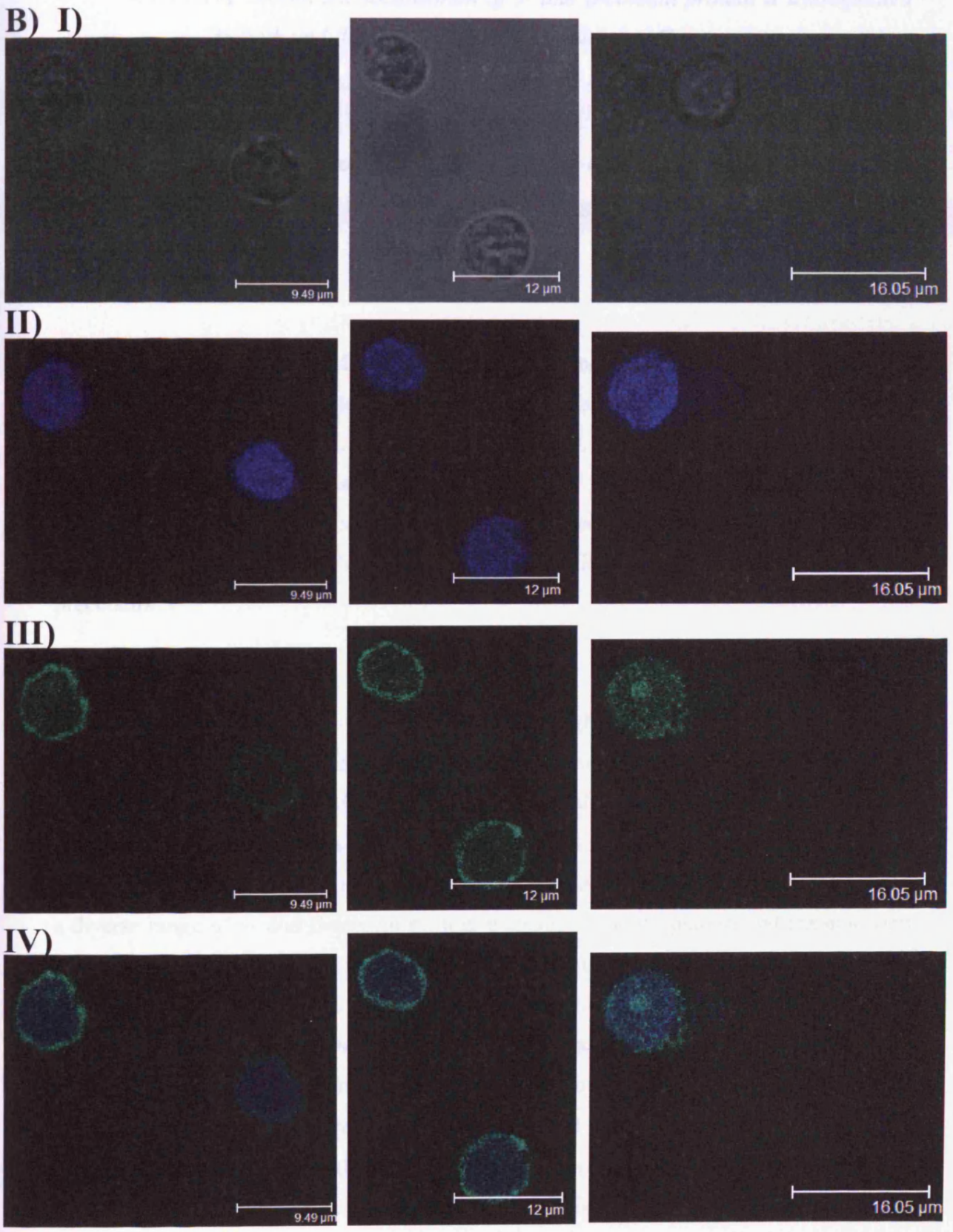


Figure 4.4 - The subcellular localisation of γ-catenin in M0/1 blasts by CLSM.

A) Summary of average nuclear γ-catenin protein localisation in normal $CD34^+$ haematopoietic progenitor cells (n=3) versus M0/1 AML blasts (n=5), as given by CLSM. *P<0.05. Error bars represent SD from each data. **B)** Various CLSM fields **(overleaf)** showing γ-catenin localisation in M0/1 AML blasts with I) phase contrast, II) nuclear (blue), III) γ-catenin (green), and IV) merged images.


B) I)
9.49 µm
12 µm
16.05 µm
II)
9.49 µm
12 µm
16.05 µm
III)
9.49 µm
12 µm
16.05 µm
IV)
9.49 µm
12 µm
16.05 µm

4.4.4.2 *The subcellular localisation of γ- and β-catenin protein is dysregulated in both undifferentiated and differentiated AML*

Once CLSM had identified nuclear γ-catenin within a small group of AML patient blast samples it was necessary to expand the number and type of AML samples analysed. N/C Western blotting provided a more time- and labour-effective method for analysing multiple AML samples, and its efficacy for detecting nuclear translocation of catenin had been validated in section *3.4.1.6*.

Figure 4.5A shows the subcellular localisation of γ and β-catenin protein expression in normal human haematopoietic cells versus the localisation in undifferentiated (*Figure 4.5B*) and differentiated AML blast samples (*Figure 4.6A* and *B*). The large cell number required for N/C fractionation meant *in vitro* expansion of normal $CD34^+$ haematopoietic progenitor cells was necessary. Day 5 cultured $CD34^+$ cells are predominantly $CD34^+$ blasts while day 13 cells consist largely of immature white cell precursors.

Figure 4.5A shows how γ- and β-catenin protein is highly expressed in day 5 and day 13 cultured haematopoietic cells which approximately encompass the range of differentiation seen in M0-M5 AML. As with freshly isolated $CD34^+$ cells this was mainly cytoplasmically localised. When compared to the expression observed in differentiated and undifferentiated AML blasts (*Figure 4.5* and *Figure 4.6*, respectively) a diverse range of γ- and β-catenin protein intensity, banding pattern and translocation can be observed among all the samples; though again the majority of catenin expression was cytoplasmically localised. Given the lack of control replicates (and thus no mean or standard deviation), an upper threshold of 5-fold the level of catenin obtained from normal cells was set as the marker for overexpression in AML cells. This value was used because the '$\bar{X}$+2SD' overexpression threshold set in the above section (*4.4.3*) actually represented 5-fold the mean obtained from normal $CD34^+$ cells. A similar variation was assumed for normal cells in this analysis. Using this cut off, it was found that 18 of 59 AML samples (31%) overexpressed cytoplasmic γ-catenin compared to normal controls, a proportion not too dissimilar (20%) to that obtained for total catenin

in the above section (*4.4.3*). A similar number of samples (18/59, 31%) were also found to have higher levels of nuclear translocated γ-catenin. The same analysis for β-catenin expression showed that a similar frequency of AML samples, 17/59 (29%) overexpressed the cytosolic form, whilst a higher proportion 22/59 (37%) exhibited elevated nuclear β-catenin. An interesting observation made for both catenins (but perhaps more accentuated for γ-catenin) was the differential migration pattern of protein bands between the cytosolic and nuclear fractions. The significance of this multiple banding is investigated and discussed further within *Chapter 5*. A more detailed FAB type breakdown (for those patients where clinical data is available (49/59)) of catenin overexpression in AML samples is featured in *Table 4.5*.

Table 4.5 - Breakdown of γ- and β-catenin overexpression between FAB types of AML samples N/C fractionated.

Feature	Overexpression in FAB type M0/1	Overexpression in FAB type M2	Overexpression in FAB type M4/5
Cytosolic γ-catenin	9/26 (35%) Examples = (*Figure 4.5B*, patient lanes c, g, h, i, k, l and n)	3/10 (30%) (*Figure 4.6A*, patient lanes c and g)	6/13 (46%) (*Figure 4.6B*, patient lanes b, d, e and g)
Nuclear γ-catenin	8/26 (31%) (*Figure 4.5B*, patient lanes h, i, j, k and l)	3/10 (30%) (*Figure 4.6A*, patient lanes b, c and e)	7/13 (54%) (*Figure 4.6B*, patient lanes c, e and g)
Cytosolic β-catenin	2/26 (8%) (*Figure 4.5B*, patient lanes g and j)	7/10 (70%) (*Figure 4.6A*, patient lanes a, b, e and g)	5/13 (38%) (*Figure 4.6B*, patient lanes d, e and g)
Nuclear β-catenin	5/26 (19%) (*Figure 4.5B*, patient lanes h, i, and j)	6/10 (60%) (*Figure 4.6A*, patient lanes b, c, d, f and g)	7/13 (54%) (*Figure 4.6B*, patient lanes b, c, e and g)

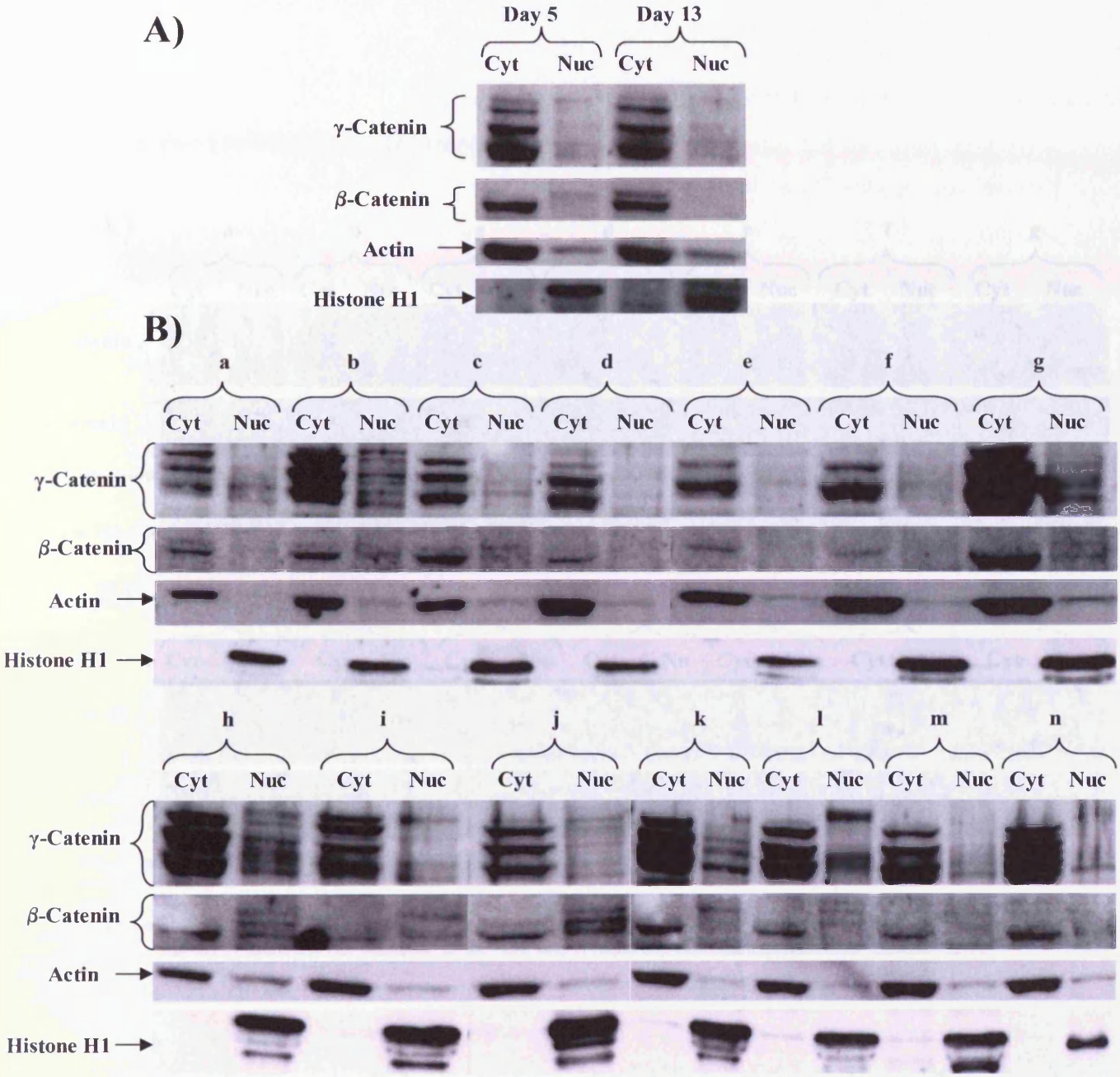


Figure 4.5 - The subcellular localisation of γ-catenin and β-catenin in normal haematopoietic progenitor cells versus undifferentiated AML.

A) The subcellular localisation of γ- and β-catenin protein within *in vitro* cultured and expanded primary human haematopoietic cells. CB-derived $CD34^+$ haematopoietic progenitor cells were purified and cultured *in vitro* for the time period indicated. **B)** Representative N/C Western blots showing the subcellular localisation of γ- and β-catenin in selected M0/1 AML blasts. Detection of β-actin and histone H1 provide an assessment of fractionation purity and loading.

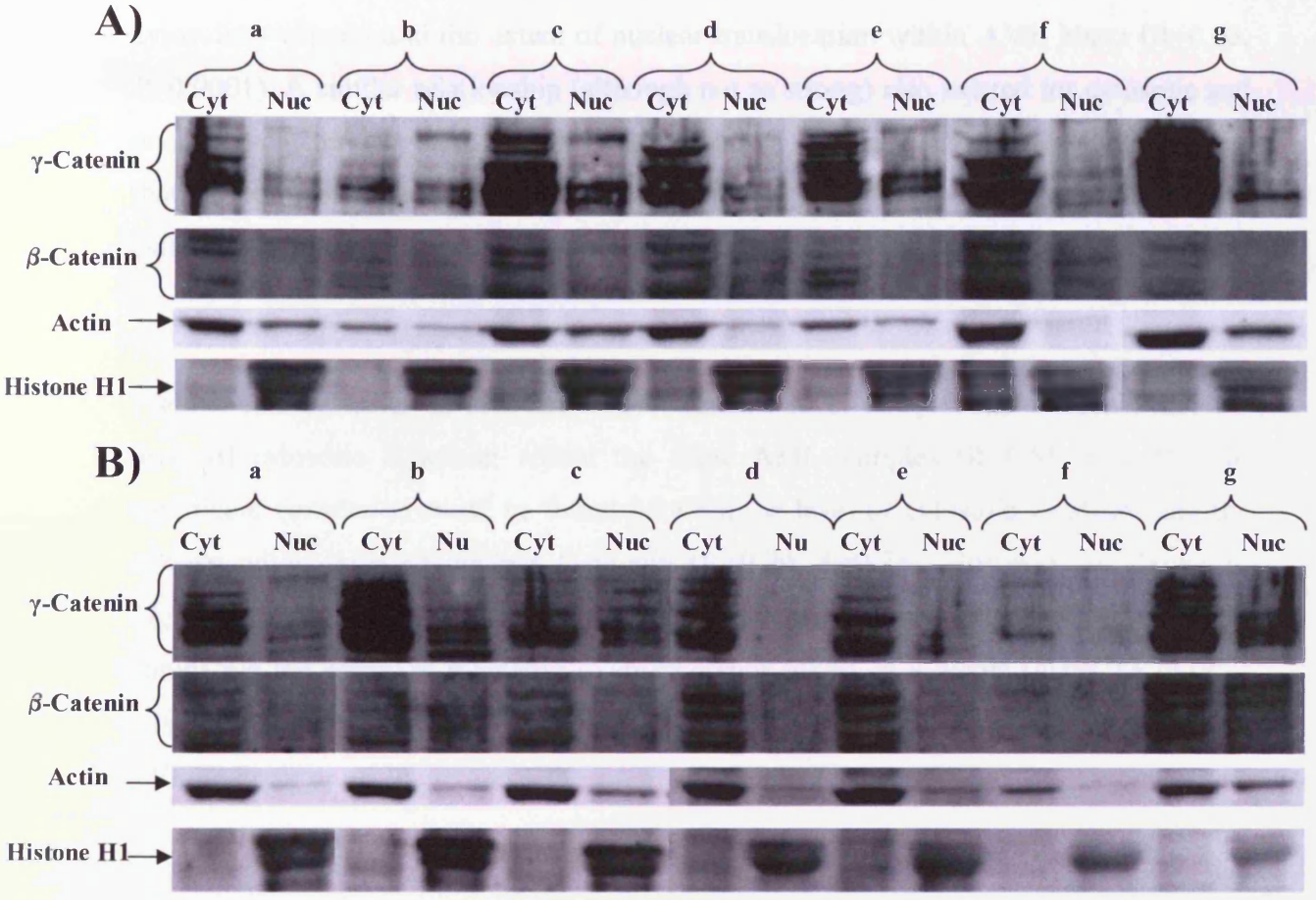


Figure 4.6 - The subcellular localisation of γ-catenin and β-catenin in differentiated AML by N/C western blotting.

Representative N/C Western blots showing the subcellular localisation of γ- and β-catenin in selected **A)** M2 and **B)** M4/5 AML blasts. Detection of β-actin and histone H1 indicate the level of purity and protein loaded within each fraction.

4.4.5 The level of γ- and β-catenin protein correlate in AML blasts

During the analysis of N/C fractionated samples in the above section (*4.4.4.2*) a number of potential relationships for γ- and β-catenin were observed. *Table 4.6* summarises the relationships analysed, the degree of correlation obtained and the level of significance for each. As illustrated in *Figure 4.7A*, a correlation existed between the level of cytosolic γ-catenin and the extent of nuclear translocation within AML blasts (R=0.63, $P<0.0001$). A similar relationship (although not as strong) also existed for cytosolic and nuclear β-catenin (R=0.56, $P<0.001$) and is shown in *Figure 4.7B*. This would suggest that, in contrast to normal haematopoiesis, the level of cytosolic expression of both catenins determines the degree of nuclear translocation.

Figure 4.8A indicates an association between the level of cytosolic γ-catenin and the level of cytosolic β-catenin within the same AML samples (R=0.51, $P<0.01$). No significant correlation could be found between the level of cytosolic γ-catenin and the corresponding level of nuclear β-catenin (R=0.29, $P=0.75$). However, as shown in *Figure 4.8B* the levels of nuclear β-catenin were significantly higher ($P<0.05$) when comparing the cytosolic γ-catenin overexpressing cohort as a whole (mean 13.1±19.2; range 0.0-80.0, median 7.3) with the rest of the cohort (5.6±6.9; range 0.0-26.3, median 3.9).

The final relationship examined was between the levels of translocated nuclear γ-catenin and nuclear β-catenin within AML samples. This relationship demonstrated a modest correlation (R=0.3, $P<0.05$). When the cohort was split (as above) and the level of nuclear β-catenin compared between samples overexpressing nuclear γ-catenin (18/59) versus otherwise normal/low levels (*Figure 4.8C)* the AML cohort exhibiting normal/low nuclear γ-catenin level demonstrated an average nuclear β-catenin level of 5.8±6.7 (range 0-26.3, median 4.9) whilst the high nuclear γ-catenin group had a statistically significantly higher mean of 15.6±22.2 (range 0-80.0, median 8.6, $P<0.05$).

Overall, the data of both this section and *4.4.4* above, suggest that both γ- and β-catenin protein expression is aberrantly localised in AML when compared to normal haematopoiesis. The results further imply that the cytosolic levels of both γ- and β-catenin are indicative of their respective nuclear level. Finally, the data has identified a correlation between γ- and β-catenin protein, in both the cytosolic and nuclear compartments.

Table 4.6 - The relationships between γ- and β-catenin in AML blasts.

Relationship	Degree of correlation (R)	Level of significance (*P*)
Cytosolic γ-catenin Vs Nuclear γ-catenin	0.63	<0.0001
Cytosolic β-catenin Vs Nuclear β-catenin	0.56	<0.001
Cytosolic γ-catenin Vs Cytosolic β-catenin	0.51	<0.01
Cytosolic γ-catenin Vs Nuclear β-catenin	0.29	0.75 (not significant)
Nuclear γ-catenin Vs Nuclear β-catenin	0.30	<0.05

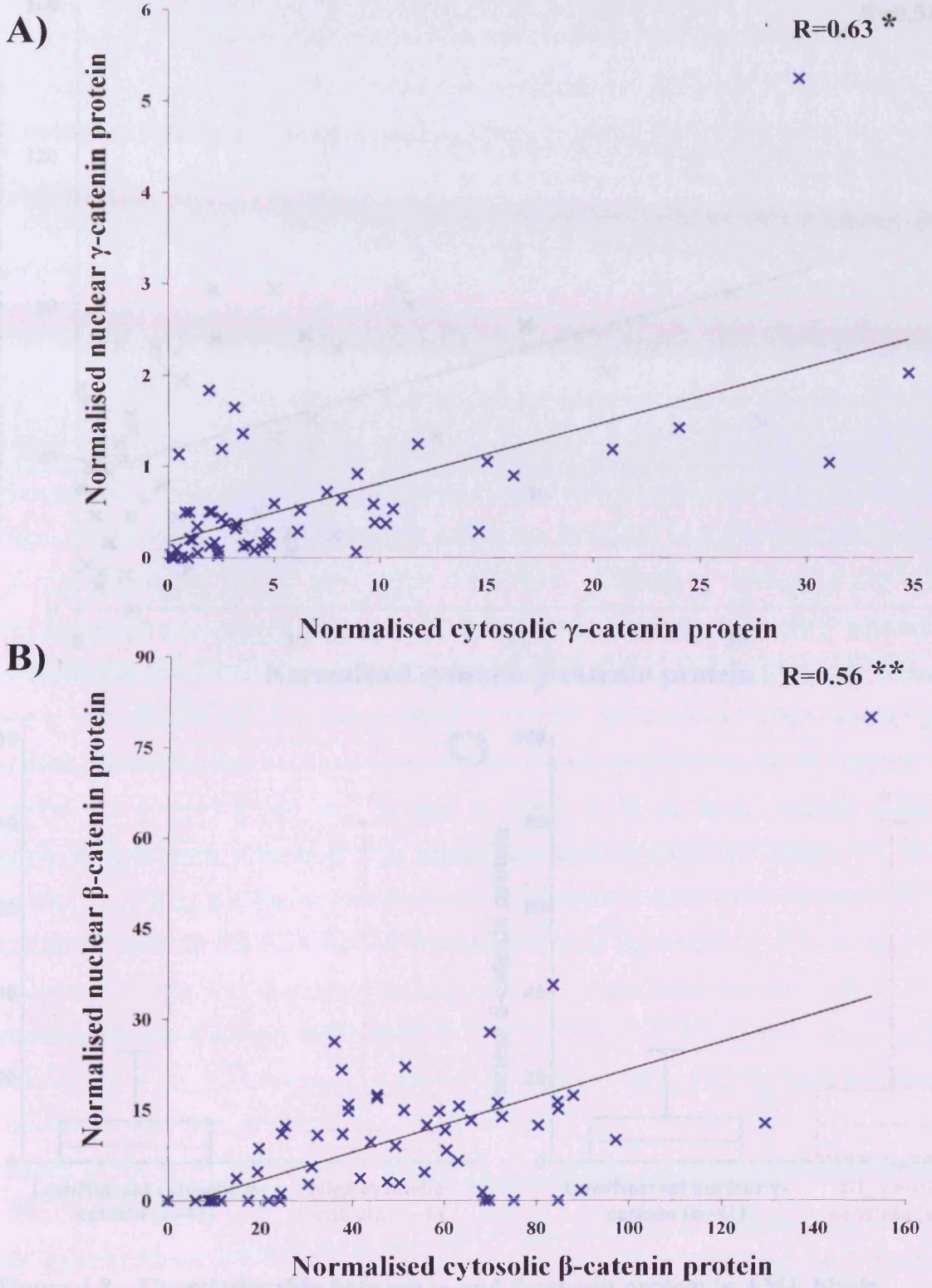


Figure 4.7 - The influence of cytosolic catenin level on nuclear translocation in AML blasts.

A) The correlation between the cytosolic level of γ-catenin and the degree of nuclear translocation in AML blasts, *P<0.0001. **B)** The correlation between the cytosolic level of β-catenin and the degree of nuclear translocation in AML blasts, **P<0.001.

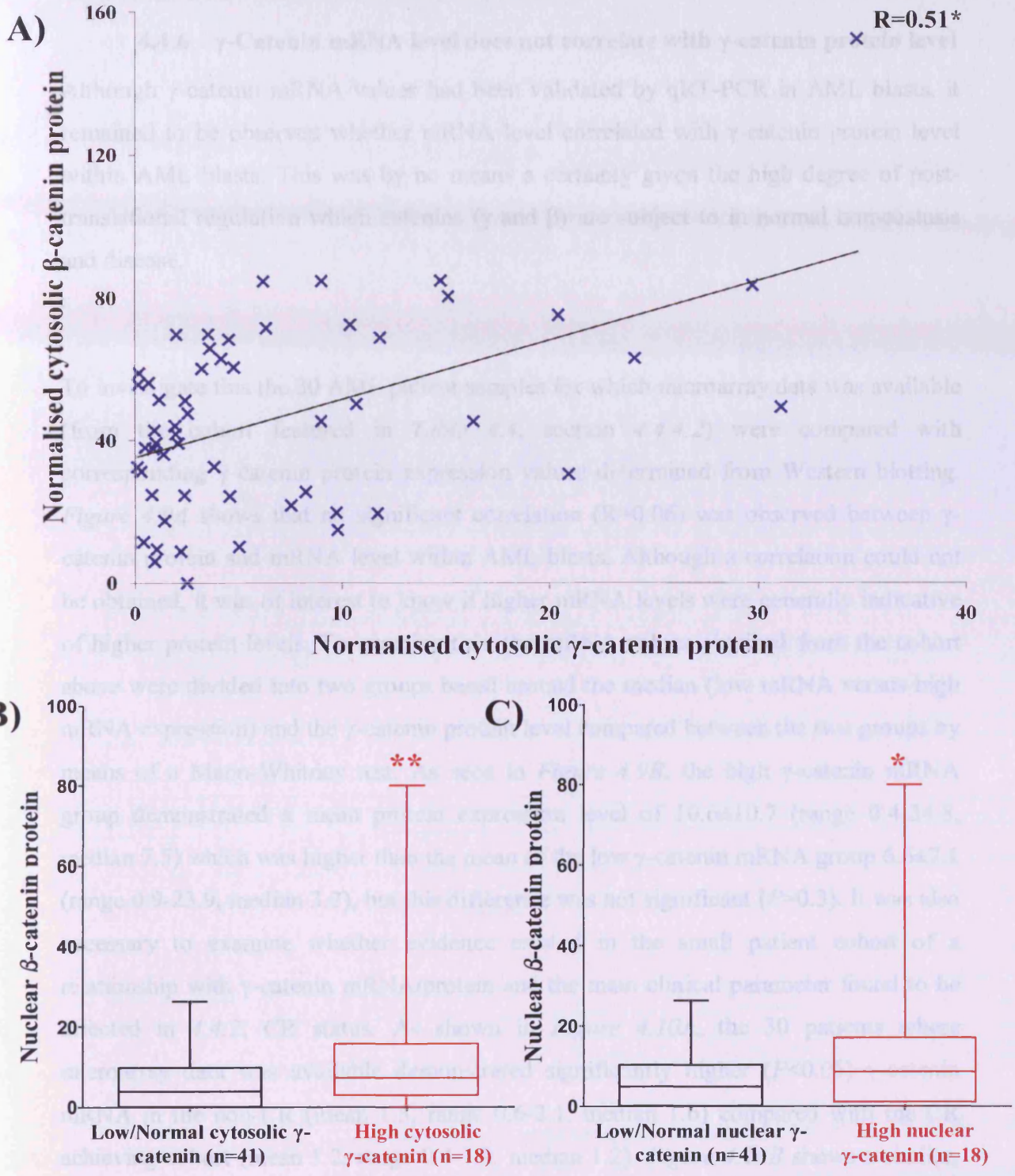


Figure 4.8 - The relationship between γ- and β-catenin protein in AML blasts.

A) The relationship between cytosolic levels of γ-catenin and β-catenin protein within AML blasts, *$P<0.01$. **B)** The level of nuclear β-catenin protein associated with AML blasts overexpressing cytosolic γ-catenin protein versus normal/low cytosolic γ-catenin protein, **$P<0.05$. **C)** The level of nuclear β-catenin protein associated with AML blasts overexpressing nuclear γ-catenin versus normal/low nuclear γ-catenin, **$P<0.05$. Units are arbitrary.

4.4.6 γ-Catenin mRNA level does not correlate with γ-catenin protein level

Although γ-catenin mRNA values had been validated by qRT-PCR in AML blasts, it remained to be observed whether mRNA level correlated with γ-catenin protein level within AML blasts. This was by no means a certainty given the high degree of post-translational regulation which catenins (γ and β) are subject to in normal homeostasis and disease.

To investigate this the 30 AML patient samples for which microarray data was available (from the cohort featured in *Table 4.4*, section *4.4.4.2*) were compared with corresponding γ-catenin protein expression values determined from Western blotting. *Figure 4.9A* shows that no significant correlation (R=0.06) was observed between γ-catenin protein and mRNA level within AML blasts. Although a correlation could not be obtained, it was of interest to know if higher mRNA levels were generally indicative of higher protein levels. To examine this, the mRNA values obtained from the cohort above were divided into two groups based around the median (low mRNA versus high mRNA expression) and the γ-catenin protein level compared between the two groups by means of a Mann-Whitney test. As seen in *Figure 4.9B*, the high γ-catenin mRNA group demonstrated a mean protein expression level of 10.6±10.7 (range 0.4-34.8, median 7.5) which was higher than the mean of the low γ-catenin mRNA group 6.6±7.1 (range 0.9-23.9, median 3.2), but this difference was not significant (P=0.3). It was also necessary to examine whether evidence existed in the small patient cohort of a relationship with γ-catenin mRNA/protein and the main clinical parameter found to be affected in *4.4.2*; CR status. As shown in *Figure 4.10A*, the 30 patients where microarray data was available demonstrated significantly higher (P<0.05) γ-catenin mRNA in the non-CR (mean 1.5, range 0.6-2.1, median 1.6) compared with the CR achieving cohort (mean 1.2, range 0.4-1.9, median 1.2). *Figure 4.10B* shows a similar, but non-significant (P=0.1), difference was observed when analysing the γ-catenin protein values. Generally, higher γ-catenin protein expression was found in the non-CR (mean 9.9, range 0.8-34.8, median 6.8) versus CR patient group (mean 5.9, range 0.2-24.0, median 3.3).

Taken together, these data suggest that although higher γ-catenin mRNA expression is generally associated with a higher γ-catenin protein level, the actual degree of correlation is poor. This would mean the survival phenotype obtained in section *4.4.2* is not necessarily related to γ-catenin protein expression, though the relatively small number of comparisons made could mean that the poor correlation arises from sampling error.

