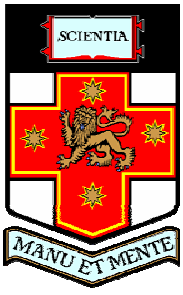
Molecular Regulation and Enhancement of Megakaryopoiesis and Thrombopoiesis by the p45 Subunit of NF-E2

Ee-ling Fock

A thesis submitted in fulfilment
of the requirements for the degree of
Doctor of Philosophy

Department of Medicine
St George Clinical School
University of New South Wales

UNSW MEDICINE
School of Medical Sciences



February 2008

THE UNIVERSITY OF NEW SOUTH WALES
Thesis/Dissertation Sheet

Surname or Family name: FOCK

First name: EE-LING Other name/s: N/A

Abbreviation for degree as given in the University calendar: PhD

School: St George Clinical School, Dept. of Medicine Faculty: Medicine

Title: Molecular Regulation and Enhancement of Megakaryopoiesis and Thrombopoiesis by the p45 Subunit of NF-E2.

Abstract 350 words maximum:

Megakaryocytes (MKs) are a rare population of haematopoietic cells, which produce platelets. Platelet production is a complex process that is tightly regulated at the transcriptional level by lineage specific transcription factors such as p45 NF-E2. Understanding how transcriptional regulators operate is imperative to advance our knowledge of disease pathophysiology and to propose novel treatment options. Therefore, the aims of this study were to: i) study the effects of p45 NF-E2 overexpression on various stages of megakaryopoiesis; (ii) elucidate the nuclear transport mechanisms of p45 NF-E2; and iii) determine the impact of a p45 NF-E2 modification called SUMOylation on thrombopoiesis.

Exogenous p45 NF-E2 was overexpressed in haematopoietic cells in culture and various aspects of megakaryopoiesis were examined. Overexpression of p45 NF-E2 enhanced multiple stages of MK differentiation such as colony forming unit (CFU)-MK formation and terminal MK maturation. Most importantly, p45 NF-E2 overexpression resulted in significant increases in proplatelet and functional platelet production *in vitro*. This latter result was confirmed *in vivo* using lethally irradiated mice transplanted with cells that overexpressed p45 NF-E2. Unexpectedly, the enhancement of MK differentiation was at the expense of myeloid development and, for the first time, identified p45 NF-E2 as a negative regulator of myeloid differentiation.

Secondly, we determined the nuclear localisation signal of p45-NF-E2 and the pathway responsible for nuclear import. We also investigated the importance of p45 NF-E2 nuclear import in thrombopoiesis. Finally, we showed that p45 NF-E2 is modified mainly by SUMO-2/3 in bone marrow cells and this process is involved in the transcriptional activation of MK-specific genes and platelet release.

Taken together, these results suggest that enforced expression of p45 NF-E2 selectively enhances many aspects of MK differentiation including early and terminal MK maturation, proplatelet formation and platelet release. Equally important, this thesis also indicates that white blood cell differentiation may be inhibited by p45 overexpression, while molecular processes such as the nuclear import and SUMOylation of p45 NF-E2 are vital for thrombopoiesis. These observations will facilitate subsequent studies into the feasibility of manipulating p45 NF-E2 protein levels for the treatment of conditions such as thrombocytopaenia and other platelet disorders.

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ABSTRACT

Megakaryocytes (MKs) are a rare population of haematopoietic cells, which produce platelets. Platelet production is a complex process that is tightly regulated at the transcriptional level by lineage specific transcription factors such as p45 NF-E2. Understanding how transcriptional regulators operate is imperative to advance our knowledge of disease pathophysiology and to propose novel treatment options. Therefore, the aims of this study were to: i) study the effects of p45 NF-E2 overexpression on various stages of megakaryopoiesis; (ii) elucidate the nuclear transport mechanisms of p45 NF-E2; and iii) determine the impact of a p45 NF-E2 modification called SUMOylation on thrombopoiesis.

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ACKNOWLEDGEMENTS

Firstly, I would like to extend my gratitude to my supervisor Prof. Chong for his support and guidance throughout the course of my PhD. I also appreciate the level of trust and freedom he has kindly given me over the past few years.

My sincere gratitude also goes to my co-supervisor, Dr Feng Yan, who has invested so much of his own time, knowledge and patience in guiding me through every aspect of my thesis. Thank you for teaching me so much and for always doing it with a smile. You have played an invaluable role in making all this happen. I would also like to make special mention of Dr José Perdomo. Thank you for helping me with not only my GST pull-downs, but also in every area of molecular biology. I respect you for your intelligence and passion for science. You somehow always have an answer for everything and I have great respect for you as a scientist.

To every single person in the lab I have had the privilege of working with…. A BIG THANK YOU! In particular, my thanks goes to Dr Shu Pan (Shuppy) for taking me under her wing in the early days and for being like a sister to me; to Dr Alana Philips (Lala) for always making me laugh and for always helping me in those moments of desperation. We made it! To Dr Juliana Kwok (Kwoky), a big thank you for all the helpful discussions and advice. You have been an inspiring person to work with. Thanks also to Daniel Carter and MJ for all the nights you helped to take out my PCR tubes or eppendorfs from the incubator. Dr Charles de Bock - without you, NF-E2 would never have been cloned in this lab. Most importantly, thank you all for your dear friendships.

To my family- Eric, Dad, Mum and Jie Jie, I could not have done this without your endless support, prayers and unconditional love. I have felt your presence each step of the way. I love you dearly and this thesis is dedicated to you all. Last but not least, to my Heavenly Father who is able to do immeasurably more than I could ever ask or imagine, to Him be all glory given!

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LIST OF ABBREVIATIONS

A	alanine
aa	amino acid
ACD	acid citrate-dextrose
AchE	acetylcholinesterase
ADP	adenosine diphosphate
AML	acute megakaryoblastic leukaemia
AMP	ampicillin
APC	allophycocyanin
ATP	adenosine triphosphate
B	B lymphocytes
BFU-MK	burst-forming unit-MK
BM	bone marrow
BMC	bone marrow cell
βME	β-mercaptoethanol
bp, kb	base pairs, kilobase pairs
BSA	bovine serum albumin
bZip	basic region-leucine zipper
°C	degrees Celsius
CBF	core binding factor
CBP	CREB binding protein
C/EBP	CAAT/enhancer binding protein
CFU-MK	colony-forming unit-MK
Ci	curie (3.7×10^{10} disintegrations/ sec)
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CNC	cap 'n collar
CO_2	carbon dioxide
cpm	counts per minute
CRM1	chromosome region maintenance 1
CSF	colony stimulating factor

ddH_2O	deionised and distilled water
DEPC	diethyl pyrocarbonate
DMEM	dulbecco's modified eagle's medium
DMS	demarcation membrane system
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
E	glutamic acid
E.coli	*escherichia coli*
EDTA	ethylene diamine tetra-acetic acid (disodium)
eg.	example
eGFP	enhanced green fluorescent protein
EMSA	electromobility shift assay
Eo	eosinophil
EPO	erythropoietin
ET	essential thrombocytopaenia
Ets	E twenty six
EXP	exportin
FACS	fluorescent activated cell sorting
FCS	foetal calf serum
FL	full length
Flt3 L	Flt-3 ligand
FOG	Friend of GATA
g	acceleration due to gravity (9.8 $msec^{-2}$)
G	granulocyte
G418	geneticin
GAPDH	glyceraldehyde-3-phophate dehydrogenase
G-CSF	granulocyte colony stimulating factor
GM	granulocyte-macrophage
GP	glycoprotein
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

HLA-DR	human leukocyte antigen DR
HPC	haematopoietic progenitor cell
HPP-CFC	high-proliferative-potential colony forming cell
HSC	haematopoietic stem cell
HSF	heat shock factor
IL	interleukin
IMP α	importin α
IMP β	importin β
IPTG	isopropyl-β-D-thiogalactopyranosid
IRES	internal ribosomal entry site
K	lysine
KAN	kanamycin
kDa	kilo daltons
L	leucine
LB	luria bertani
LIF	leukaemia inhibitory factor
Lin^-	lineage depleted (negative)
Luc	luciferase
M	macrophage
mA	milliamperes
MACS	magnetic activated cell sorting
MEP	megakaryocyte-erythroid progenitor
min	minutes
MK	megakaryocyte
mRNA	messenger RNA
MTT	3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide
μCi	microcurie
μL, mL, L	microlitre, millilitre, litre
μM, mM, M	micromolar, millimolar, molar
NA	NF-E2/AP1
NES	nuclear export signal
NF-E2	nuclear factor-erythroid 2

NK	natural killer cells
Nrf2	NF-E2-related factor 2
NLS	nuclear localisation signal
NP-40	igepal CA-630
NPC	nuclear pore complex
Nup	nucleoporin
OD-600	optical density at 600 nm
OSM	oncostatin
PAGE	polyacrylamide gel electrophoresis
PAR4	proteinase-activated receptor 4
PBS	phosphate buffered saline
PBS-T	PBS/ 0.05% Tween™-20
PBGD	porphobilinogen deaminase PBGD
PCR	polymerase chain reaction
PE	R-phycoerythrin
PGE1	prostaglandin E1
PMkB	promegakaryoblast
PMSF	phenylmethylsulfonylfluoride
PML	promyelocytic leukaemia protein
POD	PML oncogenic domain
PV	polycythaemia vera
R	arginine
RBC	red blood cell
RNA	ribonucleic acid
RNAPII	RNA polymerase II
rpm	revolutions per minute
RT	reverse transcription
Sca-1	stem cell antigen-1
SCF	stem cell factor
SDS	sodium dodecyl sulfate
siRNA	small interfering RNA molecules
SUMO	small ubiquitin-related modifier

T	T lymphocytes
TAE	tris-acetate-EDTA buffer
TE	tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TF	transcription factor
TPO	thrombopoietin
Tris	[2-amino-2-hydroxy-(hydroxymethyl)-propane-1, diol, (tris)]
TXS	thromboxane synthase
U	unit
ULP	ubiquitin-like protein
U.V.	ultra violet
V	volts
v/v, w/v	volume per volume, weight per volume
vWf	von willebrand factor
WBC	white blood cell
WT	wildtype

LIST OF PUBLICATIONS AND AWARDS

PUBLICATIONS ARISING FROM THIS THESIS

Fock E.-L., Yan F., Pan S., Chong B.H. (2008) NF-E2 Mediated Enhancement of Megakaryocytic Differentiation and Platelet Production *In Vitro* and *In Vivo*. *Exp Hematol* 36 (1): 78-92

Fock E.-L., Perdomo J., Chong B.H. (2008) Characterisation of the Nuclear Localisation Signals of NF-E2 and its Role in Thrombopoiesis. Manuscript in preparation for submission to *J Biol Chem*.

Fock E.-L., Perdomo J., Chong B.H. (2008) p45 NF-E2 SUMOylation and its Essential Role in Thrombopoiesis. Manuscript in preparation for submission to *J Biol Chem*.

CONFERENCE PRESENTATIONS

St George Hospital Medical Symposium 2005- St George Hospital, Kogarah, NSW. Abstract accepted for poster presentation: *Proplatelet Production Enhanced by NF-E2 Overexpression.*

Australian Society of Medical Research (ASMR) Annual Scientific Meeting 2006- Kerry Packer Education Centre, Royal Prince Alfred Hospital, Sydney, NSW. Abstract accepted for oral presentation: *Enhancing Platelet Production by the Overexpression of NF-E2 in Haematopoietic Cells.* (Oral Prize)

International Society for Cellular Therapy Annual Meeting 2006- Conference Centre, Berlin, Germany. Abstract accepted for poster presentation: *Enhancing Platelet Production by NF-E2 Overexpression in Haematopoietic Cells.*

International Society for Stem Cell Research Annual Meeting 2006- Toronto Convention Centre, Toronto, Canada. Abstract accepted for poster presentation: *Enhancing Platelet Production by Overexpression of NF-E2 in Haematopoietic Cells.*

Combio2006- Brisbane Convention Centre, Brisbane, QLD. Abstract accepted for poster presentation: *Enhancing Platelet Production by Overexpression of NF-E2 in Haematopoietic Cells.*

UNSW Faculty of Medicine Research Day: Merck Sharp and Dohme Poster Competition 2006- UNSW, Sydney, NSW. *Enhancing Platelet Production by Overexpression of NF-E2 in Haematopoietic Cells.* (Poster Prize)

St George Hospital Medical Symposium 2006- St George Hospital, Kogarah, NSW. Abstract accepted for oral presentation: *Enhancing Platelet Production by the Overexpression of NF-E2 in Haematopoietic Cells.*

International Society of Haematology and Stem Cells (ISEH) annual meeting 2007- Congress Centre, Hamburg, Germany. Abstract accepted for oral presentation: *NF-E2 Mediated Enhancement of Megakaryocytic Differentiation and Platelet Production.* (Travel Award)

St George Hospital Medical Symposium 2007- St George Hospital, Kogarah, NSW. Abstract accepted for oral presentation: *NF-E2 Mediated Enhancement of Megakaryocytic Differentiation and Platelet Production.* (Oral Prize)

AWARDS ARISING FROM THIS THESIS

Anthony Rothe Memorial Trust PhD Scholarship- Scholarship of $33,000 PA, 2005-2008. Awarded on the basis of academic merit.

Australian Society for Medical Research (ASMR) Elli Lilly Student Oral Prize ($500) - for best student oral presentation presented at ASMR's Annual Scientific Meeting, Kerry Packer Education Centre, Royal Prince Alfred Hospital, Sydney, NSW, 2006.

Merck Sharpe and Dohme Poster Prize ($1000) - for best poster presentation at UNSW Faculty of Medicine Research Day Poster Competition, UNSW, Sydney, 2006.

ISEH Travel Award ($1000) – Awarded on the basis of scientific merit to present at the ISEH 36th annual scientific meeting, Hamburg Congress Centre, Hamburg, Germany, 2007.

Young Investigator Award ($1000)-for best basic science oral presentation at the St George Hospital Annual Medical Symposium, Sydney, NSW, 2007.

CHAPTER 1

INTRODUCTION

HAEMATOPOIESIS

At any given moment in time, only a tiny portion of a person's stem cell pool begins to differentiate along one of eight major lineages of haematopoietic development. This elegant process that ultimately gives rise to the wide diversity of mature cells of the haematopoietic system, is referred to as haematopoiesis and the pluripotent stem cells involved, haematopoietic stem cells (HSCs). Haematopoiesis is a complex and dynamic developmental process. Quantitative or qualitative abnormalities of any blood cell type lead in a predictable manner to human disease.

HSCs are primitive cells, which are characterised by their extensive self-renewing potential and their capability to give rise to all haematopoietic cell lineages (Figure 1.1). Their quiescent nature and extensive proliferative capacity ensures the existence of an adequate pool of sustaining HSCs throughout the life-span of an individual. In an adult, the production of approximately one trillion different blood cells occurs daily to ensure normal bodily functions (Cantor and Orkin, 2001). HSCs were first purified from mice as a tiny subpopulation of cells that could completely reconstitute the haematopoietic systems of other mice, whose own marrows had been destroyed by inherited mutations or by radiation (Cheshier et al., 1999). Diverse results from numerous experimental systems also indicate that the proliferative and self-renewal capacity of a given HSC decreases progressively with differentiation (Zhu and Emerson, 2002).

Various disease states such as leukaemia and thrombocytopaenia often arise as a result of injury to the bone marrow or defects in blood cell development. A better understanding of the mechanisms and genetic hierarchies involved in haematopoiesis will provide novel insights into disease pathogenesis and potential treatment strategies.

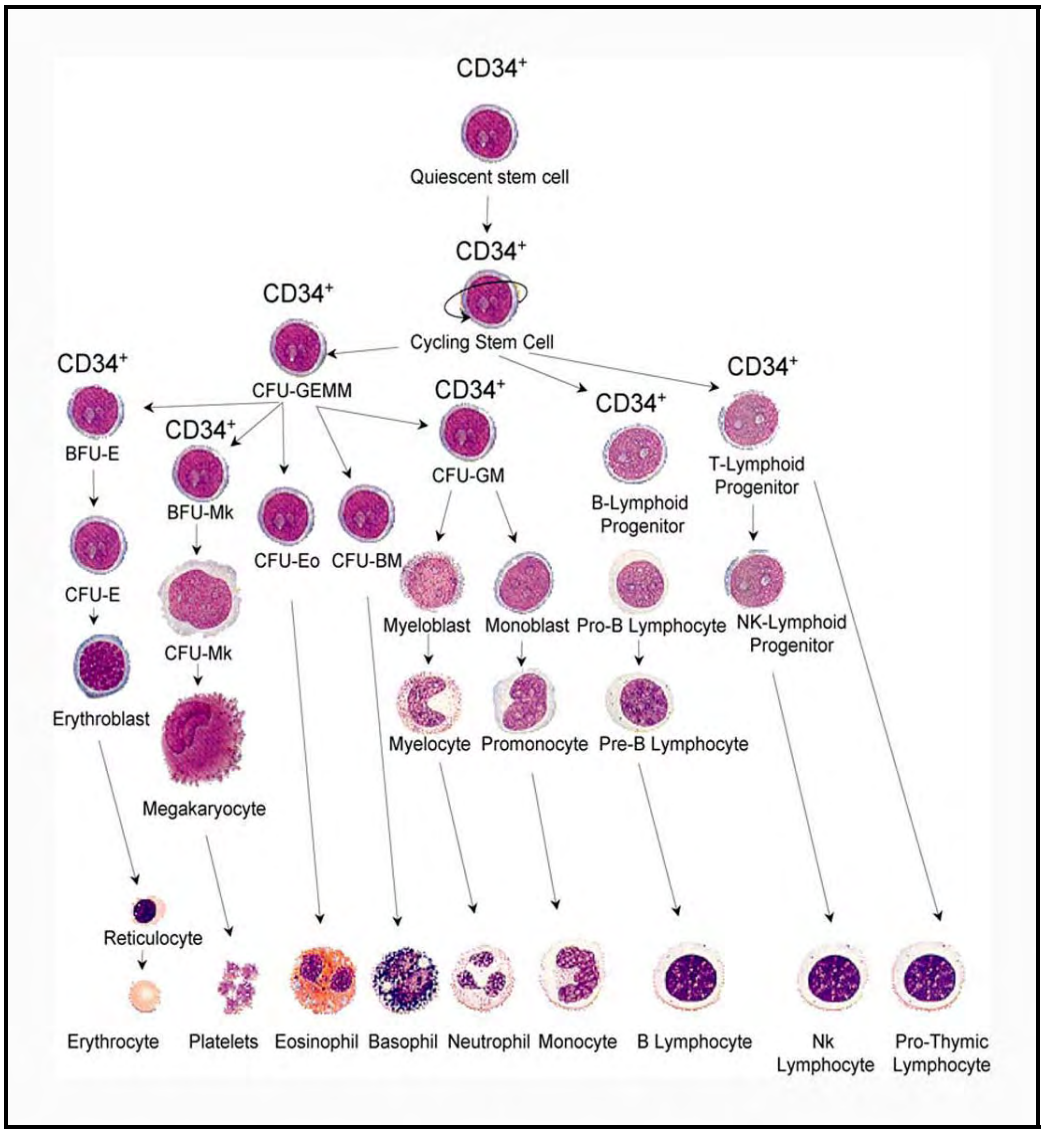


Figure 1.1 Haematopoiesis in humans. Mature cells originate from pluripotent $CD34^+$ HSCs, characterised by its extensive self-renewing capacity and its capability to give rise to all haematopoietic cell lineages as shown.

MEGAKARYOPOIESIS AND THROMBOPOIESIS

Megakayrocytes and Platelet Biology

Megakaryocytes (MKs) are large multi-nucleated cells that serve the essential function of producing platelets, which are a critical component of haemostasis. During its life-span, a mature MK can produce up to 10^4 platelets (Long, 1998). Each day the human adult produces 1 x 10^{11} platelets and this number can increase tenfold with demand (Branehog et al., 1975). Despite the biological importance of platelets in both health and disease, remarkably little is known about the molecular mechanisms involved in MK biology. This is largely due to the minimal representation (<0.5%) of MKs within the human bone marrow, which has hindered the procurement of purified primary cell populations (Murphy and Leavitt, 1999). However, since the improvement of *in vitro* systems, the cloning of thrombopoietin (TPO) and the advent of stem cell technologies, the quality of MK cultures has improved substantially (Wendling et al., 1994). Platelet production has even been observed in such cultures (Cramer et al., 1997; Fujimoto et al., 2003).

MKs possess many unique attributes. Firstly, they undergo endomitosis, a process involving successive nuclear replications without cytoplasmic divisions. The resulting MKs contain 1 to 32 nuclei (2N-64N nuclear content). MKs are distinctly large cells that can easily be distinguished on low magnification, with a size of up to 90 μm in diameter (Rozenberg, 1997). Mature MKs also assemble a unique set of organelles, including alpha granules, dense bodies, and an extensive demarcation membrane system, which eventually gives rise to platelets (Zauli and Catani, 1995).

Platelets are brought about from the fragmentation of the mature MK cytoplasm and are essential for haemostasis and thrombosis. When a blood vessel is damaged, platelets adhere to the endothelium together with other clotting factors to form a haemostatic plug that closes the vessel wall. Several events take place during this regulated process, including adhesion, activation and aggregation. In addition, platelets perform important roles in wound healing, inflammation, and tumour metastasis (Szalai et al., 2006). Deviant platelet levels can therefore lead to pathological situations such as thrombocytopaenia (too few platelets), which leads to bleeding with minimal trauma,

and thrombocytosis (too many platelets) that increases the risk of stroke, peripheral ischemia and myocardial infarction (Patel et al., 2005; Feinbloom and Bauer, 2005; Kaluzhny and Ravid, 2004). Abnormal MK development can also lead to deregulated platelet number and functionally defective platelets, as seen in myelodysplastic syndrome, myeloproliferative disorders and Bernard Soulier Syndrome (White Ii, 2006). Indeed, the hope of developing novel approaches to treat many of these disorders is driving much of the current research into MK and platelet biology.

From Megakaryopoiesis to Thrombopoiesis

The processes leading to MK maturation (megakaryopoiesis) and platelet production (thrombopoiesis) are complex and best understood when artificially divided into 3 main developmental stages (Figure 1.2). Each stage is characterised by varying degrees of proliferation, cell morphology and MK-specific cell surface marker expression. Using *in vitro* studies, the main cell types observed to be involved at each respective stage include progenitor cells, immature promegakaryoblasts (PMkB), and mature MKs respectively.

Progenitor Cells

MK progenitor cells proliferate in response to a number of mitotic signals and can be cultured at low frequency from the bone marrow *in vitro*. They progressively lose proliferative potential and consist of a heterogeneous population of cells in increasing differentiation hierarchy: MK high-proliferative-potential colony forming cells (MK-HPP-CFCs), burst-forming unit-MKs (BFU-MKs) and single colony-forming-unit MKs (CFU-MKs). As its name suggests, the most primitive progenitor cell in this lineage, MK-HPP-CFC is capable of proliferating into colonies of a few thousand MKs and is the first cell type in this lineage to be detected macroscopically *in vitro* (Long, 1989); (Bruno et al., 1989). Like all other haematopoietic progenitor cells, MK-HPP-CFCs express the pan-haematopoietic cell antigen, CD34 (Strauss et al., 1986), but lack the human leukocyte antigen DR (HLA-DR), which tends to characterise a more mature subset of haematopoietic progenitor cells (Long, 1998).

BFU-MKs are slightly more mature than the MK-HPP-CFCs, but they still retain their high proliferating capacity, developing multiple “bursts” or clusters of individual MKs

and generating approximately 100-500 MKs (Long, 1998). *In vitro*, BFU-MK colonies morphologically resemble the erythroid burst-forming cell and are resistant to 5-fluorouracil (Briddell and Hoffman, 1990; Briddell et al., 1989). Similar to MK-HPP-CFCs, BFU-MKs are also $CD34^+$/ HLA-DR^-. In addition, they are the immediate ancestors of CFU-MKs, which are the most differentiated of the MK progenitor cells. A CFU-MK has restricted proliferative capacity and can generate only 4-32 MKs (Williams and Jackson, 1982). These cells are both $CD34^+$/ HLA-DR^+ and express elevated levels of CD61, CD41 (glycoprotein (GP) GPIIb/IIIa complex) and GPVI (Lagrue-Lak-Hal et al., 2001).

Promegakaryoblasts

The second main developmental stage involves promegakaryoblasts (PMkB), which are transitional cells that bridge between proliferating progenitor cells and post-mitotic mature MKs (Long, 1998). They have restricted proliferative potential, but still continue to acquire an increased DNA content via endomitosis. Presumably, endomitosis facilitates the increased cell mass required for the production of platelets and this occurs via complex regulatory mechanisms during anaphase, which results in the abortion of cytokinesis (Matsumura and Kanakura, 2002; Italiano Jr and Shivdasani, 2003). PMkBs respond quickly to thrombopoietic demand and are the first to increase or decrease during thrombocytopaenia and thrombocytosis respectively (Long and Henry, 1979). Although these transitional cells are not easily observed *in vitro* or in bone marrow specimens, they can be identified by their loss of CD34 expression and increasing platelet factor-4 (PF-4), GPIIb/IIIa and von Willebrand factor (vWf) expression (Rabellino et al., 1981; Debili et al., 1992).

Mature MKs

Morphologically, 3-4 stages of mature MKs have been identified. The earliest recognisable cell is the megakaryoblast, which is still relatively small, but has a high nuclear to cytoplasm ratio as well as scanty, basophilic cytoplasm that reflects the large amount of protein synthesis occurring in these cells (Zauli and Catani, 1995). The second stage cell is the pro-megakaryocyte, which shows increases in both its nuclear and cytoplasmic volume and the number of platelet-specific granules (Long, 1998). Finally, there is the granular or 'platelet-shedding' MK that has the most abundant

mature cytoplasm and a large lobulated nucleus. By this stage, mature MKs express little if any CD34, but they do express other platelet related markers such as platelet factor 4, CD42a/b, thrombospondin, and thrombomodulin. There may however be considerable antigenic heterogeneity in expression levels of certain markers (Long, 1998). In general, the most widely used markers for examining MK differentiation are CD61, CD41 and CD42 (Figure 1.2). Thus, $CD34^{+}CD41^{+}CD42^{+}$ and $CD34^{-}CD41^{+}CD42^{+}$ cells represent more mature human MKs, while $CD34^{+}CD41^{+}CD42^{-}$ cells characterise intermediately mature MKs.

It is now universally accepted that the end point of MK development is the release of platelets into the blood circulation. During maturation, platelet-specific proteins and organelles, such as α-granules and dense granules are acquired along with the development of a tubular network known as the demarcation membrane system (DMS) (Italiano Jr and Shivdasani, 2003; Long, 1998). Once thought to define 'platelet territories' within the MK cytoplasm, the DMS has instead been found to function as a membrane store permitting the extension of proplatelets into the bone marrow sinuses before platelets are released into the bloodstream (Zauli and Catani, 1995; Italiano Jr and Shivdasani, 2003). Following platelet release, MKs undergo apoptosis within the bone marrow and are phagocytosed by macrophages (Zauli et al., 1997). This apoptotic process of platelet release is facilitated by apoptotic effectors such as pro-caspase-3, -9 and Bcl-2 (De Botton et al., 2002; Ogilvy et al., 1999).

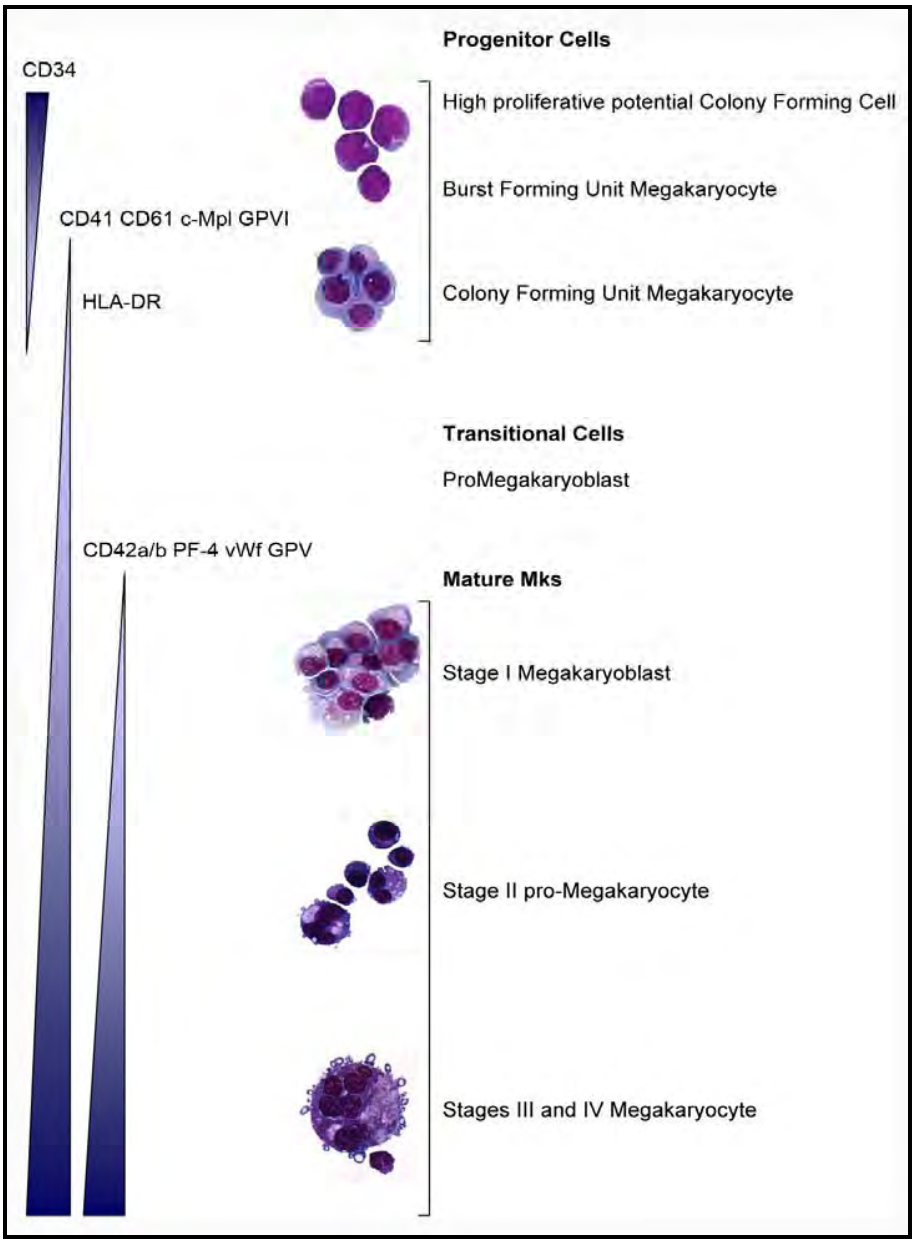


Figure 1.2 Stages of MK development. Each stage of MK differentiation is characterised by varying degrees of proliferation, cell morphology and MK-specific cell surface marker expression. CD34 is gradually lost from the progenitor cells while early MK markers such as CD41, CD61, c-Mpl and GPVI expression increases in the later progenitor cell stages. Beginning at the transitional stage, CD42a/b, vWf, PF-4 and GPV expression increases as MK maturation progresses into the terminal stages.

Platelet Release

The relative scarceness of MKs in normal bone marrow contrasts with the abundance of platelets in peripheral blood. This implies that the processes of platelet assembly and release must be exceedingly efficient and dynamic. The established model of thrombopoiesis involves the extrusion of long beaded cytoplasmic processes, termed proplatelets, from differentiated MKs (Becker and De Bruyn, 1976; Radley and Scurfield, 1980). These pseudopodial proplatelet extensions (Figure 1.3) constitute the essential intermediate structures in platelet release and have been observed to extend into bone marrow sinusoids (Lichtman et al., 1977; Tavassoli and Aoki, 1989). Proplatelets have been recognised in various mammalian species (Tablin et al., 1990; Miyazaki et al., 1992) and their absence correlates with thrombocytopaenia in genetic murine models (Lecine et al., 1998a; Onodera et al., 2000).

However, a criticism of this proplatelet phenomenon is that it has largely only been recognised and understood *in vitro* in the absence of cellular contacts or signals and frequently only after non-physiological manipulations (Choi et al., 1995b; Cramer et al., 1997; Lecine et al., 1998a). In culture, transformation of the MK cytoplasm begins with the erosion at one pole of the cell and subsequently, virtually all of the cellular contents are concentrated into proplatelet extensions. These proplatelets undergo repeated cycles of extension, retraction, bending and branching before eventually generating particles with many or all of the properties of blood platelets (Italiano Jr and Shivdasani, 2003; Choi et al., 1995b; Lecine et al., 1998a).

Not till recently, was an elegant study by Junt et al (2007) carried out to confirm a genuine correlation between proplatelet formation *in vitro* and thrombocytopoiesis *in vivo*. By multi-photon intravital microscopy, they captured MKs extending plump perivascular pseudopodia that were sheared by flowing blood, resulting in the appearance of proplatelets in peripheral blood. Although the study largely confirms what has been shown in culture, there were some differences. Instead of the proplatelets being ‘ribbon-like’ structures breaking off neatly from the MK fingers in barbell-shaped pairs, what was actually released from the MKs *in vivo* were larger, more compound structures that were further processed in the circulation (Junt et al., 2007). This study

lays to rest any doubts about the basic mechanism by which platelets form. Concurrently, it opens up new questions about how MKs release platelets and what interventions might enhance or diminish that step in the process.

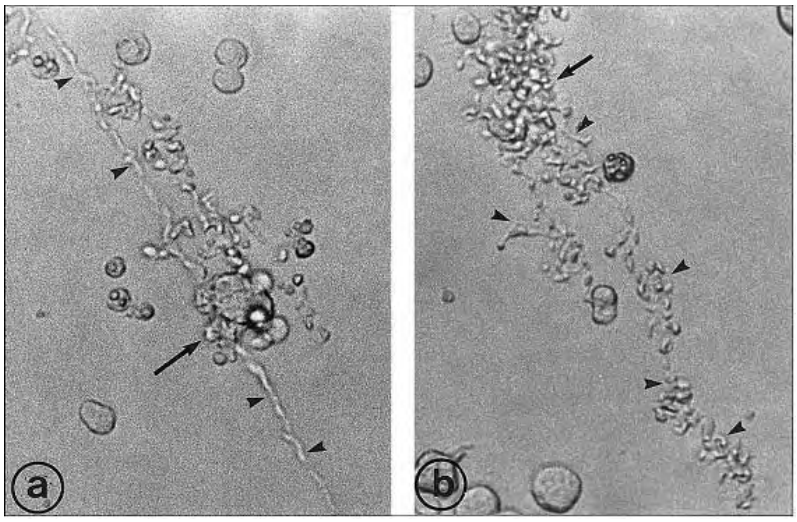


Figure 1.3 Proplatelet formation (arrowheads) as seen by phase contrast microscopy: (a-b) Proplatelet structures developing from mature MKs grown under thrombopoietin conditions. Picture taken from (Cramer et al., 1997).

REGULATION OF MEGAKARYOPOIESIS AND THROMBOPOIESIS

With the emergence of improved culture techniques and numerous genetic mouse models, came renewed interest in the cellular and molecular basis of megakaryopoiesis and thrombopoiesis. The complexity of haematopoiesis and the requirement for adaptable production of vastly different cell types from common progenitors must entail for sophisticated regulatory mechanisms. Here we review two distinct, but equally important modes of regulation. The first involves extracellular factors such as cytokines, which activate signal transduction pathways to promote cellular proliferation. Cytokines then interplay with intrinsic transcription factors that co-ordinate the expression of lineage-specific subsets of genes. Deeper insights into the underlying molecular mechanisms involved in megakaryopoiesis and platelet release will enhance the understanding of both normal haematopoiesis and disease states arising from deregulation.

Humoral Regulation of Haematopoiesis and Megakaryopoiesis

Cytokines are soluble molecules that are produced by a number of different cell types and allow communication between a wide range of targets via activation of signal transduction pathways (Alexander, 1998). Under specific haematopoietic stresses such as infection or acute blood loss, short-lived amplifications of specific cytokine secretions are overlaid upon the basal cytokine production. These soluble molecular regulators may be responsible for stem cell expansion and maintenance, as well as the modulation of lineage specific transcription factor expression (Alexander, 1998). To date, in excess of 20 secreted molecular regulators have been identified to stimulate the production or function of haematopoietic cells (Table 1.1). The abundance of cytokines clearly implies that no single haematopoietic lineage is controlled exclusively by a single regulator. A combination of cytokines can have synergistic effects on haematopoietic cells, both quantitatively and by broadening the cellular response beyond that seen with the individual factors. The following section will cover a selected number of cytokines, with more emphasis placed on the main growth factors involved in this study.

Table 1.1 Cytokines involved in haematopoiesis. Adapted from Alexander (1998)

*Regulator**	*Cell Source***	*Responding Cells#*
G-CSF	Endothelial cells, fibroblasts, BM stromal cells, monocytes, macrophages. Generally requires induction via internal stimulation.	G, M
GM-CSF	As above, including T-lymphocytes. Generally requires induction via internal stimulation.	G, M, Eo, MK
M-CSF	Endothelial cells, fibroblasts, BM stromal cells, monocytes, macrophages.	M, G
Multi-CSF (IL-3)	Activated T-lymphocytes, mast cells, NK cells. Synthesis tightly regulated.	G, M, Eo, MK, Mast, Stem
EPO	Liver (foetal life), kidney (adult life). Synthesis regulated by renal tissue hypoxia.	E, MK
TPO	Liver, kidney, smooth muscle, spleen, BM	MK, Stem
IL-1β	Macrophages, endothelial cells, keratinocytes, lymphocytes, fibroblasts, osteoblasts	T, B, Stem
IL-2	Activated T-cells	T, B
IL-4	Helper T-cells, mast cells, basophils, BM stromal cells	T, B, G, M, Mast
IL-5	Helper T-cells, mast cells	Eo, B
IL-6	Macrophages, fibroblasts, endothelial cells, T-cells	B, G, Stem, MK
IL-7	Bone marrow, thymus, spleen, gut	B, T, M
IL-9	T-cells	T, B, E, Mast
IL-10	B & T-lymphocytes, macrophages, mast cells, keratinocytes	B, E, MK, Stem
IL-11	Stromal cells	MK, B, Stem, E
IL-12	T-cells	NK, T
IL-13	T-cells	M, B
IL-14	Endothelial cells, lymphocytes	B
IL-15	Macrophages, fibroblasts, keratinocytes, endothelial cells	T, B
SCF	BM stromal fibroblasts, endothelial cells	Stem, G, E, B, MK,Mast
LIF	Endothelial cells	MK, M
OSM	Monocytes, macrophages, T-cells	MK
Flt3 L	Mesenchymal stromal cells	Stem, G, M, B

* G-CSF, granulocyte colony-stimulating factor; GM, granulocyte-macrophage; M, macrophage; Multi, multi-potential; EPO erythropoietin; TPO, thrombopoietin; IL, interleukin; SCF, stem cell factor; LIF, leukaemia inhibitory factor; OSM, oncostatin M; Flt 3 L, Flt-3 Ligand
** BM, bone marrow; NK, natural killer
# Only responsive haematopoietic cells shown. G, granulocytes; M, macrophages, Eo, eosinophils, MK, megakaryocytes; E, erythroid cells; Mast, mast cells, Stem, stem/multi-potential haematopoietic cells; T, T-lymphocytes; B, B lymphocytes; NK, natural killer cells

Stem Cell Factor (SCF)

SCF (also termed steel factor, c-kit ligand, or mast cell growth factor) is constitutively expressed in bone marrow stromal fibroblasts and endothelial cells and is clearly shown to be essential for the survival, proliferation, adhesion, migration and differentiation of HSCs (Broudy, 1997; Hartman et al., 2001). In adult mice, as early as 2 days after administration of a neutralising antibody against the SCF receptor, c-kit, all progenitor cells were depleted, resulting in the eventual absence of erythroid and myeloid cells in the bone marrow (Ogawa et al., 1991). With respect to megakaryopoiesis, SCF together with other colony stimulating factors (CSFs) such as granulocyte-macrophage CSF (GM-CSF) were shown to increase proliferation and expansion of both primary bone marrow MK progenitors and MK cell lines (Szalai et al., 2006). In addition, the administration of SCF to mice led to an expansion in the number of transplantable HSCs (Bodine et al., 1993). However, despite these results, SCF alone could not maintain the input numbers of stem cells in *ex vivo* cultures (Li and Johnson, 1994). This suggests that *in vivo* SCF may interact with other haematopoietic factors to bring about its effects. Examples of candidate growth factors thought to fill such a role are Flt-3 ligand and interleukin-3.

Flt-3 Ligand (Flt3 L) and Interleukin-3 (IL-3)

Flt3 L is widely expressed on mesenchymal stromal cells and is the ligand of the Flt-3 receptor, which has restricted expression in $CD34^+$ cells, with the exception of cells found in the bone marrow and cord blood (Broudy et al., 1996). The targeted disruption of Flt3 L results in a five-fold reduction in long-term repopulating HSCs and subsequent decreases in mature myeloid, B, NK and dendritic cells (Broudy et al., 1996). In contrast to SCF, there has been no evidence that the administration of Flt3 L increases the number of stem cells.

Several other cytokines, including IL-3, GM-CSF and granulocyte CSF (G-CSF) have been shown to be important for normal megakaryopoiesis. These cytokines affect megakaryopoiesis during the early stages of MK development (Szalai et al., 2006). IL-3 was shown to act on bone marrow progenitor cells through the CFU-MK stage of MK development (Quesenberry and McGrath, 1985; Robinson and Quesenberry, 1987). Several studies have also demonstrated that IL-3 acts in synergy with thrombopoietin (TPO) to produce colonies of multiple haematopoietic lineages, including multi-lineage colonies (Ku, 1996; Schattner, 1996). Therefore, the above mentioned cytokines play a role in regulating MK ontogeny, but probably function mainly in concert with TPO, which is still the primary physiological regulator of megakaryopoiesis (Kaushansky, 2002).