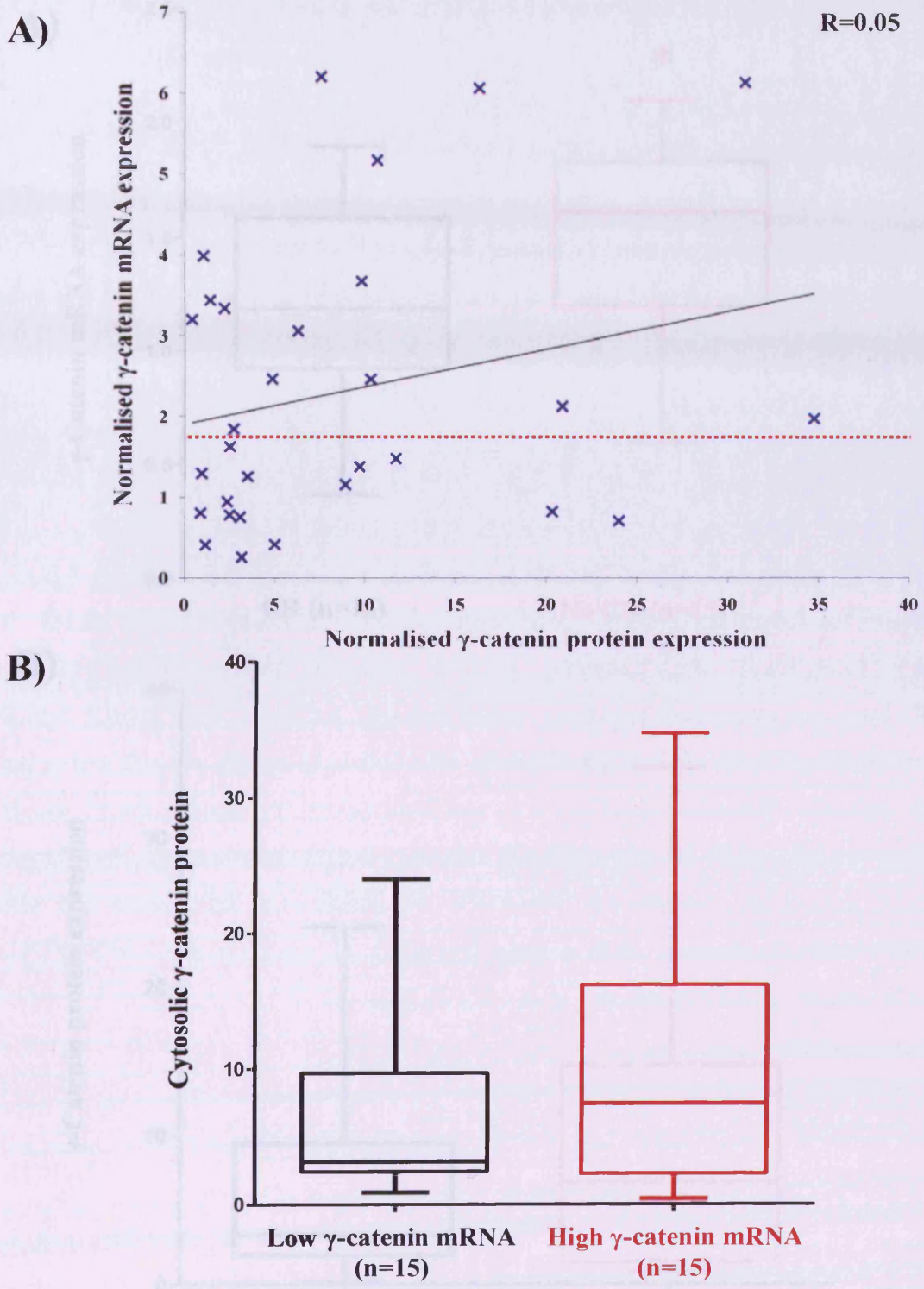


Figure 4.9 - The correlation of γ-catenin mRNA with γ-catenin protein level.

A) Scatter plot showing the lack of correlation between γ-catenin protein (derived from Western blotting) and γ-catenin mRNA expression (derived from microarray) in AML blasts. Red dotted line shows division of cohort analysed in B. **B)** Box and whisker plot showing general level of cytosolic γ-catenin protein in low γ-catenin mRNA versus high γ-catenin mRNA group.

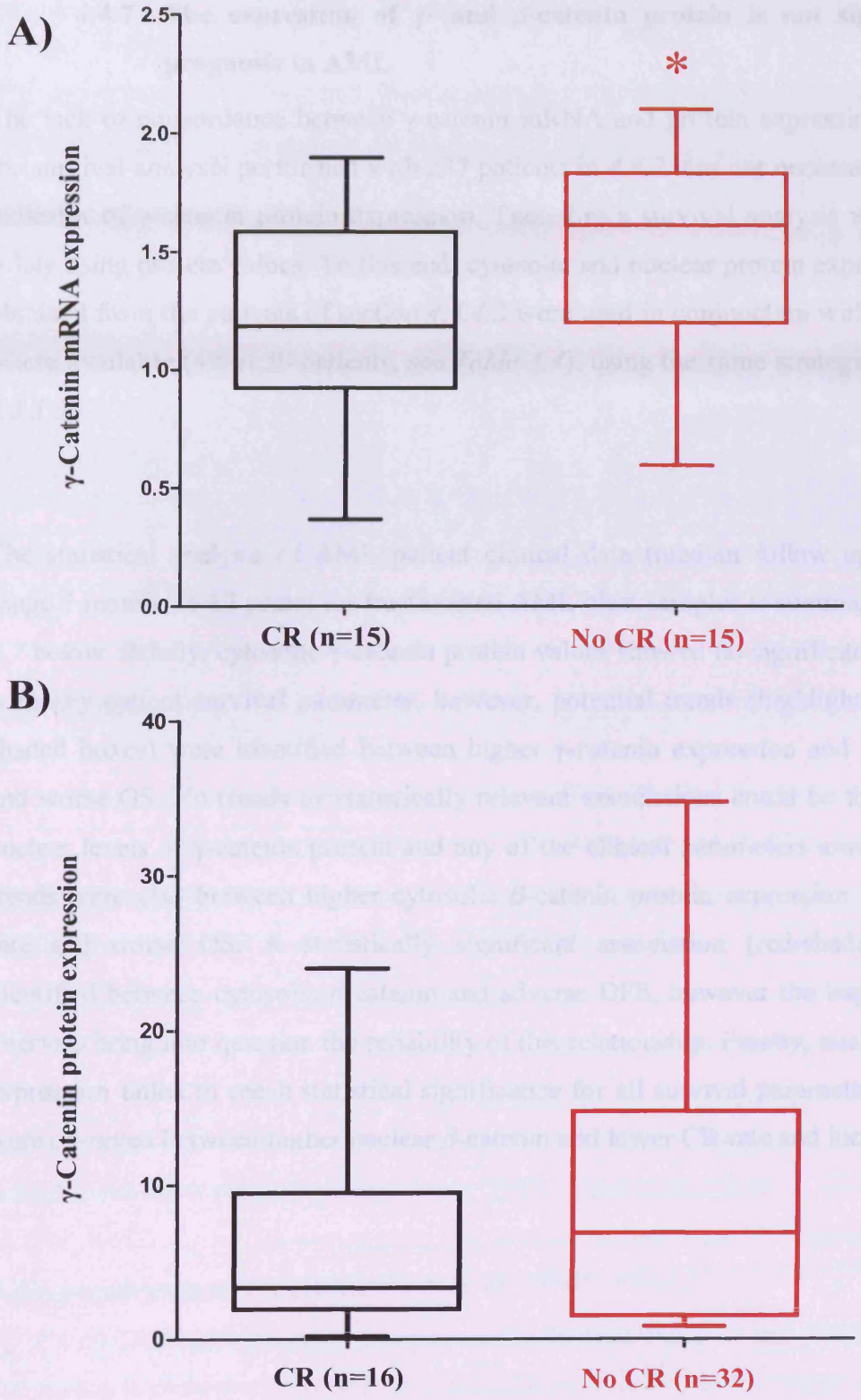


Figure 4.10 - The level of γ-catenin mRNA and protein expression associated with CR status of N/C Western blotted patients.

A) Box and whisker plot showing the γ-catenin mRNA status between patients achieving and not achieving CR ($P<0.05$). **B)** Similar plot but showing the γ-catenin protein status between CR and non-CR patient group.

4.4.7 The expression of γ- and β-catenin protein is not significant for prognosis in AML

The lack of concordance between γ-catenin mRNA and protein expression meant that the survival analysis performed with 237 patients in *4.4.2* was not necessarily a reliable indicator of γ-catenin protein expression. Therefore a survival analysis was necessary solely using protein values. To this end, cytosolic and nuclear protein expression values obtained from the patients of section *4.4.4.2* were used in conjunction with clinical data where available (49 of 59 patients, see *Table 4.4*), using the same strategies outlined in *4.3.1.3*.

The statistical analysis of AML patient clinical data (median follow up 64 months, range 7 months to 17 years) for fractionated AML blast samples is summarised in *Table 4.7* below. Briefly, cytosolic γ-catenin protein values showed no significant relationship with any patient survival parameter, however, potential trends (highlighted by orange shaded boxes) were identified between higher γ-catenin expression and poor CR rate and worse OS. No trends or statistically relevant associations could be found between nuclear levels of γ-catenin protein and any of the clinical parameters assessed. Similar trends were also between higher cytosolic β-catenin protein expression and poor CR rate and worse OS. A statistically significant association (red-shaded box) was identified between cytosolic β-catenin and adverse DFS, however the large confidence intervals bring into question the reliability of this relationship. Finally, nuclear β-catenin expression failed to reach statistical significance for all survival parameters, but trends were observed between higher nuclear β-catenin and lower CR rate and increased RD.

Table 4.7 - Summary of relevant findings from survival analysis of cytosolic and nuclear γ- and β-catenin.

	Survival parameter			
Localised catenin	**CR**	**OS**	**RD**	**DFS**
Cytosolic γ-catenin	2.11(0.43-10.37) *P*=0.3	1.59(0.60-4.27) *P*=0.3	NS	NS
Nuclear γ-catenin	NS	NS	NS	NS
Cytosolic β-catenin	1.80(0.47-7.00) *P*=0.3	1.55 (0.77-3.12) *P*=0.2	NS	12.2(1.38-108.1) *P*<0.01
Nuclear β-catenin	1.53(0.83-2.79) *P*=0.2	NS	1.72(0.74-3.98) *P*=0.2	NS

Clinical parameters include complete remission (CR), overall survival (OS), resistant disease (RD), and disease-free survival (DFS). Odds ratios and 95% confidence intervals (in parentheses) are listed with associated significance values included below. NS = not significant.

4.5 Discussion

4.5.1 γ-Catenin mRNA expression confers an adverse prognosis but does not correspond with the protein level present in AML blasts

This study sought to characterise γ-catenin protein expression in primary AML blasts for the first time, and draw comparisons with the expression previously observed in normal haematopoietic development (*Chapter 3*). Aberrant activation of the Wnt/catenin pathway has been implicated in a number of solid cancers including breast (Lin *et al.*, 2000), colon (Korinek *et al.*, 1997; Morin *et al.*, 1997) and liver (Monga, 2009). Further, whilst there is plenty of evidence suggesting haematological malignancies such as AML (Simon *et al.*, 2005; Wang *et al.*, 2010; Yeung *et al.*, 2007), chronic lymphocytic leukaemia (CLL) (Lu *et al.*, 2004), chronic myeloid leukaemia (CML) (Jamieson *et al.*, 2004; Zhao *et al.*, 2007; Abrahamsson *et al.*, 2009), acute lymphoblastic leukaemia (ALL) (Khan *et al.*, 2007; Nygren *et al.*, 2007) mantle cell lymphoma (Gelebart *et al.*, 2008) and multiple myeloma (MM) (Sukhdeo *et al.*, 2007; Qiang *et al.*, 2009) arise partly due to constitutive activation of the Wnt/β-catenin pathway, it remains to be established whether there is γ-catenin involvement in human AML. Using a cohort of 243 AML trial patients and associated clinical data, this study analysed γ-catenin mRNA gene expression for correlation with clinical outcome.

Despite the association of γ-catenin expression with a good risk sub-type (RUNX-1/ETO expression (Zheng *et al.*, 2004; Muller-Tidow *et al.*, 2004; Tonks *et al.*, 2007)), analyses of survival correcting for this bias showed that γ-catenin mRNA level was significantly associated with a poor CR rate (arising from resistant disease); there was no evidence of any association with age at diagnosis, WBC or sex. These data indicated that it may be γ-catenin that is primarily dysregulated and prognostic at mRNA level, unlike β-catenin which was not identified as significantly dysregulated in our previous microarray studies (unpublished data).

In order to validate the Affymetrix GEP data, γ-catenin mRNA was analysed by qRT-PCR which confirmed that the microarray did reflect γ-catenin mRNA level. However, analysis of γ-catenin protein expression by Western blot or flow cytometry did not correlate with mRNA expression. This is not entirely unexpected given the post-translational mechanisms which are known to regulate γ-catenin protein stability. Lack of agreement between mRNA and protein level has been previously reported for both γ-catenin (Bradley *et al.*, 1993; Kowalczyk *et al.*, 1994; Papagerakis *et al.*, 2004) and β-catenin (Aberle *et al.*, 1997; Ysebaert *et al.*, 2006; Gandillet *et al.*, 2011). The study by Ysebaert *et al.* (Ysebaert *et al.*, 2006) found β-catenin mRNA level did not correspond with protein level and attributed this to the post-translational modifications that are active in AML cells. Discordance between mRNA and protein level has also been frequently observed in other contexts (Schindler *et al.*, 1990; Ranganathan *et al.*, 1998; Gygi *et al.*, 1999; Chen *et al.*, 2002; Greenbaum *et al.*, 2003; Lee *et al.*, 2003; Pascal *et al.*, 2008). On the other hand, in model systems concordance of γ-catenin mRNA and protein levels has been demonstrated. Müller-Tidow *et al.* (Muller-Tidow *et al.*, 2004) observed γ-catenin overexpression in leukaemic cell lines expressing common fusion proteins, such as promyelocytic leukaemia/retinoic acid receptor α (PML/RARα) or RUNX-1/ETO; and in the latter case this has also been demonstrated in primary haematopoietic cells (Zheng *et al.*, 2004; Tonks *et al.*, 2007). In this study patients expressing RUNX-1-ETO were not distinguished by high γ-catenin at the protein level, however further AML samples with a CBF abnormality would be needed to be analyse this relationship further.

The lack of concordance between γ-catenin mRNA and protein may indicate that the adverse CR phenotype obtained from survival analyses either represents a chance finding, or that factors promoting γ-catenin mRNA may also influence the expression of an alternative protein impacting on CR rate. Alternatively the lack of correlation could arise from sampling error of the patients included in the correlation analysis since higher protein levels did show a trend towards reduced CR (*Figure 4.10B*). Fundamentally, in comparison with the microarray analysis (243 patients), analysis of γ-catenin protein expression and localisation (49 samples) lacked the power to significantly demonstrate any association with outcome at the protein level. It is estimated at least 100 additional patients would need to be analysed to establish whether the trends observed have biological significance, and this may reflect the heterogeneity of this disease and/or the lack of any strong influence of γ-catenin protein expression on treatment outcome. The lack of any strong survival influence from nuclear γ- or β-catenin has been noted before both in renal cell carcinoma (Aaltomaa *et al.*, 2004) and also in colon cancer where cytosolic catenin is instead found to be more prognostically significant (Maruyama *et al.*, 2000; Norwood *et al.*, 2010).

The failure to observe any survival effect for nuclear β-catenin expression is perhaps a little surprising at first given its associated role with driving self-renewal/proliferation in leukaemic cells (Jamieson *et al.*, 2004; Zhao *et al.*, 2007;Hu *et al.*, 2009; Abrahamsson *et al.*, 2009; Wang *et al.*, 2010; Yeung *et al.*, 2010; Siapati *et al.*, 2011). β-Catenin has also frequently been reported as overexpressed in primary AML blasts (Chung *et al.*, 2002; Serinsoz *et al.*, 2004; Simon *et al.*, 2005; Chen *et al.*, 2009) and invariably linked with poor survival (Ysebaert *et al.*, 2006; Xu *et al.*, 2008). Indeed, Xu *et al.* found nuclear β-catenin protein to be associated with unfavourable cytogenetics (-7/-7q), lower CR rates, and worse overall survival, whilst Ysebaert *et al.* (Ysebaert *et al.*, 2006) found more general β-catenin expression to be an independent prognostic marker predicting poor event-free survival and shorter overall survival. However it must be noted that these AML survival studies featured small patient numbers (<100) which are too small in such a heterogeneous malignancy to be truly definitive. Regardless, although these proteins serve essential roles in AML pathology, there is evidence to

suggest they do not necessarily govern chemo-sensitivity and hence influence the survival outcome (Gandillet *et al.*, 2011).

4.5.2 γ-Catenin is aberrantly localised in primary AML blasts

From *Chapter 3*, it was clear that subcellular distribution of γ-catenin may play a role in normal haematopoiesis. This study is the first to report nuclear γ-catenin expression in AML. This study observed an increase in nuclear γ-catenin expression when compared to normal $CD34^+$ progenitor cells suggesting that nuclear γ-catenin expression represents an abnormality for FAB type M0/1 (undifferentiated) AML. Since nuclear γ-catenin is a feature of more differentiated cells *3.4.3*, the same cannot be said of the differentiated AML patients analysed (M2-M5).

Understanding the significance of this nuclear γ-catenin is a more challenging proposition. *Does it represent a transcriptionally active form?* γ-Catenin's ability to bind and activate TCF/LEF complexes (albeit with less efficiency than β-catenin) has been demonstrated before in other model systems (Simcha *et al.*, 1998; Kolligs *et al.*, 2000; Williams *et al.*, 2000; Zhurinsky *et al.*, 2000a; Maeda *et al.*, 2004; Fukunaga *et al.*, 2005). Within a haematopoietic context, the study of Müller-Tidow *et al.* (Muller-Tidow *et al.*, 2004) identified direct activation of the *c-myc* promoter by increased γ-catenin expression. Alternatively could its presence in the nucleus represent a functionally redundant form that has merely escaped a saturated degradation system? Nuclear expression has been linked with suppression of Wnt mediated transcription (Miravet *et al.*, 2002; Garcia-Gras *et al.*, 2006). The former study identified binding sites on TCF-4 for both β- and γ-catenin, but demonstrated that interactions involving γ-catenin actually hindered the transcriptional activity of the complex in various epithelial cell lines.

It was less surprising to observe nuclear forms of β-catenin in fractionated AML blasts given that nuclear beta catenin has been identified in many haematological malignancies including CML (Jamieson *et al.*, 2004; Abrahamsson *et al.*, 2009), myelodysplastic syndrome (MDS) (Xu *et al.*, 2008) MM (Sukhdeo *et al.*, 2007), ALL (Khan *et al.*, 2007;

Nygren *et al.*, 2007) and CLL (Lu *et al.*, 2004). This protein is already known to be present and transcriptionally active in nuclei of normal HSCs (Reya *et al.*, 2003; Jamieson *et al.*, 2004; Scheller *et al.*, 2006; Kirstetter *et al.*, 2006; Holmes *et al.*, 2008; Congdon *et al.*, 2008). The data in *Chapter 3* also demonstrated nuclear localisation of β-catenin in progenitor cells. Nuclear β-catenin has already been identified in AML blasts (Simon *et al.*, 2005; Xu *et al.*, 2008; Wang *et al.*, 2010; Gandillet *et al.*, 2011). Interestingly, in the study by Xu *et al.* they reported a similar detection rate of nuclear β-catenin as this study (22/54 [41%] Vs 22/59 [37%]), albeit by a very different immunohistochemical method. Additionally, nuclear beta catenin has also been frequently documented within the wider cancer field including colon (Korinek *et al.*, 1997; Morin *et al.*, 1997; Brabletz *et al.*, 1998; Brabletz *et al.*, 2000; Maruyama *et al.*, 2000; Kobayashi *et al.*, 2000; Jung *et al.*, 2001) liver (Nhieu *et al.*, 1999), malignant melanoma (Rimm *et al.*, 1999) and prostate cancers (Jaggi *et al.*, 2005).

4.5.3 The expression of γ- and β-catenin protein correlate in AML

This study represents the first to identify a positive correlation between γ- and β-catenin protein expression in primary AML blasts. The finding that cytosolic γ- and β-catenin protein levels correlate should perhaps not be so surprising given that the stability of the two proteins is regulated by a common process (see introductory sections *1.3.1* and *1.3.4*). Hence, should any of these components be dysregulated in AML then a concomitant rise in both γ- and β-catenin might be expected. When observing expression of each catenin individually, it was interesting to find that cytosolic level determined the nuclear level in AML blasts. This is in contrast, to observations in normal haematopoiesis, where nuclear catenin localisation was independent of increased cytosolic expression. This implies whatever mechanism is active in normal haematopoietic cells regulating catenin translocation (described in length in *Chapter 3* discussion *3.5.3*) is relaxed or dysfunctional in AML. Indeed, loss of such tight regulation would leave the cell vulnerable to the wealth of candidate molecules (inside and outside of the Wnt signalling cascade) that have been proposed to alter catenin level and localisation, including FLT-3 (Tickenbrock *et al.*, 2005; Kajiguchi *et al.*, 2007), prostaglandin E_2 (PGE2) (Castellone *et al.*, 2005), protein kinase A (PKA) (Hino *et al.*, 2005), phosphatase and tensin homologue (PTEN) (Persad *et al.*, 2001), Ras-related C3

botulinum toxin substrate 1 (Rac-1) (Wu *et al.*, 2008), Notch (Hayward *et al.*, 2005), Roof plate-specific Spondin 1 (R-spondin-1) (Wei *et al.*, 2007), v-Akt murine thymoma viral oncogene homologue (Akt) (Monick *et al.*, 2001) and growth arrest-specific gene 6 (Gas-6) (Goruppi *et al.*, 2001).

A degree of correlation was also found between γ- and β-catenin expression in the nucleus of AML samples. It is unknown whether this relationship represents an inevitable 'side-effect' of the increased cytosolic levels of both proteins in these cells, or a co-ordinated re-localisation as has been previously reported for nuclear γ- and β-catenin in other contexts (Simcha *et al.*, 1998; Maeda *et al.*, 2004; Li *et al.*, 2007). This issue is investigated further in *Chapter 5*.

4.5.4 Multiple-banding of γ- and β-catenin is observed upon Western blotting of primary AML samples

As observed in the whole cell Western blotting of AML cell lines in *Chapter 3* (see *Figure 3.10*), multiple banding of γ-catenin and (to a lesser extent) β-catenin was exhibited in the N/C fractionated lysates of AML blasts and normal human haematopoietic cells. This type of multiple banding has been observed previously for both γ-catenin (Zheng *et al.*, 2004; Muller-Tidow *et al.*, 2004; Tonks *et al.*, 2007) and β-catenin (Chung *et al.*, 2002; Simon *et al.*, 2005; Ysebaert *et al.*, 2006) in haematopoietic cells. Indeed, the study by Simon *et al.* also N/C Western blotted primary AML blasts (albeit by a different protocol) and obtained a similar variation in intensity and migration of β-catenin protein bands. Intriguingly, the migration of catenin bands in this study was often different between nuclear and cytosolic fractions of the same patient samples. It is highly likely that this multiple-banding pattern is indicative of a key mechanism involved in the regulation of catenins. The fact the patterns are inconsistent between AML (N/C) samples implies that this process may be dysregulated in malignancy. Exactly what mechanism this could represent is unknown. One possible explanation is that these bands correspond to differentially phosphorylated catenin molecules (see *3.5.3*), as demonstrated previously for catenins in Western blotted

samples (Ruff *et al.*, 1997; Sukhdeo *et al.*, 2007). This possibility is investigated further in *Chapter 5*.

Mutations in catenin protein are unlikely to be the cause of the multiple banding, not least because only one such mutation has ever been reported for γ-catenin in cancer (Caca *et al.*, 1999). Furthermore, this was a small missense mutation to a potential phosphorylation site in the amino terminus, and is thus unlikely to seriously modify the overall protein structure to an extent where it would migrate differently through a gel compared to the *wt* form. No significant isoforms of γ- or β-catenin exist or have been reported in normal human biology either.

Finally, a less exciting possibility is that these multiple migratory forms between cytosol and nuclear fractions may actually represent artefact generated by the respective lysis buffers of the N/C fractionation kit. Such a cause is difficult to investigate given the manufacturer's secrecy over the constitution of these buffers, and is perhaps irrelevant given that other studies have observed the same banding patterns using alternative lysing techniques.

In summary, this study has shown for the first time that γ-catenin protein expression is dysregulated in some cases of AML. Patient survival data would indicate that γ-catenin mRNA expression is associated with a worse prognosis in AML, however this clinical phenotype could not be substantiated because of discordance between γ-catenin mRNA and protein level. Contrary to normal haematopoiesis, γ-catenin protein is frequently localised to the nuclei of undifferentiated AML blasts, and unlike normal cells, translocation appears to be influenced by cytosolic accumulation. A correlation between γ- and β-catenin has been identified in the cytosol (and to a lesser extent in the nuclei) of AML blasts, suggesting they are co-ordinately dysregulated. The next chapter seeks to investigate whether the relationship between γ- and β-catenin in AML blasts represents a correlative or causative association and, related to this, whether γ-catenin contributes individual or combinatory effects to AML pathology.

5 - Examination into the Functional Role of γ-Catenin in Haematopoietic Cells

5.1 Introduction

Given the limited number of studies on γ-catenin in normal haematopoiesis and AML, many of the results from *Chapters 3* and *4* have been observational in nature. Thus, the main purpose of *Chapter 5*, is to establish the functional roles of γ-catenin in both normal haematopoiesis and AML pathology.

The data of *Chapter 3* suggested that γ-catenin has a role in both the early and late stages of haematopoietic development. Further, preliminary experiments of this laboratory (Tonks *et al.*, 2007) suggested that ectopic expression of γ-catenin in normal human $CD34^+$ haematopoietic cells led to modest increases in self-renewal, although no significant effect on myeloid differentiation was observed. To complement these studies, it was necessary to assess the importance of γ-catenin for normal myeloid development by using an shRNA approach to deplete endogenous γ-catenin in normal human haematopoietic progenitors. Previous studies in mice would suggest that the loss of γ- and/or β-catenin has no consequences for normal haematopoiesis (Cobas *et al.*, 2004; Koch *et al.*, 2007; Jeannet *et al.*, 2008), however, these studies only examined the functionality of these knockout cells in a repopulation assay and did not examine the developmental capacity of these cells. Further, the consequence of γ-catenin underexpression in *human* haematopoietic cells has not been previously studied.

Chapter 4 identified a correlation between the expression of γ- and β-catenin protein in AML blasts. Although evidence exists suggesting these catenins can influence the level and subcellular localisation of one another in other settings (Salomon *et al.*, 1997; Simcha *et al.*, 1998; Zhurinsky *et al.*, 2000a; Miravet *et al.*, 2003; Maeda *et al.*, 2004; Fukunaga *et al.*, 2005; Li *et al.*, 2007; Shimizu *et al.*, 2007) no such relationship has

been reported within a haematopoietic or leukaemic context. To address this, γ-catenin was ectopically expressed in both normal human progenitors and myeloid leukaemia cell lines with the capacity to undergo chemically-induced differentiation (Tsuchiya *et al.*, 1982; Hass *et al.*, 1989). Such investigations would explore the inter-dependence between γ- and β-catenin proteins and the consequences of such a relationship for the development of these cells.

The discovery that γ-catenin translocation in normal progenitors is apparently tightly regulated raises the question of the mechanisms regulating this process. One such mechanism to be assessed is the ability of γ-catenin to respond to a Wnt stimulus. Members of the Wnt family of glycoproteins are known to be active throughout normal haematopoiesis (Austin *et al.*, 1997; Van den Berg *et al.*, 1998) and capable of stabilising β-catenin expression (mainly Wnt3A; (Dosen *et al.*, 2006; Kim *et al.*, 2009)). Interestingly, Wnt signalling proteins have also been found to be dysregulated in acute leukaemias and Wnt-mediated stabilisation (leading to nuclear translocation) of β-catenin has also been observed within this context (Simon *et al.*, 2005; Tickenbrock *et al.*, 2005; Nygren *et al.*, 2007; Tickenbrock *et al.*, 2008; Kawaguchi-Ihara *et al.*, 2008). Such a phenomenon has not been reported for γ-catenin in haematopoietic cells, and it will be interesting to observe whether this could be contributing to the correlation between β- and γ-catenin.

Related to the above, is the investigation into the cause of multiple catenin bands in Western blotting which may inform how the level and localisation of catenins are regulated in haematopoietic cells. It is outside the scope of this study to investigate all the potential causes of the multiple protein banding, however this chapter seeks to investigate one of the potential causes; differential phosphorylation. There is a wealth of evidence demonstrating that phosphorylation of catenin is crucial in regulating activity, turnover and translocation of the protein (see *3.5.3* and *4.5.3*).

5.2 Aims

In order to better understand the functional roles of γ-catenin in normal haematopoiesis and AML pathology, the aims of this chapter are:

1) To use an established model of *in vitro* haematopoiesis, to examine the effects of γ-catenin silencing on normal haematopoietic development.

2) To investigate how the modulation of γ-catenin expression affects the level and subcellular localisation of β-catenin in normal and leukaemic cells.

3) To examine the consequences of γ-catenin overexpression/silencing on the induced differentiation of monocytic cell lines.

4) To explore the possible mechanisms regulating the translocation of γ-catenin from cytosol to nucleus in haematopoietic cells.

5) To establish the possible causes of the multiple banding of catenin protein observed during Western blotting.

5.3 Materials and Methods

5.3.1 Investigation into the function of γ-catenin in normal haematopoiesis

The full strategy of this experiment and endpoints are outlined in the following sections and summarised in *Figure 5.1.*

5.3.1.1 *Modelling haematopoiesis* in vitro

The comprehensive characterisation of the HSC immunophenotype and vastly improved purification techniques for their isolation (by fluorescence activated cell sorting (FACS) or immunogenic magnetic beads) has permitted developmental studies of these cells outside of the body. Furthermore, pioneering work of researchers in the 1970's identified the medium, growth factors (GF) and cytokines necessary to culture haematopoietic cells *in vitro* (Pike and Robinson, 1970; Iscove *et al.*, 1971; Chervenick and Boggs, 1971; Metcalf, 1971; Metcalf *et al.*, 1974; Dao *et al.*, 1977a; Dao *et al.*,

1977b). Specifically, many of these studies highlighted the importance of specific soluble colony stimulating factors (CSF) in bringing about the growth and differentiation of specific haematopoietic lineages. Since these fundamental discoveries, many laboratories have optimised combinations and concentrations of growth factors necessary to model their particular area of haematological interest *in vitro*. In particular, pioneering work from this laboratory has resulted in the development of an effective model of *in vitro* human myeloid differentiation using such GFs and cytokines as IL-3, SCF, GM-CSF and G-CSF (Darley *et al.*, 1997; Darley and Burnett, 1999; Tonks *et al.*, 2004).

The *in vitro* differentiation of HSC into mature progeny such as neutrophils, monocytes, and macrophages can be effectively monitored through the cell surface cluster of differentiation (CD) expression. Such molecules not only serve important functions for the cell (e.g. cell signalling receptors), but also provide accurate information as to the cell type, function and stage of differentiation. The expression pattern of CD antigens on specific developing haematopoietic lineages has been well characterised and can be easily detected by fluorochrome-conjugated antibodies using flow cytometry. Morphological assessment can be used in conjunction with CD marker expression to confirm the extent of differentiation. The use of cytochemical stains such as May-Grünwald-Giemsa allow the differential staining of cytosol and nucleus, such that the morphological changes associated with development can be identified by basic light microscopy. Exploiting all of the aforementioned parameters, plus the relative ease with which HSCs can be retrovirally transduced, means a powerful model is available to assess the impact of single gene changes, such as γ-catenin, on normal haematopoietic development.

5.3.1.2 *In vitro culture of normal human primary haematopoietic cells*

Normal CB-derived $CD34^+$ HPC were isolated and enriched as outlined in section *2.4*. Following isolation (designated **day 0**), $CD34^+$ cells were cultured overnight at a density of $2x10^5$/ml in supplemented IMDM (containing 1% BSA fraction V, 20% FBS, 45μM BME, 360μg/ml 30% iron-saturated human transferrin, 100IU/ml penicillin, and

100μg/ml streptomycin) containing 50ng/ml huIL-3, huSCF, huFLT-3L and 25ng/ml huIL-6, huG-CSF and huGM-CSF, at 37°C with 5% CO_2. These conditions were necessary to stimulate the cell cycle in an otherwise largely quiescent population in preparation for retroviral transduction (*2.5.3*). Once retroviral transduction was complete, unless otherwise stated, $CD34^+$ cells were sub-cultured in supplemented IMDM containing 5ng/ml huIL-3, huG-CSF and GM-CSF, and 20ng/ml huSCF, and maintained at $2x10^5$/ml as before until **day 21** of *in vitro* culture. Cells were sub-cultured every 2-3 days or when cells reached confluence (~$1x10^6$/ml).