Thrombopoietin: the Primary Regulator of Megakaryocytic Differentiation

TPO was first described in 1958 to be the humoral substance responsible for platelet production following induced thrombocytopaenia (Kelemen, 1958). Since then, multiple biological properties of TPO have been predicted and reported, effects primarily restricted to MK differentiation. Whether the hormone also affects megakaryocytic progenitor cell proliferation and other haematopoietic lineages was debated until recently when TPO was successfully cloned in 1994 (De Sauvage et al., 1994; Lok and Foster, 1994; Bartley et al., 1994). Following this, it has become clear that TPO affects all aspects of MK development and plays an essential role in other lineages as well.

The precise role of TPO has been defined by a diverse range of studies inclusive of genetically engineered mice that lack either the cytokine or its receptor, c-Mpl and examination of the actions of recombinant TPO in *in vitro* and *in vivo* systems (Gurney et al., 1994; Kaushansky, 2002; Kuter and Glenn Begley, 2002). In summary, TPO stimulates the proliferation of MK progenitor cells, the expression of platelet-specific cell surface markers such as CD41, CD61 and CD42 as well as induces endomitosis (Kaushansky, 2002). TPO or c-Mpl knockout mice are severely thrombocytopaenic and produce low numbers of MKs and their progenitors (Gurney et al., 1994; De Sauvage et al., 1996).

The administration of nanogram quantities of TPO to mice leads to a massive expansion of marrow and splenic MKs and a log-linear dose-response rise in circulating mature platelets (Kaushansky, 1995). Similar effects have been noted in initial clinical trials where TPO elicits an accelerated platelet recovery in individuals who receive radiation or chemotherapy for malignancy (Fanucchi et al., 1997; Basser et al., 2002; Matsumura and Kanakura, 2002). However, while a potent stimulus of platelet production, TPO appears to have little effect on the last stages of platelet formation and shedding (Choi et al., 1995c). Some even suggest that TPO may actually inhibit platelet release (Fishley and Alexander, 2004).

Blood levels of TPO are mostly inversely related to marrow MK and platelet abundance (Koike et al., 1998), although multiple disease states are associated with unexpectedly high or low circulating levels of the hormone (Schoffski et al., 2002, Tacke et al., 2002). Two non-mutually exclusive models have been proposed to explain the regulation of TPO production. In the first, TPO production is constitutive, and its levels are controlled by a negative feedback loop. Platelets bearing TPO receptors absorb excess circulating hormones and destroy it, so that an increased platelet mass varies inversely with TPO levels (Fielder et al., 1996; Yang et al., 1999). In the second model, other investigations have also demonstrated a second level of regulation by hepatocyte and stromal cell responses to thrombocytopaenia or mediators of inflammation such as IL-6 (Kaser et al., 2001).

Besides its effects on MK and platelet development, an essential role for TPO signalling in other haematopoietic lineages has also emerged. Administration of TPO to normal animals expands the number of progenitor cells of all haematopoietic lineages, and if given during myelosuppressive therapy, accelerates their recovery to normal values (Kaushansky et al., 2001). Conversely, in TPO and c-Mpl knockouts, the number of erythroid and myeloid progenitor cells were greatly reduced by between 70-90% and only half the wildtype number of progenitor cells of essentially all lineages was observed (Carver-Moore et al., 1996). Additional evidence from limiting dilution cell transplantation analyses also showed that HSC self-renewal and expansion is reduced 10-20 fold after transplantation of normal stem cells into TPO null mice compared to

their wildtype counterparts (Kaushansky, 2003). Thus, TPO appears to be more than just a lineage restricted growth factor, but is also one of the factors that supports the survival and proliferation of pluripotent stem cells.

In addition to TPO's obvious role in MK ontogeny, later stages of megakaryopoiesis may also be directed by other chemokines or localised expression of ligands for MK surface receptors. For example, CXCL12 (stromal-derived factor 1) is a potent MK chemoattractant, and fibrinogen present along BM sinusoids promotes proplatelet formation through $\alpha_{IIb}\beta_3$ integrin on MKs (Junt et al., 2007). These recent findings further refine the humoral processes involved in regulating MK and platelet development and may someday provide additional targets for maintaining proper megakaryopoiesis or treating conditions associated with bleeding disorders.

TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION

Growth factors and their related signalling cascades have unmistakable importance in determining lineage restricted phenotype. However, the actions and properties of each cell type are also determined by the kinds and amounts of protein it contains and this is regulated largely at the transcription level. A mammalian organism is required to regulate transcription of approximately 100,000 genes in the proper spatial and temporal patterns (Carey, 2000). Knowledge of how transcription factors function during this differential gene expression can then be applied to fundamental issues in the fields of biology and medicine.

A General Model of Gene Expression

Protein production is a multi-step process that can be broadly divided into two parts (Figure 1.4). Firstly, before the synthesis of a protein begins, the corresponding messenger RNA (mRNA) molecule is produced by RNA transcription. Synthesis of mRNA requires an RNA polymerase (RNAP) to initiate transcription, polymerise ribonucleoside triphosphates complementary to the DNA coding strand and to terminate transcription. The synthesised mRNA can then be used as the template for translation of the polypeptide.

Proteins that regulate transcription are of one of three classes. Firstly there are chromatin remodelling and modification complexes such as the SWI/SNF complexes, histone acetyltransferases and histone deacetylases (Aalfs, 2000; Martens and Winston, 2003). These proteins enable the transcriptional machinery to access highly compacted DNA. Secondly, the general transcriptional machinery is required for promoter recognition and initiation of RNA synthesis. The general machinery includes RNAPII, TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH as well as co-activators and repressors (Carey, 2000; Reese, 2003).

Finally, there are transcription factors that bind to specific DNA sites either upstream or downstream from the gene's promoter, orchestrating the activation or repression of genes in a pre-programmed manner. As a result of this arrangement, transcription from a single promoter may be regulated by the binding of multiple transcription factors to alternative control elements. This process permits complex control of gene expression in different regulatory contexts. The following sections will focus on the last group of transcription factors and more specifically on the factors involved in MK-specific gene expression.

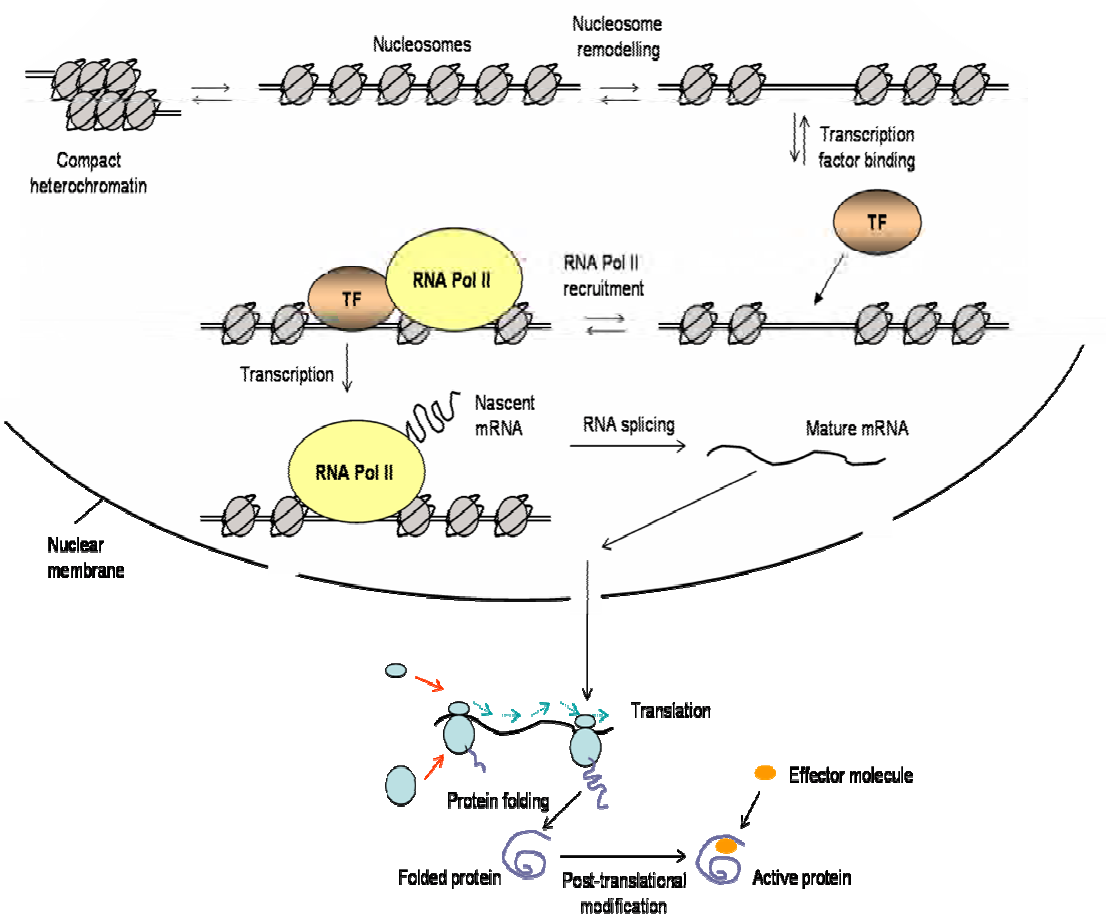


Figure 1.4 Schematic summary of the processes involved in protein production. Compact heterochromatin is decondensed to an array of nucleosomes, which then undergo remodelling by remodelling enzymes or histone acetylases. Transcription factors (TF) are then able to bind along with RNA polymerase II to control elements, which upon activation results in the transcription of nascent mRNA. Post-transcriptional RNA splicing occurs before the mature mRNA translocates across the nuclear membrane into the cytoplasm where translation of mRNA by ribosomes takes place. The protein is then folded by chaperones and post-translationally modified to produce the active protein.

Transcription Factors: Activators and Repressors of Transcription

Sequence-specific recognition of DNA by proteins is essential for transcription. Transcription factors form a growing family of regulatory proteins that can positively or negatively influence transcription by binding to regulatory elements in DNA and contacting components of the basal transcription machinery. Some transcription factors are ubiquitous and are required in all cases of transcription (eg. general transcription machinery), while others only have effect on specific genes. Expression of transcription factors may also be tissue-specific or temporal.

Transcription factors in general contain distinct domains that are physically and functionally separable (Mitchell and Tjian, 1989). Firstly, they contain a DNA-binding domain, which binds to specific DNA sequences. These DNA-binding domains contain a variety of structural motifs that have resulted in the classification of a large number of transcription factors. Some of these DNA-binding domains include the GATA domain (Shimizu and Yamamoto, 2005), E twenty six (Ets) domain (Maroulakou and Bowe, 2000; Bartel et al., 2000), the Zinc-Finger domain (Sekhon, 2006), the basic region-leucine zipper (bZip) domain (Vinson et al., 2006), the Basic Helix-Loop-Helix domain (Jones, 2004) and many others. For example, the DNA-binding domain in the bZip family of proteins consists of a basic region followed by a leucine zipper of about ~30-40 amino acids long, with a leucine at every seventh residue to form a 'heptad repeat'. The bZip class of proteins often exists in alternative heterodimeric combinations of monomers, hence enabling different transcriptional responses to be elicited depending on which specific heterodimer is expressed at that particular time (Vinson et al., 2006).

In addition to their DNA-binding domains, sequence specific transcription factors also contain a second functional domain, which determines whether the protein activates or represses transcription. Transcription activators and repressors contain either one or a few activation domains or repression domains respectively. The different domains frequently are linked by flexible polypeptide regions and can interact either directly with components of the general transcription machinery or with co-activators/ co-repressors (Chi et al., 1995). In several cases, some factors can mediate transcription by both activation and repression.

In summary, the promoter regions of most genes contain binding sites for multiple transcription factors that may form multi-protein complexes. The particular repertoire of transcription factors that is expressed and co-operatively activated in a cell at a particular time, determines whether a gene is actively transcribed or repressed.

TRANSCRIPTIONAL REGULATION OF MEGAKARYOPOIESIS

At the heart of stem cell renewal and lineage commitment lies the relative expression level of lineage-specific transcription factors. The role of specific transcription factors in HSC fate decisions has derived largely from genetic strategies such as gene-targeting and overexpression studies. These experimental findings have helped assemble a rough outline of putative transcriptional hierarchy. In the case of MKs and platelets, unique challenges in gene expression profiling are that MKs constitute a rare cell lineage that need to be expanded *ex vivo* to generate sufficient numbers for molecular analysis. In addition, platelets lack nuclei and their mRNAs represent the residue from MK differentiation. However, despite these challenges, several transcription factors have been found to play critical roles in MK development (Table 1.2). Among them, GATA-1, FOG-1 and NF-E2 stand out for their non-redundant and essential roles in megakaryopoiesis and will therefore be the focus of this section.

Table 1.2 Key identified transcriptional regulation at individual steps in MK differentiation and thrombopoiesis.

Transcription Factor	Stage of MK maturation	Key target genes or markers	Affects on MK phenotype
GATA-1-FOG-1 partnership	Lineage commitment Committed MK progenitors (2N DNA) and early maturing MKs (16-64N DNA)	CD41, p45 NF-E2, Inositol polyphosphate 4-phosphastase type 1	Mutations disrupting GATA1-FOG1 interaction leads to anaemia and macrothrombocytopaenia (Mehaffey et al., 2001; Nichols et al., 2000).
Runx1/CBFβ	Lineage commitment	CD61, c-Mpl	Conditional *Runx1* deletion in mice results in reduced platelet numbers, small, hypoploid MKs (Putz et al., 2006; Ichikawa et al., 2004). Overexpression in haematopoietic cells leads to MK differentiation (Niitsu et al., 1997).
GATA-1	Proliferation of MK precursors	Cyclin E, CD42, NF-E2, platelet factor 4	Absence of *GATA-1* in mice results in thrombocytopaenia and increased number of abnormal MKs (Vyas et al., 1999). *GATA-1* mutations promote MK leukaemia in trisomy 21 (Crispino, 2005).
Fli-1	Differentiation of early maturing MKs (16-64N DNA)	CD41, CD61, CD42a, CD42b, GPVI, GATA-1, c-Mpl,	Overexpression in K562 cells impart MK phenotype (Athanasiou et al., 1996). Absence of *Fli-1* in mice is embryonic lethal and results in severe dysmegakaryopoiesis (Hart et al., 2000; Spyropoulos et al., 2000).
NF-E2; p45 and MafG or MafK	Terminal MK differentiation Proplatelet formation and platelet release	β1-tubulin, thromboxane synthase, Rab27b, caspase 12, β-hydroxysteroid reductase, Lims1	Elaboration of cytoplasmic processes, microtubule elongation and creation of marginal bands (Shivdasani, 2001). Severe thrombocytopaenia in NF-E2 knockout mice (Shivdasani et al., 1995; Shivdasani, 1996).

GATA-1

GATA-1 is the founding member of the GATA family of zinc finger transcription factors and it is primarily expressed in the erythroid, eosinophil, mast cell and megakaryocytic lineages. In particular, GATA-1 has been shown to be essential for erythroid and MK maturation (Shivdasani et al., 1997b; Fujiwara et al., 1996; Ferreira et al., 2005). Homozygous null mutations of GATA-1 in mice resulted in embryonic lethal anaemia (Fujiwara et al., 1996), while selective knockdown of GATA-1 in MKs resulted in thrombocytopaenia and an accumulation of immature MKs (Shivdasani et al., 1997b; Vyas et al., 1999). Conversely, overexpression of GATA-1 in the myeloid line 416B resulted in a MK phenotype, with increased expression of MK markers (Visvader and Adams, 1993). Missense mutations in GATA-1 were also found to cause inherited blood disorders (Nichols et al., 2000; Freson et al., 2001), while acquired mutations were found to be associated with essentially all cases of acute megakaryoblastic leukaemia (AML) in children with Down syndrome (Crispino, 2005).

GATA-1 contains two zinc fingers. The N-terminal zinc finger interacts with FOG-1, while the C-terminal finger is required for DNA binding. Together, these domains act in a manner similar to a multitude of transcription factors that bind DNA and activate transcription factors. GATA-1 function is modulated by interactions with other proteins such as Friend-of GATA-1 (FOG-1), Sp1, EKLF, PU.1, Fli-1 and p300/CBP (Szalai et al., 2006).

Molecularly, the absence of GATA-1 leads to reduced expression of several key MK specific genes, including NF-E2, CD42, and platelet factor 4 (Vyas et al., 1999). It is likely that other GATA-1 target genes such as CD41 may be regulated by GATA-2 in the absence of GATA-1, resulting in only minimal reduction of expression in these genes (Vyas et al., 1999). Indeed, evidence showing that GATA-2 can replace GATA-1 during the early stages of megakaryopoiesis supports this theory (Chang et al., 2002). GATA sites are also essential for the activity of one of the two p45 NF-E2 promoters in developing red blood cells (Moroni et al., 2000). Expression of p45 NF-E2 is not only reduced in GATA-1 null MKs (Vyas et al., 1999), but also depends on the GATA co-activator FOG-1 (Tsang et al., 1997).

FOG-1

A second factor, Friend of GATA-1 (FOG-1), was isolated through its ability to interact with GATA-1 via a two-hybrid screen in yeast (Tsang et al., 1997). FOG-1 interacts with the N-terminal zinc finger of GATA-1 and depending on the promoter context either enhances or represses GATA-1's transcriptional activity (Wang et al., 2002). Within the haematopoietic system, FOG-1 demonstrates a similar restricted expression pattern to GATA-1 both temporally and spatially. FOG-1 knockout experiments resulted in embryonic death as a result of severe anaemia (Tsang et al., 1998). The block in erythroid maturation was found to be at a stage similar to that observed in GATA-$1^{-/-}$ mice, providing genetic evidence that FOG-1 and GATA-1 function in a co-ordinate manner in erythroid development (Fujiwara et al., 1996).

In contrast to GATA-1 deficient mice, FOG-$1^{-/-}$ mice exhibit a complete absence of MKs, suggesting a possible GATA-1 independent role of FOG-1 in early megakaryopoiesis (Chang et al., 2002). However, this discrepancy may alternatively be attributed to the compensatory FOG-dependent roles of GATA-1 by GATA-2 in early MK differentiation. More recent findings confirm that the protein interaction between FOG-1 and GATA-1 itself is crucial for MK development. A number of mutations in the N-finger region of GATA-1 (e.g. V205M, G208S, D218Y) have been identified in patients with anaemia and macrothrombocytopaenia (Mehaffey et al., 2001; Nichols et al., 2000). Interestingly, such mutations impair GATA-1's ability to interact with FOG-1. These findings have been further confirmed in murine models where compensatory mutations in FOG-1 that restore the GATA-FOG-1 interaction also restore normal MK development (Wang et al., 2002; Chang et al., 2002). An example of the GATA-FOG-1 synergy is its combined ability to activate the p45 NF-E2 promoter (Eisbacher, 2003).

NF-E2

A less well-characterised mediator of MK development is the transcription factor NF-E2 (Nuclear Factor Erythroid 2). A heterodimer consisting of haematopoietic-specific (p45) and widely expressed (p18) subunits, NF-E2 is a member of the bZip family of nuclear proteins. The p18 subunit lacks a canonical transcriptional effector domain and is an obligatory partner of the p45 subunit, which contains recognisable functional motifs, but cannot bind DNA as a monomer or homodimer (Motohashi et al., 2006). NF-E2 preferentially binds to AP-1 like consensus binding sites of its target genes (Figure 1.5). A number of *in vitro* DNA binding and *in vivo* expression studies have shown that having bound to these AP-1 consensus sequences via its bZip domains, NF-E2 then exerts its function by the recruitment of remodelling complexes and other factors to the vicinity of its binding sites (Armstrong and Emerson, 1996; Gong et al., 1996).

Figure 1.5 Schematic representation of NF-E2. The p18 and p45 subunits interact through their leucine zippers allowing NF-E2 to recognize the AP-1-like core palindrome consensus sequence. p18 and p45 recognise the larger (bold) and smaller (italic) portions of the asymmetric NF-E2 site respectively.

Molecular Understanding of NF-E2

p45 NF-E2 expression is restricted to erythroid, megakaryocytic and mast cells. Of the three small p18 Maf proteins that are free to associate with the p45 subunit, only MafG and MafK are expressed in MKs, with MafG predominating in cells with advanced differentiation (Shavit et al., 1998; Lecine et al., 1998a). p45's activation domain located within its N-terminal interacts with proteins such as the general transcription factor TAFII130 (Amrolia et al., 1997), the mammalian ubiquitin ligase Itch (Chen et

al., 2001), and CREB binding protein (CBP) (Cheng et al., 1997), a co-activator with histone acetyltransferase activity (Cheng et al., 1997; Hung et al., 2001). In the case of adult β globin promoters, p45 NF-E2 is required for histone hyperacetylation ~50 kilobases downstream of the locus control region, inducing long-range transfer of RNAPII to the promoter and resulting in transcriptional activation (Johnson et al., 2001). Acetylation of the p18/Maf NF-E2 subunit by CBP, in conjuction with the SUMOylation of the p45 subunit, is believed to augment NF-E2's DNA binding and transcription activities (Hung et al., 2001; Shyu et al., 2005).

Interestingly, two isoforms of the human p45 NF-E2 gene have been isolated (*a* and *f* NF-E2) (Chan et al., 1993; Pischedda et al., 1995). These isoforms produce the same protein, but are regulated by two different promoters and are expressed in different ratios during development. The two isoforms are co-expressed in every cell type, but the *f* form predominates in foetal liver and the *a* form is prevalent in adult bone marrow.

NF-E2's Implication in Megakaryopoiesis and Thrombopoiesis

Like GATA-1, NF-E2's primary biological influence was first thought to be in globin gene expression, but p45 deficient mice surprisingly displayed only mild abnormalities in erythropoiesis (Shivdasani and Orkin, 1995). However, these mice were found to be severely thrombocytopaenic due to arrest in late MK maturation. Although MKs were present, they showed profound cytoplasmic abnormalities, including a dramatic reduction in granule numbers, disorganised demarcation membranes, failure to elaborate proplatelets in culture and failure to delimit platelet territories. While these findings do not pinpoint the thrombopoietic defect to the failure of initiating proplatelet formation, it is fair to consider that essential NF-E2 functions are manifested at or very close to the point at which MKs prepare to release their platelet load. In particular, it is the p45 subunit of NF-E2, which is postulated to play an essential role in platelet production (Kotkow and Orkin, 1995; Shivdasani et al., 1995). Targeted disruption of either the Maf G/ Maf K protein alone, results in animals with no discernible abnormalities (Kotkow and Orkin, 1996), whereas MafG $^{-/-}$ MafK $^{-/-}$ compound mutant mice result in a similar phenotype as that of the p45$^{-/-}$ mice (Onodera et al., 2000).

In a past study, the abolishment of p45 NF-E2 in the human megakaryoblastic cell line, Meg-J inhibited induced polyploidisation, leading to the conclusion that p45 NF-E2 may be essential for MK endomitosis (Kobayashi et al., 1998). Contrarily, in separate studies, in the absence of NF-E2 function, endomitosis and subsequent expansion of cytoplasmic volume were found to be overtly normal (Shivdasani et al., 1995), although proliferation of MK progenitors was mildly reduced (Levin et al., 1999). Molecular and cellular studies show a differentiation arrest that occurs late in MK maturation and does not directly involve TPO signalling (Shivdasani et al., 1995). A proposed hypothesis is that although NF-E2 expression is present early in MK ontogeny, its target genes are passively or actively silenced by small-Maf dimers, which compete for the same DNA binding sites (Motohashi et al., 2006). These genes are only activated when protein stability or post-translational modifications, favour the activity of p18-p45 heterodimers over those of other small-Maf protein complexes (Shivdasani, 2001; Motohashi et al., 2006).

In terminally differentiated cells, NF-E2 carries out its most important functions probably through co-ordinate activation of many genes that reorganise the cytoskeleton and transport organelles into proplatelets, enabling platelet release. To date, identified megakaryocytic target genes of NF-E2, include thromboxane synthase (TXS) (Deveaux et al., 1997), β1-tubulin (Lecine et al., 2000), Rab27b (Tiwari et al., 2003), caspase 12 (Kerrigan et al., 2004), 3β-hydroxysteroid dehydrogenase (Nagata et al., 2003) and Lims1 (Chen et al., 2007).

NF-E2's Role in Disease Processes

To date, there have been three publications associating NF-E2 with human disease. The p45 gene represents a common integration site for the Friend erythroleukaemia virus in mice. This suggests that the loss of p45 function may contribute to leukaemogenesis in that model system (Lu et al., 1994). In addition, erythroleukaemia cells lacking p45 fail to produce globin proteins, supporting the conclusion that in spite of the knockout phenotype, p45 is important in erythropoiesis.

In malignant MKs from essential thrombocytopaenia (ET) patients, real-time RT-PCR showed that both isoforms of NF-E2 were significantly reduced compared to their

normal counterparts (Catani et al., 2002). Both the NF-E2*a*/ MafG mRNA ratio and TXS mRNA expression were also found to be significantly reduced in MKs from ET patients, raising the possibility that NF-E2 could play a role in MK transformation (Catani et al., 2002).

Another recent example of NF-E2's possible impact on disease is in polycythaemia vera (PV) patients. PV is a myeloproliferative disorder, which is clinically defined by erythrocytosis and thromboembolic complications. Gene expression profiling of PV patients by microarray, revealed a 2-40 fold overexpression of NF-E2 in these patients (Goerttler et al., 2005). This suggests that elevated concentrations of NF-E2 in PV patients may lead to an overproduction of erythroid and, in some cases, MK/ platelets. NF-E2 concentration may therefore be a determinant of both the severity of erythrocytosis and the concurrent presence or absence of thrombocytosis in such patients.

REGULATION OF GENE EXPRESSION BY NUCLEAR PROTEIN TRANSPORT

The passage of macromolecules between the nuclear and cytoplasmic compartments through the nuclear pore complex (NPC) is fundamental to eukaryotic cell processes such as growth, proliferation, signalling, cell cycle control, and gene expression (Suntharalingam and Wente, 2003). For example, proteins synthesised in the cytoplasm need to be imported to the nucleus, while mRNA needs to be exported to the cytoplasm. As a result, mechanisms for nuclear entry of transcription factors are not only a key way by which gene regulation can be controlled (Jans et al., 2000), but it is also central to longer term processes of cellular phenotype change such as differentiation during development.

Proteins and nucleic acids are transported actively between the nucleus and cytoplasm along the central axis of the NPC (Feldherr et al., 1984; Dworetzky and Feldherr, 1988). Proteins containing a nuclear localisation sequence (NLS) are imported into the nucleus while proteins containing a nuclear export sequence (NES) are exported to the cytoplasm. The various different nuclear trafficking pathways are generally facilitated

by soluble carrier molecules that bind their cargo macromolecule in one compartment and release it in the other, before being recycled back to the original compartment to participate in further rounds of transport (Izaurralde and Adam, 1998; Stewart, 2003). This process is orchestrated and energised by the nucleotide state of the Ras-family GTPase Ran (Bayliss et al., 2000) and will be described in further detail in the following sections.

Nuclear Pore Complex

All molecular traffic between the nucleus and the cytoplasm travels through the NPCs. These large, proteinaceous, pore structures are embedded into the nuclear envelope and are made up of 40-50 distinct nuclear pore proteins (nucleoporins; Nups) (Jans et al., 2000). Thanks to the improvements in electron microscopy methods, the three-dimensional structure of the NPC has been refined over the years (Yang et al., 1998; Stoffler et al., 2003; Beck et al., 2004). As illustrated in Figure 1.6, the NPC is minimally characterised as possessing a central channel surrounded by 8 spokes, nucleoplasmic filaments, which conjoin to form a basket-like structure, and cytoplasmic filaments that project into the cytoplasm (Suntharalingam and Wente, 2003).

In addition, a recent study by Beck et al. (2004) has shown that the NPC is a highly dynamic structure, with several of the nucleoporins being mobile and particular components of the NPC able to change their morphology. The NPC has an effective diameter of 9 nm that allows passive exchange of ions, small molecules and small proteins ~ 20–40 kDa (Lyman et al., 2002). Larger macromolecules >50 kDa require NLSs or NESs together with facilitative translocation pathways to actively pass through the NPC (Moroianu, 1999).

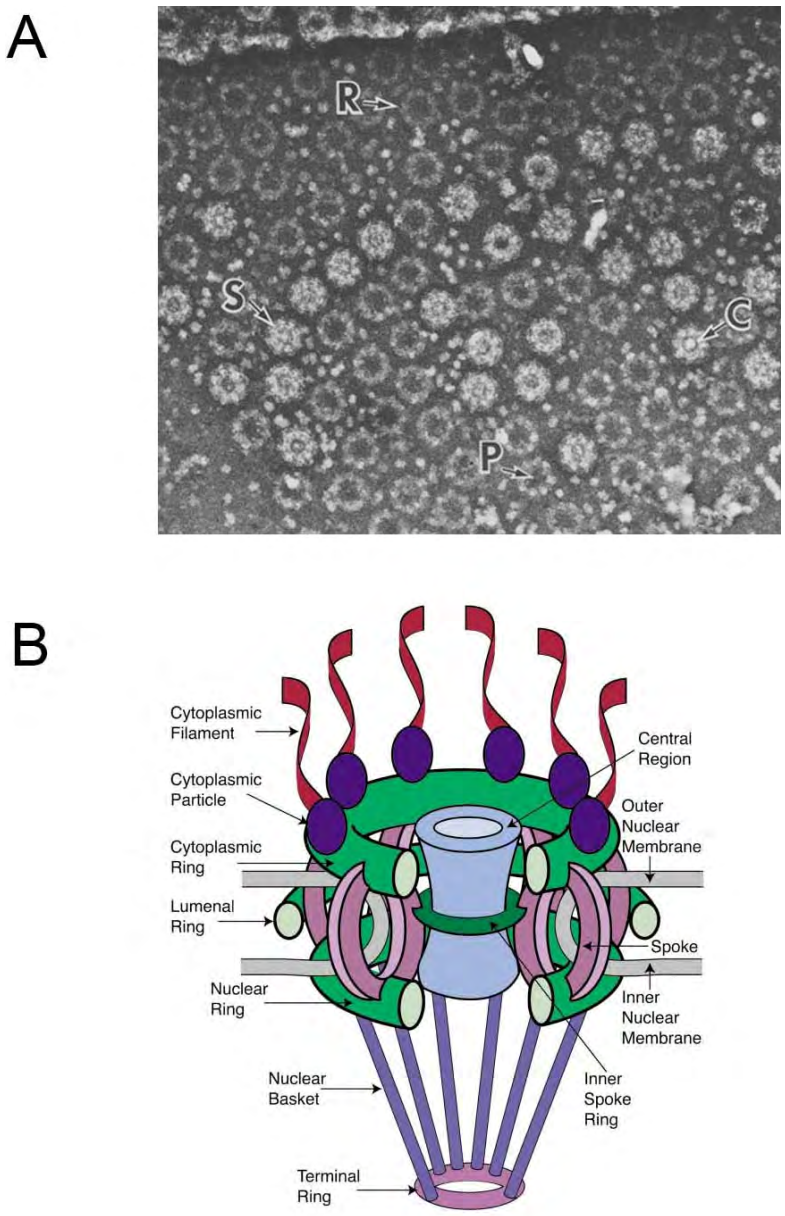


Figure 1.6 The nuclear pore complex. (A) Electron micrographs of NPCs released from the nuclear envelope following treatment with detergent. Constituents most related to the pore complex are rings (R); central plugs (C); spokes (S) and particles (P), occasionally observed around the rings. Magnification: x 60,000 (Unwin and Milligan, 1982). (B) Schematic diagram of the NPC substructures (Suntharalingam and Wente, 2003).

Nuclear Transport Pathways

Active protein import is a multi-step process that requires nucleocytoplasmic shuttling proteins known as importins and exportins that recognise NLSs and NESs respectively. Although there is no strict consensus sequence, NLSs are typically short stretches of positively charged amino acids, arranged either as monopartite (a single cluster) or bipartite (two clusters separated by a 10-12 amino acid spacer) sequences (Dingwall and Laskey, 1998). NESs on the other hand are generally short stretches of hydrophobic leucine-rich amino acids (Fukuda et al., 1997).

In the classical nuclear import pathway (Figure 1.7A), the NLS of the target protein is recognised by importin α (IMP α), which is then bound by a soluble carrier molecule of the importin β (IMP β) family (Adam, 1999). This trimeric complex is then translocated through the NPC by transient IMP β-mediated interactions with nucleoporins (Bayliss et al., 2000). It is believed that transport of the importin-cargo complex is facilitated by an affinity gradient between the different nucleoporins from the cytoplasmic to nuclear side of the NPC (Crowley et al., 2002). Translocation is then followed by the displacement of the cargo from the carrier molecule within the nucleus by Ran in its GTP-bound state. The carrier, complexed with RanGTP is recycled back through the NPCs to the cytoplasm, where RanGTP is hydrolysed and the carrier released. In some cases, import is via a non-classical pathway (Figure 1.7B), requiring only IMP β (Moroianu, 1998). The NLS of the target protein can even interact directly with the nucleoporins of the NPC (Moroianu, 1998; Ten Dijke and Hill, 2004; Marg et al., 2004).

Nuclear protein export (Figure 1.7C) is analogous to nuclear import, except that distinct family members of IMP β are involved. The most well described exportin (EXP) being CRM1 (chromosome region maintenance 1; also know as exportin 1 or Xpo1) (Stade et al., 1997). Dependent on RanGTP, exportins recognise NESs, move through the NPC via nucleoporin interactions, before being dissociated in the cytoplasm by the hydrolysis of RanGTP (Poon and Jans, 2005).

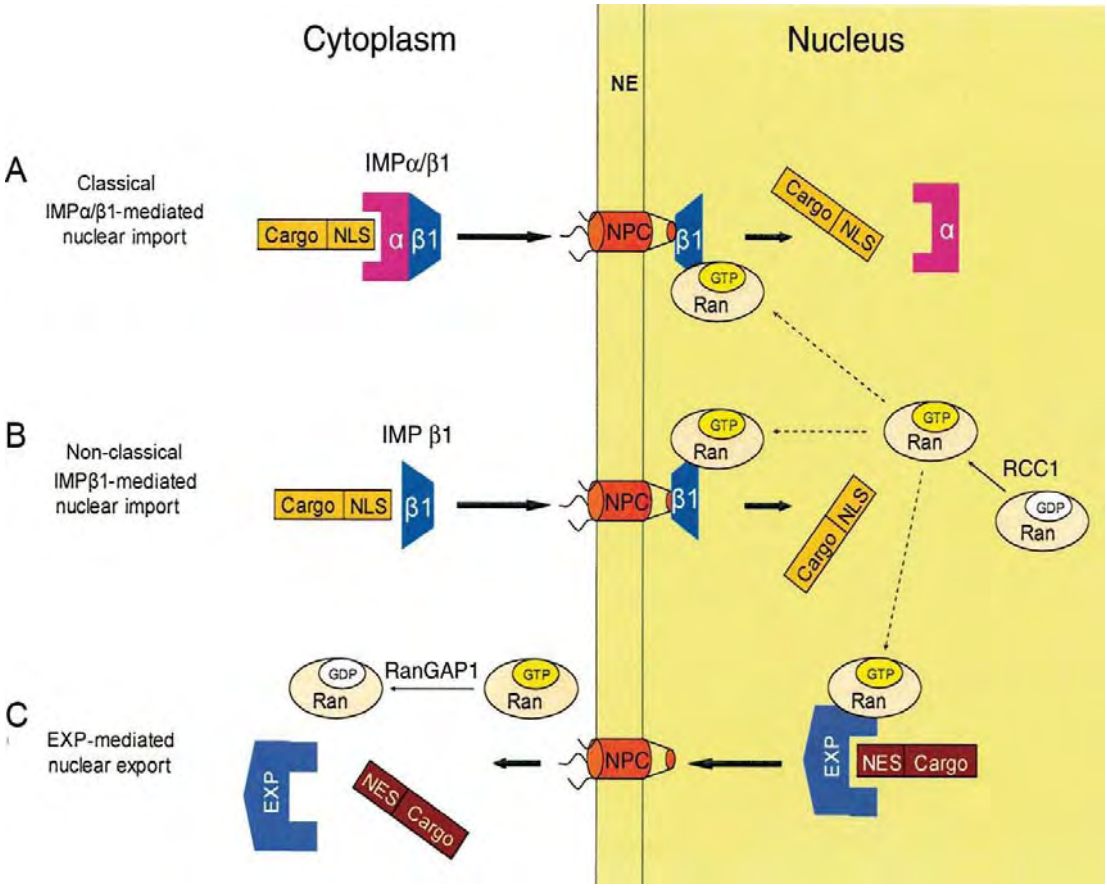


Figure 1.7 Nuclear transport pathways. (A) Classical nuclear import pathway: mediated by importin α (IMP α)/ importin β1 (IMP β1) bound to the NLS and effected by RanGTP hydrolysis. (B) Non-classical import pathway: mediated by IMP β1 bound directly to the NLS and effected by RanGTP hydrolysis. (C) Nuclear export pathway: mediated by exportins (EXP) bound to NESs and RanGTP. In all cases, upon passing through the nuclear pore complex (NPC), the shuttling carriers are recycled back to their original compartments. Adapted from Poon and Jans (2005).

Maintaining normal gene expression by regulating nuclear import and export of transcription factors is crucial. Indeed, impairment of the nuclear localisation of proteins has been associated with a range of diseases such as Saethre-Chotzen syndrome (El Ghouzzi et al., 2000), Holt-Oram syndrome (Fan et al., 2003), Leri-Weill syndrome (Sabherwal et al., 2004) and DiGeorge syndrome (Stoller and Epstein, 2005). A number of specific mechanisms regulate nuclear transport precisely, including responses to a variety of signals such as hormones, cytokines, cell cycle signals, developmental signals, immune challenges and stress (Poon and Jans, 2005). To date, post-translational modification of signalling molecules via phosphorylation/ dephosphorylation is the best understood mechanism to regulate nuclear transport (Jans et al., 2000). Elucidation of unknown nuclear transport signals and mechanisms will further add to the understanding of specific gene expression regulation.

SUMOYLATION

Another key mechanism for achieving dynamic gene regulation is via post-translational modifications such as phosphorylation, acetylation, methylation, ubiquitination and SUMOylation. These processes can cause rapid changes in protein activity, subcellular localisation, stability, as well as protein interactions. Protein modifications also regulate and orchestrate protein functions in response to changes in a cell's state or its environment, without having to alter their synthesis or turnover rates (Jurgen Dohmen, 2004).

A growing list of transcription factors, including promoter-specific transcription factors, cofactors, and regulators of chromatin structure, have been shown to be post-translationally modified by conjugation to the Small Ubiquitin-related Modifier, SUMO. There are at least four identified SUMO isoforms in mammals, SUMO-1 (also known as sentrin, PIC1, GMP-1, Ub11 and Smt3c, hSmt3), SUMO-2 (sentrin-2 and smt3a), SUMO-3 (sentrin-3 and Smt3b) and SUMO-4. SUMO-2 and SUMO-3 share high sequence homology (~97%) and are therefore commonly referred to as SUMO-2/3. SUMO-1 appears to be the most prominently conjugated isoform under normal conditions and exhibits an overall three-dimensional structure similar to its protein degradation counterpart, ubiquitin (Bayer et al., 1998). SUMO-1 however differs from

ubiquitin in terms of charge and a flexible protruding N-terminal domain. *In vivo*, the majority of SUMO-1 is conjugated to proteins, unlike SUMO-2/3 (Hoege et al., 2002). The latter generally appears to be conjugated to proteins during stress (Saitoh and Hinchey, 2000), although there are examples such as topoisomerase II (Azuma et al., 2003) and CAAT/ enhancer-binding protein (C/EBP)- beta (Eaton and Sealy, 2003) that are modified by SUMO-2/3 under normal physiological conditions. SUMO-4 is encoded by an intron of the human TAB2 gene (Bohren et al., 2004). However, SUMO-4 lacks the capacity to be appropriately processed for conjugation to substrates and has not been studied in detail (Owerbach et al., 2005).

SUMOylation Pathways

All SUMO isoforms are conjugated via a conserved enzymatic cascade in an ATP-dependent manner (Figure 1.8). SUMO is first activated by the formation of a thioester bond between its C-terminal glycine and the catalytic cysteine of the heterodimeric E1 activating enzyme (Aos1/Uba2, also named SAE1/SAE2). SUMO is then transferred to the catalytic cysteine of the E2 conjugating enzyme, Ubc9. Finally, SUMO is attached to the ε-amino group of a specific lysine in the target protein, resulting in an isopeptide bond formation.

The majority of SUMO accepting lysine residues (K) have been shown to lie within the consensus sequence ψKXE, where ψ is a large hydrophobic residue, preferably leucine, isoleucine or valine (Johnson, 2004). The hydrophobic core is a quintessential component for securing interaction with SUMO while the electrostatic interactions promoted by the acidic amino acid residue/s (aspartic and glutamic acid (E)) play a role in the affinity, orientation, and functionality of the substrate-SUMO association (Song et al., 2005; Hecker et al., 2006). Ubc9 binding to the SUMO acceptor site is sufficient for efficient transfer of SUMO to the selected target *in vitro*, but a specific SUMO E3 ligating enzyme might be required for efficient targeted modification *in vivo* (Verger et al., 2003). Several E3 ligases have been identified, including PIAS1 (Kahyo et al., 2001), PIASy (Sachdev et al., 2001) and RanBP2 (Pichler et al., 2002). These enzymes are thought to also confer substrate specificity to the SUMOylation reaction. For example, RanBP2 stimulates SUMO modification of RanGAP1 and Sp100, but not p53 (Pichler et al., 2002).