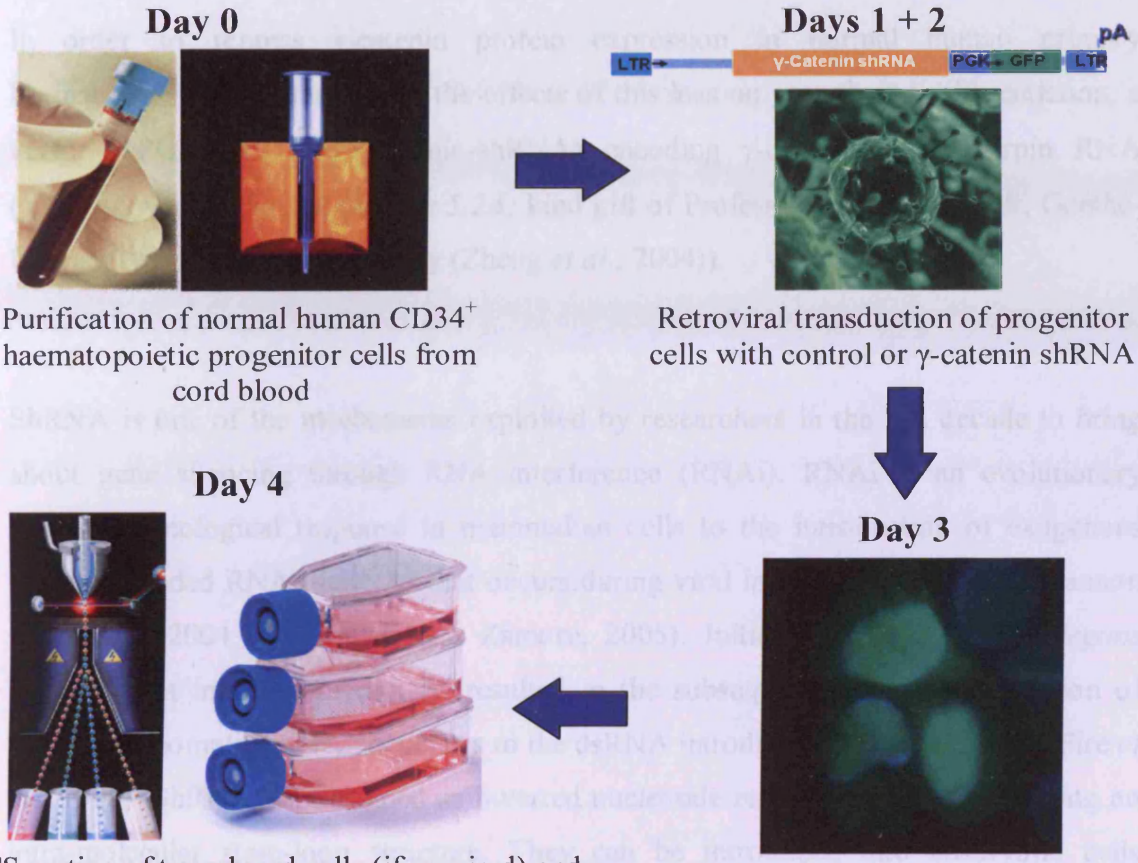


Figure 5.1 - Strategy for investigation into effect of γ-catenin silencing on haematopoietic development.

Summary flow diagram showing the experiments performed and time points used in examining the effect of γ-catenin knockdown on development of human CB-derived haematopoietic progenitor cells.

5.3.1.3 *Establishment of γ-catenin silencing system in normal human primary haematopoietic cells*

In order to repress γ-catenin protein expression in normal human primary haematopoietic cells and follow the effects of this loss on growth and differentiation, a vector (vPGKpuroU3U6-γ-catenin-shRNA) encoding γ-catenin short hairpin RNA (shRNA) was employed. (*Figure 5.2A;* kind gift of Professor Martin Ruthardt, Goethe-University of Frankfurt, Germany (Zheng *et al.*, 2004)).

ShRNA is one of the mechanisms exploited by researchers in the last decade to bring about gene silencing through RNA interference (RNAi). RNAi is an evolutionary conserved biological response in mammalian cells to the introduction of exogenous double stranded RNA (dsRNA) that occurs during viral invasion (reviewed by Hannon and Rossi, 2004 and Tomari and Zamore, 2005). Initial discoveries in *C. elegans* revealed that injection of dsRNA resulted in the subsequent repressed expression of genes with complimentary sequences to the dsRNA introduced (Fire *et al.*, 1991; Fire *et al.*, 1998). ShRNA are designed as inverted nucleotide repeat sequences containing an intra-molecular stem-loop structure. They can be introduced into eukaryotic cells through retroviral expression constructs and are continually expressed from RNA polymerase II or III (U6) promoters. The subsequent hairpin structure produced is recognised and cleaved at the 'loop' by the enzyme Dicer generating 30bp (or less) small interfering RNA (siRNA). These siRNA are then incorporated into the RNA-induced silencing complex (RISC) which unwind the double strands (ATP-dependent) and use the antisense siRNA to target and continually degrade the complimentary mRNA (in this case γ-catenin mRNA).

The vector described above utilised puromycin resistance as a selectable marker of gene transduction, which although appropriate for selection in cell lines, is not suitable for use in developing primary human haematopoietic cells. This is due to the artefactual effects on development arising from drug selection, which could mask any potential phenotype generated by a gene of interest (as observed in pilot studies, data not shown). To circumvent this problem, the puromycin selection cassette and associated PGK

promoter were excised from the retroviral backbone and replaced with a PGK promoter driving expression of GFP (originating from pRRLSIN.cPPT.PGK/GFP.WPRE; *Figure 5.2B*, kind gift of Dr. James Matthews, Cardiff University). The sub-cloning process involved is summarised within *Figure 5.2*, and essentially used many of the sub-cloning techniques fully described in methods section *3.3.1.*

Retrovirus encoding γ-catenin shRNA (vPGK/GFP-γ-catenin shRNA) was generated as described in section *2.5.2* and subsequently used to infect normal human $CD34^+$ haematopoietic cells on **day 1 and 2** of *in vitro* culture as described in section *2.5.3.* After 2 rounds of retroviral infection, transduced $CD34^+$ cells were harvested into fresh culture medium as in *5.3.1.2* **(day 3)** and allowed to recover. Cells were analysed by flow cytometry to determine infection frequency as below in *5.3.1.5.*

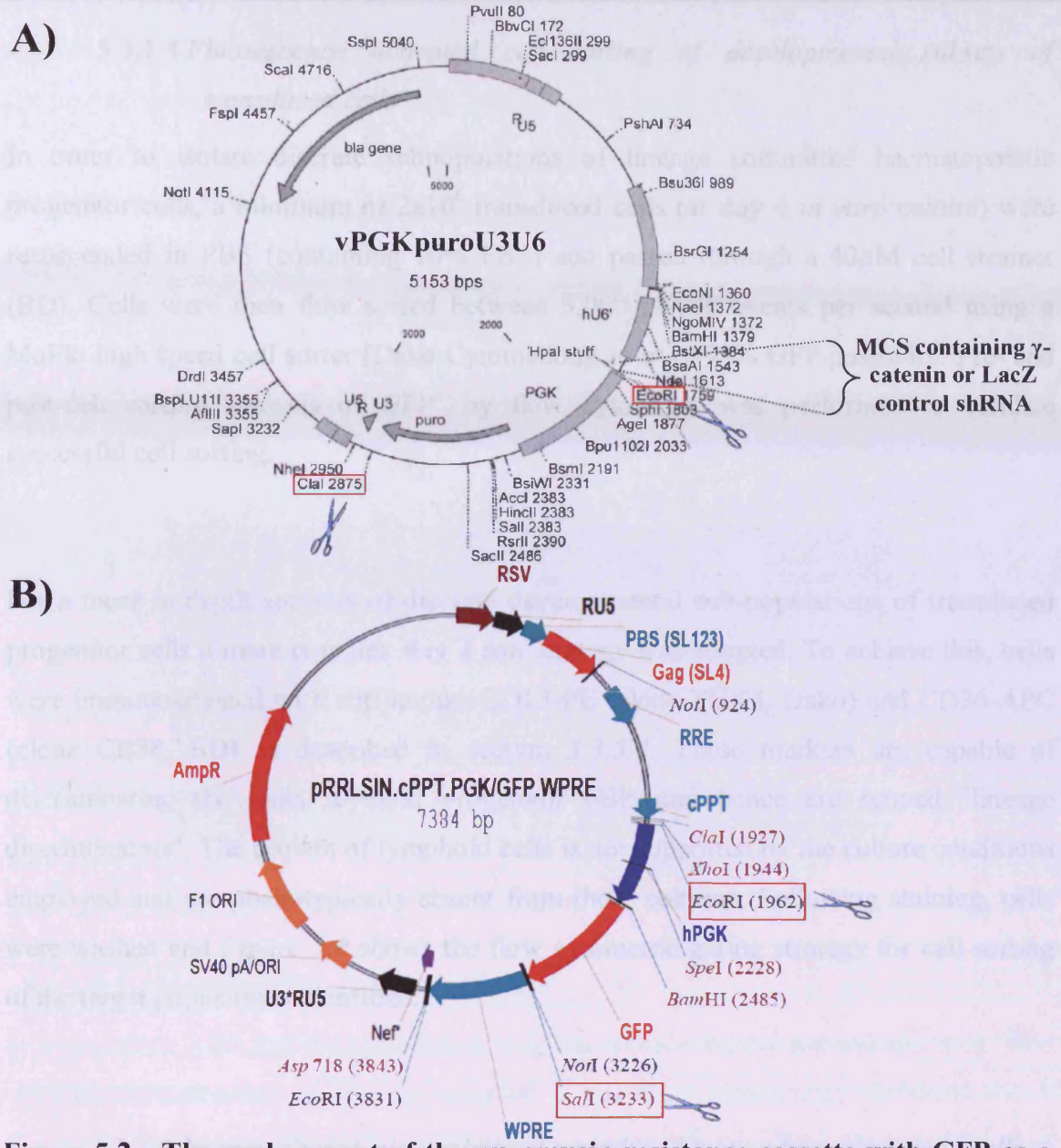


Figure 5.2 - The replacement of the puromycin-resistance cassette with GFP as a reporter gene for vPGKU3U6.

A) The vPGKpuroU3U6-γ-catenin-shRNA plasmid contains inverted repeats of 21 nucleotides (corresponding to 1862-1882 bp of the human γ-catenin gene) separated by a 10 nucleotide spacer (Zheng *et al.*, 2004). The associated control plasmid contains shRNA to the LacZ gene (not present in the human genome). The puromycin resistance cassette was excised using *Eco*R1 and *Cla*1 restriction endonucleases (red boxes) by first cutting with *Cla*1 and blunting with DNA polymerase I large (Klenow) fragment and then subsequently cutting with *EcoR*1 to create open vector with heterologous ends (*EcoR*1-blunt). **B)** PGK/GFP was removed from pRRLSIN.cPPT.PGK/GFP.WPRE using a double *Sal*1 *EcoR*1 digest (red boxes) by first cutting with *SalI* and blunting (as A) and then subsequently cutting with *EcoR*1 to create ends compatible with the processed vector. The *EcoR*1-blunt PGK/GFP cassette was then ligated into vPGKpuroU3U6 with DNA ligase and transformed in competent *E.coli*. The newly created plasmid was designated vPGK/GFP-γ-catenin shRNA (or -LacZ-shRNA for control).

5.3.1.4 *Fluorescence activated cell sorting of developmental subsets of transduced cells*

In order to isolate discrete subpopulations of lineage committed haematopoietic progenitor cells, a minimum of $2x10^6$ transduced cells (at **day 4** *in vitro* culture) were resuspended in PBS (containing 10% FBS) and passed through a 40μM cell strainer (BD). Cells were then flow sorted between 5,000-10,000 events per second using a MoFlo high speed cell sorter (Dako Cytomation), to over 98% GFP positivity. Pre- and post-cell sorting analysis of GFP^+ by flow cytometry was performed to validate successful cell sorting.

For a more in-depth analysis of discrete developmental sub-populations of transduced progenitor cells a more complex **day 4** sort strategy was adopted. To achieve this, cells were immuno-stained with anti-human CD13-PE (clone TÜK4, Dako) and CD36-APC (clone CB38, BD) as described in section *3.3.5.2.* These markers are capable of discriminating the main myeloid progenitor cells and hence are termed 'lineage discriminators'. The growth of lymphoid cells is not supported by the culture conditions employed and are phenotypically absent from these cultures. Following staining, cells were washed and *Figure 5.8* shows the flow cytometric gating strategy for cell sorting of the target populations identified.

5.3.1.5 *Immunophenotypic analysis of transduced human haematopoietic cells*

Retrovirally transduced $CD34^+$ haematopoietic cells were cultured *in vitro* for 21 days under cytokine conditions favourable for myeloid cell growth. On **days 7, 10, 14, 17** and **21**, cultures were analysed for expression of specific cell surface markers indicative of lineage and maturation state. All replicates were stained with the lineage discriminator antibodies CD13 (Clone WM15, Biolegend) and CD36 (Clone SMO, Ancell, Minnesota, USA), plus one additional PE-conjugated antibody of the selection listed in *Table 5.1*, which served as specific markers of differentiation. FSC and SSC characteristics were also recorded for all cells analysed. Cells were immunostained as outlined in section *3.3.5.2.* Multi-parameter flow cytometric measurements were acquired on the Accuri® C6 flow cytometer in conjunction with CFlow software

version 7.5 (Accuri Cytometers, Cambridgeshire, UK). Post-acquisition analysis of flow cytometric data was performed using FCS Express V3 (De Novo Software, California, USA). Debris was excluded from all analyses on the basis of forward- and side-scatter characteristics, whilst specific individual sub-populations were identified using the gating strategies illustrated in *Figure 5.5.*

Table 5.1 - Antibodies used in immunophenotypic analysis of *in vitro* cultured human haematopoietic cells.

Antibody	Target
IgG-PE (Clone MOPC-21, BD)	Non-specific binding
CD15-PE	Granulocytes
CD14-PE (Clone TÜK4, Dako)	Monocytes
CD163-PE (Clone GHI/61, BD)	Macrophages
GlyA-PE	Erythrocytes
CD34-PE	Progenitor cells

5.3.1.6 *Assessment of cell morphology*

In conjunction with immunophenotypic analysis, morphological assessment of *in vitro* cultured haematopoietic cells also occurred at the fixed time-points identified above (*5.3.1.5*). Approximately $2x10^4$ cells were resuspended in 250μl of 1 x PBS and centrifuged at 60 x *g* for 5 minutes onto a glass slide using a Cytospin 3 (Thermo Shandon, Cheshire, UK). Slides were stained with May-Grünwald-Giemsa using an ABX Pentra DX120 automated cell stainer (Horiba Medical Diagnostics, Northampton, UK). With this stain, nuclei and basophilic granules appear a dark blue or purple, whilst the cytoplasm and eosinophilic granules appear a pale pink/red. The cytospun cells were mounted in one drop of Dibutyl Phthalate in Xylene mountant (DPX) and coverslipped. Cells were viewed with a Zeiss AXIOSKOP microscope (Carl Zeiss Ltd, Hertfordshire, United Kingdom) using 40X and 100X oil immersion objectives.

5.3.2 The influence of γ-catenin expression on β-catenin subcellular localisation

To assess how the level and subcellular localisation of γ-catenin protein may affect the level and localisation of β-catenin protein in normal and leukaemic cells, the following subcellular fractionation procedures were carried out.

5.3.2.1 *In primary cell cultures*

In order to provide ectopic expression of γ-catenin protein in $CD34^+$ haematopoietic progenitor cells the PINCO vector described in section *3.3.1* (*Figure 3.1*) was used. CB-derived $CD34^+$ progenitor cells were purified and transduced with GFP (control) or γ-catenin DNA as in sections *2.4.2* and *2.5.3*, respectively. Transduced cells were cultured up to 5 days *in vitro* (last day at which cultures were substantially $CD34^+$) and N/C fractionated as described in *2.6.2*. Cytosolic and nuclear homogenate were immuno-blotted (sections *2.6.3-2.6.7*), for respective levels and localisation of γ- and β-catenin. The conditions for γ-catenin detection are described in section *3.3.3*. However a different mouse monoclonal to β-catenin (Clone 14, BD) was used at 250ng/ml. For assessment of fraction purity and protein loading β-actin and histone H1 were employed as in section *3.3.7.2*.

5.3.2.2 *In leukaemia cell lines*

Leukaemic cell lines are immortalised, clonal cultures of cells that were originally transformed from leukaemia patients. They provide a tractable and unlimited source of haematopoietic cell material for which to analyse the effects of exogenously expressed proteins. The background of existing genetic mutations within these cells makes them an appropriate model for studying proteins of interest within a leukaemic context. However, given that many these genetic abnormalities remain uncharacterised and are extremely heterogeneous between individual cell lines means there are limitations to any experimental conclusions which may be drawn from them.

The following leukaemic cell lines were used:- HEL, THP-1, U937 and K562 pBabe-Puro control and γ-catenin cell lines (created in *3.3.2*, and shown in *Figure 3.10*). Each cell type was N/C fractionated (alongside the respective pBabe-Puro control), and Western blotted as outlined above (*5.3.2.1*). In addition to the above, a K562 cell line expressing the original γ-catenin silencing construct (vPGKpuroU3U6-γ-catenin-shRNA, featured in *Figure 5.2A*) was also processed as above.

5.3.3 The effect of γ-catenin and β-catenin expression on induced differentiation of monocytic cell lines

The monocytic cell lines U937 and THP-1 can be induced to differentiate into monocytes/macrophages upon addition of phorbol esters such as 12-O-Tetradecanoylphorbol 13-acetate (TPA) (Tsuchiya *et al.*, 1982; Hass *et al.*, 1989). The pBabe-Puro control and pBabe-Puro-γ-catenin transgenic lines of THP-1 and U937 created in section *3.3.2* were again utilised. In addition, THP-1 and U937 cell lines were transduced (as in section *2.5*) to express γ-catenin shRNA (vPGKpuroU3U6-γ-catenin-shRNA, featured in *Figure 5.2A*) and the β-catenin degradation-resistant mutant (β-catΔN89) described in *3.3.4.2* (giving a total of 8 cell lines that were studied).

5.3.3.1 *Establishment of cell line morphology and immunophenotype prior to TPA treatment*

Prior to TPA-induction (see *5.3.3.2* immediately below), $2x10^6$ cells from each cell line were N/C fractionated and Western blotted for γ- and β-catenin (as per *5.3.2.1*) to assess the level and location of each protein at the start of the assay. Cells were cytospun as above (*5.3.1.6*) to establish cell morphology prior to TPA-induction. Each cell line was assessed immunophenotypically for monocytic/macrophage cell surface expression markers to establish the extent of differentiation before TPA treatment. As described in section *3.3.5.2*, cells from each cell line were immunostained with anti-human CD14-FITC (Clone TÜK4, Dako), CD11b-PE (Clone 2LPM19c, Dako) and CD13-APC. Immuno-labelled cells were analysed as above (*5.3.1.5*) with the threshold for cell surface staining set with the isotype- and manufacturer-matched irrelevant control antibodies.

5.3.3.2 *TPA treatment of cell lines*

A total of $2x10^6$ cells from each transgenic cell line were resuspended in 10ml of the appropriate culture medium (see *Table 2.1*) at a density of $2x10^5$/ml, in a $25cm^2$ tissue culture flask. All flasks were inoculated with 40nM TPA, mixed well, and cultured overnight at 37°C with 5% CO_2. The following day, all TPA treated cell lines received the identical morphological, protein, and immunophenotypic assessment that was performed pre-TPA-induction (*5.3.3.1*).

5.3.4 Investigation into the effect of a Wnt stimulus on γ-catenin expression

To investigate whether γ-catenin, like β-catenin, was capable of responding to a Wnt signal, levels and localisation of both proteins were observed after treatment with recombinant Wnt glycoproteins. Recombinant murine/human Wnt3A (concentration unknown) was obtained by harvesting conditioned medium from Wnt3A-transduced murine L-cells (generously donated by Dr. Kenneth Ewan, Cardiff University (Willert *et al.*, 2003)). Recombinant human Wnt5A was purchased from R&D systems (Abingdon, UK). Each test condition is outlined in *Table 5.2* below. In each case, wild-type K562 cells were resuspended at a density of $4x10^5$/ml in the appropriate medium, and incubated overnight at 37°C with 5% CO_2. The following day, $2x10^6$ K562 cells from each test condition were N/C fractionated and Western blotted as above (*5.3.2.1*).

Table 5.2 - The test conditions used on K562 cells to ascertain γ-catenin response to a Wnt stimulus.

Test condition number	Test	Volume of culture medium	Additions
1	Untreated (control)	5ml	N/A
2	Wnt3A	2.5ml	2.5ml Wnt3A conditioned medium
3	Wnt5A	5ml	500ng/ml recombinant Wnt5A
4	Wnt3A + Wnt5A	2.5ml	2.5ml Wnt3A conditioned medium, plus 500ng/ml recombinant Wnt5A

5.3.5 Determination of cause of multiple banding pattern of γ-catenin in Western blotting

To investigate whether the differential phosphorylation status of γ-catenin protein was responsible for the multiple banding pattern observed in Western blotting, γ-catenin protein was immunoprecipitated and then de-phosphorylated.

5.3.5.1 *Immunoprecipitation of γ-catenin protein*

Whole cell protein homogenate was generated from $1x10^6$ K562 cells as outlined in section *2.6.1*, except the homogenisation buffer was replaced with a calf intestinal alkaline phosphatase (CIP) extraction buffer (25mM HEPES pH 7.8, 300mM NaCl, 1.5mM MgCl2, 1% Triton® X-100, 0.1mM dithiothreitol, 1 protease inhibitor tablet) to provide optimal conditions for the CIP (New England Biolabs) used in the de-phosphorylation step below (*5.3.5.2*). Protein was quantified using the Bradford's assay of *2.6.3*.

Immobilised Protein G sepharose beads were washed in 1ml of TBS with centrifugation at 16,000 x *g* for 1 minute. Pelleted beads were resuspended in TBS to generate a 50% bead slurry prior to 'pre-clearing' of the K562 protein lysate. 'Pre-clearing' removes the

non-specific protein binding to the sepharose beads, thus improving the signal-to-noise ratio. To achieve this, 100μl of Protein G slurry was incubated with 500μl (containing 600μg protein) K562 lysate, under gentle agitation at 4°C for 1 hour.

At the same time, the Protein G bead: γ-catenin antibody complex was generated by taking 100μl of Protein G bead slurry and combining with 3.2μg of rabbit polyclonal γ-catenin antibody (#2309s, Cell Signalling) under gentle agitation for 1 hour at 4°C. Upon completion of 'pre-clearing', beads were pelleted by centrifugation as above, and the cleared lysate supernatant aspirated and split between four Eppendorfs. Three of the cleared lysate tubes each received 33μl of Protein G bound γ-catenin antibody, the other remaining free of antibody to serve as a negative control. All tubes were incubated overnight under gentle agitation at 4°C. The following day, the three tubes containing conjugated beads were washed 3 times with 500μl ice cold TBS each followed by centrifugation as above. Pelleted beads were resuspended in 30μl TBS before proceeding to de-phosphorylation of immuno-precipitated protein.

5.3.5.2 *De-phosphorylation of immunoprecipitated γ-catenin protein*

Of the three tubes containing bead-bound protein, tube 1 was designated the 'control' and received 5μl PBS. Tube 2 was designated 'low-de-phosphorylation' and received 10U (1μl) CIP, whilst Tube 3 represented 'high de-phosphorylation' and received 100U (10μl) CIP. CIP dephosphorylates serine, threonine and tyrosine residues on proteins. Tube 4 served as a 'Cleared lysate' control and also received 5μl PBS. All tubes were incubated at 37°C for 2 hours. The de-phosphorylation reaction was stopped by adding 200μl TBS to each tube (not tube 4) and pelleting the beads by centrifugation as above. CIP contaminating supernatant was removed and discarded before resuspending the bound protein in TBS, and Western blotting all tubes as in steps *2.6.4-2.6.7.* Purified γ-catenin was detected using the mouse monoclonal of *3.3.3*, whilst efficiency of dephosphorylation was assessed using a rabbit polyclonal antibody to phosphotyrosine (#T1325, Sigma).

5.4 Results

5.4.1 The function of γ-catenin in normal haematopoietic development

5.4.1.1 *γ-Catenin protein knockdown perturbs human monocyte/macrophage development*

In order to determine the role of γ-catenin in normal haematopoiesis it was necessary to knockdown endogenous levels of γ-catenin protein within a model of *in vitro* human haematopoiesis and examine the consequences for normal development. To optimise and validate this approach K562 cells were used because of their tractability and high endogenous levels of γ-catenin. The time-course Western blot of *Figure 5.3* reveals how the γ-catenin shRNA retroviral construct (featured in *Figure 5.2*) was capable of stable knockdown of 76%±5% of γ-catenin protein in K562 cells over the 31 days tested.

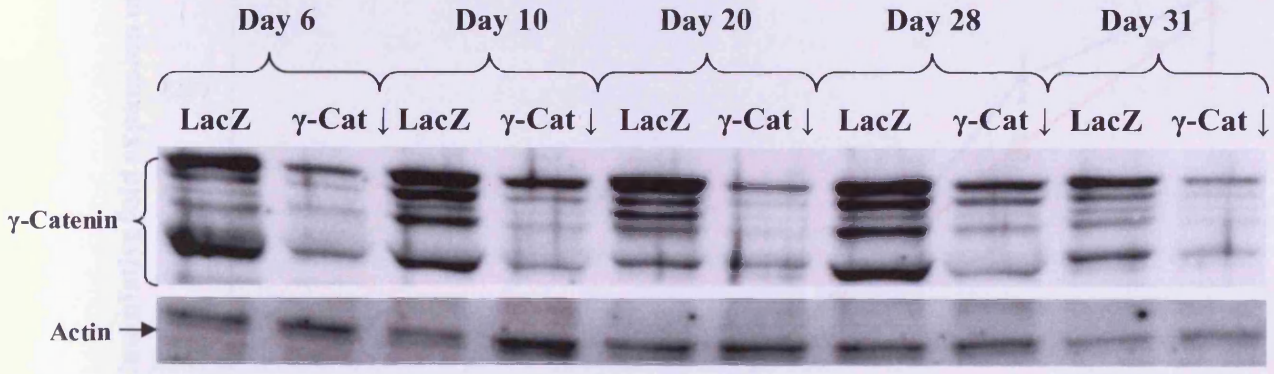


Figure 5.3 - The use of γ-catenin shRNA to knockdown γ-catenin protein expression in K562 cells.

Western blotting of K562 whole cell lysates retrovirally transduced with either γ-catenin shRNA or corresponding LacZ shRNA control. Change in γ-catenin protein expression in knock-down cultures can be observed compared to mock-transduced control cells. Actin was used as a measure of equal protein loading between sample pairs.

Following successful validation of γ-catenin protein knockdown, $CD34^+$ haematopoietic progenitor cells were retrovirally transduced with either LacZ control or γ-catenin shRNA and cultured *in vitro* for 21 days. Morphological, immunophenotypic and developmental assessments were performed at regular time-points throughout the experiment as summarised in *Figure 5.1*. In terms of growth rate, no significant difference was observed between control and γ-catenin knockdown (γ-cat KD) cells throughout the entire 21 days, as depicted by the cumulative fold expansions featured in *Figure 5.4* below.

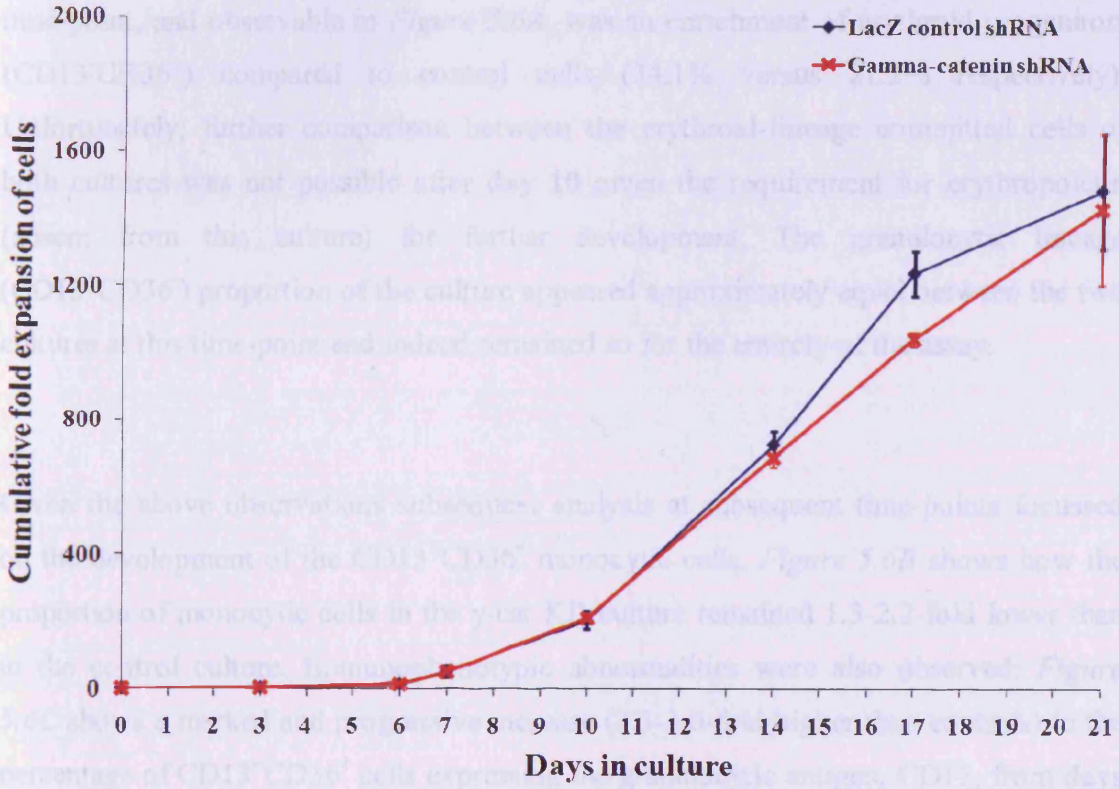


Figure 5.4 - The effect of γ-catenin silencing on growth of haematopoietic cells.

Growth curve comparing the cumulative fold expansion of haematopoietic cultures retrovirally transduced with either LacZ control (blue line) or γ-catenin shRNA (red line). Data indicate mean ± 1 SD.

In order to closely monitor the differentiation of transduced haematopoietic cells, specific cell surface marker expression was assessed by flow cytometry. The analytical strategy is outlined in *Figure 5.5*.

Using this strategy a number of key developmental abnormalities were identified in the γ-cat KD cells and are summarised in *Figure 5.6*. At the earliest time-point assessed (**day 7**) there appeared to be negligible differences between the two cultures as deduced from all the differentiation parameters previously described (*5.3.1.5*). At **day 10** a lower frequency of $CD13^+CD36^+$ (monocytic) events were present in the γ-cat KD culture (14.6% versus 25.9%, respectively) as shown in *Figure 5.6A*. Also identified at this time-point, and observable in *Figure 5.6A*, was an enrichment of erythroid progenitors ($CD13^-CD36^+$) compared to control cells (34.1% versus 21.2% respectively). Unfortunately, further comparison between the erythroid-lineage committed cells of both cultures was not possible after **day 10** given the requirement for erythropoietin (absent from this culture) for further development. The granulocytic lineage ($CD13^+CD36^-$) proportion of the culture appeared approximately equal between the two cultures at this time-point and indeed remained so for the entirety of the assay.