SUMOylation is a dynamic process that can be readily reversed by specific proteases, such as members of the ubiquitin-like protein (ULP) processing family of enzymes (Li and Hochstrasser, 1999; Li and Hochstrasser, 2000). These proteases play a dual role by first having to process the precursor form of SUMO prior to SUMOylation to expose a di-glycine motif, which is only present in its mature form (Verger et al., 2003). The subsequent activity then releases SUMO from its conjugates and enables it to re-enter the conjugation cycle. Several other related SUMO specific proteases such as SENP2 and SMT3Ip1 have also been shown to have distinct localisation patterns (Nishida et al., 2000; Hang and Dasso, 2002). This distinct feature is thought to be an important determinant of substrate specificity *in vivo* (Gill, 2003).

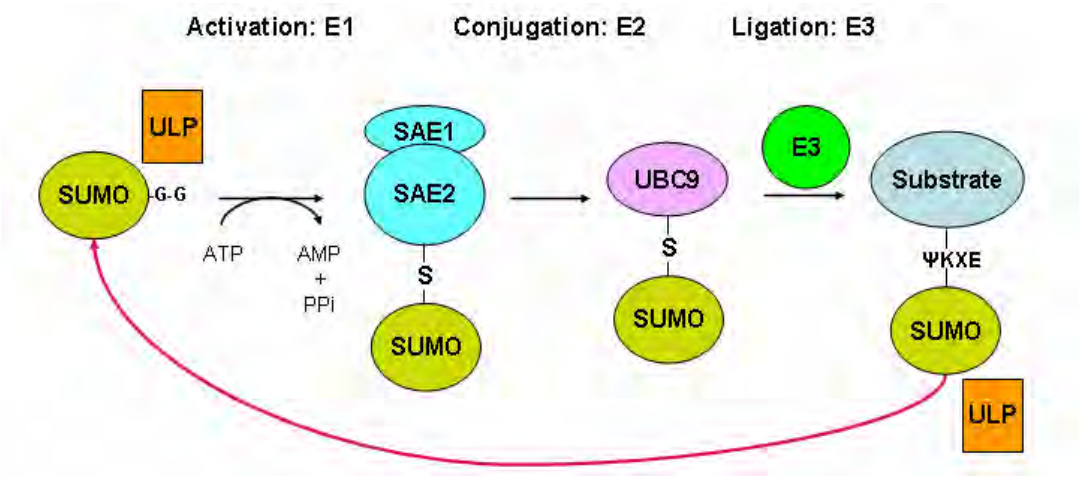


Figure 1.8 The SUMOylation Pathway. The SUMO precursor is hydrolysed by ULPs to expose the double-glycine motif to conjugation. SUMO is then conjugated to target proteins by means of the E1 activating (SAE1/SAE2), E2 conjugating (Ubc9) and E3 ligating enzymes in the order as shown above. The resulting isopeptide bond is stable, requiring de-SUMOylation by an ULP.

Functional Implications of SUMOylation

Despite SUMO's importance, its effects were discovered relatively late, as most SUMO targets are modified at very low steady state levels *in vivo* and upon cell lysis, most SUMOylated proteins are susceptible to de-modification by isopeptidases (Bossis and Melchior, 2006). Nevertheless, with more SUMO targets being identified, their corresponding SUMO mutants have now identified a diverse range of effects resulting from SUMO modification. Indeed, SUMO has been implicated as a possible antagonist of ubiquitin (Desterro et al., 1998; Hoege et al., 2002), and in the regulation of protein-protein interactions (Matunis et al., 1998), subcellular nuclear localisation (Seeler and Dejean, 2001; Pichler and Melchior, 2002), DNA binding (Goodson et al., 2001; Hong et al., 2001) and enzymatic activity (Hardeland et al., 2002).

In the majority of cases studied to date, SUMOylation has been found to repress the activity of transcriptional activators. In the case of transcription factors such as Sp3, GR, Myb and C/EBP, mutations carried out in either the acceptor lysine or other residues in the SUMO consensus sequence led to a dramatic increase in transcriptional activity (Ross et al., 2002; Sapetschnig et al., 2002; Le Drean et al., 2002; Tian et al., 2002; Bies et al., 2002; Kim et al., 2002a). SUMO may alter transcription factor activity by modulating protein-protein interactions important in transcriptional activation or by competing with other post-translational modifications, which share the same target lycine residue (Desterro et al., 1998; Braun et al., 2001). Moreover, there has also been evidence suggesting that transcriptional repression is proportional to the number of SUMOylation sites present, and these inhibitory domains are highly potent and portable. This indicates that SUMOylation does not merely modulate, but may actually provide inhibitory domain functions (Iniguez-Lluhi and Pearce, 2000; Kim et al., 2002a; Yang et al., 2002).

Despite the general trend that SUMOylation decreases transcriptional activity, there have been a few examples in which SUMO modification enhances transcription factor activity. Post-translational modification of heat shock factor-1 and -2 (HSF1; HSF2) by SUMO-1 increases the DNA binding ability of these proteins, while mutation of their SUMO sites reduces transcriptional activity (Hong et al., 2001; Goodson et al., 2001).

In certain instances, including Sp3, LEF-1, HSF1 and TEL, SUMOylation has also been found to regulate subcellular localisation (Ross et al., 2002; Sachdev et al., 2001; Hong et al., 2001; Chakrabarti et al., 2000). SUMOylated Sp3 or LEF-1, which is repressed, accumulates at the nuclear periphery and in nuclear dots, whereas the activated form, lacking SUMO-1 has a more diffused nuclear localisation (Ross et al., 2002; Sachdev et al., 2001).

Some substrates are also SUMOylated and de-SUMOylated upon their passage through the NPC (Seeler and Dejean, 2003; Pichler et al., 2002). Interestingly, SUMOylation of the promyelocytic leukaemia protein (PML) is involved in the formation of nuclear sub-domains called PML oncogenic domains (PODs), and in the recruitment of other nuclear-body associated proteins such as Sp100, Daxx and SUMO-1 itself (Seeler and Dejean, 2001). PODs have proposed roles in transcription, cellular transformation, cell cycle regulation and viral infection, suggesting that SUMOylation may serve to organise specific protein complexes within nuclear bodies, thus regulating their activities negatively or positively (Sachdev et al., 2001; Best et al., 2002).

As more SUMO substrates are identified at a rapid pace, further questions about the affects of SUMOylation on substrate protein function, the determination of substrate-dependent activities of SUMO or the regulation of SUMOylation, are imminent. Answers to these questions will depend on not only the identification of SUMO targets, but more importantly, on experiments that probe for the function of their SUMOylation.

AIMS OF THIS THESIS

There are two major steps in the birth of platelets: first the commitment and differentiation of MKs, followed then by the elaboration and release of their contents, platelets. A single defect at any stage of this process may result in a variety of pathological states, including thrombocytopaenia. An approach that is currently under investigation to treat patients with thrombocytopaenia is the development of drugs that mimic the effects of TPO. However, TPO only stimulates the initial step of growing MKs and not the later step of making proplatelets and platelets. This latter stage of thrombopoiesis is an attractive target for new therapies.

The mechanisms involved in megakaryopoiesis and thrombopoiesis are not fully defined. However, it is widely accepted that transcriptional regulation by lineage specific transcription factors is a major determinant of megakaryopoiesis. In particular, the p45 subunit of NF-E2 plays an essential role in the terminal stages of MK differentiation and platelet production. Therefore, the major aims of this thesis were (1) to enhance MK maturation and platelet release by the overexpression of p45 NF-E2 and (2) to develop a better understanding of the molecular mechanisms, which drive p45 NF-E2 regulation of thrombopoiesis. Such understanding may then enable improved treatment options for platelet disorders.

In comparison to other essential transcription factors such as GATA-1, relatively fewer studies have been carried out on NF-E2. In light of the growing evidence of NF-E2's essential role in thrombopoiesis and selected blood disorders, a dedicated examination of NF-E2 in the context of a primary cell system is fitting. As such, the primary aim was to demonstrate stable expression of exogenous p45 NF-E2 in murine bone marrow cells via a retroviral gene delivery system and study its functional effects on various stages of MK development. More specifically, the aspects of megakaryopoiesis analysed included: (i) progenitor cell proliferation; (ii) CFU-MK colony formation; (iii) MK-specific marker expression; (iv) MK maturation as determined by MK-specific AchE staining; (v) endomitosis; (vi) NF-E2 target gene expression; (vii) proplatelet formation and (viii) platelet release.

After demonstrating the effects of p45 NF-E2 overexpression on MK differentiation *in vitro*, our next aim was to study the effects of enforced p45 NF-E2 expression *in vivo*. This study was carried out under true physiological conditions in immunocompromised mice using a transplant model. The platelet population derived from genetically modified donor cells in the recipient mice, were analysed along with the erythroid and white blood cell populations. In particular, the effects of p45 NF-E2 overexpression on the granulocyte/ myeloid lineage were also studied to some degree both *in vitro* and *in vivo*.

Another facet of gene expression regulation is the precise shuttling of transcription factors in and out of the nucleus. This process is vital for normal cell function as changes in subcellular localisation of transcription factors can result in disease and developmental abnormalities (El Ghouzzi et al., 2000; Fan et al., 2003; Sabherwal et al., 2004; Stoller and Epstein, 2005). Therefore, in Chapter 5, a third aim was to determine: i) the specific sequence that targets p45 NF-E2 to the nucleus; ii) the molecular mechanisms involved in facilitating this process; and iii) the biological impact of mutating p45 NF-E2's nuclear import signal on thrombopoiesis.

Finally, the biological role of p45 NF-E2 SUMOylation in MKs was considered. SUMOylation is a dynamic post-translational modification process that has been implicated in many different biological processes. The SUMOylation target site of p45 NF-E2 has recently been identified, but its role in MK-specific gene regulation is still unknown (Shyu et al., 2005). The SUMO mutant of p45 NF-E2 therefore provides an interesting candidate for further study. It was our final aim to establish the biological role of p45 NF-E2 SUMOylation in MKs by investigating how SUMOylation affects: i) nuclear import; (ii) transcriptional regulation of MK-specific genes; and iii) thrombopoiesis.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

Cell Lines

Phoenix and PT67 cells are both retroviral packaging cell lines derived from 293T and TK-NIH/3T3 cells respectively. PT67 cells were obtained from the American Type Culture Collection (ATCC) and the phoenix packaging cell line was a kind gift from Dr Mark Hulett (Australian National University, Canberra, Australia). The WEHI-3 cell line was purchased from ATCC and WEHI-3 conditioned media was harvested from the cells after 48-72 hours of culture. The following non-haematopoietic cell lines were used in nuclear localisation studies and do not express endogenous p45 NF-E2 (Andrews et al., 1993; Onishi and Kiyama, 2003). HeLa cells are a cervical adenocarcinoma cell line that was obtained from the ATCC. COS-7 cells were originally derived from African green monkey kidney cells that had been immortalised with a version of the SV40 genome (Gluzman, 1981). These cells were obtained from our laboratory stock.

Mice

Purified haematopoietic stem cells were obtained from the bone marrow of C57BL/6J mice. All mice were purchased from the Animal Resource Centre in Perth, Australia and subsequently maintained at the St George Hospital animal facility. The mice were treated with 5-fluorouracil purchased from Mayne Pharma Pty Ltd., VIC, Australia.

Cell Culture Reagents

Dulbecco's Modified Eagle's Medium (DMEM), RPMI 1640, trypsin/ EDTA solution, penicillin-streptomycin solution and geneticin (G418) were purchased from Invitrogen (Grand Island, NY, USA). Media were supplemented with foetal calf serum (FCS; Invitrogen), as indicated. The cell culture plastic-ware was purchased from Nunc™ (Roskilde, Denmark).

The following mouse cytokines were used during the course of this thesis:

Recombinant mouse Flt-3 ligand	R&D Systems, Inc
Recombinant mouse interleukin-3 (IL-3)	R&D Systems, Inc
Recombinant mouse SCF	R&D Systems, Inc

Recombinant human TPO	Genentech, Inc

Other reagents used for cell culture or experiments using mammalian cells are listed below, along with their suppliers.

Lineage Cell Depletion Kit	Miltenyi Biotec GmbH
Lipofectamine	Invitrogen
Lipofectamine 2000	Invitrogen
Luciferase Assay System	Promega
MethoCult® collagen based media	Stem Cell Technologies
Methylcellulose culture medium	Stem Cell Technologies
MTT	Promega
Passive Lysis Buffer	Promega
Polybrene	Sigma
Propidium Iodide	Sigma
Prostaglandin E1 (PGE1)	Sigma
Proteinase-Activated Receptor 4 (PAR4) Thrombin Receptor-Activating Peptide (AYPGFK)	BioScientific Pty Ltd
Streptavidin Microbeads	Miltenyi Biotec GmbH

Bacterial strains

The following bacterial strains were obtained from Invitrogen and used for plasmid DNA preparations and cloning manipulations:

Escherichia Coli (*E.coli*) DH5α: F- Φ80dLacZΔM15 Δ(LacZYA-argF)U169 deoR recA1 endA1 hsdR17 (r_k^-, m_k^+) phoA supE44 thi-1 gyrA96 relA1, λ^-.

E.coli TOP10: F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG.

The following bacterial strain was purchased from Stratagene and used for site-directed mutagenesis transformations:

Ultracompetent *E.coli* XL10 Gold: Tet^R Δ(mcrA) 183 Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac The [F' ProAB lacIqZΔM15 Tn10 (Tet^R) Amy Cam^R].

Enzymes

Restriction enzymes were purchased from either New England BioLabs or Promega. Other enzymes used during this project include:

Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT)	Invitrogen
PfuUltra™ High-Fidelity DNA Polymerase	Stratagene
Platinum SYBR® Green qPCR Supermix UDG	Invitrogen
Taq DNA Polymerase	Promega
T4 DNA Ligase	Promega
T4 polynucleotide kinase	Promega

Molecular Biology Reagents/ Kits

Reagents used during the experimental manipulation of DNA/ RNA were as follows:

2-Log DNA Ladder (0.1-10kb)	New England BioLabs
Maxi Plasmid Purification kit	Invitrogen
Microspin™ G-25 Columns	Amersham Biosciences
QIAquick® Gel Extraction kit	Qiagen
QuikChange® II XL Site-Directed Mutagenesis kit	Stratagene
SYBR Safe® DNA Gel Stain	Invitrogen
TRIzol® Reagent	Invitrogen
Wizard® *Plus* SV miniprep kits	Promega

Protein Reagents/ Kits

The following reagents were required for protein purification and detection:

Benchmark™ Prestained Protein Ladder	Invitrogen
Bio-Rad Protein Assay Reagent	Bio-Rad
Glutathione Sepharose™ 4B Beads	GE Healthcare
Immobilon™-P polyvinilidene (PVDF) Membranes	Millipore
PageRuler™ Prestained Protein Ladder	Fermentas Life Sciences
PageBlue™ Protein Staining Solution	Fermentas Life Sciences
Ponceau S Solution	Fluka Biochemika
SeeBlue® Prestained Protein Ladder	Invitrogen
TNT® T7 Coupled Reticulocyte Lysate System	Promega

Western Lightening™ Chemiluminescence Reagent *Plus*	PerkinElmer

General Chemicals and Reagents

The general chemicals and reagents used are listed below, along with their suppliers.

Acetic Acid	Ajax Fine Chemicals
Acetylthiocholine Iodide	Sigma
Acid Citrate-Dextrose (ACD)	Sigma
Adenosine Diphosphate (ADP)	Sigma
Adenosie Triphosphate (ATP)	Promega
Agarose (DNA grade)	Progen Industries Ltd
30% Acrylamide/ Bis Solution, 29:1 (3.3% C)	Bio-Rad
Albumin, Bovine Serum (BSA)	Sigma
Ampicillin (AMP)	CSL Ltd
Ammonium Persulphate	Sigma
β-mercaptoethanol (βME)	Sigma
BigDye® terminator version 3.1	Applied Biosystems
Bacto Agar	Difco
Boric Acid	Sigma
Chloroform	Sigma
Crystal Violet	Sigma
Cupric Sulphate	Sigma
Dextran Sulphate	Sigma
Diethylpyrocarbonate (DEPC)	Sigma
Deoxynucleotide triphosphates (dNTPs)	Promega
Dimethylsulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
Ethylenediaminetetraacetic Acid (EDTA)	Sigma
Ethanol	AnalaR® BDH
Formaldehyde	Sigma
Glycerol	Sigma
Glycine	Amresco® Astral Scientific
Glucose	Sigma

Isopropanol	AnalaR® BDH
Igepal CA-630 (NP-40)	Sigma
Isopropyl-β-D-thiogalactopyranosid (IPTG)	Progen Industries Ltd
Kanamycin (KAN)	Sigma
Luria Bertani (LB) Media Capsules	MP Biomedicals
Magnesium Chloride	Sigma
Methanol	AnalaR® BDH
N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES)	Sigma
Phenylmethylsulfonide Fluoride (PMSF)	Sigma
Phosphate Buffered Saline (PBS) tablets	Amresco® Astral Scientific
Poly (dI-dC)•poly (dI-dC)	Sigma
Polyoxyethylenesorbitanmonolaurate (Tween™20)	Sigma
Potassium Chloride	Sigma
Potassium Ferricyanide	Sigma
Protease Inhibitor Cocktail	Sigma
RNAase A	Invitrogen
S.O.C. medium	Invitrogen
Sodium Acetate	Sigma
Sodium Bicarbonate	Sigma
Sodium Citrate	Sigma
Sodium Chloride	Sigma
Sodium Dodecyl Sulfate (SDS)	Sigma
Sodium Phosphate	Sigma
N,N,N',N'-tetramethylethylenediamine (TEMED)	Sigma
Tris (ultra pure grade)	MP Biomedicals
Triton® X-100	Sigma
Trypan Blue Solution (0.4%)	Sigma

Antibodies

The following antibodies were used to examine protein expression by western blotting (dilutions are shown in brackets):

β-actin (1:5000)	Sigma
Goat anti-rabbit IgG-HRP (1:3000)	Santa Cruz Biotechnology
Goat IgG-HRP (1:3000)	DakoCytomation
GMP-1 (SUMO-1) (1:300)	Invitrogen
Karyopherin (importin) β1 (sc-1863; 1:200)	Santa Cruz Biotechnology
p45 NF-E2 (sc-291;1:200)	Santa Cruz Biotechnology
Rabbit anti-mouse IgG-HRP (1:3000)	DakoCytomation
SUMO 2/3 (1:1000)	abCam

The antibodies listed below were used at their respective dilutions in cellular assays including magnetic cell separation (MACS) and the examination of cell surface/ cellular proteins, via flow cytometry or confocal microscopy:

Alexa Fluor® 594 goat anti-rabbit IgG (1:1000)	Invitrogen
Annexin V-PE (1:20)	BD Pharmingen
Allophycocyanin (APC)-conjugated anti-rat IgG (1:50)	BD Pharmingen
Biotin-conjugated goat anti-rat IgG	BD Pharmingen
Rabbit anti-mouse CD62P (P-Selectin)	Chemicon Australia
Rabbit anti-V5 (1:1500)	Sigma
Rat anti-mouse CD41 (1:100)	BD Pharmingen
Rat anti-mouse CD42a (1:100)	Emfret Anlytics
Rat anti-mouse CD42b (1:100)	Emfret Analytics
Rat anti-mouse CD61 (1:100)	Emfret Analytics
Rat anti-mouse 11b (Mac-1) (1:2000)	BD Pharmingen
Rat anti-mouse Ly-6G (Gr-1) (1:2000)	BD Pharmingen
Rat anti-mouse CD45R/B220 (1:2000)	BD Pharmingen
Rat anti-mouse CD5 (Ly-1) (1:2000)	BD Pharmingen
Rat anti-mouse Ter-119 (Ly-76) (1:2000)	BD Pharmingen
Rat IgG_1, κ isotype control (1:100)	BD Pharmingen
R-phycoerythrin (PE) labelled goat anti-rat Ig (1:100)	BD Pharmingen

R-PE-conjugated anti-rabbit IgG (1:100) Sigma

Radioisotopes

[α-^{32}P]-dATP (540 μCi, 3000 Ci/mM) was used for EMSA and purchased from PerkinElmer Pty Ltd. Redivue PRO-mix [^{35}S]-methionine (>37 TBq/mM, >1000 Ci/mM) was obtained from Amersham Biosciences (GE Healthcare) and used for *in vitro* translation reactions.

Plasmids

The names and sources of the plasmids used in this thesis are listed in Table 2.1.

Table 2.1 Details of plasmids used during the course of this thesis.

Plasmid Name	Vector Backbone	Insert Details	Source
pKMV	pKMV-eGFP	Retroviral vector cloning vector containing bicistronic eGFP reporter cDNA	SP
pKMV-NFE2	pKMV	Human p45 NF-E2 cDNA corresponding to aa 1-373	EF
pKMV-K368R	pKMV	As above, with mutation	EF
pKMV-V5	pKMV	V5 tag cDNA corresponding to aa 1-15 cloned into KMV via EcoRI and Xho I multiple cloning sites	EF
pKMV-V5NFE2	pKMV	cDNA of V5 tag fused upstream of Human p45 NF-E2 cDNA	EF
pKMV-V5R271-273A	pKMV	As above, with mutation	EF
pKMV-V5RK284-287A	pKMV	As above, with mutation	EF
pKMV-V5K368R	pKMV	As above, with mutation	EF
pDONR™	-	Gateway vector	AP
pDEST47	pcDNA3	Gateway vector for C-terminal GFP fusion	AP
pDEST47-NFE2	pcDNA3	Human p45 NF-E2 cDNA corresponding to aa 1-373 fused upstream to GFP	EF
pDEST47(1-209)	pcDNA3	Human p45 NF-E2 cDNA corresponding to aa 1-209	EF
pDEST47(1-209)R167-169A	pcDNA3	As above, with mutation	EF
pDEST47(210-266)	pcDNA3	Human p45 NF-E2 cDNA corresponding to aa 210-266	EF
pDEST47(267-293)	pcDNA3	Human p45 NF-E2 cDNA corresponding to aa 267-293	EF
pDEST47(294-373)	pcDNA3	Human p45 NF-E2 cDNA corresponding to aa 294-373	EF
pDEST53	pcDNA3	Gateway vector for N-terminal GFP fusion	AP
pDEST53-NFE2	pcDNA3	Human p45 NF-E2 cDNA corresponding to aa 1-373 fused downstream to GFP	EF
pDEST53-R271-273A	pcDNA3	As above, with mutation	EF
pDEST53-RK284-287A	pcDNA3	As above, with mutation	EF
pDEST53-R271A	pcDNA3	As above, with mutation	EF
pDEST53-R272A	pcDNA3	As above, with mutation	EF
pDEST53-R273A	pcDNA3	As above, with mutation	EF

Plasmid Name	Vector Backbone	Insert Details	Source
pDEST53-E295STOP	pcDNA3	Human p45 NF-E2 cDNA fragment corresponding to aa 1-295 fused downstream to GFP	EF
pDEST53-E295STOP-R271-273A	pCDNA3	As above, with mutation	EF
pRL-Tk	-	Renilla luciferase reporter plasmid	JK
pRBGP2-Luc	pGL2-basic	Contains three copies of the NF-E2 binding sequence from the chicken β-globin enhancer upstream of the TATA box and luciferase gene	MY
pcDNA3-GAL4-SUMO1	pcDNA3	cDNA of GAL4 fused upstream to SUMO1 cDNA	JP
pGEX-2T-IMPα (PTAC58)	pGEX-2T	GST fused to importin α cDNA corresponding to aa 1-529	JP
pGEX-2T-IMPβ (PTAC97)	pGEX-2T	GST fused to importin β cDNA corresponding to aa 1-876	JP

Abbreviations: AP=Alana S Philips; EF=Ee-ling Fock; JK=Juliana Kwok; JP= Jose Perdomo; MY=Masayuki Yamamoto (Tsukuba University, Japan); SP=Shu Pan

Oligonucleotides

All oligonucleotides used in this thesis were synthesised by Sigma-Genosys as purified, desalted stocks and were resuspended at a final concentration of 100 μM in Milli-Q water. The oligonucleotides were then further diluted in Milli-Q water to 10 μM working stocks for PCR. The sequences of specific oligonucleotides are shown in the appropriate sections.

METHODS

Standard Molecular Biology Techniques

The range of general molecular biology techniques such as agarose gel electrophoresis, SDS-polyacrylamide gel electrophoresis, restriction enzyme digestion, PCR, sub-cloning of cDNAs, isolation and purification of plasmid DNA and RNA, bacterial transformation and DNA sequencing were performed by the standard protocols. Besides the above mentioned techniques, other methodologies of specific importance to this research are outlined under the headings below.

Cell Culture

All cell lines and primary murine cells used in this thesis were maintained in DMEM medium, supplemented with 10% FCS. The cells were treated with cytokines as indicated in the following sections and appropriate figure legends. Generally, primary cells were supplemented with 10 ng/mL of mouse IL-3, SCF, and Flt-3 Ligand prior to and during infection. Polybrene (8 μg/mL) was added during retroviral infection and the medium was supplemented with 50 ng/mL TPO and 10 ng/mL IL-3 during MK differentiation. All cells were maintained in a humidified incubator at 37°C, with 5% CO_2.

Murine Bone Marrow Cell (BMC) Isolation and Culture

Murine BMCs were harvested from C57BL/6J mice as previously described (Persons et al., 1999). Briefly, BMCs were harvested from 8-10 week old C57BL/6J mice either 2 or 8 days after treatment with 150 mg/kg 5-fluorouracil (5FU) intraperitoneally (hereafter referred to as 2 day 5FU and 8 day 5FU BMCs, respectively). 5-fluorouracil is a cytotoxic drug commonly used in chemotherapy to kill dividing cells. Mice treated with 5FU have the majority of their cycling progenitor cells eliminated and their stem cells driven out of rest state. Depending on the day of harvest after 5FU treatment, either an enriched population of primitive stem cells (2 day 5FU) or an enriched population of CFU-MK progenitor cells (8 day 5FU) (Bradley et al., 1989) can be obtained. The marrow was obtained by flushing the femurs and tibias with DMEM containing 10% FCS. Mature red blood cells were lysed by incubating with red blood

cell lysis buffer (Sigma). The remaining BMCs were subjected to magnetic activated cell sorting (MACS) depletion of lineage positive cells (Randall and Weissman, 1997). Briefly, cells were resuspended to 10^7 cells/mL in PBS containing 0.5% BSA and 2 mM EDTA and incubated with a cocktail of biotinylated antibodies: Ly-6G (Gr-1), CD11b (Mac-1), CD45R (B220), CD5 and Ter-119. These antibodies recognise myeloid-, lymphoid- and erythroid-specific cell surface antigens and were incubated with the BMCs at a 1:2000 dilution. After washing, the cells were incubated with streptavidin magnetic beads, and applied to a LS Type MACS separation column, resulting in a purified population of 5FU lineage depleted (Lin^-) cells. The purified 5FU Lin^- BMCs were maintained in DMEM/ 10% FCS medium with the appropriate cytokines.

Retroviral Overexpression of p45 NF-E2

Transient Transfection of Pheonix and PT67 cells

Retroviral overexpression of p45 NF-E2 was carried out using a two-step 'ping-pong' method with primary and secondary viral packaging cell lines. Firstly, transient transfections of phoenix packaging cells were carried out in 6 well plates. On the day prior to transfection, phoenix cells were seeded at 2 x 10^5 cells/mL in 2 mL DMEM/ 10% FCS. The following day, the cells were transiently transfected with 4 μg of the appropriate retroviral vector, using Lipofectamine 2000, as per the manufacturer's instructions. On the same day, PT67 cells were seeded at a density of 2 x 10^5 cells/mL in 2ml of DMEM/ 10% FCS such that cells were at 70-80% confluency the following day. Viral supernatant was collected from phoenix cells 24 hours post-transfection and filtered through a 45 μm filter directly onto the PT67 monolayer. Polybrene was then added to the PT67 cells at a concentration of 8 μg/mL. The PT67 cells were repeatedly infected as described, every 24 hours for 2-3 days. Following infection, the percentage of $eGFP^+$ cells was analysed by flow cytometry and collected by fluorescence-activated cell sorting (FACS) if higher infection efficiency was required. The infected PT67 cells were grown to confluency before being frozen down in liquid nitrogen or used directly for co-cultivation with the 5FU Lin^- BMCs.

Retroviral Transduction of 5FU Lin$^-$ BMCs

Retroviral transduction of Lin$^-$ BMCs was carried out two days after or on the day of harvesting for 2 day 5FU and 8 day FU cells respectively. The BMCs were co-cultured with transduced PT67 cells for 48 hours in the presence of 8 μg/mL polybrene, 10 ng/mL of mouse IL-3, SCF, and Flt-3 Ligand. Non-adherent BMCs were then rinsed off the PT67 monolayer, pelleted, and resuspended in fresh culture medium with 50 ng/mL TPO and 10 ng/mL IL-3. When required, eGFP$^+$ viable cells were sorted by fluorescent activated cell sorting (FACS) using a FACStar Plus cell sorter (BD Biosciences, CA, USA). Cells were differentiated for 5-7 days before further analyses were performed.

Megakaryocytic Differentiation Assays

Phenotype Analysis by Immunostaining

Cultured BMCs were harvested, washed and incubated with either purified rat anti-mouse CD41, CD42a or CD42b monoclonal antibody for 30 minutes at 4°C, followed by PE-conjugated anti-rat IgG antibody for another 30 minutes. An isotype antibody was used simultaneously as a negative control. A total of 10^4 eGFP$^+$ cells were then analysed by flow cytometry performed by a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA).

MK-Specific Acetylcholinesterase (AchE) Staining

AchE staining to identify MKs was carried out as originally described by Karnovsky and Roots (Karnovsky and Roots, 1964). Briefly, 10^4 transduced cells were transferred onto glass slides by cytospin then incubated at room temperature for 3-4 hours in 0.1 M sodium phosphate pH 6.0 containing 10 mg acetylthiocholine iodide, 0.05 M tri-sodium citrate, 3 mM copper sulphate and 0.5 mM potassium ferricyanide. The samples were then fixed with ethanol and looked at under the microscope. A total of 10^4 cells were counted.

DNA Ploidy Analysis

DNA content of differentiated cells was analysed by flow cytometry as previously described (Fujimoto et al., 2003). Sorted cells were gently collected from culture after 5 days and labelled with purified rat anti-mouse CD41 monoclonal antibody followed by APC-conjugated anti-rat IgG. The cells were then washed and incubated overnight at

4°C in hypotonic sodium citrate (0.1%) solution containing 50 μg/ml propidium iodide. RNAase A (20 μg/ml; Invitrogen, CA, USA) was added 30 minutes before flow cytometry analysis.

Cell Proliferation Assays

To evaluate the effects of p45 NF-E2 overexpression on cell proliferation, transduced 8 day 5FU Lin^- cells were sorted by FACS and seeded at a density of 3.5 x 10^4 cells/well in a 24 well plate. The cells were grown in 50 ng/mL TPO and 10 ng/mL IL-3 liquid culture for 7 days with cell counts taken on day 3, 5 and 7 using trypan blue exclusion of non-viable cells. Concurrently at the same time points, MTT assays were carried out in triplicate on the same batch of BMCs. Cells were seeded into four 96 well plates at a density of 5 x 10^3 cells/ well and grown in the presence of TPO and IL-3. At each time point, a MTT assay was carried out on a different plate as per manufacturer's instructions (Promega).

Colony Forming Assays

Lin^- BMCs were harvested from 2 day 5FU treated mice and stimulated in 10 ng/mL of IL-3, SCF and Flt-3 Ligand for 2-4 days before retroviral transduction. Transduced cells were sorted by FACS and resuspended in MethoCult® collagen based media with 50 ng/mL TPO and 10 ng/mL IL-3 or methylcellulose culture medium containing 10% WEHI-3 conditioned media for CFU-MK and CFU-GM assays respectively. CFU-MK and CFU-GM cultures were plated out in quadruplicate according to the manufacturer's instructions. Cultures were incubated at 37˚C in a 5% CO_2 humidified atmosphere and colonies were enumerated after 7 days. AchE staining was carried out to identify CFU-MK colonies. Colonies were photographed using a Leica IM50 v1.20 microscope (Leica Microsystems AG, Germany).

Quantitative Real-time Reverse Transcription (RT) PCR.

Total RNA was extracted using TRIzol® from 1-2 x 10^6 transduced 8 day 5FU BMCs as per the manufacturer's instructions. cDNA for real-time PCR was then generated from 1 μg RNA using M-MLV reverse transcriptase and oligo(dT), as per manufacturer's protocol. Real time PCR was carried out using the Platinum SYBR® Green qPCR supermix, as per manufacturer's instructions. Absolute gene expression levels were

calculated by generating standard curves for each gene. Data were collected using software from Corbett Research, Australia, and analysed using Michael Pfaffl's relative quantification method. Target quantities were normalised against the expression of the housekeeping gene, (GAPDH). All quantities were expressed as n-fold relative to the calibrator (pKMV), which was defined, as a value of 1. The primers were designed to span exon-exon boundaries, so as to avoid amplification of genomic DNA. Water controls were run in parallel with each reaction. The sequences of the primers used (5'-3') in this thesis were as follows:

β1- tubulin
Sense: GCTGCTGTCCATTCAGACAA
Antisense: GCTCAGAGACCCTGGTGAAG
Rab27b
Sense: CCAGACCAAAGGGAAGTCAA
Antisense: GTGTCCGGAACCTGTGTCTT
Thromboxane Synthase (TXS)
Sense: GAGGTGCTGGGACAACGTAT
Antisense: AAAGGGCAGGTATGTGAACG
Caspase 12
Sense: TGGCTCTCATCATCTGCAAC
Antisense: GGATGCCATGGGACATAAAC
p45 NF-E2
Sense: TTATCACAGCTGCCTGTTGG
Antisense: TTCTGGGACATGGGGAGTAG
GAPDH
Sense: CCTGCACCACCAACTGCTT
Antisense: GGCCCATCACGCCACACTTT

In Vitro Platelet Analysis by Flow Cytometry

Platelets released into culture supernatant were determined by flow cytometry using a method adapted from Fujimoto et al (2003). Following viral infection, equal numbers of $eGFP^+$/ $CD41^+$ 8 day 5FU Lin^- cells were seeded in fresh culture medium containing 50 ng/mL TPO. On day 7 of differentiation, the culture medium was centrifuged at 150g for 20 minutes to remove nucleated large cells. The supernatant was then centrifuged at 900g for 10 minutes and the pellet resuspended in PBS containing 0.5% BSA and 2 mM EDTA. The cells were then incubated with purified rat anti-mouse CD41or CD42b monoclonal antibodies, followed by PE-goat anti-rat IgG. Finally, the cells were washed and analysed by flow cytometry. A single platelet gate was created based on the forward- and side-scatter profiles of adult mouse peripheral platelets. By flow cytometry, data from 100 µL aliquots were collected several times during a continuous flow, and the number of platelet sized and $eGFP^+$/ $CD42b^+$ particles were determined.

Functional Studies of In Vitro Produced Platelets

The day prior to performing the assay, the equivalent of 1 µM prostaglandin E1 (PGE1) was added to the BMC culture medium. On the following day, the culture medium was collected and a 1:9 volume of ACD solution added before centrifugation at 150g for 20 minutes. The supernatant was then centrifuged at 900g for 10 minutes and the pellet resuspended in 10 mM HEPES/ NaOH (pH 7.4) buffer containing 140 mM NaCl and 2.5 mM $CaCl_2$. Platelets were stimulated with 10 µM ADP or 500 µM proteinase-activated receptor 4 (PAR4) thrombin receptor-activating peptide AYPGFK for 15 minutes at room temperature in the dark. After fixation with 2% paraformaldehyde for 1 hour, the cells were washed and incubated with anti-P-selectin rabbit polyclonal serum followed by PE-conjugated anti-rabbit antibody. The cells were then analysed by flow cytometry.

To determine Annexin V binding, the cells were stimulated and fixed as previously mentioned. The cells were washed and incubated with 5 µL Annexin V-PE for 15 minutes at room temperature in the dark before being analysed by flow cytometry. For all functional assays, mouse peripheral blood platelets were stimulated, stained and analysed simultaneously as positive controls. Negative control cells were resuspended

in the presence of 2 mM EDTA, instead of $CaCl_2$, fixed without stimulation, stained and analysed simultaneously.

In Vivo Platelet Analysis of Transplanted Mice

To study the effects of p45 NF-E2 overexpression *in vivo*, transplantation experiments using lethally irradiated mice were performed. C57BL/6J mice were administered a single dose of 8.5 Gy total body irradiation using a linear accelerator. In each experiment, the equivalent number of (2-3.8 x 10^5) $eGFP^+$ 8 day 5FU Lin^- cells/ animal were washed and resuspended in DMEM containing 10% FCS before being transplanted by tail vein injection into the lethally irradiated mice. Beginning 6 days post-transplantation, peripheral blood samples from the recipients were obtained by venous puncture of the lower limb and full blood counts for each sample determined by an automated haematology analyser (Sysmex Asia Pacific, Woodlands, Singapore). Based on the forward- and side-scatter profiles of normal adult mouse peripheral blood, the various cell and platelet populations were gated by flow cytometry. The red blood cell (RBC) and platelet populations were confirmed by Ter-119 and CD41, CD42b immunostaining respectively. White blood cell (WBC) populations were obtained by lysis of the RBCs and confirmed using Mac-1, Gr-1, CD5 and B220 immunostaining. eGFP expression within each population was also analysed by flow cytometry .

Western Blotting

Preparation of Whole Cell Lysates

Whole cell extracts from BMCs or HeLa cells were prepared from >1 x 10^6 cells. Cells grown in 25cm^2 tissue culture flasks were washed twice in 10 mL of ice-cold PBS containing protease inhibitors. The cells were pelleted at 1250g for 10 minutes at 4ºC. All subsequent steps were performed on ice using pre-chilled reagents and equipment in a 4ºC cold-room. The supernatant was removed and the cell pellet resuspended in a volume of 1x Laemmli buffer (0.25 mM Tris/ 1.92 mM glycine/ 0.1% SDS/ 40 µL /mL 25x protease inhibitor cocktail solution) equal to that of the cell pellet. The homogenate was then transferred to an eppendorf tube for successive steps. Cells were vortexed and sonicated before being frozen at -80°C for 10 minutes and subsequently thawed on ice. The cell debris was then pelleted by centrifugation at 12,000g for 15 minutes at 4°C.

The supernatant was transferred to a fresh eppendorf tube and stored at -80ºC until required. The protein concentration of the extracts was determined using the Bio-Rad protein assay, as per manufacturer's instructions.

Western blotting

For western blotting experiments, protein samples were subject to SDS- polyacrylamide gel electrophoresis (PAGE) on 8% or 10% Tris-glycine polyacrylamide gels, at 30mA for 60-90 minutes. The proteins were then transferred to Immobilon®-P PVDF membranes for 1 hour at 100V or overnight at 30V, 4°C. Following transfer, membranes were blocked for 1-2 hours in 5% skim milk/ PBS/ 0.05% Tween™-20 (PBS-T) at room temperature. The primary antibodies were diluted in the blocking solution and incubated with the membrane for two hours. Four 10 minute washes were then carried out on the membrane using PBS-T before the membrane was incubated for one hour with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000 dilution in blocking solution). Another four 10 minute washes were carried out before the immunoreactive bands were visualised using the Western Lightening™ Chemiluminescence Reagent *Plus* and developed with Kodak reagents. To re-probe the membrane, it was incubated in 50 mM Tris/ 2% SDS/ 100 mM βME for 30 minutes followed by extensive PBS-T washing before being re-blocked overnight in 5% skim milk/ PBS-T. The membrane was then incubated with fresh antibodies as normal. The immunoreactive signals were quantified by densitometry (Model GS-700, Bio-Rad). To ensure equal loading, β-actin was either used as an internal control or the membrane was stained with Ponceau S Solution, as per manufacturer's instructions.

Mutagenesis

Mutagenesis was carried out using the QuickChange® II XL Site-Directed Mutagenesis Kit as per manufacturer's instructions. Briefly, a PCR reaction was carried out using PfuUltra™ High-Fidelity DNA Polymerase and specifically designed primers containing the desired mutations. Following cycling, the non-mutated parental DNA was digested with 1 μL of DpnI for 1 hour at 37°C, before being transformed into XL10-Gold® ultracompetent cells as per manufacturer's protocol. DNA sequencing was subsequently carried out on minipreps to ensure the desired mutations were achieved.

Transient Transfections

Transient transfections of HeLa and COS-7 cells were carried out in 6-well plates using Lipofectamine 2000 at a concentration of 2.5 μL per 1 μg of DNA according to the manufacturer's instructions. Cells were seeded on coverslips at 1 x 10^5 cells/mL, in 2 mL DMEM/ 10% FCS. The following day 2-4 μg of plasmid DNA in 250 μL of optiMEM was combined with 10 μL of Lipofectamine 2000 in 250 μL of optiMEM for each transfection. This mixture was incubated for approximately 30 minutes at room temperature, before being added directly to the cells in culture. $eGFP^+$ cells were analysed by confocal microscopy 48 hours after transfection. Briefly, the cells were washed twice with PBS, fixed with 4% paraformaldehyde for 20 minutes at room temperature and washed again with PBS. Coverslips were mounted onto slides using gelatin glycerol (Sigma) and the cells were viewed with an Olympus confocal microscope (Olympus, Tokyo, Japan). Images were acquired using Olympus Fluoview software, version 4.3, FV300 (Olympus Optical Co. Ltd.).

Luciferase Assays

COS-7 cells were seeded at 3 x 10^5 cells per well in 6-well plates. The following day cells were transiently transfected with 15 ng phRG-Tk renilla control reporter vector, 600 ng of the pRBGP2 reporter plasmid and 1.4 μg of wildtype or mutant p45 NF-E2 expression vectors using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen). The cells were assayed for luciferase activity 48 hours post-transfection using the Dual Luciferase Assay Kit (Promega) as per manufacturer's protocol. Briefly, cells were washed with PBS and lysed with 0.5x Passive Lysis Buffer (Promega) containing complete protease inhibitor. 50 μL of the cell lysate was assayed with 50 μL of the luciferase assay reagent with a TD20/20 luminometer (Turner Designs, Sunnyvale, CA). Each experiment was performed at least three times and in duplicate. The results were normalised against the renilla luciferase control readings and are presented as average fold increases in firefly luciferase, compared to the empty pDEST53 vector. To confirm p45 NF-E2 protein was expressed at similar levels in transiently transfected cells, western blot analysis was carried out on the cell lysates as previously described. Membranes were probed using the rabbit anti-p45 NF-E2

antibody (sc-291; 1:200 dilution), followed by an anti-rabbit-HRP antibody (DakoCytomation; 1: 3000 dilution).