Given the above observations subsequent analysis at subsequent time-points focussed on the development of the $CD13^+CD36^+$ monocytic cells. *Figure 5.6B* shows how the proportion of monocytic cells in the γ-cat KD culture remained 1.3-2.2-fold lower than in the control culture. Immunophenotypic abnormalities were also observed: *Figure 5.6C* shows a marked and progressive increase (2.5-3.0-fold higher than controls) in the percentage of $CD13^+CD36^+$ cells expressing the granulocytic antigen, CD15, from **days 14-21**. In contrast, *Figure 5.6D* shows little difference in the expression of the monocytic marker, CD14. Finally, it was noted from around **day 14** onwards that γ-cat KD monocytes exhibited lower FSC and SSC (*Figure 5.6E* and *F* respectively), suggesting smaller and less granular cells than present in control cultures.

Analysis of cell morphology by May-Grünwald-Giemsa staining of leukocytes was used in conjunction with flow cytometric measurements to assess the differentiation of cells. At **day 21** of culture, total GFP^+ events were FACS sorted from both cultures (nearly all $CD13^+$ at this point), cytospun onto glass slides, stained, and examined by light microscopy. The morphology, as represented in *Figure 5.7A* and *B*, appeared to corroborate the immunophenotypic data described above. As anticipated LacZ shRNA control cultures (*A*) were dominated by macrophages with rare mature granulocytes (with hyper-segmented nuclei). Conversely, the γ-cat KD culture (*B*) was deficient in macrophages instead exhibiting predominantly cells of granulocytic morphology (band-from and hyper-segmented neutrophils). The prevalence of granulocytic cells within this culture validates the increase in CD15 expression (see above) and the lower SSC and FSC measurements are in keeping with the predominance of smaller cells in the γ-cat KD culture. To see further morphology images of both cultures please consult ***sub-folder 1*** of ***Folder 1*** from the main ***Chapter 5*** section of the ***supplementary CD***.

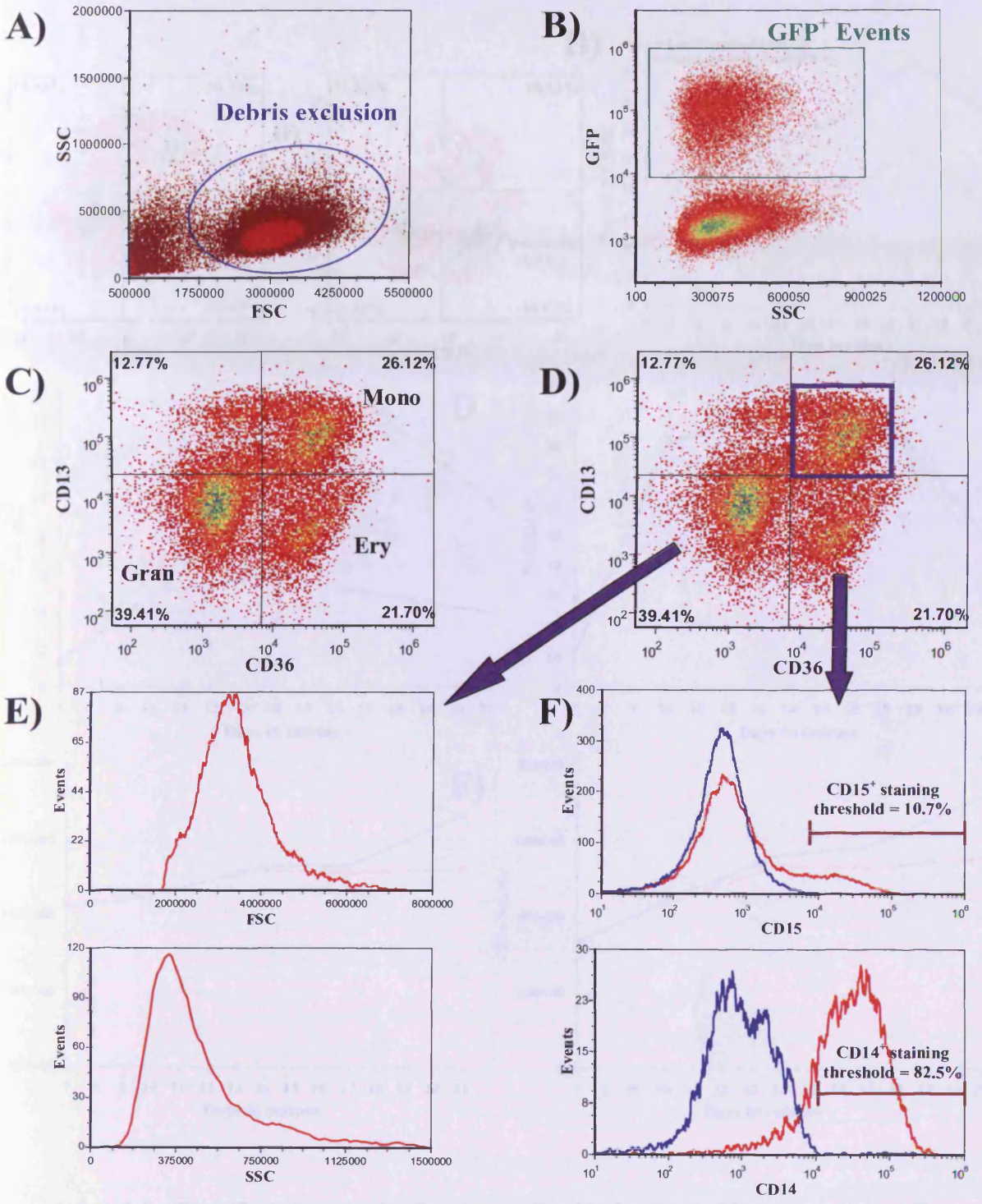


Figure 5.5 - Strategy for immunophenotyping of transduced haematopoietic cells.

Preliminary steps involved **A)** exclusion of debris and **B)** gating of GFP positive cells. **C)** Use of 'lineage discriminators' (CD13 and CD36) to identify 3 sub-populations of lineage-committed haematopoietic cells: monocytic (mono: $CD13^{+}CD36^{+}$), erythroid (ery: $CD13^{-}CD36^{+}$) and granulocytic (gran: $CD13^{-}CD36^{-}$). **D-F)** Each of these subsets was then gated and examined for cellular characteristics such as size (FSC) and granularity (SSC) or for specific cell surface markers of differentiation such as CD15 or CD14.

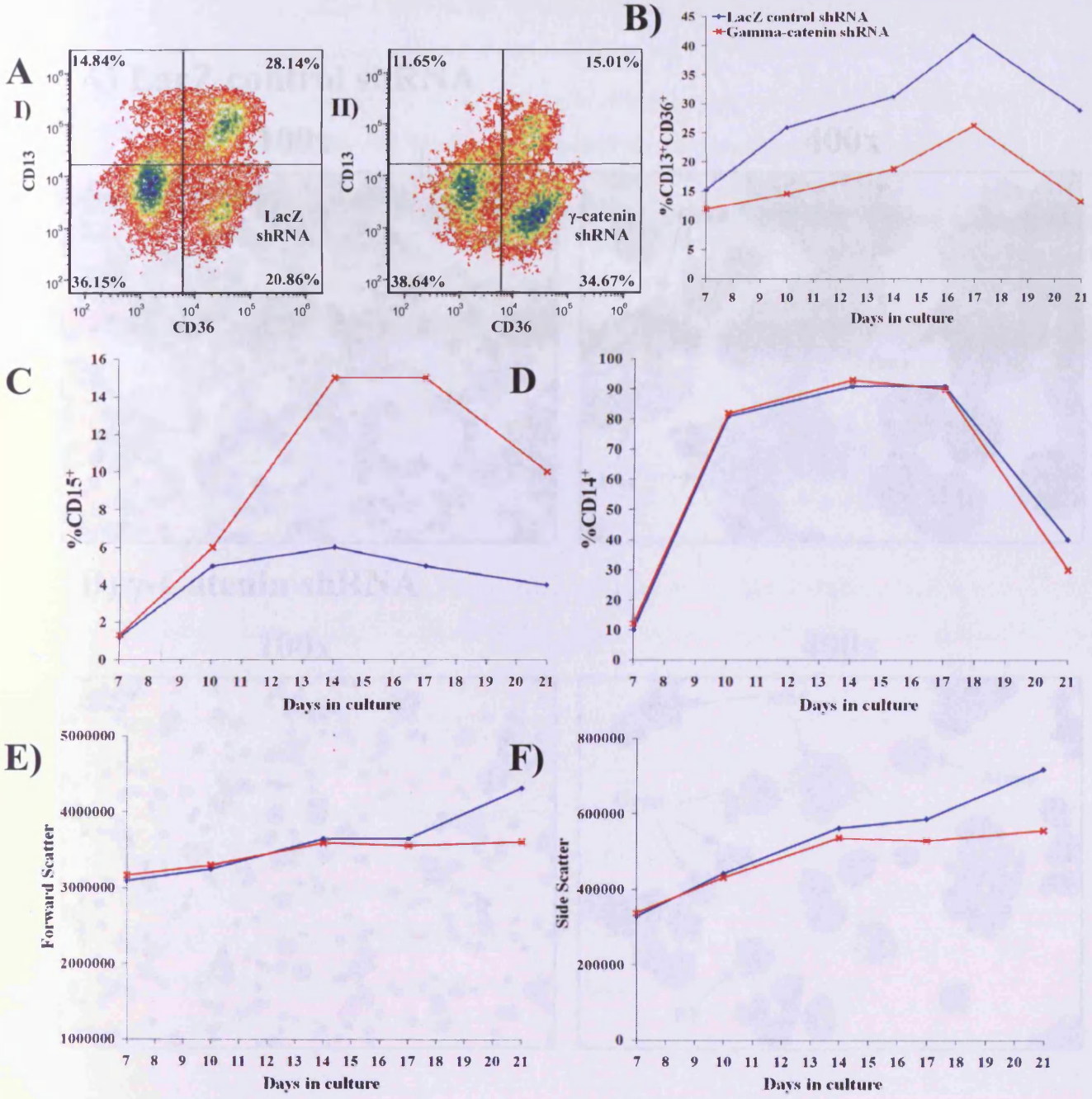


Figure 5.6 - The effect of γ-catenin silencing on the development of haematopoietic cells.

A) Representative CD13 versus CD36 contour plots from **day 10** of culture comparing lineage frequency between I) LacZ control and II) γ-catenin shRNA transduced haematopoietic cells. **B)** Frequency of $CD13^{+}CD36^{+}$ events in LacZ control and γ-catenin shRNA transduced cultures over 21 days. **C-F)** Differentiation profile of LacZ and γ-catenin shRNA transduced monocytic cells ($CD13^{+}CD36^{+}$) showing direct comparison of percentage CD15 and CD14 cells present in each culture, followed by forward and side scatter comparison (n=1 for all).

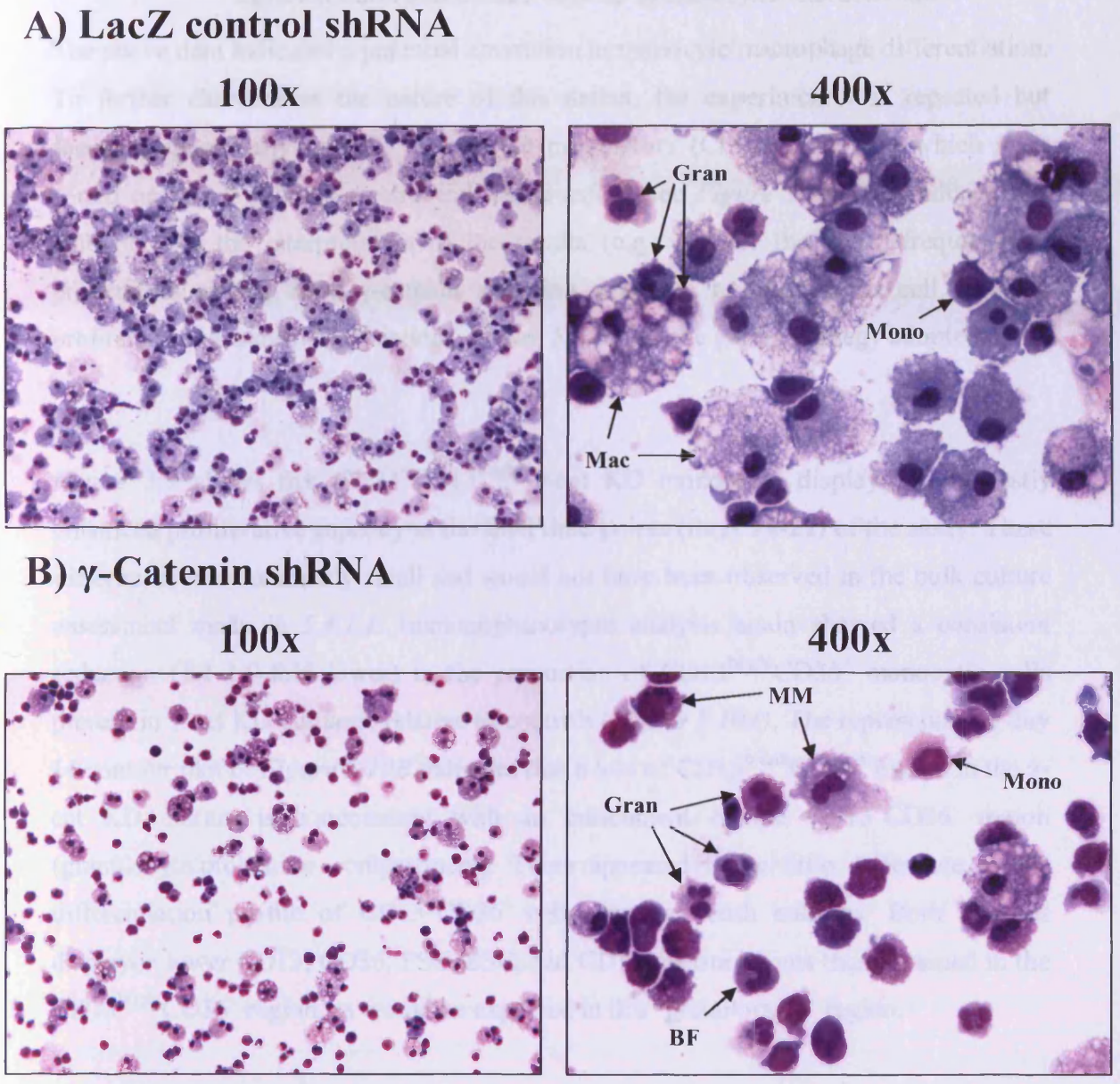


Figure 5.7 - The effect of γ-catenin silencing on differentiation of haematopoietic cells.

Representative cell morphology of **A)** LacZ control shRNA and **B)** γ-catenin shRNA transduced haematopoietic cells following 21 days of *in vitro* culture. Cells were cytospun onto glass slides and May-Grünwald-Giemsa stained. Morphology is shown at 100x and 400x magnification. Annotations denote: **Mac**=macrophage, **Mono**=monocyte, **Gran**=granulocyte, **MM**=metamyelocyte and **BF**=band-form neutrophil.

5.4.1.2 *γ-Catenin deficient monocytes fail to complete monocytic/macrophage differentiation and instead develop granulocytic characteristics*

The above data indicated a potential aberration in monocyte/macrophage differentiation. To further characterise the nature of this defect, the experiment was repeated but focusing specifically on GFP$^+$ monocyte progenitors (CD34$^+$CD13^{bright}) which were sorted on day 4 following retroviral transduction (see *Figure 5.1*). This reduced any ambiguity in the interpretation of the results (e.g. whether the lower frequency of progenitors arising from γ-catenin silencing was due to changes in cell survival, proliferation or transdifferentiation). *Figure 5.8* shows the gating strategy adopted.

Figure 5.9 shows that CD34$^+$CD13^{bright} γ-cat KD monocytes displayed a modestly enhanced proliferative capacity in the final time-points (**days 14-21**) of the assay. These differences were relatively small and would not have been observed in the bulk culture assessment made in *5.4.1.1.* Immunophenotypic analysis again showed a consistent reduction (1.1-1.9-fold lower) in the proportion of CD13^{bright}CD36$^+$ monocytic cells present in γ-cat KD cultures, relative to controls (*Figure 5.10A)*. The representative **day 14** contour plot of *Figure 5.10B* indicates that a loss of CD13^{bright}CD36$^+$ events in the γ-cat KD culture is concomitant with an enrichment of the CD13$^+$CD36$^-$ region (granulocyte/progenitor compartment). There appeared to be little difference in the differentiation profile of CD13$^+$CD36$^-$ cells between both cultures. Both cultures displayed lower CD13, CD36, FSC, SSC, and CD14 measurements than obtained in the CD13^{bright}CD36$^+$ region, as would be expected in this 'granulocytic' region.

In the CD13^{bright}CD36$^+$ region, a higher proportion of cells expressed the granulocytic antigen, CD15, in γ-cat KD cultures from **day 7** onwards (starting 4-fold higher, peaking at 16-fold higher on **day 14**, *Figure 5.10C*). The identity of these cells remained ambiguous however, as they also maintained high expression of the monocytic marker, CD14, which was not dissimilar from controls (except for a 1.3-fold higher level at **day 17**, *Figure 5.10D*). Finally, as also identified in the previous assay and demonstrated in *Figure 5.10E* and *F* respectively, FSC and SSC measurements were again lower in γ-cat KD monocytes particularly at later time-points (**days 14-21**).

It should also be noted that no significant retention of CD34 expression, or aberrant expression of GlyA (for mature erythrocytes) was detected in either monocyte culture. Unfortunately, immuno-staining with CD163 (a marker of macrophages) failed at each of the time-points assayed, despite the presence of macrophages in mature monocyte cultures. This was probably due to reagent quality issues. A colony assay was also performed immediately after the **day 4** FACS sort (not described in methods) to explore the developmental fate of single progenitor cells, however, this assay failed due to a preparative error in the media formulation arising from inadequate deionisation of the BSA component.

As before, the assessment of cell morphology appeared to support the immunophenotypic data. To observe the full range of developing monocyte morphology from **day 7** to **21**, consult ***sub-folders 2-6*** of ***Folder 1*** from the main ***Chapter 5*** section of the ***supplementary CD***. Briefly, very minor morphological differences between the two transduced monocyte cultures were present at **day 7** and **10** assessments. The morphology observed in *Figure 5.11* from **day 17** shows that LacZ shRNA control transduced cells (*A*) exhibited classic macrophage or monocyte morphology, as seen in previously (*Figure 5.7A*) and shows that under normal conditions $CD13^{bright}$ progenitors give rise to predominantly cells of the monocyte/macrophage lineage. Conversely, the γ-catenin shRNA transduced monocytes (*B*) was dominated by the presence of cells exhibiting a pale 'macrophage-like' cytoplasm, but with a ruffled, uneven cell membrane and displaying granulocytic (often hyper-segmented) nuclear morphology. These cells appeared smaller, less granular and more vacuolated (an indicator of *in vitro* culture physiological stress) than their normal developmentally-matched counterparts. These observations confirmed the biphenotypic staining pattern seen above in that the cells appeared to have both granulocytic and monocytic features. Taken with the previous section, these data would imply that γ-catenin expression is important for normal monocytic development. γ-Catenin deficiency appears to prevent macrophage differentiation instead resulting in dysplastic cells with granulocytic/monocytic characteristics.

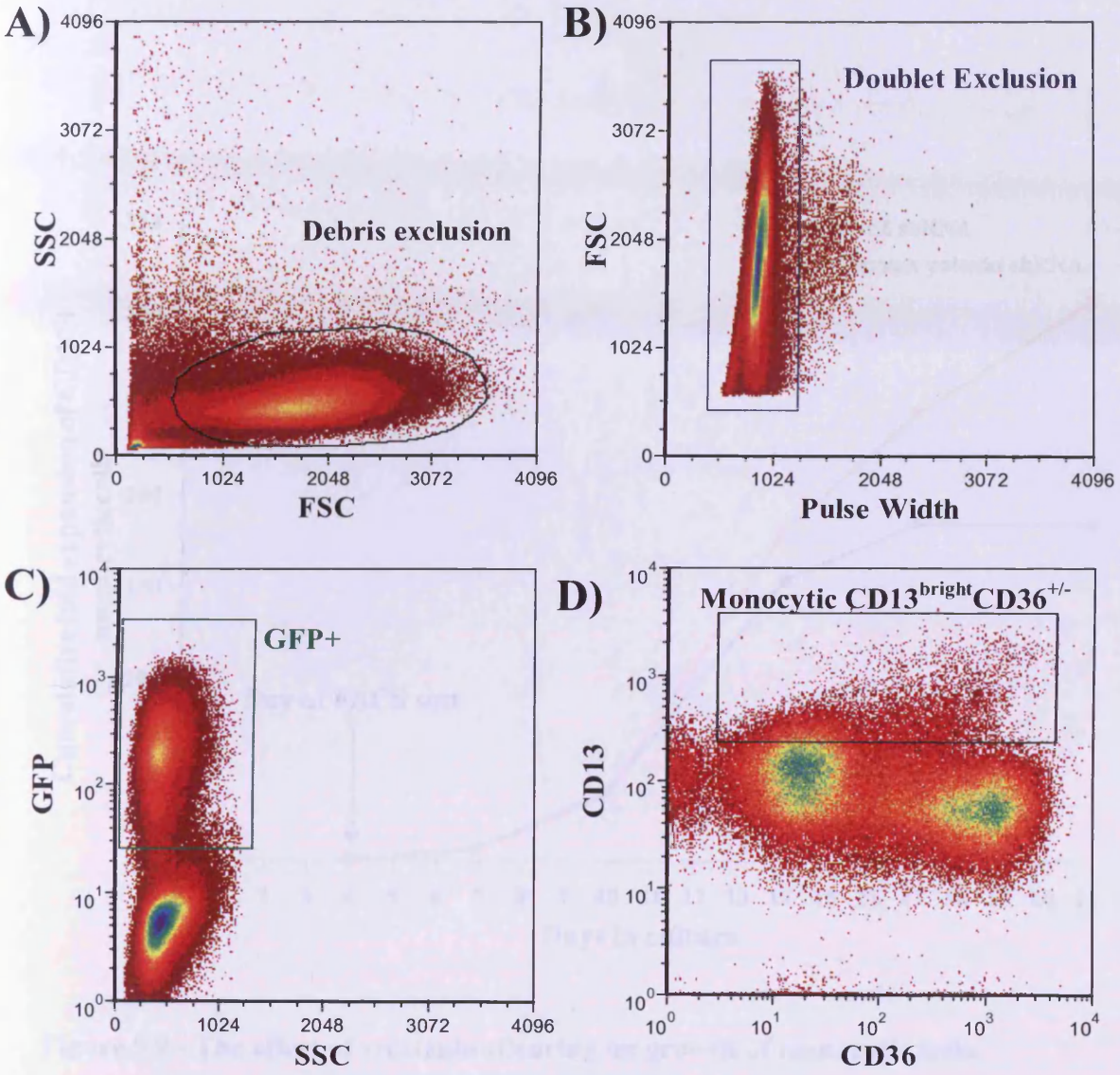


Figure 5.8 - The isolation and purification of monocytic lineage-committed haematopoietic progenitor cells.

The sequential gating strategy adopted using the high speed cell sorter to purify a monocyte committed subpopulation of retrovirally transduced $CD34^+$ haematopoietic progenitor cells following retroviral transduction (**day 4**). **A)** Firstly, cell debris was excluded using FSC versus SSC parameters before, **B)** exclusion of doublets by use of pulse width. **C)** Retrovirally transduced cells were gated on the basis of GFP positivity. **D)** Monocytic lineage-committed haematopoietic progenitors were identified by cell-high surface expression of CD13 (using the $CD13^{bright}CD36^+$ definitive monocyte population to set the CD13 threshold (Tjonnfjord *et al.*, 1996)).

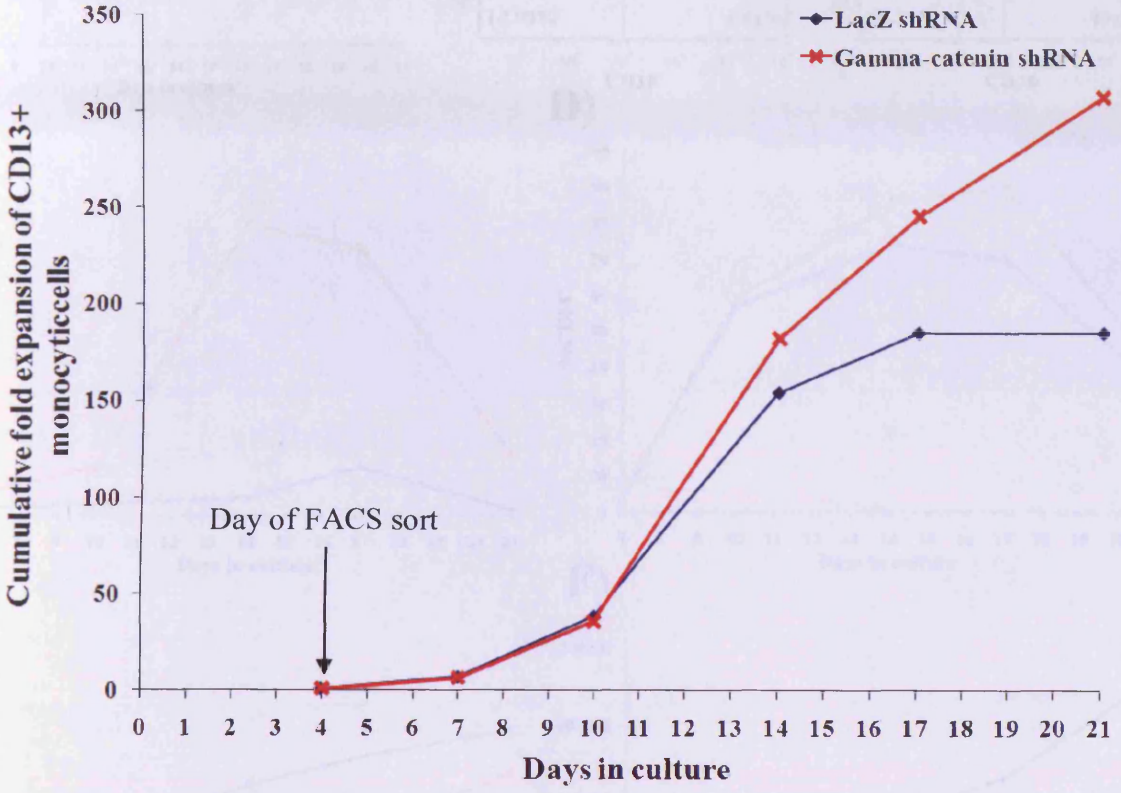


Figure 5.9 - The effect of γ-catenin silencing on growth of monocytic cells.

Growth curve comparing the cumulative fold expansion of **day 4** FACS sorted $CD13^{bright}(CD36^{+/-})$ monocytic cells retrovirally transduced with either LacZ control or γ-catenin shRNA (n=1).

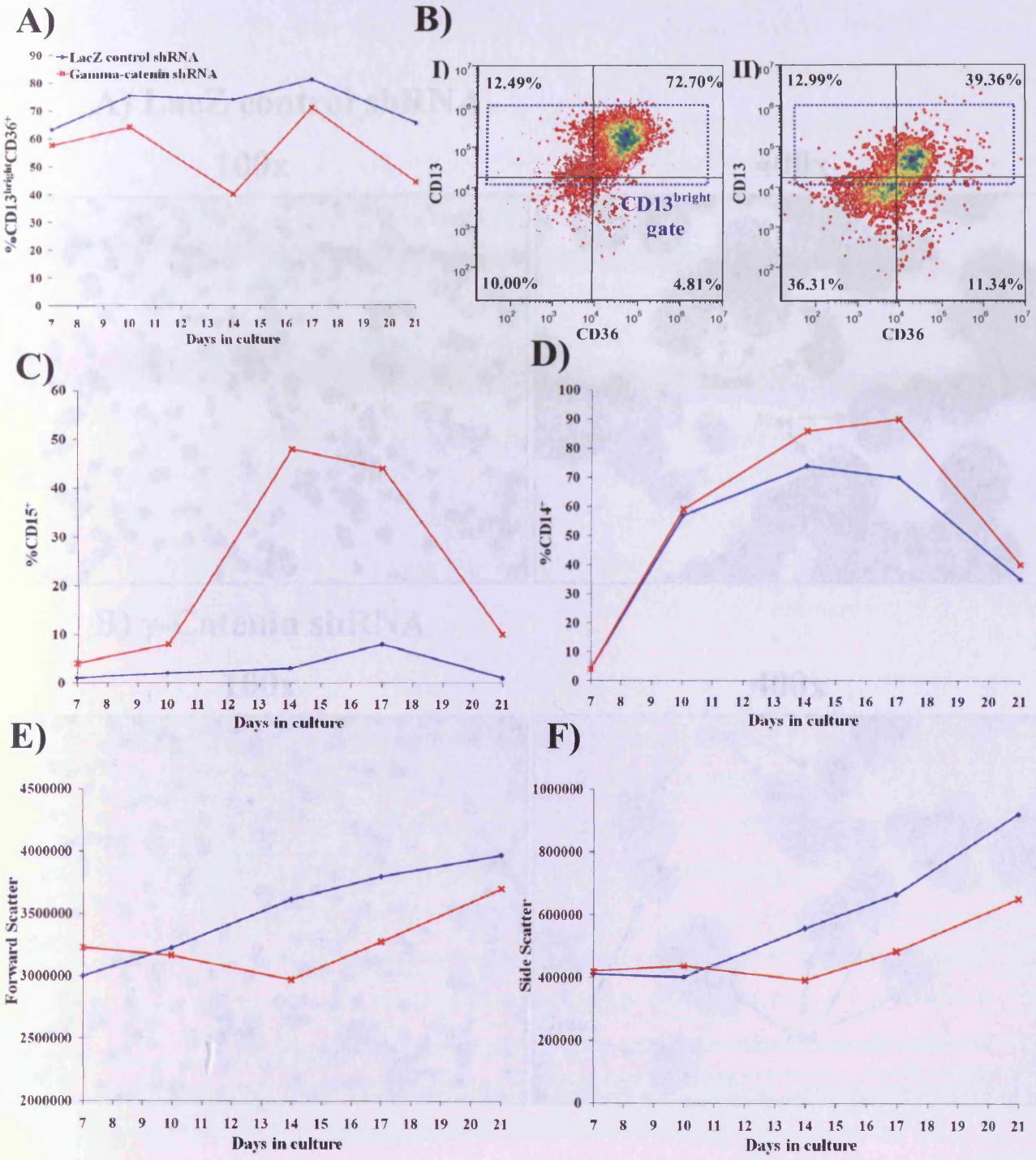


Figure 5.10 - The effect of γ-catenin silencing on the development of monocytic cells.

A) Comparison of percentage $CD13^{bright}CD36^{+}$ events detected in LacZ control and γ-catenin shRNA transduced monocyte populations over 21 days. **B)** Representative contour plots from **day 14** comparing CD13 and CD36 expression in day 4 sorted differentiating monocyte populations transduced with I) LacZ control and II) γ-catenin shRNA. **C-F)** Differentiation profile of LacZ and γ-catenin shRNA transduced monocytic cells (using $CD13^{bright}$ gate illustrated in **A**) showing direct comparison of percentage CD15 and CD14 cells present in each culture, followed by forward and side scatter comparison (n=1 for all).