Immunofluorescence Staining

Nuclear localisation of exogenous p45 NF-E2 was visualised in transduced Lin^- BMCs by immunofluorescence detection of the V5 tag, which was fused upstream to p45 NF-E2. Transduced cells were harvested from culture, washed twice and resuspended in PBS at a density of 10^2 cells/μL. Cells were then either transferred to glass slides by cytospin or air dried directly onto chamber slides. The cells were fixed in 70% ethanol and blocked in 2% rabbit serum/ 1% BSA solution for 30 minutes at room temperature. The primary anti-V5 antibody was diluted (1:1500) in 5% rabbit serum and incubated with the cells for 1 hour. Following four washes with PBS, the secondary Alexa Fluor 594 antibody diluted in 1% BSA (1:1000) was applied for 45 minutes in the dark. The slides were washed 4x with PBS and coverslips were mounted onto the cells using glycerol. The cells were viewed with an Olympus confocal microscope (Olympus, Tokyo, Japan) and images were acquired using Olympus Fluoview software, version 4.3, FV300.

siRNA

Small interfering RNA molecule (siRNA) knockdown experiments targeting exon 3 and exon 9 of the human importin β gene (Ambion, Austin, TX, USA) were carried out as previously described (Philips et al., 2007). A negative control siRNA that has been shown not to bind to any known human sequence was also purchased (Ambion). The sequences of the sense strands (5'-3') are shown below:

Importin β exon 3 siRNA: GGAAACAGUCAGGUUGCCAdTdT
(Catalogue #AM16706; siRNA ID #11125)
Importin β exon 9 siRNA: GCACACCAGCAAGUUUUAUdTdT
(Catalogue #AM16706; siRNA ID #145041)
Negative control siRNA: sequence not provided
(Catalogue #4611)

The annealed double stranded siRNAs were resuspended in the provided nuclease-free water to 25 μM. Transfection of the siRNAs was performed using siPORT NeoFX

(Ambion) according to the manufacturer's instructions. Briefly, 2.3 x 10^5 HeLa cells were transfected with 100 pmol siRNA and 5 μL NeoFX reagent for 48 hours. The cells were subsequently transfected with GFP-p45 NF-E2 expression vector using Lipofectamine 2000, as previously described. After 24 hours, the siRNA effects on p45 NF-E2 nuclear localisation were examined by confocal microscopy. Western blot analyses were performed to assess importin β knockdown 48 and 72 hours after siRNA transfection.

GST Pull-down Assays

Protein Expression and Purification

Importin α and importin β proteins were produced by soluble expression in the *E.coli* strain BL21 using standard techniques. A small scale expression protocol was utilised to express the GST-fusion proteins. Briefly, 5 mL of Luria Bertani (LB) broth containing 100 ng/mL AMP (Sigma) was inoculated with a single bacterial colony clone of the expression vector of interest and allowed to grow overnight at 37°C with shaking (225 rpm). The following day, the culture was used to inoculate 250 mL LB-AMP, and grown to an OD of 0.4-0.6 before being induced with 50 μL IPTG (0.5M). The culture was then returned to the shaker and grown overnight with shaking at 25°C. The culture was then pelleted by centrifugation at 5000g and washed twice with PBS.

The pellet was resuspended in 5 mL of chilled lysis buffer (50 mM Tris-HCl pH 7.5/ 150 mM NaCl/ 0.1 mM DTT/ 0.5 mM PMSF/ 40 μL/mL 25x protease inhibitor cocktail/ 0.5% Triton X-100) and the suspension was left on ice for 15 minutes. Subsequently, the cells were then lysed by 6-8 x 20 seconds of sonication on ice. Cell debris was removed by centrifugation at 10,000g for 15 minutes. The supernatant, containing the soluble protein was transferred to a fresh 15 mL tube and incubated with 200 μL Glutathione Sepharose beads for 2 hours at 4°C, on a rotary wheel. The beads, now bound with the GST-tagged protein, were washed 4x by repeated resuspension (in cold lysis buffer) and centrifugation (10,000g for 10 seconds). The purity and integrity of the GST-bead bound proteins were assessed by SDS-PAGE prior to carrying out GST pull-down assays to ensure equal protein input.

In Vitro Transcription and Translation

^{35}S-labelled p45 NF-E2, deletion constructs and mutants were translated *in vitro* using the TNT T7 Quick Coupled Transcription/ Translation System (Promega) as per manufacturer's instructions. Prior to use, the translated proteins were assessed by SDS-PAGE, followed by autoradiography to ensure equal protein expression.

GST Pull-down

GST pull-down assays were performed using GST-bound importin α/β proteins and ^{35}S-labelled p45 NF-E2 proteins. Equal amounts of GST- proteins (10-15 μL) were incubated with 5 μL of ^{35}S-labelled protein in 300 μL of pull-down buffer (150 mM NaCl, 20 mM Tris [pH 7.5], 1% NP-40, 2.5 μg/ml BSA, 1.5 mM PMSF, 0.1 μL/ml βME) for 1 hour at 4°C, on a rotary wheel. The beads were then pelleted by centrifugation at 10,000g for 10 seconds and washed three times with 1 mL cold GST-pulldown buffer. After the final wash, the supernatant was discarded and the beads were resuspended in 20 μL 2x SDS-PAGE loading buffer. The samples were boiled for five minutes and subjected to SDS-PAGE on 10% tris-glycine gels. Following electrophoresis, the gels were dried and subjected to autoradiography.

Electromobility Shift Assay (EMSA)

The double stranded DNA probe (5' CCT CCA GTG ACT CAG CAC AGG TTC T 3') containing a p45 NF-E2 binding site (underlined) was annealed and end-labelled with [^{32}P]-dATP using the T4 polynucleotide enzyme (Promega) for 1 hour at 37°C. Following labelling, unincorporated radiation was removed using Microspin™ G-25 columns according to the manufacturer's instructions. The purified probe was further diluted on the day of gel-shift to give a working concentration of 1 x 10^5 cpm/μL .Wild-type p45 NF-E2 and its mutants were *in vitro* translated using the TNT T7 Quick Coupled Transcription/ Translation System as previously described and allowed to bind to the probe (2 x 10^5 cpm) in the presence of 0.5 ng/μL poly (dI-dC) (Sigma), for 30 minutes at room temperature. For supershift studies, samples, as indicated in the figure legend, were then incubated for a further 30 minutes with a rabbit anti-NF-E2 antibody (sc-291; Santa Cruz; 1:20 dilution). The samples were subjected to PAGE on 5% polyacrylamide gels, which were then dried before autoradiography was performed. In parallel, to ensure equal expression of the *in vitro* translated proteins, samples were

labelled with ^{35}S-methionine and subjected to SDS-PAGE. The gels were dried and exposed to Kodak BioMax MR-1 X-ray film (GE Healthcare Bioscience).

Statistics

All statistical analyses were carried out by Excel software using the student *t* test (**= $p<0.05$; ***= $p<0.001$).

CHAPTER 3

NF-E2 MEDIATED ENHANCEMENT OF MEGAKARYOCYTIC DIFFERENTIATION AND PLATELET PRODUCTION *IN VITRO*

INTRODUCTION

Thrombocytopaenia frequently occurs in many haematological diseases and also following intensive chemotherapy or radiotherapy. Haematopoietic reagents such as TPO are used to treat this problem, but the development of anti-TPO antibodies in some patients makes its clinical advantages unclear (Linker, 2000; Neumann and Foote, 2000). Since then, alternative therapeutic approaches toward the treatment of thrombocytopaenia have been investigated, including the ability to produce functional platelets *in vitro* for the study of platelet biology and for possible cellular therapy purposes.

Since the discovery of TPO (Wendling et al., 1994; Kaushansky et al., 1994), *in vitro* MK culture techniques have also greatly improved (Debili et al., 1995; Guerriero et al., 1995; Hagiwara et al., 1998; Choi et al., 1995a). In several cases, platelet production has even been observed in such systems (Choi et al., 1995a; Cramer et al., 1997). However, current culture techniques still present the difficulty of obtaining a sufficiently high number of terminally differentiated MKs, capable of generating adequate amounts of platelets for basic research or clinical application. The forced expression of factors, which enhance MK differentiation and stimulate proplatelet formation and platelet release from MKs could provide a useful method for improving the efficiency of platelet production in these systems.

As described in Chapter 1, one of the key transcription factors involved in MK differentiation is NF-E2. Like GATA-1 (Shivdasani et al., 1997b; Vyas et al., 1999), NF-E2's primary role was first thought to be in erythroid development. Instead, knockout mice (Shivdasani and Orkin, 1995; Shivdasani et al., 1995) have implicated this nuclear protein as a prime regulator of MK terminal differentiation and platelet release. Mice lacking either the p45 subunit or both of the alternative p18 subunits, were found to be severely thrombocytopaenic despite having an abundance of MKs (Shivdasani and Orkin, 1995; Shivdasani et al., 1995; Onodera et al., 2000). However, in the absence of NF-E2 function, early MK differentiation, including endomitosis and subsequent expansion of cytoplasmic volume, were found to be normal (Shivdasani et al., 1995) while proliferation of MK progenitors was mildly reduced (Levin et al.,

1999). In addition, expression pattern analyses of human MKs have also identified NF-E2 as an important gene involved in megakaryopoiesis (Kim et al., 2002b; Shim et al., 2004).

MKs possess unique hallmark features that enable each stage of differentiation to be analysed. Firstly, the maturation of a MK from the HSC goes through different progenitor stages with varying proliferative capacities and cell marker expression (Figure 1.2). The expression of characteristic CD antigens such as CD61, CD41 and CD42a/b are typically used to analyse different stages of megakaryopoiesis. MK differentiation is characterised by the expression of CD61 and increasing expression of CD41 (Debili et al., 2001; Mathur et al., 2004), which together form a heterodimeric receptor complex known as glycoprotein (GP) IIb/IIIa (Phillips et al., 1988). This receptor is present on the surface of cells from the MK lineage and its expression levels increases as cells mature (Rabellino et al., 1981). CD42 expression is slightly later than that of CD41 (Debili et al., 1992), but expression levels of the two correlate with MK maturity (Debili et al., 2004). Thus, $CD41^{+}CD42^{+}$ cells represent more mature MKs, while $CD41^{+}CD42^{-}$ cells represent intermediately mature MKs. Megakaryocytes also undergo a characteristic endomitosis process to increase in size and ploidy to a DNA content in excess of 16N. MKs are morphologically large cells with single multi-lobed, polyploid nuclei (Schulze and Shivdasani, 2005) and distinctive acetylcholinesterase activity, which enables easy identification by simple histochemical staining (Karnovsky and Roots, 1964).

Perhaps most remarkable among the processes associated with MK differentiation, and certainly specific for this lineage, is that by which MKs extrude long cytoplasmic proplatelets that fragment into thousands of individual platelets (Choi et al., 1995b; Cramer et al., 1997). The microtubule cytoskeleton plays a central role in thrombopoiesis (Tablin et al., 1990) and further evidence suggests that platelet assembly occurs de novo within proplatelet extensions (Italiano et al., 1999). β1-tubulin, an apparent NF-E2 target gene (Lecine et al., 2000), is a major component of these microtubuli and is required to assemble platelets in proper number and shape (Schwer et al., 2001). Two other known NF-E2 targets include thromboxane synthase (TXS)

(Deveaux et al., 1997) and caspase 12 (Kerrigan et al., 2004), which are also found to be involved in platelet function. The latter is an effector of apoptosis and demonstrates reduced fibrinogen binding and aggregation upon stimulation with thrombin and ADP (Kerrigan et al., 2004). The absence of signalling factors such as Rab27b causes severe quantitative and qualitative defects in proplatelet formation that resemble findings in the NF-E2 mutant mice (Tiwari et al., 2003). Once again, NF-E2 is recruited to the Rab27b promoter and is responsible for its expression (Tiwari et al., 2003).

Overexpression studies have proven to be an effective method for highlighting the importance of transcription factor concentrations and the plasticity of the haematopoietic system. In addition, such studies also provide valuable information about novel transcription factor functions (Lako et al., 2001; Lulli et al., 2006; Rice et al., 2007). For example, by the overexpression of Wnt3 in embryonic stem (ES) cells, Lako et al. (2001) demonstrated Wnt3's importance in haematopoietic commitment during *in vitro* differentiation of ES cells. In addition, this ectopic Wnt3 expression resulted in a marked upregulation of Brachyury expression. Thus, providing evidence that Brachyury may be one of the target genes involved in the Wnt3 signalling pathway. Another recent study revealed that the overexpression of Ets-1 in erythroid cells blocks cell maturation while increasing GATA-2 and decreasing both GATA-1 and erythropoietin receptor expression (Lulli et al., 2006). Conversely, Ets-1 overexpressing MKs were characterised by enhanced differentiation and maturation, coupled with the upregulation of GATA-2 and MK-specific genes. These findings led Lulli et al. (2006) to prove that Ets-1 binds to and activates the GATA-2 promoter, *in vitro* and *in vivo,* indicating that one of the pathways through which Ets-1 blocks erythroid and promotes MK differentiation is via increased GATA-2 expression.

A p45 NF-E2 overexpression study focusing on erythroid development has also successfully shown the reprogramming of a monoblastoid cell line to promote erythroid development and increased erythroid colony formation from primary haematopoietic progenitors (Sayer et al., 2000). However, even with the emergence of morphologically mature erythroid cells, these cells failed to synthesise haemoglobin in the presence of EPO (Sayer et al., 2000).

In light of the clear importance of p45 NF-E2 in MK differentiation and eventual platelet release; the work in this chapter aimed to investigate the hypothesis that haematopoietic cells may have the propensity to enhance MK maturation and platelet production if the concentration of NF-E2 is increased. Potential long-term gains from such outcomes would be therapeutic applications, such as transfusions or cell transplantations.

In this report, exogenous p45 NF-E2 was delivered into BMCs enriched for MK progenitors via retroviral means and multiple stages of megakaryopoiesis were proven to be enhanced *in vitro*. These included haematopoietic stem cell (HSC)-derived progenitor cell development, terminal MK differentiation, extrusion of proplatelets, and finally functional platelet release. One of the mechanisms found to be responsible for this augmentation in megakaryopoiesis and thrombopoiesis is the accompanying upregulation of NF-E2 target MK genes. Furthermore, this is the first report where p45 NF-E2 expression was found to inhibit myeloid differentiation.

RESULTS

Characterisation of Retroviral-Transduced Murine Bone Marrow Cells

A strategy for achieving ectopic expression of p45 NF-E2 in murine BMCs was required to elucidate the role of p45 overexpression in enhancing megakaryopoiesis and thrombopoiesis. An efficient retroviral gene delivery system previously established in our laboratory (Pan, 2007) was adopted, and stable simultaneous expression of p45 NF-E2 and eGFP was attained in murine BMCs. p45 NF-E2 cDNA was inserted into a retroviral vector (pKMV) (Figure 3.1A). The p45 NF-E2 sequence was followed by an internal ribosome entry site (IRES) and the cDNA encoding eGFP (pKMV-NF-E2). $eGFP^+$ PT67 packaging cell lines were generated as described in the Materials and Methods (Chapter 2, page 49), for both pKMV-NF-E2 and the control vector, pKMV (Figure 3.1A). Both retrovirus producer cell lines efficiently expressed eGFP as assessed by FACS and fluorescent microscopy (Figure 3.1B; C) and were subsequently used to infect primary BMCs.

The cells used to study biological function in this thesis were a HSC/ HPC enriched population (Lin^-) obtained from 5FU treated mice (Chapter 2, pages 48-49). After co-cultivation with the respective viral packaging cells, 42-44% of the BMCs were also positive for eGFP expression (Figure 3.2A). Similar transduction efficiencies were consistently observed in replicate experiments.

BMCs transduced with pKMV alone (KMV) or pKMV-NF-E2 were allowed to differentiate for 5-7 days in the presence of TPO and IL-3. The cells were then lysed and p45 NF-E2 detected by Western blot analysis. As shown in Figure 3.2B and confirmed by densitometry, approximately a 2 to 3.5-fold overexpression of p45 NF-E2 was achieved in pKMV-NF-E2 transduced marrow cells (hereafter referred to as NF-$E2^{++}$ cells) harvested from mice either 2 or 8 days after 5-fluorouracil treatment (referred to as 2 day 5FU and 8 day 5FU BMCs respectively).

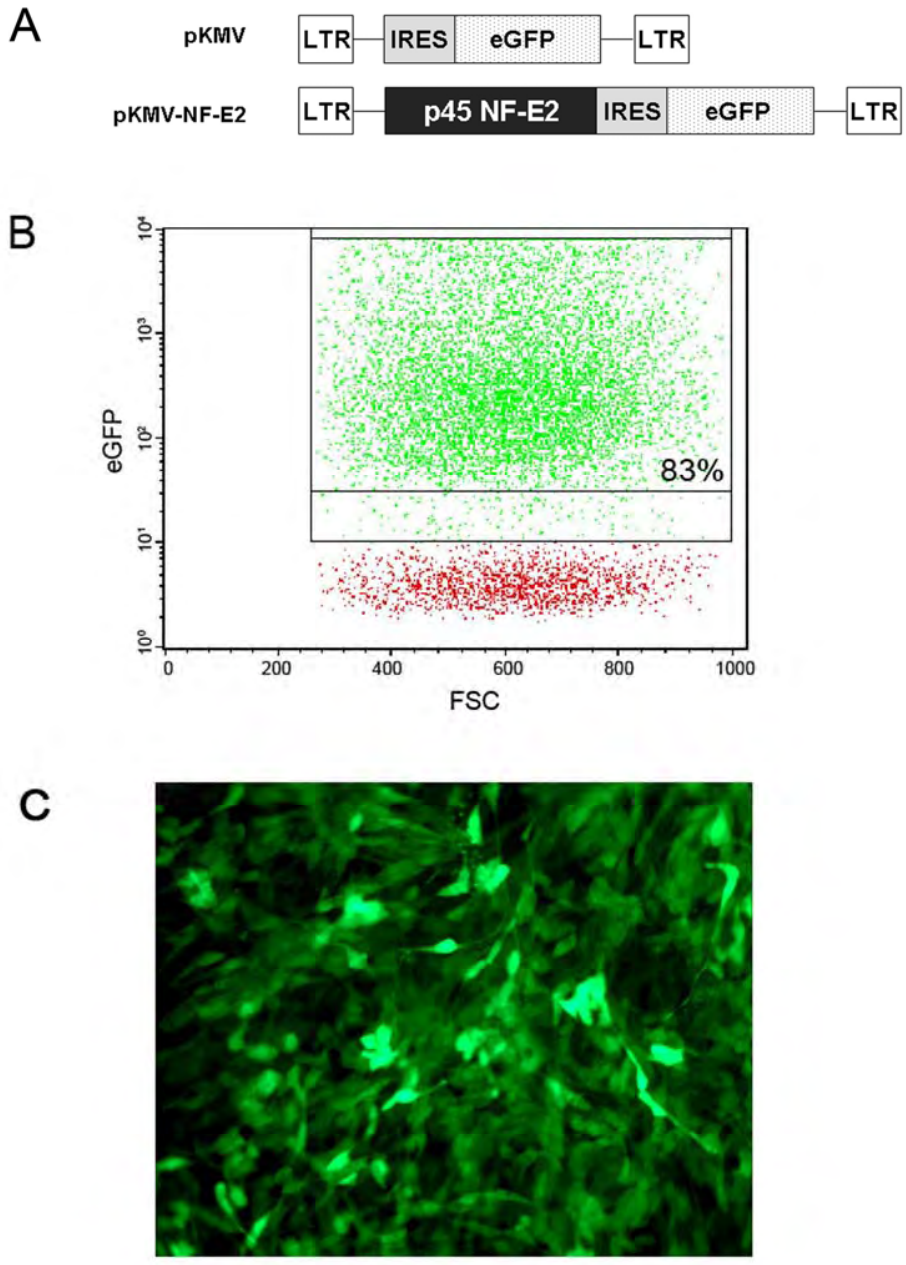


Figure 3.1 Retroviral constructs and transgene expression in viral packaging cells. (A) Schematic of the control retroviral vector, pKMV, and the bicistronic vector containing the p45 NF-E2 cDNA, pKMV-NF-E2. Both contain the eGFP reporter gene. LTR: Long terminal repeat, IRES: Internal ribosomal entry site. PT67 packaging cells were retrovirally transduced with the above constructs and subsequently analysed for the expression of eGFP by (B) flow cytometry and (C) fluorescent microscopy.