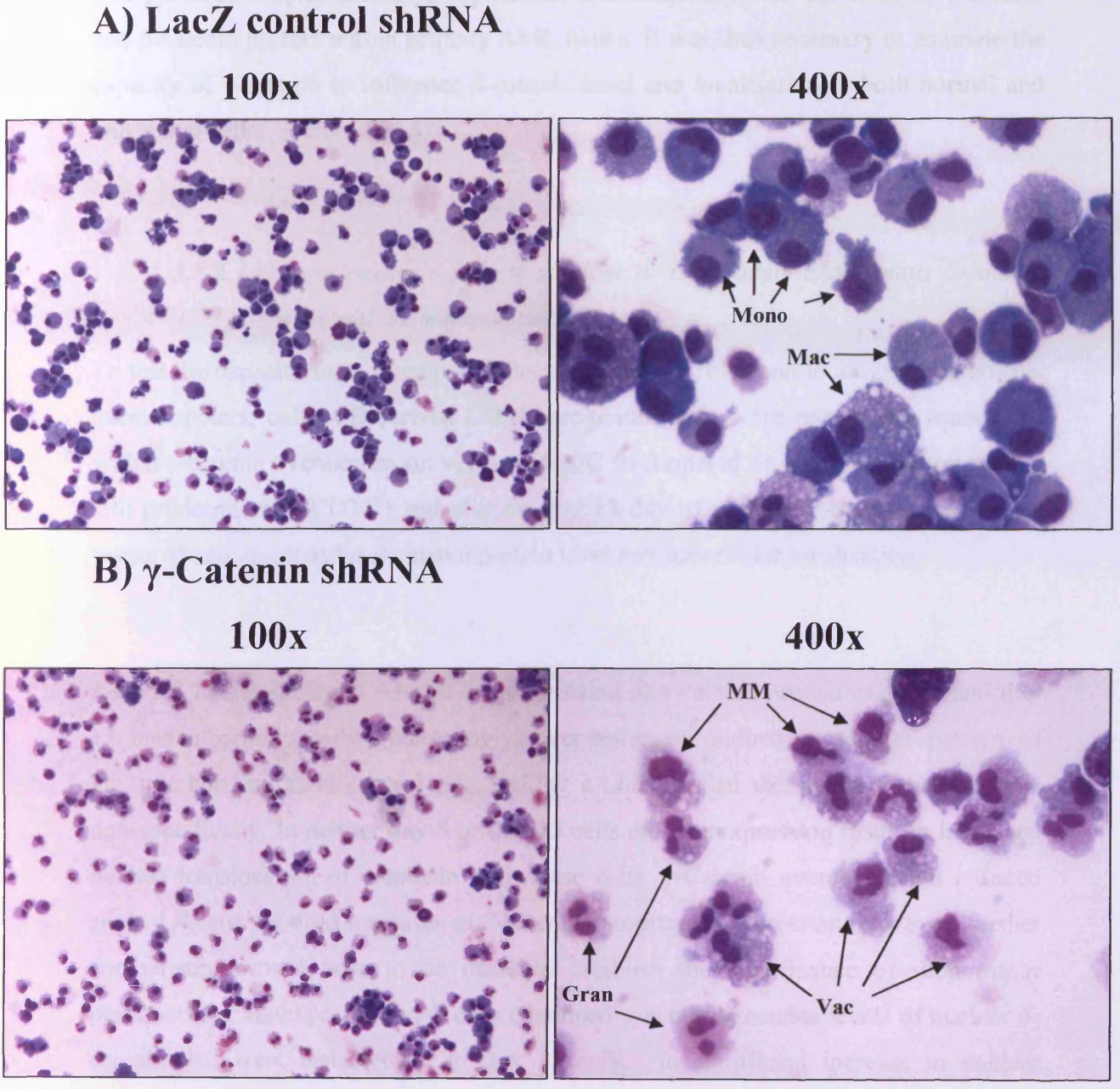


Figure 5.11 - The effect of γ-catenin silencing on differentiation of monocytic cells.

Representative **day 17** cell morphology showing differences between **A)** LacZ control shRNA and **B)** γ-catenin shRNA transduced cells. Cells were cytospun onto glass slides and May-Grünwald-Giemsa stained. Morphology is shown at 100x and 400x magnification. Annotations denote: **Mac**=macrophage, **Mono**=monocyte, **Gran**=Granulocytes with hyper-segmented nuclei, **MM**=metamyelocyte, and **Vac**=vacuolated cytoplasm.

5.4.2 The influence of γ-catenin expression on β-catenin localisation

The previous chapter identified a potential correlation between the level of γ-catenin and β-catenin protein within primary AML blasts. It was thus necessary to examine the capacity of γ-catenin to influence β-catenin level and localisation in both normal and leukaemic cells.

5.4.2.1 *Ectopic γ-catenin fails to stabilise or translocate endogenous β-catenin in normal haematopoietic cells*

To test the capacity for γ-catenin to influence β-catenin level and localisation in normal haematopoietic cells, CB-derived $CD34^+$ progenitor cells were retrovirally transduced with a γ-catenin overexpression vector and N/C fractionated on **day 5** of culture (whilst still predominantly $CD34^+$) and also on **day 13** day of culture. Protein extracts were prepared and assessed for β-catenin protein level and subcellular localisation.

Figure 5.12 demonstrates ~3-fold overexpression of γ-catenin protein in **day 5** and **day 13** haematopoietic cells. Interestingly, overexpression induced a similar pattern of multiple banding as in control cells making it unlikely that this banding pattern arose non-specifically. In neither **day 5** or **day 13** cells did overexpression result in increased nuclear translocation of γ-catenin with these cells. γ-Catenin overexpression induced slight 1.4- and 1.1-fold increases in β-catenin cytoplasmic expression, however, further comparisons would have to be made to establish the significance of such minor perturbations. As expected **day 5** cells contained low but detectable levels of nuclear β-catenin that were undetectable in **day 13** cells. No significant increase in nuclear translocation of β-catenin protein was observed in γ-catenin overexpressing cells at either time-point.

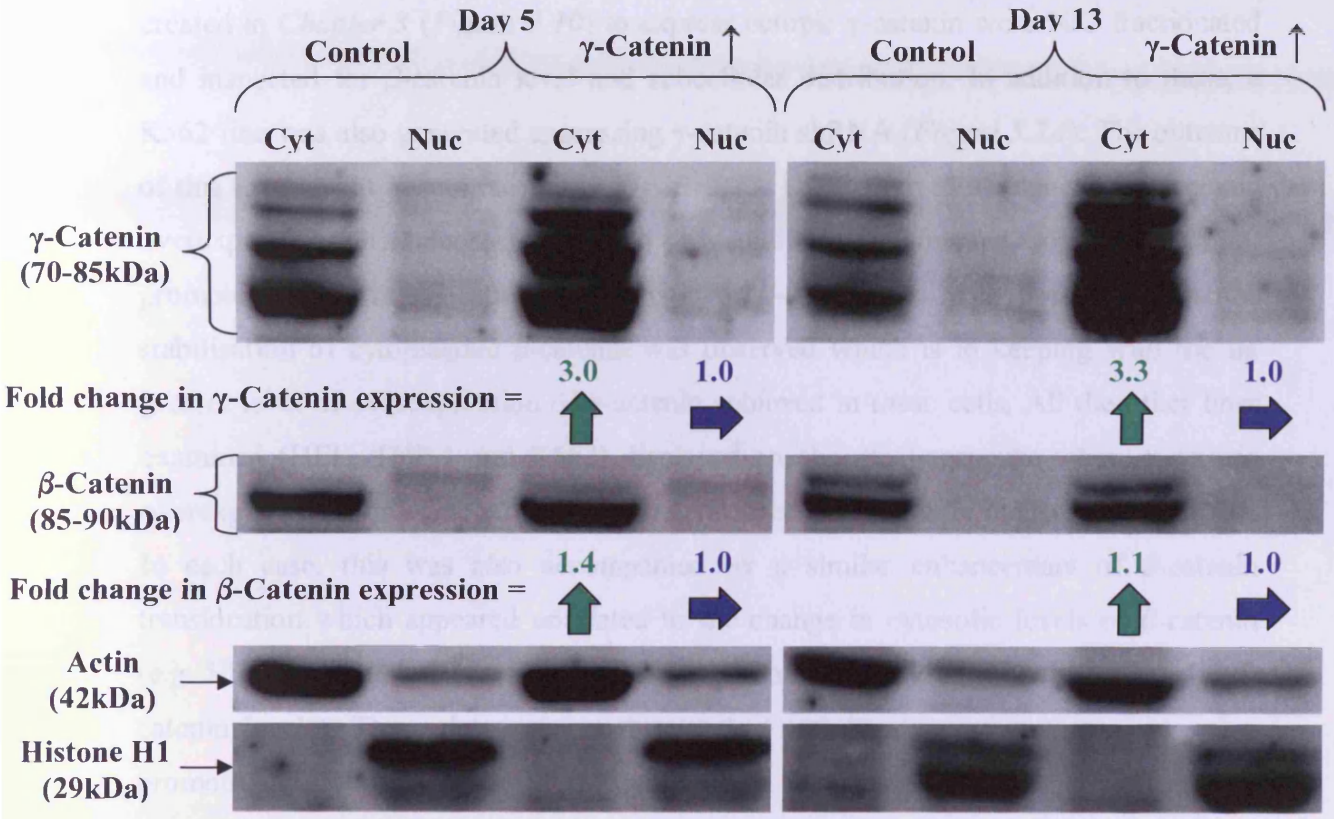


Figure 5.12 - The effect of γ-catenin overexpression on level and subcellular localisation of β-catenin in normal haematopoietic cells.

Western blot of cytosolic and nuclear homogenate from **day 5** and **day 13** *in vitro* cultured haematopoietic progenitor cells demonstrating the localisation of γ-catenin protein and the subsequent localisation of β-catenin protein. Arrows and associated numerals show the fold-change in expression versus transduced control cells. Actin and Histone H1 show the purity and loading of each fraction.

5.4.2.2 *Ectopic γ-catenin serves mainly to stabilise nuclear β-catenin in leukaemic cell lines*

It was also necessary to test the extent to which γ-catenin could influence β-catenin level and localisation within a leukaemic setting. To this end, the leukaemic cell lines created in *Chapter 3* (*Figure 3.10*) to express ectopic γ-catenin were N/C fractionated and inspected for β-catenin level and subcellular distribution. In addition to these, a K562 line was also generated expressing γ-catenin shRNA (*Figure 5.2A*). The outcome of this experiment is summarised in *Figure 5.13*. U937 showed a response to γ-catenin overexpression characteristic of normal progenitors i.e. augmented expression did not promote nuclear translocation of γ-catenin or β-catenin. As with normal cells some stabilisation of cytoplasmic β-catenin was observed which is in keeping with the far greater level of overexpression of γ-catenin achieved in these cells. All the other lines examined (HEL, THP-1 and K562) displayed an abnormal response where γ-catenin overexpression was associated with various degrees of γ-catenin nuclear translocation. In each case, this was also accompanied by a similar enhancement of β-catenin translocation which appeared unrelated to the change in cytosolic levels of β-catenin (e.g. HEL and U937 cells where β-catenin translocation is independent of cytosolic β-catenin levels). These data suggest that nuclear translocation of γ-catenin may also promote or stabilise β-catenin in the nucleus. Conversely, the γ-catenin shRNA construct reduced K562 cytosolic and nuclear γ-catenin by 90% but had only a modest effect on β-catenin expression and translocation. However, endogenous levels of nuclear β-catenin were very low in this cell line, making further reductions or fold-changes difficult to reliably quantitate. It is also possible that any loss of β-catenin could be fatal for K562 cells and so the cells which have been puro-selected and assayed could represent cells were able to maintain β-catenin expression.

The effect of γ-catenin knock-down on β-catenin expression was also analysed in the context of THP-1 and U937 cells (see *Figure 5.14* and *Figure 5.17* in section *5.4.3* below). These cell lines expressed much lower levels of γ-catenin and so the level of knock-down was less pronounced (see also below). In this context there was no consistent effect on cytosolic levels of β-catenin. Nuclear levels of β-catenin were however reduced, though in all lines the endogenous levels of nuclear β-catenin were

low, making quantification of fold-changes difficult to accurately determine. Interestingly, ectopic expression of the mutant form of degradation-resistant β-catenin (β-catΔN89) in these cell lines also lead to the stabilisation and translocation of γ-catenin (*Figure 5.14* and *Figure 5.17*). This suggests a reciprocal relationship between the two catenins within a leukaemic setting, although U937 cells again behaved like 'normal' cells demonstrating limited translocation and weak influence of catenins on one another.

To summarise the two preceding sections of data, while it would appear that ectopic γ-catenin expression is incapable of significantly modulating β-catenin localisation in normal haematopoietic cells, in a leukaemic context γ-catenin expression is much more influential in regulating both β-catenin level and localisation. Generally, an increase in γ-catenin protein frequently led to stabilisation of β-catenin protein. More consistently, changes in the nuclear translocation of γ-catenin gave rise to corresponding changes in the expression in the localisation of β-catenin. Therefore, the independent regulation of translocation that is apparent in normal haematopoiesis (see *Chapter 3*) is not evident in leukaemic cells.

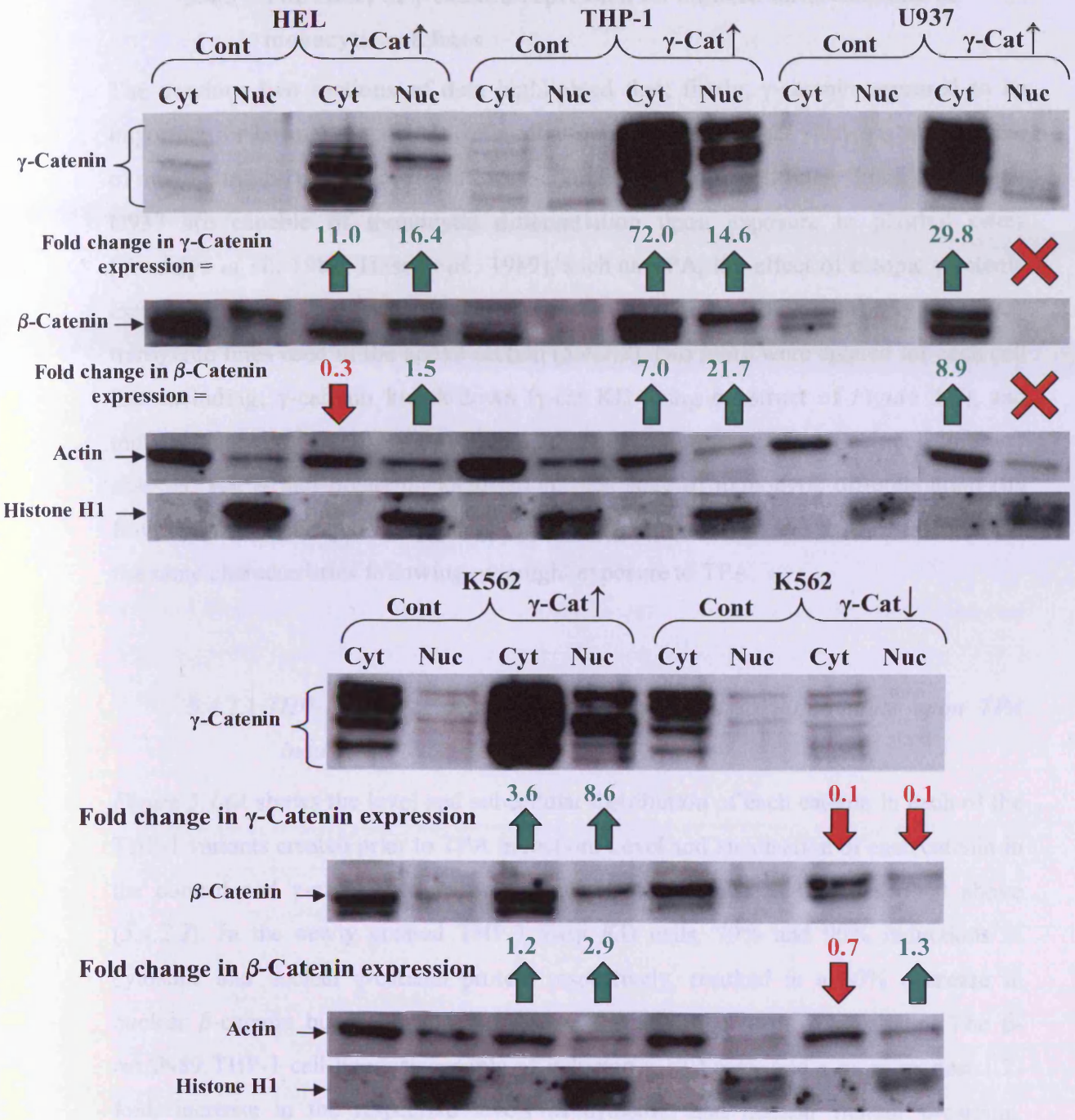


Figure 5.13 - The effect of γ-catenin overexpression on the level and subcellular localisation of β-catenin in leukaemic cell lines.

Cytosolic and nuclear homogenates from various leukaemic cell lines expressing ectopic/silenced γ-catenin protein or respective controls. Level and localisation of γ-catenin is shown along with the resulting level and localisation of β-catenin. Arrows and associated numerals show the respective fold-changes in expression versus transduced control cells. Red cross represents no fold-change or unquantifiable due to difficulties in band detection. Actin and Histone H1 show the purity and loading of each fraction.

5.4.3 The effect of γ-catenin expression on induced differentiation of monocytic cell lines

The previous two sections of data highlighted that; firstly, γ-catenin appeared to be important for normal monocytic differentiation and secondly that γ-catenin was capable of modulating β-catenin level and localisation in leukaemic cell lines. Since THP-1 and U937 are capable of monocytic differentiation upon exposure to phorbol esters (Tsuchiya *et al.*, 1982; Hass *et al.*, 1989), such as TPA, the effect of ectopic γ-catenin expression on this response was examined. In addition to the THP-1 and U937 transgenic lines used in the above section (*5.4.2.2*), two more were created for each cell line including; γ-catenin knock-down (γ-cat KD using construct of *Figure 5.2*), and mutant β-catenin (β-catΔN89, section *3.3.4.2*) overexpressing cells. Each cell line was assessed for γ- and β-catenin localisation, plus state of monocytic differentiation (by flow cytometry and cell morphology) prior to TPA treatment, and then re-assessed for the same characteristics following overnight exposure to TPA.

5.4.3.1 *THP-1 cells overexpressing γ-catenin fail to differentiate upon TPA induction*

Figure 5.14A shows the level and subcellular distribution of each catenin in each of the THP-1 variants created prior to TPA induction. Level and localisation of each catenin in the control and γ-catenin overexpressing lines was similar to that described above (*5.4.2.2*). In the newly created THP-1 γ-cat KD cells, 70% and 90% reductions in cytosolic and nuclear γ-catenin protein respectively, resulted in a 60% decrease in nuclear β-catenin but a 3.6-fold increase in the cytosolic form of β-catenin. The β-catΔN89 THP-1 cell line was capable of inducing a 10.4-fold, and a more modest 1.2-fold, increase in the respective levels of cytosolic and nuclear mutant β-catenin. Interestingly this corresponded to a 3.6-fold stabilisation of cytosolic γ-catenin along with a 2.6-fold increase in nuclear γ-catenin.

Figure 5.14B shows the level and localisation of catenin proteins in THP-1 cells following overnight treatment with TPA. A subtle TPA-mediated increase in β-catenin

protein was observed in THP-1 cells upon treatment, but this did not significantly alter the localisation of both proteins as observed in the cell lines prior to treatment.

Figure 5.15 shows the immunophenotypic profile of each THP-1 cell line prior to and following TPA treatment. The THP-1 control line, as seen in *Figure 5.15A*, was found to already express the monocytic marker CD14, which was unaffected upon TPA treatment. Cell surface expression of the myeloid protein, CD11b, was at background levels on untreated control cells, but was strongly induced (56-fold) following TPA treatment. In contrast, CD14 was not detectable on THP-1 pBabe-Puro-γ-catenin cells (*Figure 5.15B*) and was only weakly induced by TPA. Similarly CD11b could not be significantly induced upon phorbol ester treatment. The THP-1 γ-cat KD cells, featured in *Figure 5.15C*, differed from control in that these cells expressed CD11b prior to induction a feature also shared by THP-1 β-catΔN89 cells (*Figure 5.15D*), but in other respects both these lines were similar to control cells. CD13 cell surface expression was also examined (data not shown) and could not be induced by TPA in any of the THP-1 lines assayed.

May-Grünwald-Giemsa staining was used in assistance with flow cytometric data to assess the extent of monocytic differentiation in THP-1 cell lines. *Figure 5.16A* shows the normal morphology of THP-1 cells, which were dominated by dark staining monoblasts with a high nuclear: cytosol ratio. After an overnight incubation with TPA these cells differentiated into larger, mature monocyte or macrophage looking cells with pale cytoplasm and a lowered nuclear: cytosol ratio. The cytosol of these cells also appeared vacuolated, an indicator of the physiological stress. The morphology displayed in *Figure 5.16B*, for γ-catenin overexpressing THP-1 cells appeared to validate the flow cytometric analysis. The culture prior to TPA treatment seemed to contain smaller, very dark staining blasts which looked more primitive than the monoblasts present in the control culture consistent with the reduced expression of CD14. These cells failed to differentiate following TPA treatment, and maintained blast-like morphology again consistent with the reduced CD14 and absent CD11b expression. Unlike control cells, these cells also failed to become plastic-adherent following differentiation induction.

Although the THP-1 γ-cat KD cells appeared different in terms of colour (due to differences in uptake of May-Grünwald-Giemsa) the actual morphology was not dissimilar to that of control THP-1's (*Figure 5.16C*). The THP-1 γ-cat KD culture appeared to differentiate similarly to the controls, becoming adherent and increasing the presence of large vacuolated macrophage-like cells. The final THP-1 cell line overexpressing mutant β-catenin (*Figure 5.16D*) appeared darker in colour, smoother in shape with a higher nuclear:cytosol ratio (untreated). Upon TPA-induction, visible signs of differentiation were apparent as before, however, there appeared to be a large number of undifferentiated monoblasts still present in the culture. To see the full range of THP-1 cell line morphology, pre- and post-TPA induction, please refer to ***Sub-Folders 1-4*** of ***Folder 2*** from the main ***Chapter 5*** section of the ***supplementary CD***.

To summarise at this point, it appeared the capacity of THP-1 cells to undergo induced monocytic differentiation is inversely related to the extent of nuclear catenin expression. Given that the expression of nuclear γ- and β-catenin is interconnected, it is difficult to confidently conclude which catenin (or if both) is dominant in driving the differentiation block. The case for each catenin being responsible is discussed further in section *5.5.3*. Also, it seems the γ-catenin overexpression/silencing phenotypes exhibited in THP-1 cell lines are not consistent with the observations in primary haematopoietic cells (discussed further in *5.5.4*).

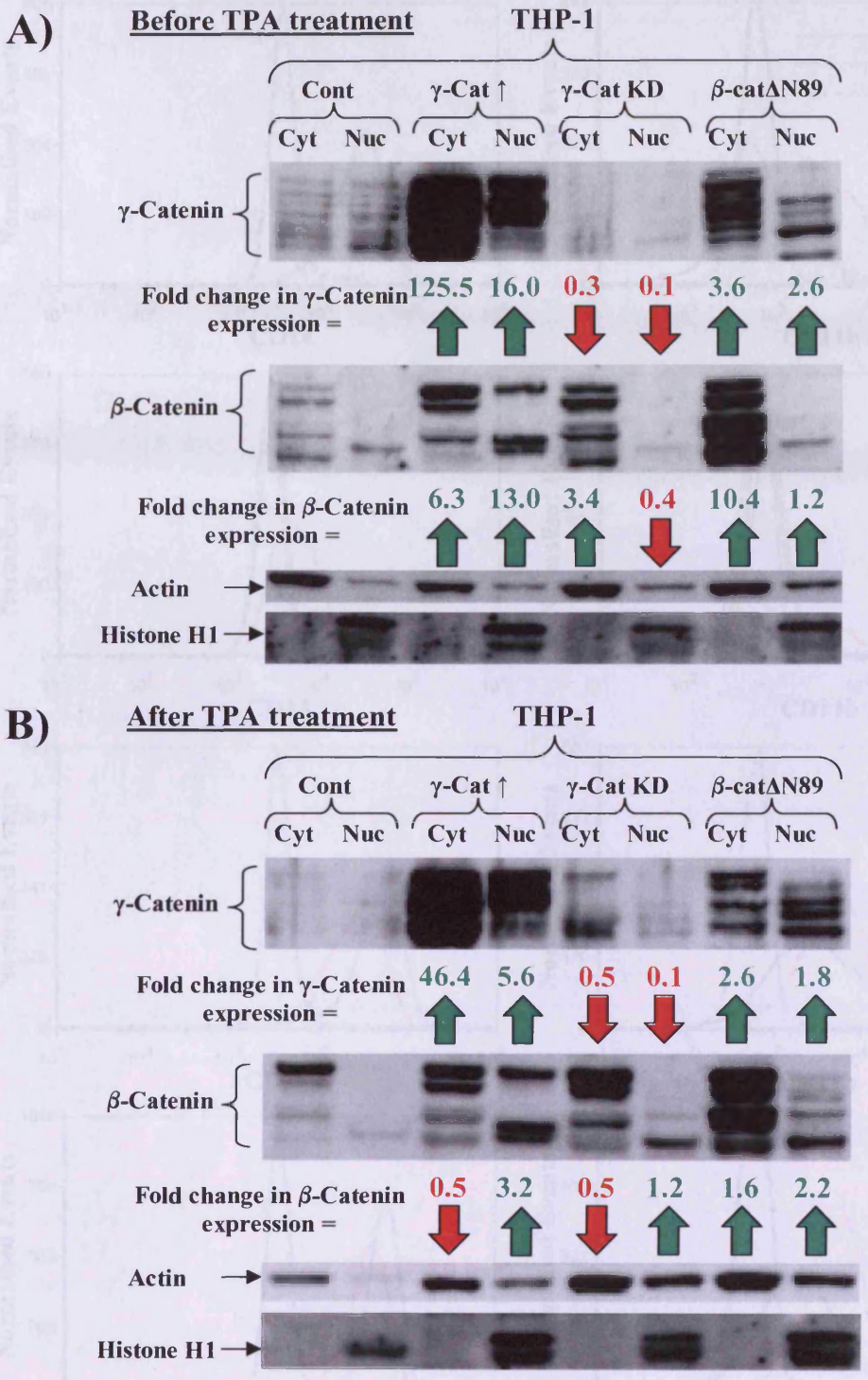


Figure 5.14 - Subcellular localisation of γ-catenin and β-catenin in THP-1 cells before and after TPA treatment.

N/C Western blots showing the level and localisation of γ- and β-catenin protein in transduced THP-1 cell lines **A)** prior to and **B)** after TPA treatment. Arrows and associated numerals show the respective fold-changes in expression versus transduced control cells, once normalised from the amount of protein loaded on each lane. Actin and Histone H1 show the purity and loading of each fraction. Note that control is under-loaded in B so banding intensity does not correspond with corrected fold change values.

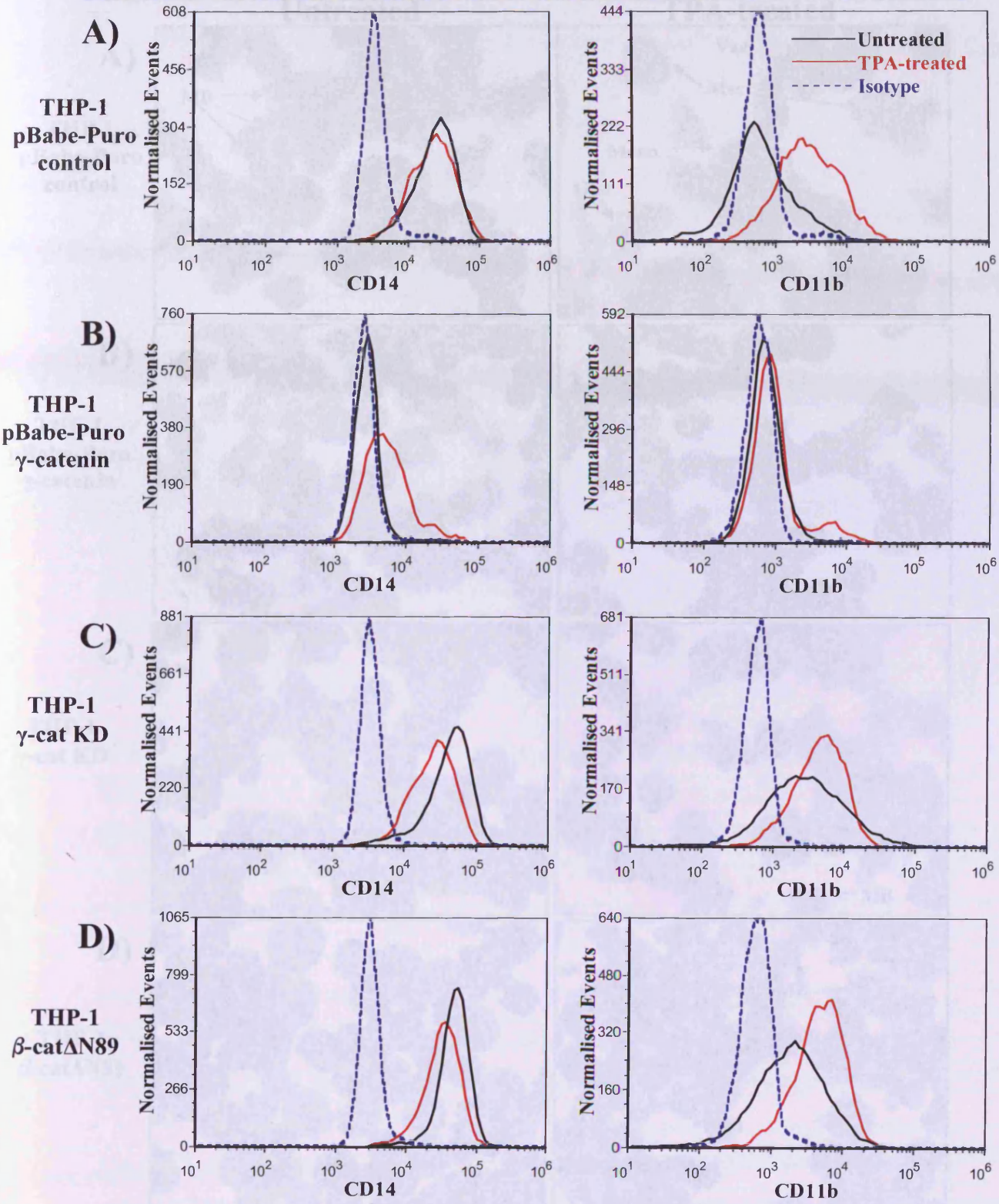


Figure 5.15 - Immunophenotyping of transduced THP-1 cells following TPA treatment.

Histograms showing the cell surface expression of CD14 and CD11b on THP-1 cells: **A)** control, **B)** γ-catenin overexpressing, **C)** γ-cat KD and **D)** β-catΔN89. Black histograms represent staining on untreated THP-1 cells, red histograms TPA-treated cells, and blue histograms indicate staining from isotype-matched control antibody.

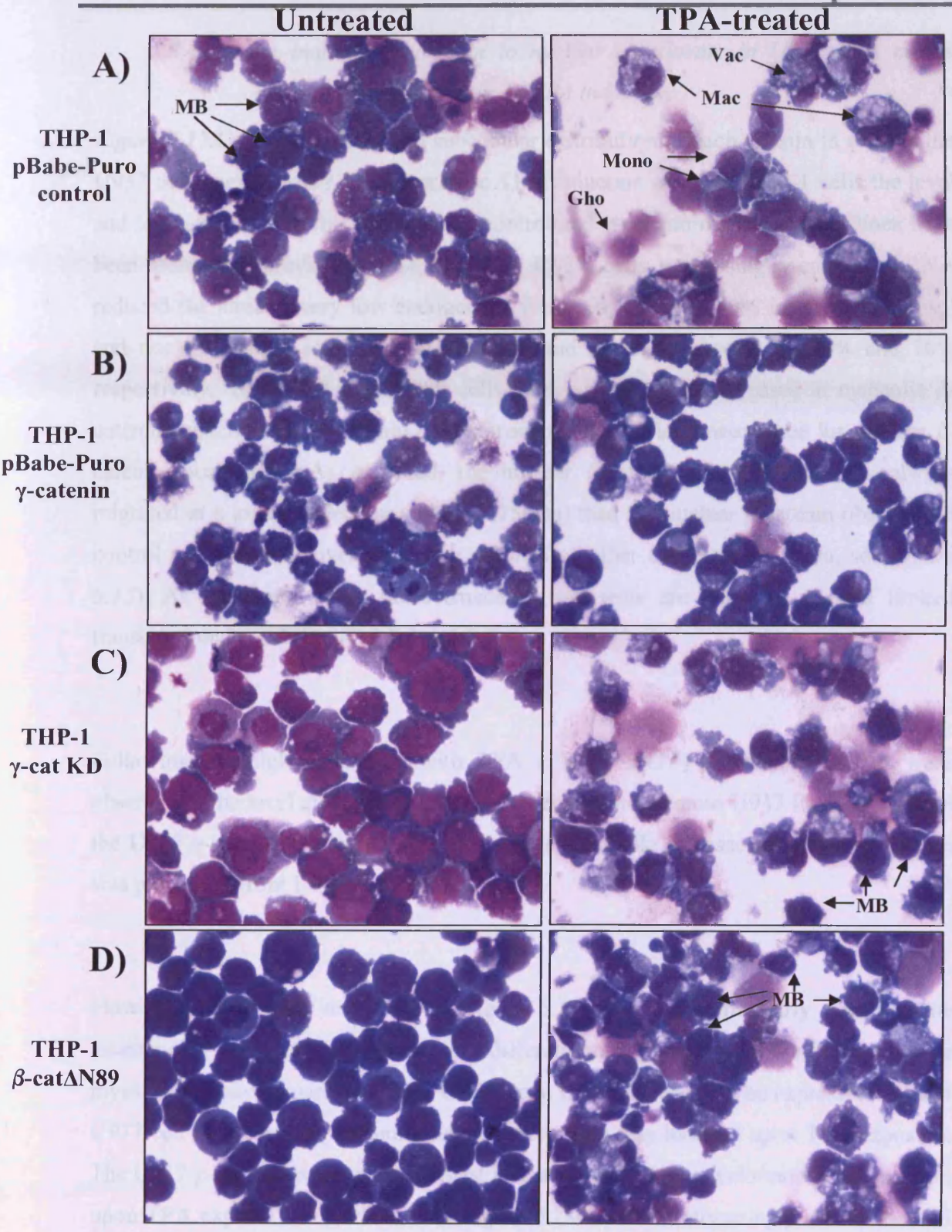


Figure 5.16 - Cell morphology of transduced THP-1 cells following TPA treatment.

Untreated and TPA-treated THP-1 cells cytospun and May-Grünwald-Giemsa stained. Morphology shown for THP-1 cells: **A)** control, **B)** γ-catenin↑, **C)** γ-cat KD and **D)** β-catΔN89 DNA. Annotations as in *Figure 5.11* except **MB**=monoblast and **Gho**=Ghost cells (smeared isolated nuclei with no cytosol). Morphology is shown at 400x.

5.4.3.2 *The inability of catenin to nuclear translocate in U937 cells means normal differentiation upon TPA induction*

Figure 5.17A shows the level and subcellular distribution of each catenin in each of the U937 cell lines variants created prior to TPA induction. As with THP-1 cells the level and localisation of each catenin in the control and γ-catenin overexpressing lines have been described previously (*5.4.2.2*). The U937 cells expressing γ-catenin shRNA reduced the already very low endogenous γ-catenin levels by 40% in both the cytosol and nucleus, which also lowered cytosolic and nuclear β-catenin by 40% and 70% respectively. The U937 β-catΔN89 cells induced a 6.3-fold increases in cytosolic β-catenin (which as for γ-catenin overexpression) had little consequence for nuclear β-catenin localisation. As expected, the nuclear β-catenin in all U937 cells always migrated to a lower molecular weight (~75kDa) than the nuclear β-catenin observed in control and γ-catenin overexpressing variants of other cell lines (~85kDa, see *Figure 5.13*). As mentioned later, these truncated fragments are likely to possess limited transcriptional capability (*5.5.3.1* and *5.5.6*).

Following overnight treatment with TPA (*Figure 5.17B*) no major changes were observed in the level and localisation of γ-catenin protein across U937 lines, apart from the U937 γ-catenin overexpressing line where a notable increase in nuclear γ-catenin was prevalent (from 1.9- to 6.8-fold).

Flow cytometric data, as shown in *Figure* 5.18, were not particularly useful in the assessment of U937 monocytic differentiation upon TPA-induction. The myeloid/monocytic markers CD14, CD11b and CD13 appeared to be expressed on most U937 cell lines assayed, but none seemed to be markedly induced upon TPA exposure. The U937 γ-cat KD exhibited the largest increases in all three myelo/monocytic markers upon TPA exposure as evidenced in *Figure* 5.18*C*, which interestingly also harboured the lowest level of nuclear β-catenin among these cell lines.

The cell morphology, as given in *Figure 5.19*, was more useful in interpreting the extent of TPA-induced monocytic differentiation in each U937 line. To see the full range of U937 cell line morphology, pre- and post-TPA induction, please refer to ***Sub-Folders 1-4*** of ***Folder 3*** from the main ***Chapter 5*** section of the ***supplementary CD***. Normal U937 morphology (untreated image of *Figure 5.19A*) shows how the majority of the cells are monoblasts similar to THP-1 cells except for a slightly lower nuclear: cytosol ratio. The morphological differences occurring through TPA exposure are quite subtle but include a further lowering of the nuclear: cytosol ratio and a paler vacuolated cytoplasm. There was an increase in the frequency of adherent macrophages and mature monocytes in the culture, although not as pronounced as for THP-1 cells. This extent of monocytic differentiation generated by TPA treatment appeared to be approximately equal in all U937 cultures as depicted in *Figure 5.19A*, *B*, *C*, and *D*. If anything the U937 γ-cat KD culture demonstrated a slightly increased frequency of differentiated monocytic/macrophage cells, however more replicates and differential morphology counts would be required to validate this observation.

To summarise, this section of data shows there is a very clear difference in the respective abilities of THP-1 and U937 cells to differentiate upon TPA stimulation. This correlates with a clear difference in the abilities of the two cell lines to translocate full-length nuclear γ-or β-catenin upon manipulation of these proteins. This would be consistent with the hypothesis that THP-1 cells have a much greater ability to influence nuclear catenin levels which in turn influences the differentiation potential. Conversely, U937 cells cannot modulate nuclear catenin to the same degree, and hence have reduced influence on differentiation potential.

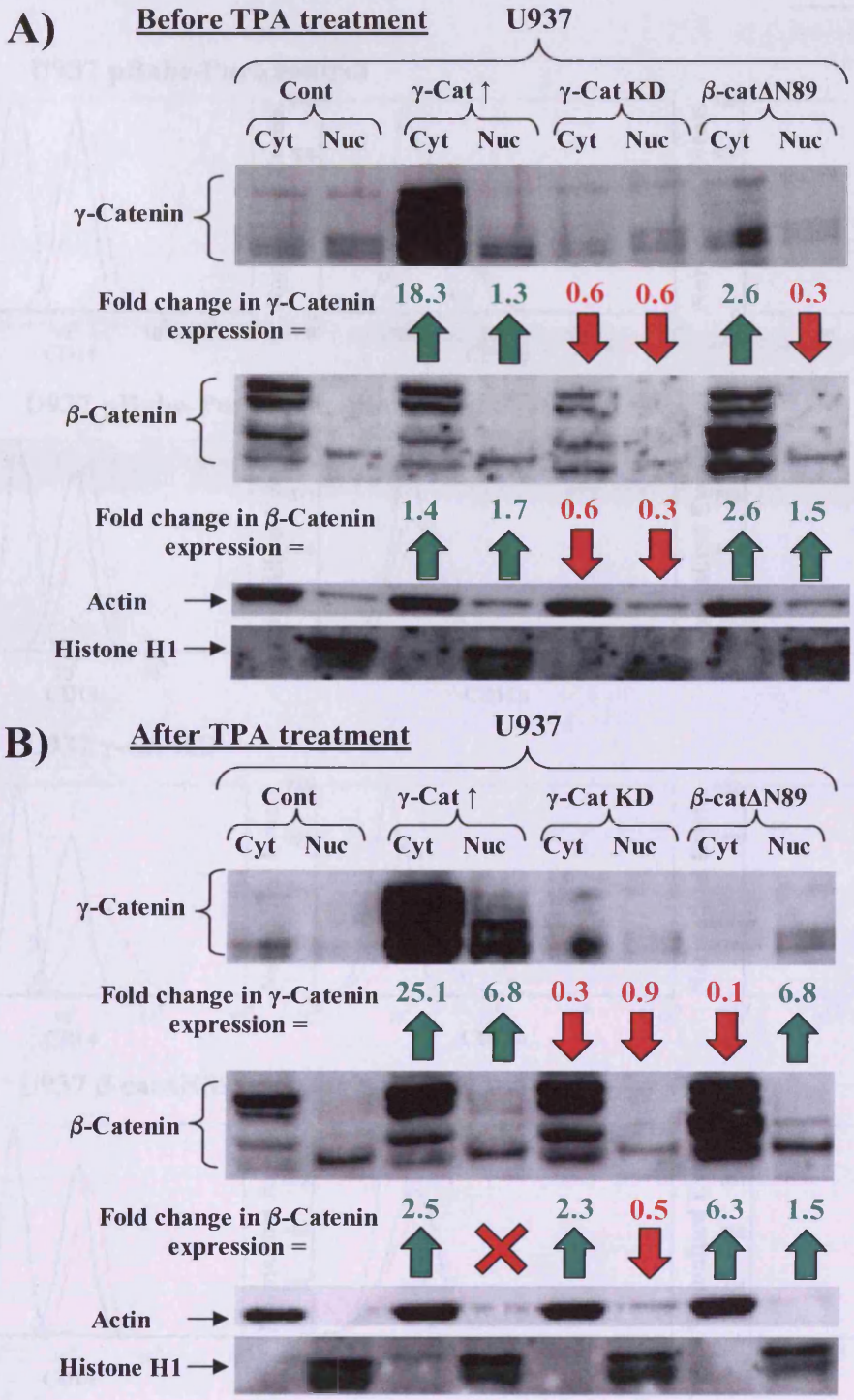


Figure 5.17 - Subcellular localisation of γ-catenin and β-catenin in U937 cells before and after TPA treatment.

N/C Western blots showing the level and localisation of γ- and β-catenin protein in transduced U937 cell lines **A)** prior to and **B)** after TPA treatment. Arrows and associated numerals show the respective fold-changes in expression versus transduced control cells once normalised from the amount of protein loaded on each lane. Red cross represents no fold-change Actin and Histone H1 show the purity and loading of each fraction.

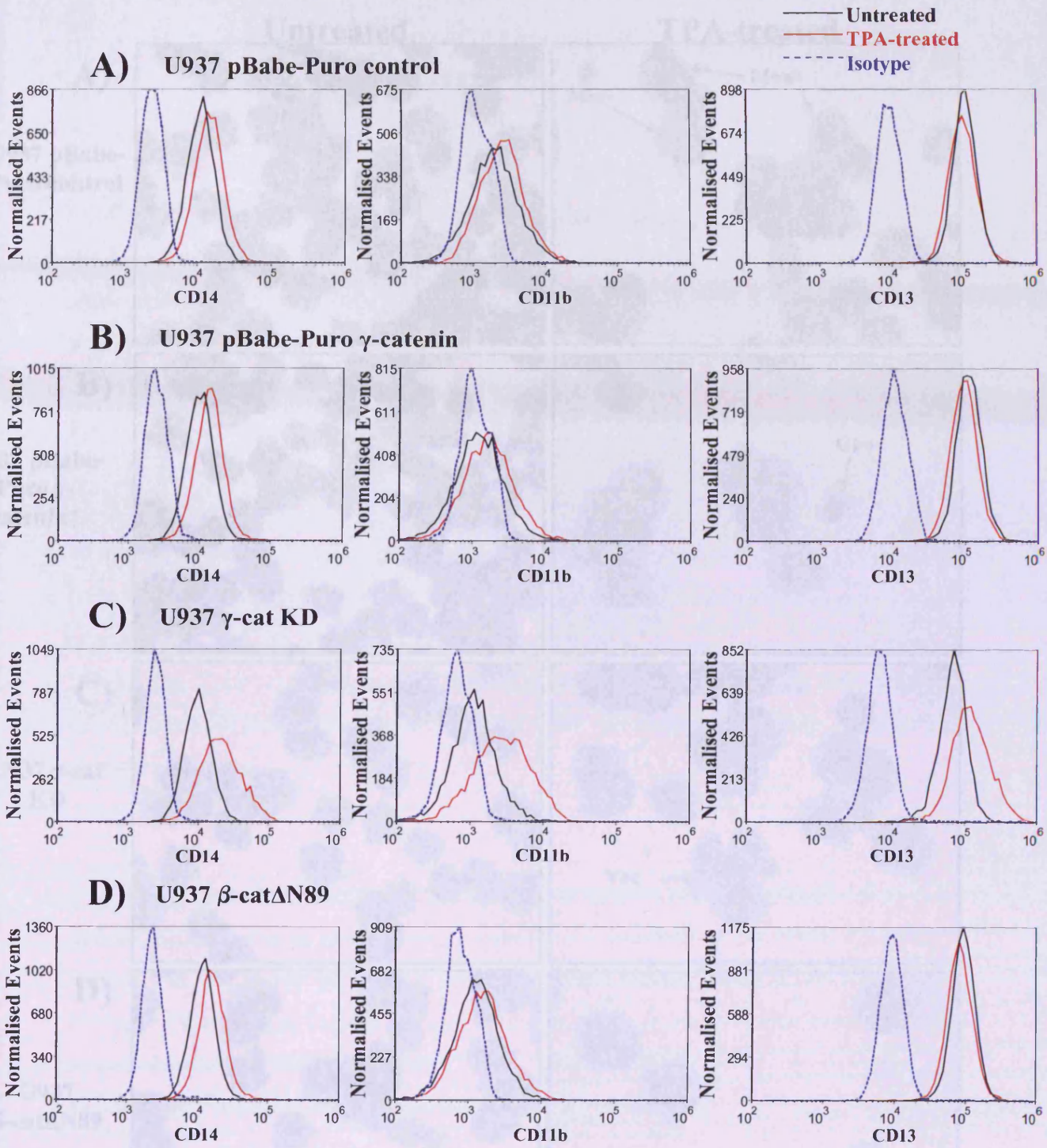


Figure 5.18 - Immunophenotyping of transduced U937 cells following TPA treatment.

Histograms showing the cell surface expression of CD14, CD11b and CD13 on U937 cells transduced with **A)** control, **B)** γ-catenin overexpressing, **C)** γ-cat KD and **D)** β-catΔN89. Black histograms represent staining on untreated U937 cells, red histograms TPA-treated cells, and blue histograms indicate staining from isotype-matched control antibody.

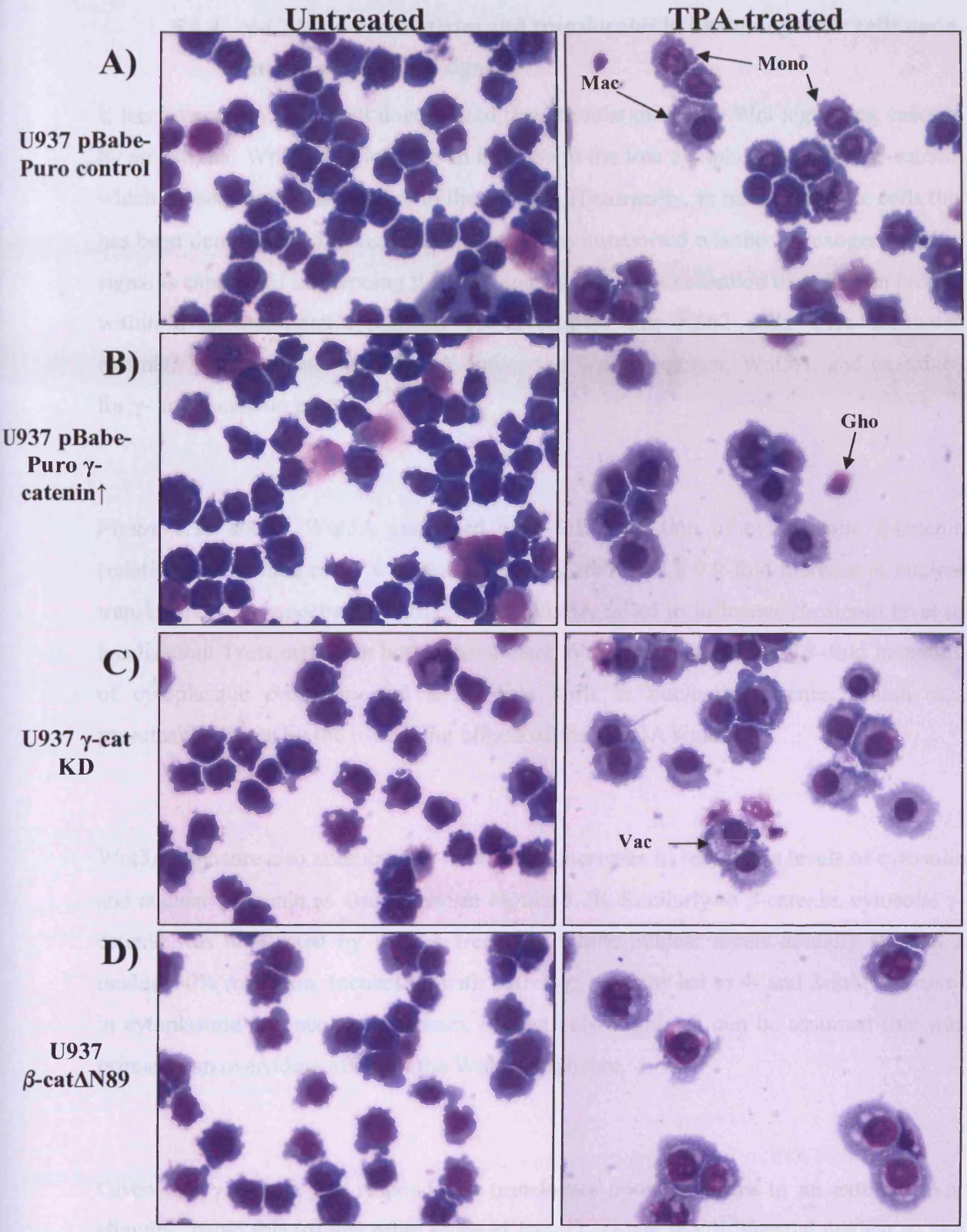


Figure 5.19 - Morphology of transduced U937 cells following TPA treatment.

Untreated and TPA-treated U937 cells cytospun and May-Grünwald-Giemsa stained. Morphology shown for U937 cells retrovirally transduced with **A)** control, **B)** γ-catenin overexpressing, **C)** γ-cat KD and **D)** β-catΔN89 DNA. Annotations as in *Figure 5.16.*

5.4.4 γ-Catenin accumulates and translocates in haematopoietic cells upon an exogenous Wnt signal

It has been previously well documented that stimulation of the Wnt signalling cascade by an external Wnt ligand leads to an increase in the free cytoplasmic pool of β-catenin which subsequently translocates to the nucleus. Historically, in haematopoietic cells this has been demonstrated using Wnt3A. It remains unreported whether an exogenous Wnt signal is capable of influencing the level and subcellular localisation of γ-catenin protein within a haematopoietic context. To investigate this K562 cells were incubated overnight with recombinant Wnt3A and/or the Wnt antagonist, Wnt5A, and examined for γ- and β-catenin protein.

Figure 5.20 shows Wnt3A generated a 15-fold induction of cytoplasmic β-catenin (relative to untreated cells) which subsequently stabilised a 9.9-fold increase in nuclear translocation, as expected. Treatment with Wnt5A failed to influence β-catenin level or localisation. Treatment with both recombinant Wnt factors caused an 18–fold induction of cytoplasmic β-catenin and a 9.9–fold shift in nuclear β-catenin, which was presumably driven by the overriding effects of the Wnt3A signal.

Wnt3A exposure also stimulated 4- and 2-fold increases in respective levels of cytosolic and nuclear γ-catenin as also shown in *Figure 5.20.* Similarly to β-catenin, cytosolic γ-catenin was unaffected by Wnt5A treatment whilst nuclear levels actually showed a modest 60% reduction. Incubation with both Wnt proteins led to 4- and 2-fold increases in cytoplasmic and nuclear γ-catenin, respectively. Again, it can be assumed this was primarily an overriding effect of the Wnt3A exposure.

Given that γ-catenin can respond and translocate upon exposure to an external Wnt stimulus, could this (or any other secreted factor) explain the differential nuclear γ- and β-catenin translocation observed between THP-1 and U937 cell lines (see *5.4.2* and *5.4.3*)? To examine this, growth medium from THP-1 control and pBabe-Puro-γ-catenin cultures was harvested at confluence, mixed equally with fresh medium and incubated

with U937 control cells overnight. The following day, U937 cells were N/C fractionated and inspected for γ- and β-catenin level and localisation. *Figure 5.21* shows how an overnight incubation of U937 cells with THP-1 medium did not significantly increase the degree of nuclear translocation of γ- or β-catenin proteins.

Taken together, this section of data shows for the first time that exogenous Wnt signals are capable of influencing the level and subcellular distribution of γ-catenin within haematopoietic cells. However, this finding cannot explain the discrepancy between the respective abilities of THP-1 and U937 cells to nuclear translocate γ- and β-catenin protein.

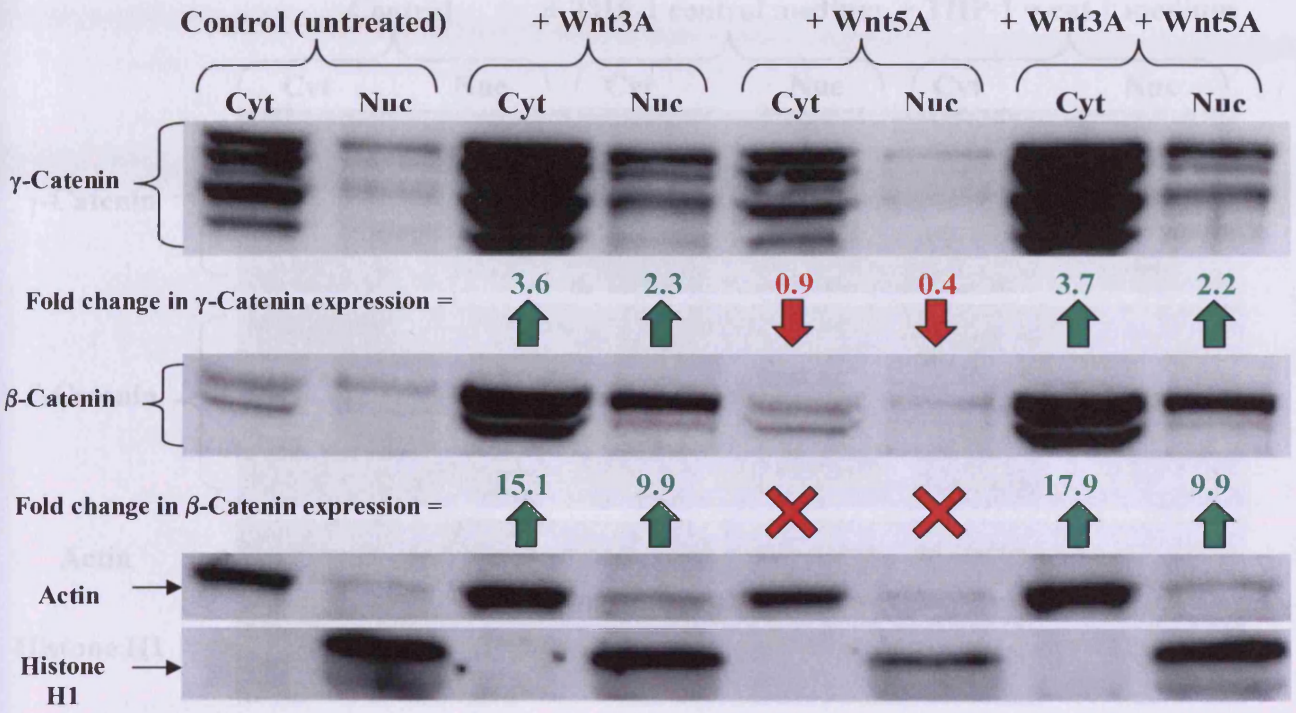


Figure 5.20 - The effect of Wnt3A and Wnt5A on the subcellular localisation γ- and β-catenin expression.

Fractionation of K562 cells into cytosolic and nuclear lysates to determine the subcellular localisation of γ- and β-catenin protein in response to Wnt stimuli. Arrows and associated numerals show the fold-change in expression versus untreated control cells. Red cross represents no fold-change or unquantifiable due to difficulties in band detection. Actin and Histone H1 show the purity and loading of each fraction.

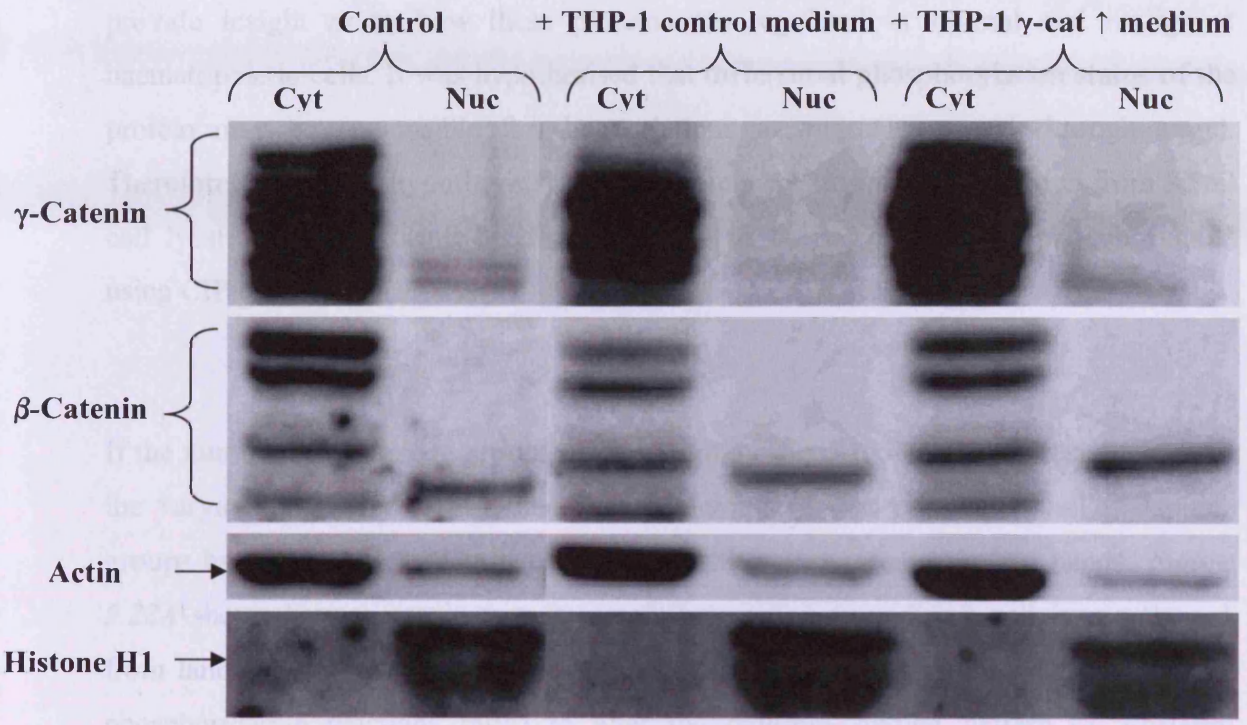


Figure 5.21 - The effect of THP-1 conditioned medium on the level and localisation of catenins in U937 cells.

Medium from confluent THP-1 pBabe-Puro control and pBabe-Puro-γ-catenin cells was harvested and mixed 1:1 with fresh RPMI medium, and incubated with U937 pBabe-puro-γ-catenin cells overnight. Western blot shows cytosolic and nuclear fractions from U937 cells from each condition and the respective level and localisation of γ- and β-catenin. Detection of Actin and Histone H1 provided an assessment of purity and protein loading of each fraction.

5.4.5 The multiple-banding of γ-catenin is unlikely due to differential phosphorylation status

It had been frequently observed during this project that Western blotting of γ-catenin protein (and β-catenin to a lesser extent) had exhibited a multiple-banding pattern in both normal and leukaemic cells. Knowledge of the cause of this banding was likely to provide insight as to how these proteins are regulated in normal and malignant haematopoietic cells. It was hypothesised that differential phosphorylation status of the protein may be responsible for the variations in migratory speed through a gel. Therefore, to test this hypothesis, γ-catenin protein was immunoprecipitated from K562 cell lysate (where multiple-banding is known to be present) and de-phosphorylated using CIP enzyme.

If the number of phosphate groups present on the catenin molecule was responsible for the varying migratory forms, then it was anticipated that removal of all phosphate groups by CIP would lead to a uniform formation of γ-catenin protein bands. *Figure 5.22A* shows how γ-catenin was successfully purified from K562 cell lysate (lane 1 from lane 4), but how both low (10U CIP, lane 2) and high (100U CIP, lane 3) de-phosphorylation reactions failed to alter the multiple-banding pattern of γ-catenin protein when Western blotted. A dose-dependent increase in the intensity of γ-catenin protein bands (relative to untreated controls) was observed between protein lanes treated with low and high CIP concentrations however the significance of this is unknown. To validate the efficacy of the de-phosphorylation reaction, the immunoprecipitated protein was probed with a phosphotyrosine antibody. Phosphorylated tyrosine residues, could not be detected around the migratory region of untreated (still phosphorylated) γ-catenin protein. Faint bands were detected in the CIP-treated lanes, however this is likely to represent residual γ-catenin protein signal that was remaining from the initial detection of purified γ-catenin protein. Thus, there is no confirmation that the specific de-phosphorylation of γ-catenin protein actually occurred. However, and shown in *Figure 5.22B* and *C*, phosphotyrosine was detected on an unknown high molecular weight protein which had immunoprecipitated with γ-catenin. This protein was around 180-200kDa in size and exhibited clear phosphotyrosine signal in the untreated lane (1)

which became heavily reduced in the high and low CIP-treated lanes. This finding would suggest that the dephosphorylation step by CIP was effective.

In conclusion, these data would suggest that differential phosphorylative forms of γ-catenin molecule are not responsible for the multiple-banding of γ-catenin protein observed in Western blotting of normal and malignant haematopoietic cells. However, these experiments are unable to decisively confirm whether specific de-phosphorylation of γ-catenin protein truly occurred.

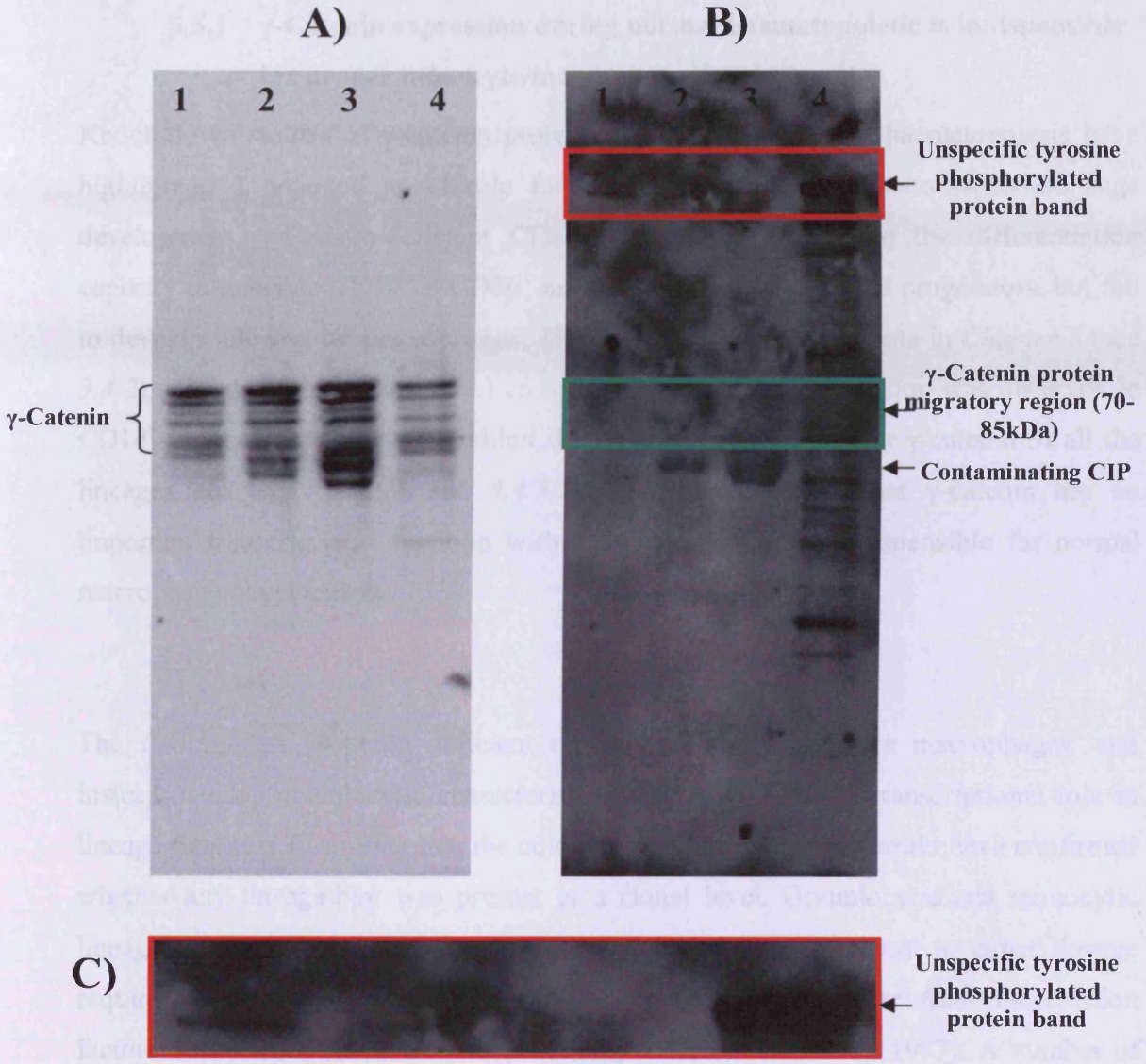


Figure 5.22 - The isolation and de-phosphorylation of γ-catenin protein.

A) Whole cell lysate Western blots of K562 cells demonstrating immuno-precipitated γ-catenin protein (lanes 1-3) and otherwise unpurified but cleared K562 lysate (lane 4). Lanes 2 and 3 were treated with low (10U) and high (100U) concentrations of CIP, respectively. Control lanes 1 and 4 were treated with PBS only. **B)** The same Western blot but probed with a phospho-tyrosine antibody. The efficiency of the CIP-induced de-phosphorylation step was assessed by observation of an unknown, non-specific protein band (red box) which is further magnified in **C)**. Phospho-tyrosine residues appeared to be undetectable within the migratory region of γ-catenin protein.

5.5 Discussion

5.5.1 γ-Catenin expression during normal haematopoietic is indispensible for proper monocyte/macrophage development

Knock-down studies of γ-catenin protein in a model of normal haematopoiesis have highlighted a potential novel role for γ-catenin in effective monocyte/macrophage development. γ-Catenin-deficient $CD34^+$ progenitor cells retain the differentiation capacity to generate $CD13^{bright}CD36^+$ monocyte-lineage committed progenitors, but fail to develop into mature macrophages. These findings support the data in *Chapter 3* (see *3.4.2*), whereby (unlike β-catenin) an increase in γ-catenin expression was observed in $CD14^+$ monocytes which also exhibit the highest levels of nuclear γ-catenin of all the lineages analysed (*3.4.3.5* and *3.4.3.7*). This would imply that γ-catenin has an important transcriptional function within these cells that is indispensible for normal macrophage development.

The finding that γ-catenin deficient monocytes fail to generate macrophages, and instead develop granulocytic characteristics is consistent with a transcriptional role in lineage fate. It is frustrating that the colony assays failed, as this would have confirmed whether any lineage-bias was present at a clonal level. Granulocytic and monocytic lineages are closely related and there is evidence that commitment to either lineage requires both expression and suppression of lineage-specific or -irrelevant transcription factors, respectively, as evidenced previously in HSC (Hu *et al.*, 1997). A number of transcription factors have been implicated in normal monocyte/macrophage differentiation including CCAAT-enhancer-binding proteins (CEBP), PU.1 and RUNX-1 as reviewed by Valledor *et al.*, 1998. These data would imply that lineage-enforcement occurs throughout haematopoietic development, and that γ-catenin could be involved in this process to promote macrophage development. Catenins have been implicated in such a process previously by Baba *et al.* whereby a constitutively active form of β-catenin in normal myeloid or lymphoid progenitors generated uncommitted progenitors with multi-lineage differentiation potential (Baba *et al.*, 2005). Inappropriate gene expression was observed with β-catenin directly increasing the expression of CEBPα, but reducing early B-cell factor (EBF), and paired box protein 5

(Pax-5) in order to generate myeloid cells from lymphoid progenitors. This would insinuate a degree of plasticity in late haematopoietic development for which there is some evidence in monocytic cells (Montanari *et al.*, 2005; Ungefroren *et al.*, 2010). It would be tempting to speculate that γ-catenin could have an influence on some of the aforementioned transcription factors, however such a claim could only be affirmed by γ-catenin knock-down studies incorporating gene expression profile microarrays. Similar studies have been conducted in this laboratory previously albeit using the γ-catenin overexpression model. In these studies (Liddiard *et al.*, submitted) γ-catenin positively regulated the activity of *myc-target protein 1* (myc-T1), which was found to preferentially promote monocytic over granulocytic colony formation. This gene represents an obvious candidate to monitor in future γ-catenin knock-down studies.

These data, and those from *Chapter 3*, are in conflict with mice studies whereby constitutive deletion of γ- and or β-catenin was of no consequence to short-term or long-term haematopoiesis (Cobas *et al.*, 2004; Jeannet *et al.*, 2008; Koch *et al.*, 2008). These studies principally focused on the ability of γ-/β-catenin knock-out (KO) HSC/progenitor cells to reconstitute sub-lethally irradiated mice, rather than analysing the developmental capacity of specific lineages. Also, despite armadillo proteins being evolutionary conserved between species it is conceivable that catenins may have different functions in a mouse compared to that of a human. The study by Koch *et al.* seemed to concentrate on absolute numbers of cell lineages, and observed no difference in the proportion of long term-HSC (LT-HSC), short term-HSC (ST-HSC), or multi-potent progenitor cells (MPP) between normal and KO mice. However they failed to examine the detailed morphological and immunophenotypic changes associated with individual lineages as this study did, and such an *in vivo* model would be incapable of detecting macrophage abnormalities, since they do not circulate but normally reside in tissues. A similar study by Jeannet *et al.* also examined the reconstitution efficiency of catenin KO cells in mice, with the same limitations as described above. In addition, they found the Wnt signalling pathway remained active in the combined absence of γ- and β-catenin, and attributed this to the presence of other 'catenin-like' with Wnt transducing capability. However, the data of this study would imply that the specific presence of γ-catenin is still required for effective macrophage development.

5.5.2 γ-Catenin significantly modulates the level and localisation of β-catenin in cell lines, but not primary human haematopoietic cells

The correlation between γ- and β-catenin in primary AML blasts, revealed in *Chapter 4*, led us to hypothesise that the expression of these proteins may be in some way inter-connected. Ectopic expression of γ-catenin in leukaemic cell lines has shown this protein to be causative in modulating both the level and subcellular localisation of β-catenin in leukaemic cells. Generally, the effect of stabilising cytosolic and nuclear γ-catenin in cell lines appeared to be a concomitant stabilisation of cytosolic and nuclear β-catenin respectively. These findings would agree with a number of other studies in differing contexts whereby γ-catenin was competent in influencing the level and localisation of β-catenin. (Salomon *et al.*, 1997; Miller and Moon, 1997; Simcha *et al.*, 1998; Zhurinsky *et al.*, 2000a; Maeda *et al.*, 2004; Li *et al.*, 2007). To our knowledge, this study represents the first to demonstrate this process is active in haematopoietic cells.

Some of the above studies have postulated that γ-catenin may be able to stabilise β-catenin expression by saturating the degradation components (catenin destruction complex (CDC) and proteasome) thus increasing the signalling competent pool of β-catenin within the cell. This hypothesis remained applicable to three of the four cell lines tested, with HEL cells displaying the only exception of decreased cytosolic β-catenin in response to ectopic γ-catenin. Although the majority of high γ-catenin expressing AML samples co-express higher β-catenin (*Figure 4.8A*), HEL cells may represent the AML cases whereby γ- and β-catenin levels do not correlate. Indeed there are many other molecules and mechanisms capable of stabilising/degrading β-catenin in leukaemic cells (other than γ-catenin) as previously covered in the *Chapter 4* discussion (*4.5.3*). This may explain why a higher degree of correlation was not achieved between γ- and β-catenin protein expression in primary AML blasts. A study by Solomon *et al.* (Salomon *et al.*, 1997) using an inducible model of γ-catenin expression actually demonstrated a 3-5 fold decrease in β-catenin protein which was further attributed to no change in RNA levels, but an increased rate of post-translational turnover instead.

A consistent observation across all cell lines analysed, was that nuclear γ-catenin level dictated the degree of nuclear translocated β-catenin regardless of cytosolic level. This degree of correlation was absent in primary AML blasts, although there remained a significant association between higher nuclear β-catenin expression and high nuclear γ-catenin expressing samples (*Figure 4.8C*). The enforced translocation of β-catenin to the nucleus upon ectopic γ-catenin expression has been previously reported in other non-haematopoietic contexts (Simcha *et al.*, 1998; Maeda *et al.*, 2004; Li *et al.*, 2007). None of these studies formally demonstrate a mechanism by which γ-catenin achieves this, but Maeda and Simcha *et al.* identified LEF-1 as a chaperone of γ- and β-catenin nuclear translocation. The reports by Simcha and Li *et al.* both hypothesised that the increase in nuclear β-catenin was due to γ-catenin's ability to displace membrane-tethered β-catenin from its junctional partners. Indeed, Li *et al.* strengthened this theory by demonstrating a reduction in the amount of two such partners (N-cadherin and α-catenin) in the nucleus of γ-catenin overexpressing cells, thus reducing their capacity to sequester β-catenin from the nucleus. An investigation by Solomon *et al.* also corroborated this hypothesis by showing γ-catenin can effectively substitute with β-catenin for N-cadherin binding in adherens junctions. Despite the evidence for this mechanism in other contexts, it cannot be assumed such a process is active in haematopoietic cells given the degree of uncertainty as to whether such adhesion complexes or junctional proteins are relevant in this tissue. Indeed evidence exists to suggest that such junctional components are expressed poorly in haematopoietic cells but are not functional (Yang *et al.*, 2009).

Whichever mechanism is responsible for co-localising γ- and β-catenin to the nucleus in most leukaemic cell lines, it is clearly not active in U937 cells. Despite a large influx of cytosolic γ-catenin (which in turn stabilised cytosolic β-catenin), neither catenin is significantly translocated to the nuclei of these cells. In this sense, U937 cells more closely resemble the tight control exhibited in normal $CD34^+$ haematopoietic progenitors, rather than leukaemic cells where expression level governs the degree of nuclear translocation. It is likely that γ-catenin-mediated stabilisation of nuclear β-catenin represents a pathological phenomenon since this relationship was absent in normal **day 5** or **day 13** primary haematopoietic cells. Ectopic expression of γ-catenin

failed to nuclear translocate and did not affect the subsequent localisation of β-catenin protein, suggesting a stringent regulatory mechanism remained functional. This would fit well with *Chapter 3* CLSM data whereby β-catenin could be found independently of γ-catenin in the nuclei of $CD34^+$ haematopoietic progenitor cells. Clearly there is another degree of complexity to this regulatory system however, given that CLSM studies also demonstrated γ- and β-catenin appeared to translocate in a reciprocal manner upon myeloid differentiation. It is conceivable that a finely tuned balance between nuclear catenin import and export mechanisms (discussed in detail in *Chapter 3* discussion section *3.5.3*) is present in normal haematopoietic tissues, which is either disturbed or lost altogether in leukaemic cells.

Finally, it was observed that ectopic β-catenin (albeit a truncated stabilised form) could also increase γ-catenin expression and nuclear translocation in leukaemic cells. Clearly this phenomenon must also be context-dependent, given that the studies by Maeda and Simcha (Simcha *et al.*, 1998; Maeda *et al.*, 2004) failed to detect any influence of β-catenin on γ-catenin level and localisation. In Simcha's study, they attributed this failure to the fact that the MDCK, 293T and SK-BR-3 used in their experiments all express desmosomes, which is an exclusive location for γ-catenin for which β-catenin cannot substitute (Bierkamp *et al.*, 1996; Ruiz *et al.*, 1996; Bierkamp *et al.*, 1999). These structures are absent from haematopoietic cells and so may explain the apparent discrepancies between the studies. The finding that β-catenin induction can also raise the level of cytosolic γ-catenin is consistent with the concept outlined above. That is, ectopic expression of either γ- or β-catenin in the cytosol is capable of saturating the degradation components, and causing a concomitant increase in endogenous levels of the other catenin. Evidence has been presented showing that γ- and β-catenin levels correlate in primary AML blasts, however identifying which of the two catenins is the primary abnormality is much more difficult to address. Alternatively it cannot be excluded that in primary AML generally high levels of γ and β-catenins arise from a defect in the common degradation machinery; indeed, if the above hypothesis is correct, the fact that the degradative mechanism is so easily saturated in leukaemic cells (compared with the lack of any significant effect of ectopic γ-catenin on β-catenin levels in normal cells) implies that this process is generally compromised in AML.

5.5.3 Enforced nuclear translocation of full-length β-catenin mediated by γ-catenin is capable of blocking differentiation in leukaemic cells

The experiments of this chapter have not only shown the correlation between γ-and β-catenin may arise from cross-stabilisation in leukaemic cells, but have also provided some insight as to how this relationship may contribute to the leukaemogenic process. Ectopic expression of γ-catenin in THP-1 cells resulted in significant nuclear translocation of both catenins which subsequently failed to undergo monocytic differentiation upon TPA-induction, whilst U937- γ-catenin cells (with barely detectable nuclear β or γ-catenin) underwent monocytic differentiation as 'normal'. If it is truly the nuclear contribution of catenin that dictates this phenotype, then a similar question to that raised above is evoked: *Is the developmental block a direct consequence of heavily translocated nuclear γ-catenin, or an indirect effect of stabilising significant levels of nuclear β-catenin?* There is convincing evidence for both cases:

5.5.3.1 *Evidence for nuclear β-catenin mediating the primary differentiation block*

The degree of monocytic differentiation achieved in each cell line seemed to be inversely proportional to the amount of nuclear β-catenin present. Increased capacity for self-renewal and loss of differentiation potential is a commonly reported phenotype of nuclear β-catenin. It has been shown to be a potent mediator of self-renewal in normal HSC (Reya *et al.*, 2003; Baba *et al.*, 2006; Zhao *et al.*, 2007; Holmes *et al.*, 2008; Nemeth *et al.*, 2009; Yeung *et al.*, 2010) and is strongly believed to do the same in LSC (Jamieson *et al.*, 2004; Zhao *et al.*, 2007; Hu *et al.*, 2009; Abrahamsson *et al.*, 2009; Wang *et al.*, 2010; Yeung *et al.*, 2010). Simon *et al.* previously reported a perturbation in myelomonocytic differentiation both morphologically and immunophenotypically when human primary $CD34^+$ progenitor cells were transduced with a constitutively active (S37A mutant) form of β-catenin (Simon *et al.*, 2005). Like us, they also observed an absence of normal CD14 induction and the morphological absence of macrophages. A disturbance was noted in CD14 and CD11b expression in TPA-treated THP-1 cells, not just overexpressing γ-catenin, but also the THP-1 line expressing mutant β-catenin (β-catΔN89, which had an increased nuclear presence).

If nuclear β-catenin was the primary instigator of the developmental block, then one might have expected the γ-cat KD or β-catΔN89 to recapitulate the phenotype observed in the γ-catenin overexpressing line. There were signs that TPA-induced monocytic differentiation in these transgenic lines was not as efficient as observed in the control line (i.e. lower induction of CD14/CD11b and the presence of residual blasts). However these cell lines (γ-cat KD and β-catΔN89) were incapable of inducing the same quantity of nuclear β-catenin as evident in the γ-catenin overexpressing THP-1 cells. The pool of nuclear β-catenin induced by γ-catenin was representative of where a full-length, transcriptionally active form would be expected to migrate (~85kDa). However the nuclear β-catenin species present in γ-cat KD and β-catΔN89 THP-1 cells was less abundant and much smaller (~70kDa), and it has been questioned previously whether these smaller fragments are even capable of binding or activating TCF/LEF promoters with the same efficiency (see *5.5.6* below). One way to definitively resolve this issue would be to transduce the same cell lines with a full-length β-catenin construct capable of inducing large volumes of full-length nuclear β-catenin, but not γ-catenin, and repeat the experiment.

5.5.3.2 *Evidence for nuclear γ-catenin being the primary initiator of differentiation block*

γ-Catenin has previously been associated with increased self-renewal capacity when ectopically expressed in primary haematopoietic progenitor cells (Muller-Tidow *et al.*, 2004; Zheng *et al.*, 2004; Tonks *et al.*, unpublished data; see *Appendix 6*). Specifically, the study by Müller-Tidow showed transduction of γ-catenin was able to enhance the proliferation and clonal growth of myeloid 32D cells. The same experiment in primitive murine haematopoietic progenitor cells preserved an immature phenotype during colony growth, indicative of enhanced self-renewal. However none of the above studies examined the co-level or co-localisation of β-catenin meaning the phenotype could have arisen through indirect stabilisation of β-catenin. Data of this study would suggest that increased γ-catenin expression is incapable of significantly translocating β-catenin in these primitive cells, however it is conceivable that a small quantity of translocated nuclear β-catenin (below the detection threshold) could be responsible for self-renewal.

Alternatively, the self-renewal phenotype may originate from a relatively small subpopulation of $CD34^+$ with the capacity for more extensive β-catenin translocation.

Regardless, γ-catenin itself has been shown by Müller-Tidow to directly bind the *c-myc* promoter (in the presence of RUNX-1/ETO fusion protein, an inducer of γ-catenin protein), indicating that γ-catenin does have transcriptional capability independent of β-catenin (Muller-Tidow *et al.*, 2004). More recently, γ-catenin has been shown to directly mediate the transcription of *survivin* on a β-catenin null background, by complexing with TCF and CREB binding protein (CBP) (Kim *et al.*, 2011). Transcriptional independence has also been demonstrated outside of the field of haematology. Kolligs *et al.* (Kolligs *et al.*, 2000) employed an epithelial cell line (RK3E) to demonstrate γ-catenin had TCF-LEF-dependent transforming capability through activation of *c-myc*, which was independent of β-catenin. γ-Catenin can bind TCF/LEF complexes in cell lines of malignant mesothelioma (NCI-H28, (Maeda *et al.*, 2004)), human embryonic kidney (293T, (Williams *et al.*, 2000; Zhurinsky *et al.*, 2000a)), teratocarcinoma (F9, (Fukunaga *et al.*, 2005)) and canine kidney (MDCK, (Simcha *et al.*, 1998)). Furthermore γ-catenin has shown the capacity to bind and activate the promoters of *cyclinD1* and *neuronal cell adhesion molecule* (*Nr-CAM*) genes in β-catenin-null embryonic stem cells (Conacci-Sorrell *et al.*, 2002). However, many of these studies acknowledge that γ-catenin binds and activates TCF/LEF with less affinity and potency than β-catenin.

5.5.4 Disparities, although explainable, are present between the γ-catenin knockdown phenotypes observed in primary cells and cell lines

The phenotypes observed from γ-catenin overexpression and knock-down were inconsistent between primary cells and leukaemic cell lines. γ-Catenin overexpression appeared to block monocytic differentiation in inducible THP-1 cells, whilst producing no detectable phenotype in primary haematopoietic cells. Disparity was also observed in γ-catenin knock-down which perturbed macrophage development in primary cells, yet did not affect the differentiation capacity of THP-1 and U937 cells. These discrepancies can be reconciled as follows. Firstly, ectopic expression of γ-catenin is unable to

detectably influence the expression or localisation of either catenin in primary human haematopoietic cells whereas in leukaemic cell lines the effect of γ-catenin cannot be separated from its effect on the nuclear translocation of β-catenin; indeed the differentiation block was only observed where ectopic γ-catenin expression promoted nuclear translocation of β-catenin (i.e. in THP-1 but not in U937). Secondly, the lack of effect of γ-catenin knock-down in cell lines may arise from the fact that TPA induction may override the normal control of monocyte/macrophage differentiation. Further, in the human primary model, the knock-down phenotype appeared to arise from a lineage-bias toward developing granulocytic characteristics over macrophage differentiation. THP-1 cells and U937 cells are not known to have the capacity for granulocytic development; therefore cues to reinforce monocyte/macrophage development through γ-catenin may be superfluous.

5.5.5 γ-Catenin can respond to a Wnt signal in haematopoietic cells

This study has shown for the first time that γ-catenin is capable of responding and translocating upon induction of a Wnt stimulus in haematopoietic cells. Unsurprisingly Wnt3A was shown to mediate this, which is in keeping with its proposed association to maintain active Wnt signalling both within normal HSC (Austin *et al.*, 1997; Van den Berg *et al.*, 1998; Reya *et al.*, 2003; Willert *et al.*, 2003; Luis *et al.*, 2009; Kim *et al.*, 2009; Luis and Staal, 2009; Luis *et al.*, 2010) and malignant haematopoietic cells (Nygren *et al.*, 2007; Tickenbrock *et al.*, 2008; Kawaguchi-Ihara *et al.*, 2008). Indeed, Wnt3A was shown to modulate β-catenin level and localisation in many of these studies. Subsequent studies from this laboratory have shown that mature differentiated haematopoietic cells (22 days of *in vitro* culture) also remain sensitive to Wnt signals as evidenced by an increase in γ-catenin expression, but not translocation, when cultured in the presence of Wnt3A conditioned medium (data not shown).

It is known that other Wnt ligands (other than Wnt3A) are active in normal haematopoiesis (Austin *et al.*, 1997; Van den Berg *et al.*, 1998; Brandon *et al.*, 2000; Sercan *et al.*, 2010; Gallagher *et al.*, 2010), and it is conceivable that they could also influence γ-catenin level and translocation. Wnt1 has been shown in mouse and rat cell lines to mediate such an effect on γ-catenin (Bradley *et al.*, 1993; Papkoff *et al.*, 1996)

and Wnt10B has been shown to increase the growth of haematopoietic precursors in mice through activation of β-catenin-mediated Wnt signalling (Congdon *et al.*, 2008). It is also likely that members of this family are dysregulated in AML (Majeti *et al.*, 2009), and both Wnt1 and Wnt10B as previously mentioned, plus Wnt2B, have been shown by Simon *et al.* to be constitutively active in primary AML patient blasts (Simon *et al.*, 2005). It is possible that these Wnt ligands could be contributing to the translocation of γ-catenin in AML blasts, such as those observed in *Chapter 4*. It was also unsurprising to observe little effect of Wnt5A induction on catenin level and localisation. The action of this Wnt family member has been more closely affiliated with the Ca^{2+}-dependent non-canonical Wnt signalling cascade, and its activity has even been shown to inhibit the canonical pathway in haematopoietic cells (Liang *et al.*, 2003; Nemeth *et al.*, 2007).

Although Wnt3A promoted γ-catenin accumulation and nuclear translocation, a question remains over the Wnt-mediated transcriptional capabilities of γ-catenin independent of β-catenin: *Is the rise and shift in γ-catenin merely a by-product of β-catenin induction by Wnt3A?* Evidence exists showing Wnt3A-mediated TCF/LEF-dependent transcription in F9 cells was dependent on β-catenin, but not γ-catenin (Shimizu *et al.*, 2008). This paper acknowledges that γ-catenin has transcriptional function in F9 cells, as previously evidenced by Fukunaga (Fukunaga *et al.*, 2005), but shows it is insufficient for Wnt3A signal transduction. To fully elucidate this issue in a haematopoietic context, Wnt mediated stimulation of γ-catenin would be required on a β-catenin null background (of which *in vivo* models exist).

Despite demonstrating Wnt glycoproteins can affect the level and localisation of both catenins, this mechanism did *not* explain the differing inherent abilities of THP-1 and U937 cells to nuclear translocate catenin. It was initially postulated that perhaps THP-1 cells, and not U937, secrete an exogenous factor (most likely a Wnt factor) that promotes the para- and/or autocrine mediated nuclear translocation of catenin within these cells. However, incubation of U937 cells with THP-1 conditioned medium exhibited no significant effect on either catenin. Therefore, there is no evidence to support that a soluble factor was responsible for governing nuclear import/export within

these cells. This leaves an internal mechanism responsible for regulating translocation and the many proposed mechanisms have already been discussed in length in *Chapter 3* (*3.5.3*).

5.5.6 The multiple banding of catenin is not due to phosphorylation

Preliminary data from this chapter suggests the cause of multiple catenin banding observed in Western blotting is *not* due to differing states of catenin phosphorylation. Such a theory (initially postulated in the *Chapter 4* discussion, *4.5.4*) might have fitted well given the documented role of phosphorylation in regulating catenin translocation (*3.5.3*), and the presence of only specific higher molecular weight bands in the nuclear fractions of primary AML blast samples and leukaemic cell lines.

The next most likely cause, backed by substantial evidence, is that these multiple bands arise from proteolytic cleavage products. Evidence for such a constitutive process is supported by the observation that the pattern of banding remains the same, and only the intensity changes, when γ-catenin is overexpressed/silenced in leukaemic cell lines (*Figure 5.13*). Such a theory has been formally demonstrated previously for β-catenin in leukaemic cells (Chung *et al.*, 2002; Hwang *et al.*, 2002) and is highly plausible given both γ- and β-catenins association with the ubiquitin-proteasome degradation system (Aberle *et al.*, 1997). Indeed, further studies have identified ubiquitinated bands of γ- and β-catenin using Western blotting (Salomon *et al.*, 1997; Kitagawa *et al.*, 1999; Sadot *et al.*, 2000; Nilkovitch-Miagkova *et al.*, 2001; Song *et al.*, 2003; Yang *et al.*, 2006; Asimaki *et al.*, 2007). However data from Simcha *et al.* would imply that γ- and β-catenin's association with the proteasome is not equivalent given that they observed only a modest increase in γ-catenin protein (relative to β-catenin protein) upon inhibition of the proteasome using MG132 treatment (Simcha *et al.*, 1998). Calpain is also known to cleave β-catenin (Hwang *et al.*, 2002; Li and Iyengar, 2002; Rios-Doria *et al.*, 2004; Benetti *et al.*, 2005). In these studies various calpain inhibitors (growth arrest specific protein 2 (Gas2), calpastatin, calpeptin) or calpain inducers (thapsigargin, ionomycin) were deployed and found to influence β-catenin level and molecular weight. However whether this system is also applicable to γ-catenin, or even operational in

haematopoietic cells, is thus far unknown. Caspases have also been shown to cleave and generate multiple bands of both γ- and β-catenin (Herren *et al.*, 1998; Steinhusen *et al.*, 2000; Ling *et al.*, 2001; Hwang *et al.*, 2002).

If these bands do represent truncated degradation fragments, it evokes the question of *why such forms should be permitted nuclear entry in leukaemic cell lines and primary AML blasts? What possible survival advantage could be conferred to a malignant cell by allowing nuclear import of a truncated form of catenin?* Of course these fragments may represent an inevitable consequence of catenin overexpression whereby saturated or dysregulated degradation and nuclear import/export systems fail to regulate such translocation. Sufficient evidence exists to suggest these smaller fragments are transcriptionally inept (Steinhusen *et al.*, 2000; Jeannet *et al.*, 2008). Studies of Jeannet showed these smaller fragments to be functionally-redundant and inefficient at binding and mediating TCF/LEF-dependent transcription in mice. Some studies have even suggested that the presence of these truncated catenin forms even act in a dominant negative manner to transcription mediated by the full length forms (Munemitsu *et al.*, 1996; Shimizu *et al.*, 2008). For γ-catenin specifically, it is known that truncations of the NH_2-termini are sufficient to stabilise the protein however all such mutants are inferior to the full length in activating TCF (Kolligs *et al.*, 2000). In this same study all large deletions of the NH_2-terminus, COOH-terminus or armadillo repeat domains 3-8 rendered *wt* γ- or β-catenin deficient in binding and activating TCF. Indeed the flanking termini of catenins are known to be crucial for proper function of the molecule (Solanas *et al.*, 2004), yet Zhurinsky *et al.* has shown that removal of them does not prevent the central armadillo domain binding DNA even if transcriptionally inactive complexes with LEF-1 result (Zhurinsky *et al.*, 2000a).

Conversely, it may be plausible that a truncated form of catenin might confer some oncogenic potential from which a malignant cell could benefit. Such a truncation could deprive the molecule of its CK1/GSK3-β consensus site thus inhibiting its phosphorylation-mediated degradation, and leading to constitutive activation. Findings by Rios-Doria *et al.* support this possibility (Rios-Doria *et al.*, 2004). In this study, a

novel 75kDa β-catenin fragment was identified in metastatic primary prostate tumour samples and cell lines, as well as breast cancer cells. This fragment was shown to accumulate in the nucleus where it mediated TCF-dependent transcription and even exhibited transforming potential.

In summary, this chapter has provided some valuable insight as to the functional role of γ-catenin in normal haematopoietic development. Contrary to existing evidence, γ-catenin expression appears to be relevant for normal haematopoietic development and is required for macrophage development. In leukaemic cells, γ-catenin can mediate β-catenin expression and its nuclear translocation which may promote the developmental block observed in differentiation-inducible leukaemic cell lines. γ-Catenin has been shown to be sensitive to an external Wnt stimulus in haematopoietic cells, though this stabilisation/translocation could have arisen indirectly though stabilisation of β-catenin. Finally, it is unlikely that differential phosphorylation of γ-catenin gives rise to multiple-banding in Western blotting.

6 - Final Discussion

Wnt signalling is an evolutionary conserved pathway critical to normal development of blood cells and has been found dysregulated in many haematological malignancies including AML (reviewed by Mikesch *et al.*, 2007). Specifically, aberrant expression/activity of the Wnt cascade central mediator, β-catenin, has been implicated in such cancers (Chung *et al.*, 2002; Serinsoz *et al.*, 2004; Simon *et al.*, 2005; Ysebaert *et al.*, 2006; Xu *et al.*, 2008; Chen *et al.*, 2009; Siapati *et al.*, 2011). Data from this laboratory and others have also identified the close homologue γ-catenin to be dysregulated in primary human AML (Muller-Tidow *et al.*, 2004; Tonks *et al.*, 2007). Furthermore, over-expression of γ-catenin in cell lines and in the human cord blood haematopoietic cell model results in increased self-renewal (Tonks, unpublished data), suggesting this homologue could have a role in AML pathology. The underlying mechanisms of this phenotype remain poorly characterised, and understanding the significance of its dysregulation in AML has been hampered by limited knowledge of γ-catenin's expression and function in normal haematopoiesis.

6.1 γ-Catenin in normal haematopoiesis

The aim of *Chapter 3* was to develop an assay capable of detecting intracellular catenin (by flow cytometry or confocal microscopy) within discrete subsets of normal haematopoietic development. This allowed a comprehensive assessment of γ- and β-catenin expression and subcellular localisation throughout normal human haematopoiesis. The data demonstrated that β-catenin was highly expressed and nuclear-localised in normal human primitive $CD34^+$ haematopoietic cells, supporting the existing evidence that β-catenin mediates self-renewal in these cells (Reya *et al.*, 2003; Baba *et al.*, 2006; Zhao *et al.*, 2007; Holmes *et al.*, 2008; Nemeth *et al.*, 2009). β-Catenin expression in lymphocytes was also confirmed, supporting studies linking active Wnt/β-catenin signalling within these cells (Reya *et al.*, 2000; Pongracz *et al.*, 2006; Dosen *et al.*, 2006). This study also showed that γ-catenin is highly expressed in HSC and has a similar expression profile to that of β-catenin in subsequent developmental subsets (*Figure 3.21* versus *Figure 3.22*). Unlike β-catenin, γ-catenin

was nuclear excluded in this cell type (*Figure 3.23* versus *Figure 3.25*) suggesting independent regulation of nuclear translocation in early haematopoiesis. The degree of structural homology between these molecules and the many shared protein partners, led to the hypothesis that γ-catenin translocation within the cell would also be similar to β-catenin. However, the independent regulation of nuclear localisation suggests that these catenins have independent transcriptional roles in haematopoietic development. Interestingly, this study has also shown that neither catenin has HSC-restricted expression, given their previous association with self-renewal. Expression of both catenins apparently increased in granulocytic and monocytic development and their nuclear localisation would imply a transcriptional role in these differentiated subsets. However independence of function is also indicated by the apparent reciprocal direction of catenin translocation during myeloid development where nuclear β-catenin levels declined whilst nuclear γ-catenin increased. Interestingly, a sub-population of $CD14^+$ monocytes expressing almost entirely nuclear translocated γ-catenin was observed, implying a particular transcriptional relevance for γ-catenin within this subset. This observation may explain the developmental abnormalities obtained upon γ-catenin protein knockdown within this lineage in the *in vitro* model of haematopoiesis (see section *5.4.1* and discussion *6.3*).

6.2 γ-Catenin in AML

Previously, data from this laboratory identified overexpression of γ-catenin mRNA in AML patient blasts by Affymetrix gene expression profiling (Tonks *et al.*, 2007). The AML Affymetrix gene expression profile data is associated with a highly characterised clinical database allowing the analysis and correlation of gene expression with a number of clinical parameters including DFS, OS, CR rate etc. *Chapter 4* of this study correlated γ-catenin mRNA expression with these survival parameters and found a significant association with poor patient outcome (reduced CR rate arising from resistant disease). These findings, despite using mRNA level, are in keeping with the small amount of survival data regarding the prognostic significance β-catenin protein expression in AML. Ysebaert *et al*, identified β-catenin protein expression (by immunoblotting) as an independent prognostic marker predicting poor event free survival (EFS) and shortened OS (Ysebaert *et al.*, 2006). The study of Xu *et al*, also

identified an association of nuclear β-catenin (by immunohistochemistry) with a reduced CR rate (Xu *et al.*, 2008). Although these studies feature small patient cohorts and varied analytical techniques they would generally indicate that catenin expression is unfavourable in AML. This highlights the context-dependent nature of these proteins given that γ-catenin appears to serve a tumour suppressor role in other disease settings (Aberle *et al.*, 1995; Simcha *et al.*, 1996; Charpentier *et al.*, 2000; Polychronopoulou *et al.*, 2002; Breault *et al.*, 2005; Misaki *et al.*, 2005; Shiina *et al.*, 2005). This is likely due to the differing adhesive function γ-catenin serves within an epithelial context, where loss of such a protein and overall tissue integrity could promote metastasis in epithelial tumours.

Catenins are subject to significant post-translational regulation (Zhurinsky *et al.*, 2000b) and therefore the degree of γ-catenin *protein* dysregulation in primary AML cells was also analysed. This study shows for the first time that γ-catenin protein is dysregulated compared with levels in normal HPC. Interestingly, around 20% of AML blast samples overexpress γ-catenin relative to normal HPC, with a similar proportion also demonstrating under expression of the protein. However, γ-catenin mRNA correlated poorly with the level of γ-catenin protein, thus compromising the reliability of the mRNA survival data. This observation was not entirely unexpected given the post-translational mechanisms active in regulating catenin protein stability, and this study is not the first to report such a discrepancy for γ-catenin (see *Chapter 4* discussion, *4.5.1*). Despite this, there is some evidence that higher mRNA expression was associated with higher γ-catenin protein, and a lower CR rate even within the smaller patient cohort. Extending these studies into a larger cohort may provide stronger statistical evidence.

This chapter also investigated the subcellular localisation of γ-catenin protein in AML blasts given that a full localisation profile had been established for normal haematopoietic cells. In undifferentiated (FAB M0/1) leukaemic blasts γ-catenin was found aberrantly localised to the nuclei which is in stark contrast to its nuclear exclusion displayed in developmentally matched normal $CD34^+$ HPC (*Figure 3.23* and *Figure 4.5A*). These observations suggest, again for the first time, that γ-catenin may contribute an aberrant transcriptional function in AML pathology. Nuclear translocation was also prevalent in differentiated M2/4/5 FAB types, however the degree of dysregulation is hard to define given γ-catenin's increasing nuclear presence in normal differentiated

myeloid cells. The specific localisation of γ- and β-catenin protein was found to carry no statistically significant prognostic relevance, although sample number was small for such a heterogeneous disease (<50 patients). Small patient numbers also prevented individual survival analyses of γ- and β-catenin expression within specific developmental subsets (FAB types) of AML. Finally, these data identified a positive correlation between the levels of γ- and β-catenin protein in primary AML blasts. Although γ-catenin has been shown to be capable of influencing β-catenin level in epithelial cell lines (Rubinfeld *et al.*, 1995; Salomon *et al.*, 1997; Miller and Moon, 1997; Simcha *et al.*, 1998; Sadot *et al.*, 2000; Zhurinsky *et al.*, 2000a; Maeda *et al.*, 2004; Li *et al.*, 2007), no such relationship has been demonstrated in primary human tumour samples or haematopoietic cells. These data demonstrate that any association of γ-catenin protein level with clinical outcome in AML may arise indirectly through its stabilisation of β-catenin.

6.3 Functional roles of γ-catenin

As discussed above (see *6.1*), the findings of *Chapter 3* suggested γ-catenin expression has a role in normal haematopoiesis. Further, given that γ-catenin protein and subcellular localisation is dysregulated in AML, it was important to establish whether this had any consequence on haematopoietic development. To examine this hypothesis, γ-catenin protein expression was suppressed in preliminary experiments utilising an *in vitro* model of normal human haematopoietic development. HPC committed to monocytic development ($CD13^{bright}CD36^{+}$) failed to undergo macrophage differentiation and instead developed bi-phenotypic, dysplastic, granulocytic traits (CD15 surface expression, hyper-segmented nuclear morphology within a pale monocytic like cytoplasm). These findings implicated γ-catenin in a potential transcriptional function with respect to lineage fate, potentially mediating the suppression of pro-granulocytic differentiation genes and/or the activation of pro-monocytic genes. Such a role fits with the increased expression of γ-catenin observed during normal development of myeloid cells (*Figure 3.21*), and the increased nuclear localisation observed in granulocytes and monocytes (see *Figure 3.31A*). The potential genes regulated by γ-catenin in this context are unknown, however a few candidates may be shortlisted. For example, CEBPα is a transcription factor crucial for

granulocytic differentiation (Smith *et al.*, 1996; Ford *et al.*, 1996) that is specifically upregulated in the granulocytic lineage whilst downregulated in the monocytic pathway (Radomska *et al.*, 1998). The Wnt/β-catenin signalling pathway has previously been linked with its repression in adipogenesis (Ross *et al.*, 2000; Kawai *et al.*, 2007) and furthermore CEBPα is known to be repressed in RUNX-1-ETO$^+$ AML (Pabst *et al.*, 2001), a context where γ-catenin expression is also high. It is tempting to speculate that the loss of γ-catenin in normal development permits re-expression of CEBPα in the monocytic lineage thus leading to the generation of granulocytic traits. Another target which γ-catenin is known to positively regulate is *myc-T1* which preferentially promotes monocytic over granulocytic colony formation (Tonks, unpublished data). Again, loss of *myc-T1* induction in haematopoietic cells through a lack of γ-catenin may reverse this phenotype so the bias is shifted towards the granulocytic lineage. Both of these genes represent potential candidates to monitor in future γ-catenin knock-down studies.

Evidence from this study (*Chapter 4*) suggested that a correlation exists between the levels of γ- and β-catenin protein in primary human AML blasts. *Chapter 5* sought to investigate whether γ-catenin could possibly be causal in influencing the level and localisation of β-catenin protein in these cells. In normal cells it would appear this is not the case; despite the induction of high levels of ectopic γ-catenin into CD34$^+$ HPC, the protein does not translocate to the nucleus and cannot significantly influence the level or localisation of β-catenin. This finding supports *Chapter 3* data which demonstrated that translocation of catenins are independently regulated during normal haematopoiesis, and accumulation of protein alone does not dictate the level of nuclear translocation. It was also shown that normal human CD34$^+$ HPC transduced with the PINCO-γ-catenin vector was not a suitable model to investigate γ-catenin dysregulation in haematopoietic development, given the inability to replicate the degree of nuclear translocation observed in many primary AML blasts. The failure to translocate γ-catenin to the nucleus may explain the lack of immunophenotypic or morphological phenotype present in the primary haematopoiesis model upon γ-catenin overexpression.

In contrast, ectopic γ-catenin expression in most myeloid leukaemia cell lines results in stabilisation of β-catenin protein (*Figure 5.13*). This difference indicates that the

processes regulating catenin stability are dysfunctional in leukaemic cells. Other studies have previously shown this effect within an epithelial context (Salomon *et al.*, 1997; Zhurinsky *et al.*, 2000a; Maeda *et al.*, 2004; Li *et al.*, 2007), and have suggested that γ-catenin overexpression may saturate the catenin degradation machinery thus allowing the cytoplasmic accumulation of signalling competent β-catenin (Miller and Moon, 1997; Simcha *et al.*, 1998; Klymkowsky *et al.*, 1999). Another difference observed amongst leukaemic cell lines, in contrast to normal cells, is that nuclear translocated γ-catenin promoted the level of nuclear localised β-catenin independently of overall expression level (see *Figure 5.13*). Again, such an observation has been made previously in epithelial settings (Simcha *et al.*, 1998; Maeda *et al.*, 2004; Li *et al.*, 2007) yet the mechanism by which it occurs remains unestablished. Indeed it is not even known whether such an observation represents an enhanced ability to translocate β-catenin or simply retain it within the nucleus. The many proposed mechanisms which regulate catenin nuclear localisation have already been discussed (see *3.5.3*), yet none suggest an ability of γ-catenin to directly nuclear localise β-catenin. Whichever mechanism is responsible, it likely represents a pathological feature of leukaemic cells given the strict and independent regulation of catenins observed in normal blood cells. This relationship could also explain the differentiation block observed in γ-catenin overexpressing THP-1 cells where substantial levels of nuclear β-catenin are indirectly stabilised (*5.4.3*).

Chapter 5 also examined whether γ-catenin expression could be influenced by exogenous soluble Wnt factors such as Wnt3a (as shown in other contexts (Bradley *et al.*, 1993; Papkoff *et al.*, 1996)). Although this study represents the first to report γ-catenin induction by a Wnt signal in haematopoietic cells (Wnt3A, see *Figure 5.20*), it is unable to confirm its independent ability to transduce this signal in the nucleus given the interdependence with β-catenin. It is also unlikely that exogenous Wnt factors are the mechanism responsible for translocating catenin in some cells (e.g. THP-1) and not others (e.g. U937), since the localisation of catenins in U937 cells remains unaffected despite overnight culture in THP-1 conditioned cell medium. The wealth of catenin regulatory molecules that reside inside the cell, probably means a dysregulated internal mechanism is responsible for the aberrant catenin localisation in leukaemic cells. The dysregulated component is likely to constitute either part of the machinery responsible

for catenin degradation (see *1.3.1*) or part of the mechanism directly responsible for catenin localisation (see *3.5.3*).

Finally, *Chapter 5* also aimed to investigate one of the potential causes of the multiple γ-catenin banding observed in Western blotting throughout this project; phosphorylation. Preliminary data from this study would suggest this is not the source leaving proteolysis as the next most likely cause. A number of systems have been linked with the degradation of catenin including the well established ubiquitin/proteasome (Aberle *et al.*, 1997; Orford *et al.*, 1997), calpain (Hwang *et al.*, 2002; Li and Iyengar, 2002; Rios-Doria *et al.*, 2004; Benetti *et al.*, 2005) and caspases (Herren *et al.*, 1998; Steinhusen *et al.*, 2000; Ling *et al.*, 2001). Which of these degradation systems is active in haematopoietic cells is currently unknown, as is the functional relevance of such proteolytic γ-catenin fragments to the cell biology.

6.4 Conclusion

In conclusion, this study has demonstrated that γ-catenin could be important for normal haematopoietic development, where protein level and subcellular localisation is tightly regulated independently of β-catenin. Many mechanisms have been proposed to regulate γ-catenin nuclear localisation, however the exact mechanisms active in haematopoietic cells are not known. Data presented here would suggest they must be defective in AML leaving two-fold consequences for the cell; *firstly*, the overexpression of γ-catenin leading to its nuclear localisation and *secondly*, the ability of dysregulated γ-catenin to influence the level and nuclear localisation of β-catenin. Clearly, this relationship is not the only one by which β-catenin becomes stabilised in AML since evidence exists in the cell line and primary AML blast data, where β-catenin expression is high independently of γ-catenin (and *vice versa*). Rather, the heterogeneity of AML, and the context-dependent nature of these proteins, make it likely that the pre-existing background of molecular defects are highly determinant of γ-catenin's contribution to AML pathology. For instance, THP-1 cells seem to provide a sufficiently aberrant molecular background whereby dysregulated γ-catenin can promote the level and nuclear localisation of β-catenin. Which catenin is directly responsible for imposing the subsequent

differentiation block observed in these cells, remains unknown. Previous evidence would suggest β-catenin is the main transcriptional driver, however a co-operative role can not be excluded given the proven ability of γ-catenin to bind and activate TCF/LEF transcription factors. The evidence for γ-catenin having any independent pathological role in AML is now questionable however given its interdependence with β-catenin level and localisation. Indeed, previous studies implicating γ-catenin in self-renewal of murine/human HPC and induction of AML-like disease *in vivo* all failed to co-assess β-catenin expression. Preliminary data of this study would suggest that γ-catenin could serve important transcriptional roles in normal haematopoiesis by governing lineage fate (between monocyte/macrophage and granulocytic development). Therefore, any attempts to therapeutically target γ-catenin directly in AML must exercise caution. However, even taking this into consideration, targeting the γ-catenin/β-catenin pathological axis could represent a therapeutically viable target since data exists *in vivo* showing normal haematopoiesis can continue in their combined or individual absence.

6.5 Further work

It will be important to firstly substantiate incomplete preliminary data generated from this study. Of particular importance, will be an expansion of the primary AML patient cohort for which γ-catenin and β-catenin protein expression has been assessed. Ideally, analysis of over 100 AML samples should provide more statistical power for identifying any prognostic significance. Further experimental repeats will also be necessary to fully characterise the phenotype of γ-catenin knockdown in normal haematopoietic development using the *in vitro* model of haematopoiesis.

Data from this study led to the hypothesis that a mechanism must exist in normal haematopoietic cells governing the translocation of catenins, which appears to be dysfunctional in AML blasts. The full characterisation of this mechanism will be essential in any attempts to therapeutically remedy it in AML. The use of a biochemical approach such as immuno-precipitation and mass spectrometry could be used to identify potential chaperone proteins, or nuclear import/export systems, active in γ-catenin translocation in healthy and malignant haematopoiesis. A few obvious candidates such

as LEF-1, TCF-4 or APC may associate with γ-catenin, but other novel protein partners cannot be excluded (see *3.5.3*).

The discovery of significant γ-catenin nuclear localisation in leukaemic cells has highlighted the limitations of our group's previous attempts to model γ-catenin in AML by over-expression in normal human $CD34^+$ HPC. This model is incapable of recapitulating the degree of nuclear γ-catenin present in many primary AML blasts or leukaemic cell lines, and ectopic expression fails to significantly modulate the level or localisation of β-catenin as it does within a leukaemic setting. Therefore an alternative model is required encompassing the aforementioned features. It is possible to artificially target γ-catenin to the nucleus using an N-terminally fused NLS (Li *et al.*, 2007). Should such a strategy be viable in haematopoietic cells, then this model would allow the examination of how nuclear γ-catenin translocation affects normal haematopoietic development. Further still, this may permit the identification (using microarrays) of the prospective monocytic and granulocytic genes γ-catenin could transcriptionally govern in late haematopoiesis (see *6.3*). However, before such investigations are performed, it will be critical to confirm the subcellular localisation of β-catenin within this NLS model, before any transcriptional independence can be attributed to γ-catenin.

The co-localisation of γ- and β-catenin in the nucleus makes it impossible to discriminate which protein has primary transcriptional activity in leukaemic cells. Typically, the TOPFLASH reporter (created by Hans Clevers laboratory) is employed in experiments aiming to measure the output of β-catenin mediated Wnt signalling. The TOPFLASH reporter is an expression construct that emits a detectable fluorescence signal when TCF/LEF transcription factors are bound and activated. However, γ-catenin has also been shown to activate this reporter on β-catenin null backgrounds (Conacci-Sorrell *et al.*, 2002; Kim *et al.*, 2011) making it unlikely such a system could discern between γ- or β-catenin mediated transcription in a nucleus where both are abundant. Resolution of this issue may only be possible by assessing γ-catenin transcriptional activity on a β-catenin-null background, however data from this study would suggest such a haematopoietic setting is unlikely to exist. The TOPFLASH reporter could be incorporated into the *in vitro* model of normal human haematopoiesis in order to

confirm whether TCF/LEF activity is elevated in the cell types identified to harbour high levels of nuclear γ-catenin (e.g. monocyte subsets). This may aid in establishing the activity and function of γ-catenin in more differentiated haematopoietic subsets.

Finally, many of the investigations of this study have been tailored toward understanding γ-catenin's transcriptional potential, however its prospective role as a cell adhesion component has been largely neglected. This function is likely to be irrelevant for haematopoietic cells in circulation (except maybe for trans-endothelial migration of monocytes as described in *3.5.1*) but may be more important in the HSC BM niche. Indeed, this study has shown γ-catenin to be nuclear excluded in $CD34^+$ HSC/HPC better suiting a structural role at this level. If γ-catenin does mediate a pro-adhesive or migratory effect in the BM niche then such a phenotype would be desirable for both normal LT-HSCs propagating healthy haematopoiesis, and LICs establishing a malignant clone and evading chemotherapy. The interaction with cadherin might also be worth investigation, not only for its association with cell adhesion, but also in the documented ability of this molecule to sequester γ-catenin from the nucleus (Fukunaga *et al.*, 2005). *Could this mechanism explain the nuclear exclusion of γ-catenin protein in primitive $CD34^+$ cells?* Future studies incorporating an inducible model of E- or N-cadherin in human $CD34^+$ HSC will be useful to assess the affect on adhesion, migration and catenin re-localisation.

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Appendices

Appendix 1

Purification of DNA from gel using a QIAquick™ gel extraction kit

Gel slices were weighed in colourless tubes and 3 volumes of 'Buffer QG' added for every 1 volume of gel. The gel slices were completely dissolved by incubating at 50°C for 10 min and vortexing every 2–3 min to promote solubilisation. Upon full dissolution of the gel slices, the colour of the mixtures was inspected to ensure a yellow colour, indicating an optimal pH (≤ 7.5) for adsorption of DNA onto the QIAquick™ membrane. To increase the yield of DNA fragments, one gels volume of isopropanol was added to the samples and mixed. The mixtures were applied to QIAquick™ spin columns and centrifuged in a microcentrifuge at 17,900 x *g* for 1 minute to bind DNA. At the end of centrifugation, flow through was discarded and 0.5ml of 'Buffer QG' applied, before centrifugation for another minute. The waste was discarded and the columns washed by the addition of 0.75 ml of 'Buffer PE' and two 1 minute centrifugations at 17,900 x *g*. The QIAquick™ columns were transferred to clean 1.5ml microcentrifuge tubes, and the respective DNAs eluted from the membranes by the direct addition of 50μl Buffer EB (10mM TrisCl, pH 8.5) and centrifugation as above.

Appendix 2

Purification of Plasmid DNA using a QIAprep® Miniprep kit

Pelleted *E.coli* cells were resuspended in 250μl 'Buffer P1' and mixed with 250μl of 'Buffer P2' by tube inversion. Then, followed the addition of 350μl 'Buffer N3' and centrifugation at 17,900 x *g* for 10 minutes. Supernatants were transferred to QIAprep spin columns which were centrifuged as above for 1 minute with the resulting eluent being discarded. After washing of the spin columns by sequential addition of 500μl 'Buffer PB' and 750μl 'Buffer PE' each followed by centrifugation as above, the

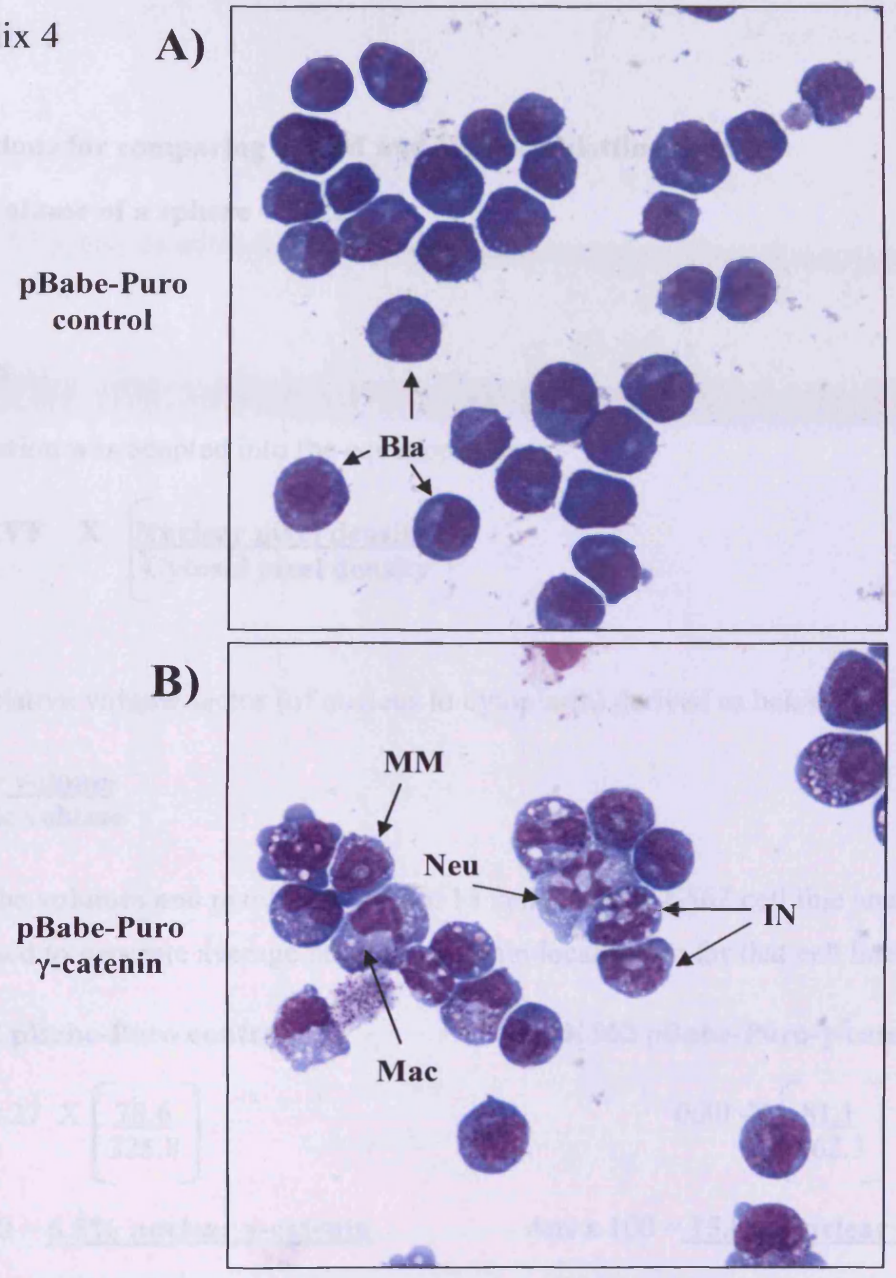
columns were placed in fresh microcentrifuge tubes. Respective DNA was eluted from columns by addition of 50μl 'Buffer EB' and a 1 minute RT incubation followed by a 1 minute centrifugation as above. The eluted DNA from each colony was quantified using a NanoDrop® to establish if DNA (and to which concentration) had been successfully purified.

Appendix 3

Purification of Plasmid DNA using a QIAprep® Maxiprep kit

Briefly, pelleted bacteria were resuspended in 10ml 'Buffer P1' before mixing with 10ml of 'Buffer P2', and allowing to stand for 5 minutes at RT. Next, 10ml 'Buffer P3' was added followed by incubation on ice for 15 minutes, and a 30 minute centrifugation at 20,000 x *g* at 4°C. Supernatant containing plasmid DNA was removed and centrifuged again, as above, to remove insoluble material. During this time a 'QIAGEN-tip 500' was equilibrated with 10ml 'Buffer QBT'. The supernatant was transferred to the 'QIAGEN-tip' where plasmid DNA was bound to the column and washed twice with 30ml 'Buffer QC'. DNA was eluted from the column using 15ml 'Buffer QF' and precipitated by the addition of 10.5ml RT isopropanol (Fisher) and centrifugation at 15,000 x *g* for 30 minutes (4°C). The DNA pellet was finally washed in 5 ml 70% ethanol before final centrifugation at 15,000 x *g* for 10 min. The supernatant was decanted with care, and the pellet air-dried for 10 minutes before resuspending in 1ml 'TE Buffer'. Plasmid DNA was then quantified using the NanoDrop® and stored at -20°C.

Appendix 4



The effect of γ-catenin overexpression on FDCP mix cells - The murine haematopoietic progenitor cell line FDCP mix was retrovirally transduced with γ-catenin or control cDNA and placed into standard culture medium containing no exogenous growth factors following puromycin selection. **A)** Control cells maintained a normal FDCP mix morphology throughout culture dominated by dark-staining, undifferentiated blasts (Bla). **B)** Conversely, γ-catenin overexpressing cells underwent spontaneous myeloid differentiation around 14 days post transduction. These cultures displayed a heterogeneous mix of differentiated myeloid cells including metamyelocytes (MM), macrophages (Mac), immature neutrophils (IN) and mature neutrophils (Neu).

Appendix 5

Calculations for comparing CLSM and Western blotting data.

- **Volume of a sphere = $4/3\pi r^3$**

Where;

r = radius

This equation was adapted into the equation below:

- **RVF X** $\left[\frac{\text{Nuclear pixel density}}{\text{Cytosol pixel density}}\right]$

Where;

RVF = relative volume factor (of nucleus to cytoplasm) derived as below:

$$\frac{\text{Nuclear volume}}{\text{Cytosolic volume}}$$

- The volumes and pixel values from 13 cells of each K562 cell line analysis were used to generate average nuclear γ-catenin localisation for that cell line:

K562 pBabe-Puro control

$$0.27 \text{ X } \left[\frac{78.6}{325.8}\right]$$

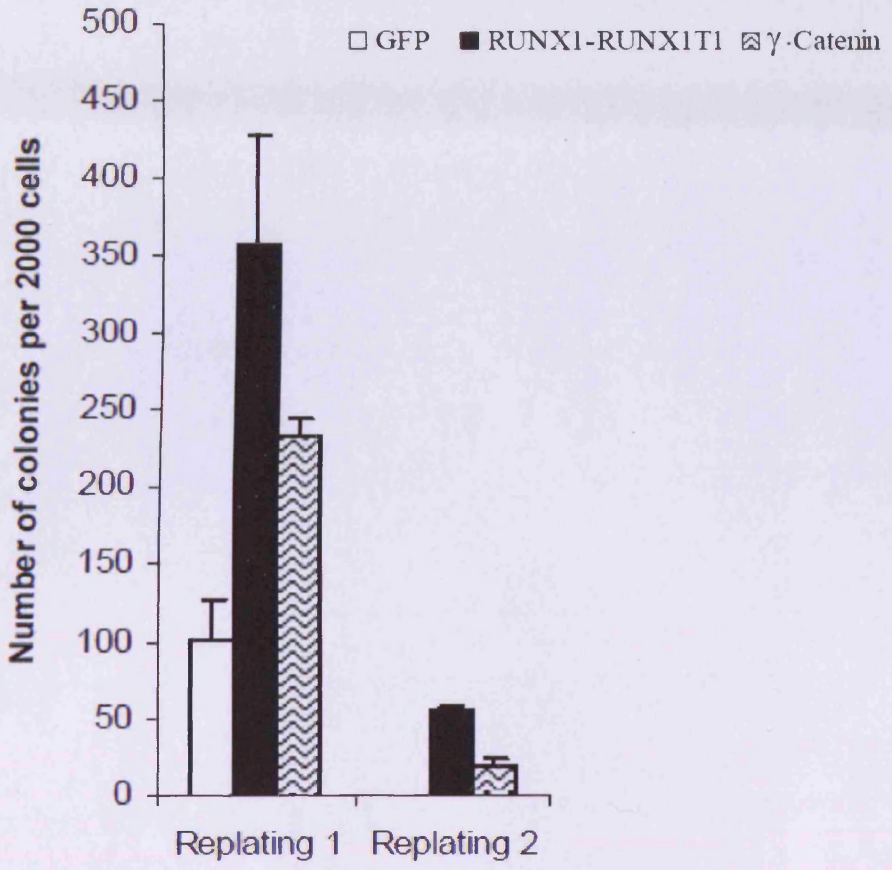
Ans x 100 = **6.5% nuclear γ-catenin**

K562 pBabe-Puro-γ-catenin

$$0.30 \text{ X } \left[\frac{81.1}{162.3}\right]$$

Ans x 100 = **15.0% nuclear γ-catenin**

Appendix 6



γ-Catenin enhances the self-renewal of CD34$^+$ haematopoietic progenitors - Cord-blood derived CD34$^+$ haematopoietic progenitors retrovirally transduced with γ-catenin (and RUNX-1/ETO (aka RUNX-1/RUNX-1T1)) displayed enhanced self-renewal capacity, compared with mock-transduced GFP$^+$ cells, as demonstrated by increased replating efficiency of myeloid colony-forming cells (Tonks *et al.* unpublished data).