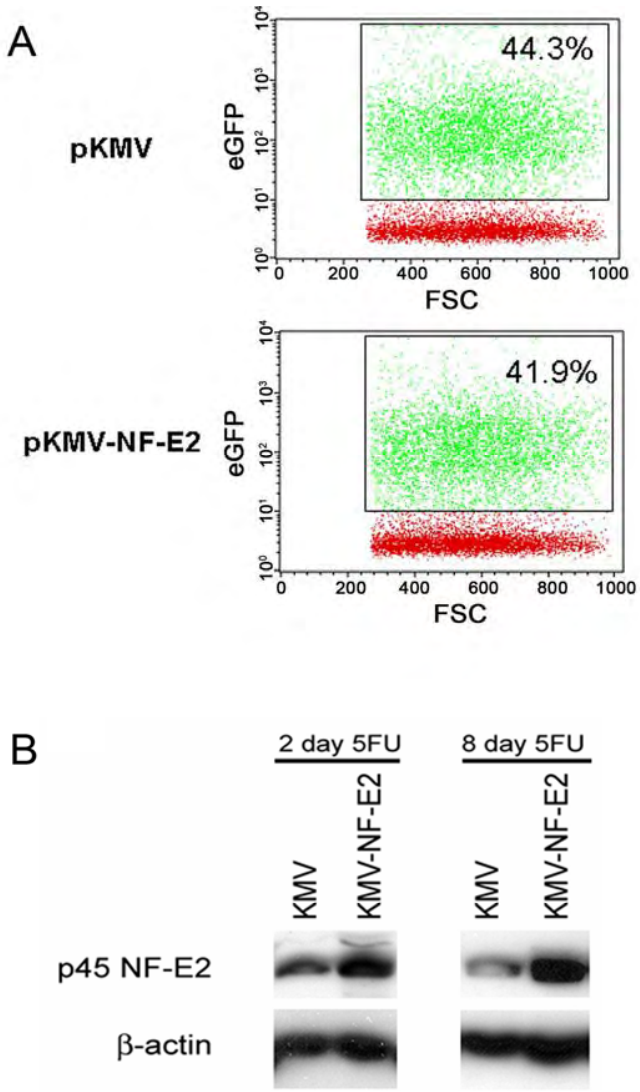


Figure 3.2 p45 NF-E2 and eGFP expression in murine bone marrow cells. (A) Flow cytometric analysis of eGFP expression in BMCs 48 hours after transduction with pKMV and pKMV-NF-E2. The transduced populations are shown on representative dotplots and the percentage of cells expressing eGFP is indicated. (B) Immunoblot analysis for p45 NF-E2 expression in transduced BMCs after 5-7 days of differentiation. Whole cell lysates of 4 x 10^6 sorted BMCs transduced with the indicated vectors were electrophoretically separated, blotted, and probed sequentially with an anti-p45 NF-E2 antibody and anti β-actin monoclonal antibody. Representative blots are shown. Overexpression was observed in both 2 day 5FU and 8 day 5FU transduced BMCs cultured for 5-7 days in TPO and IL-3.

Enforced p45 NF-E2 Expression Elicits an Increase in MK Phenotype, Without Affecting Endomitosis

Having confirmed high expression of exogenous p45 NF-E2 in our transduced BMCs, we proceeded to assess whether MK differentiation would be influenced by p45 NF-E2 overexpression. Upon liquid culture in TPO and IL-3 for 5 days, initial characterisation of unsorted transduced BMCs was performed by examining cell surface expression of both early (CD41) and late (CD42a/b) MK-specific markers.

Expression profiles obtained by flow cytometry revealed a significant increase in MK markers within the NF-E2^{++} population compared to the pKMV-transduced control group (Figure 3.3A). FACS analysis showed that pKMV-transduced eGFP^{+} populations contained only 25.4%, 17.5% and 16.3% of cells expressing CD41, CD42a and CD42b respectively (Figure 3.3A). In sharp contrast, NF-E2^{++} GFP^{+} populations contained 42.3%, 28% and 28.2% of cells expressing the same respective MK markers. These results suggest that enforced expression of p45 NF-E2 favours MK differentiation as assessed by marker expression.

To determine if the above increases in CD41 and CD42 expression were indeed a result of enhanced terminal MK differentiation, further characterisation of various stages of MK maturation was performed. Using a histochemistry technique called acetylcholinesterase (AchE) staining (Chapter 2, page 50), validation of mature MKs in mouse cells was carried out (Karnovsky and Roots, 1964). After sorting, transduced BMCs were grown in liquid culture containing TPO and IL-3 for 5 days. MK-specific AchE activity was then examined as an early marker of terminal MK differentiation. On average, a 50% increase in AchE^{+} cells occurred in NF-E2^{++} populations compared to pKMV groups (Figure 3.3B). Presumably, AchE^{+} cells would undergo the process of endomitosis, followed by cytoplamic maturation and organisation, which culminates in platelet release.

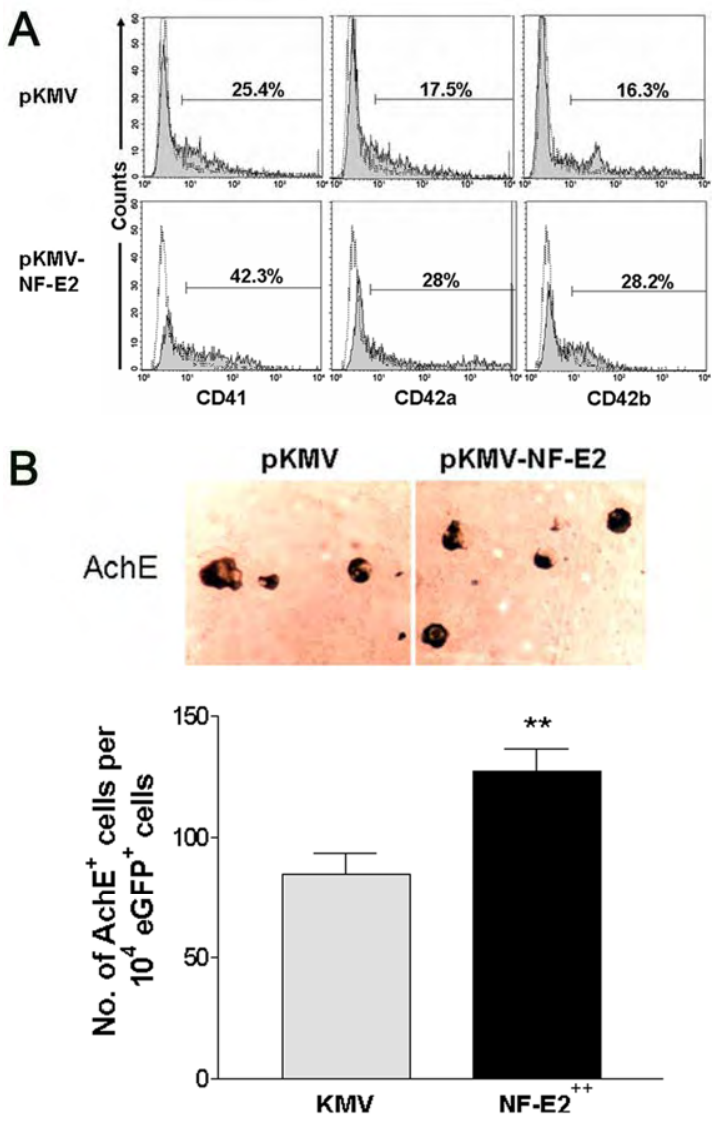


Figure 3.3 Characterisation of the various stages of MK differentiation in p45 NF-E2 transduced BMCs. (A) Expression of early and late megakaryocytic markers CD41, CD42a and CD42b were examined by flow cytometry in unsorted 8 day 5FU Lin^- BMCs cultured for 5 days in TPO and IL-3. pKMV and pKMV-NF-E2 transduced populations are shown in histograms gated on $eGFP^+$ cells. The percentage of $CD41^+$, $CD42a^+$ or $CD42b^+$ cells from one representative experiment is indicated. Broken line: control antibody. (B) Sorted 8 day 5FU retrovirus-transduced Lin^- cells were cultured for 5 days before AchE activity assays were carried out. $AchE^+$ cell frequency was calculated. The data are representative of 3 independent experiments (mean ± SD).

To verify whether endomitosis was affected by p45 NF-E2 overexpression, we performed DNA ploidy analysis on transduced cells. The modal ploidy class of both the pKMV and NF-E2^{++} groups was found to be 8N (Figure 3.4), indicating that endomitosis was not affected by p45 NF-E2 overexpression. Taken together, these experiments demonstrate that p45 NF-E2 overexpression does not influence endomitosis, but does enhance other early phases of MK development in NF-E2^{++} cells.

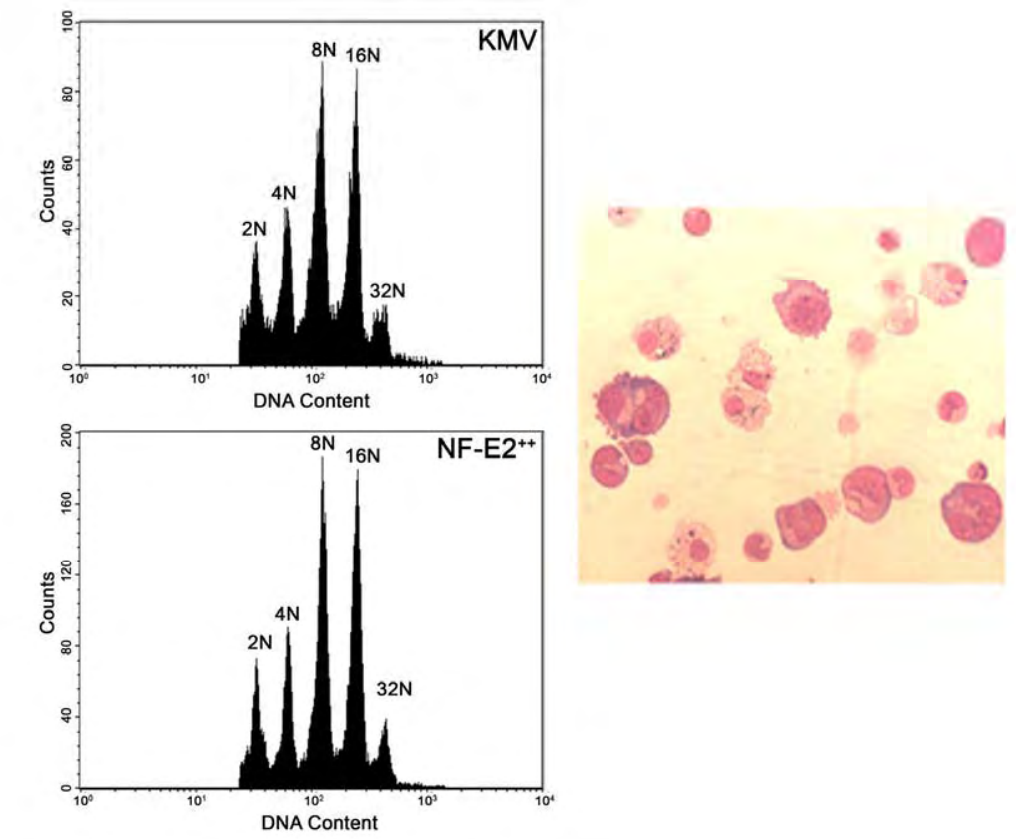


Figure 3.4 DNA ploidy analysis of transduced BMCs. The DNA content of sorted pKMV (top: KMV) and NF-E2^{++} (bottom) MKs on day 5 as analysed by flow cytometry. A representative Wright-Giemsa staining of the typical cells analysed from either group is shown on the right (x 200).

Cell Proliferation is Affected by an Increase in p45 NF-E2 Expression

p45 NF-E2 is required for the normal proliferation of MK progenitor cells (Levin et al., 1999). Therefore, to analyse cell proliferation, sorted BMCs were cultured in TPO and IL-3 for 7 days and cell counts were carried out every 2-3 days using trypan blue exclusion of non-viable cells. Only a modest increase in NF-E2^{++} cell number was observed on day 5 ($p>0.05$) while final cell numbers obtained on day 7 were indistinguishable from the control group (Figure 3.5A).

To corroborate the previous observations, we used a standard colorimetric MTT assay, which measures the metabolic activity of viable cells. Yellow MTT (3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide) is reduced to a purple formazan only in active mitochondria. This conversion is measured spectrophotometrically and is directly related to the number of viable cells present. MTT cell proliferation assays performed on the same batch of cells used in trypan blue exclusion counting showed a similar positive effect on cell proliferation in NF-E2^{++} cells on day 5 ($p<0.05$) (Figure 3.5B). Taken together, these data suggest that p45 NF-E2 overexpression has a moderate affect on total HPC proliferation, which may be of particular significance for the MK lineage in question.

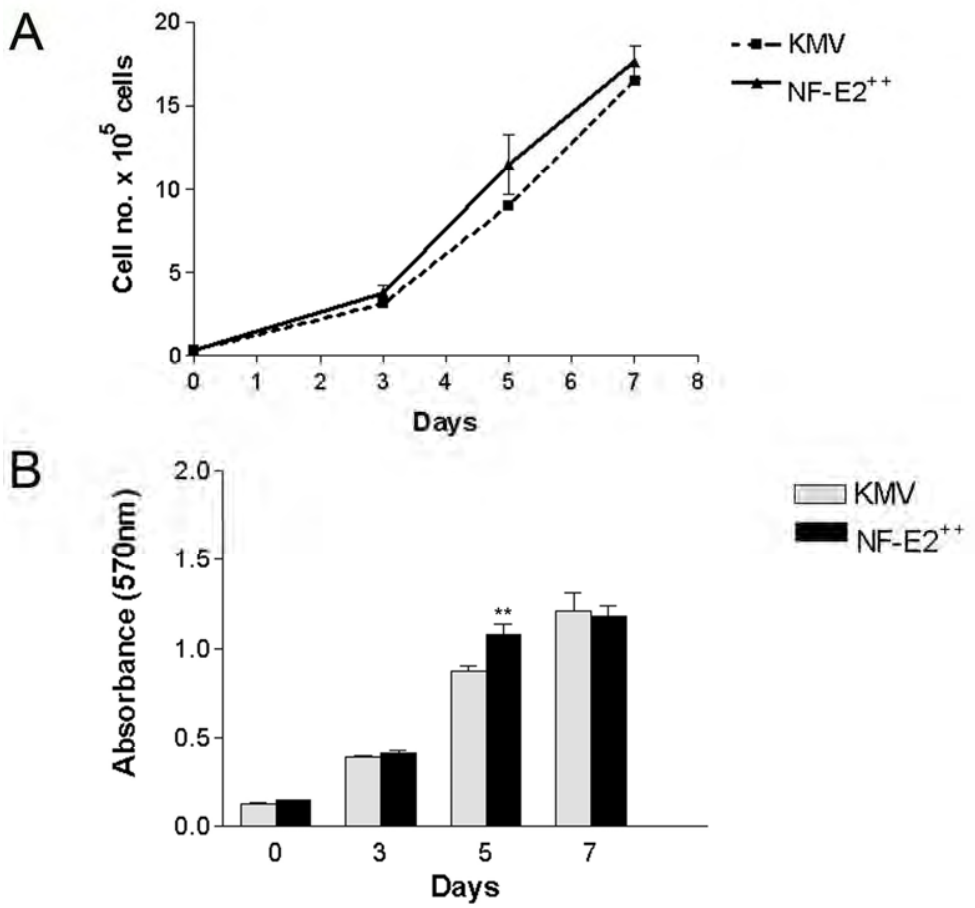


Figure 3.5 Enforced expression of p45 NF-E2 moderately increases progenitor cell proliferation. (A) Transduced 8 day 5FU Lin$^-$ BMCs were sorted by FACS and seeded at a density of 3.5 x 10^4 cells/ well in a 24 well plate. The cells were grown in TPO and IL-3 liquid culture for 7 days and cell counts using trypan blue exclusion were carried out every 2-3 days. This result is representative of 2 independent experiments. (B) The same sorted cells were seeded into four 96 well plates and allowed to grow for 7 days. An MTT assay was carried out every 2-3 days on a different plate. Data at each time point are the mean result of 6 wells and is representative of 2 independent experiments.

NF-E2 Overexpression Promotes CFU-MK and Inhibits CFU-GM Progenitor Production

We have established that ectopic expression of p45 NF-E2 favours MK differentiation (Figure 3.3A; B). However, the enhanced phenotype could either be a result of selective expansion and differentiation of already committed MK progenitors or an increased generation of HSC-derived MK progenitor cells and the direct inhibition of other lineages. To clarify this, both MK and myeloid (granulocyte/ macrophage) precursor frequencies were quantified.

An enriched population of HSCs and early progenitor cells were harvested from the bone marrow of 2 day 5FU treated mice. Transduced BMCs were sorted and subjected to CFU-MK and CFU-GM colony assays. On day 7, cells at varying stages of MK development were scored and confirmed by MK-specific AchE staining. Colonies of CFU-MK possessing uniformly larger cells with morphology typical of MKs were observed along with large single or doublet $AchE^{+}$ cells (Figure 3.6A), presumed to be more mature MKs with decreased proliferation capacity. The mean frequency of $AchE^{+}$ single or doublet MKs was increased ~2.3 fold in the $NF\text{-}E2^{++}$ cells when compared to the pKMV cells (Figure 3.6B). Surprisingly, a comparable augmentation (~1.6 fold) in CFU-MK formation was also observed in $NF\text{-}E2^{++}$ cells compared to pKMV cells (Figure 3.6B). Erythroid progenitor frequency has already been shown to increase with p45 NF-E2 overexpression in primary cells (Sayer et al., 2000). These data suggest that overexpression of p45 NF-E2 not only promotes MK maturation, but may also enhance early megakaryopoiesis.

Investigations into the effects of p45 NF-E2 overexpression on the myeloid lineage resulted in a consistent decrease in CFU-GM colony formation (includes CFU-G, CFU-M and CFU-GM colonies) in $NF\text{-}E2^{++}$ populations compared to pKMV cultures (Figure 3.7). Interestingly, these data identify p45 NF-E2 as a possible negative regulator of myeloid differentiation.

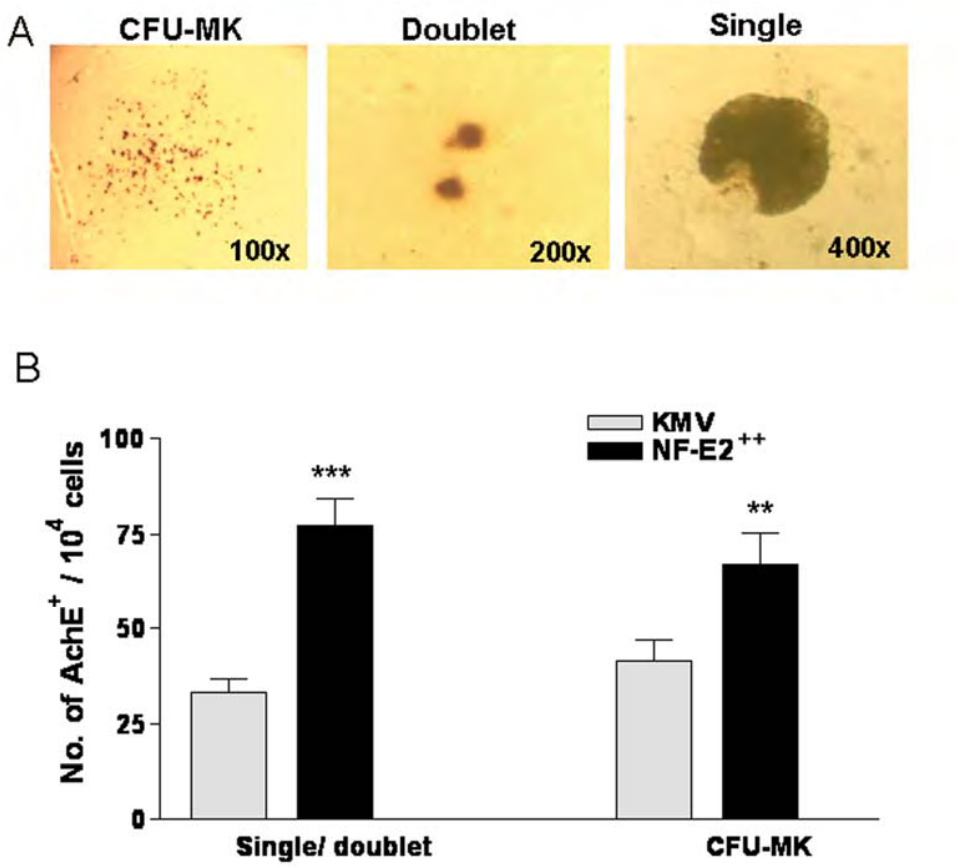


Figure 3.6 p45 NF-E2 overexpression promotes megakaryocytic (CFU-MK) colony formation. (A) Transduced 2 day 5FU Lin^- BMCs were sorted by FACS and cultured for 7 days in collagen-based media under TPO and IL-3 conditions. MK-specific AchE staining was carried out and $AchE^+$ CFU-MK colonies, doublets and large single MKs were scored. (B) $AchE^+$ single cells, doublets and CFU-MK frequencies were calculated by dividing their respective numbers by the total number of input cells (10^4 cells). Data are representative of 2 independent experiments carried out in quadruplicate (mean ± SD).

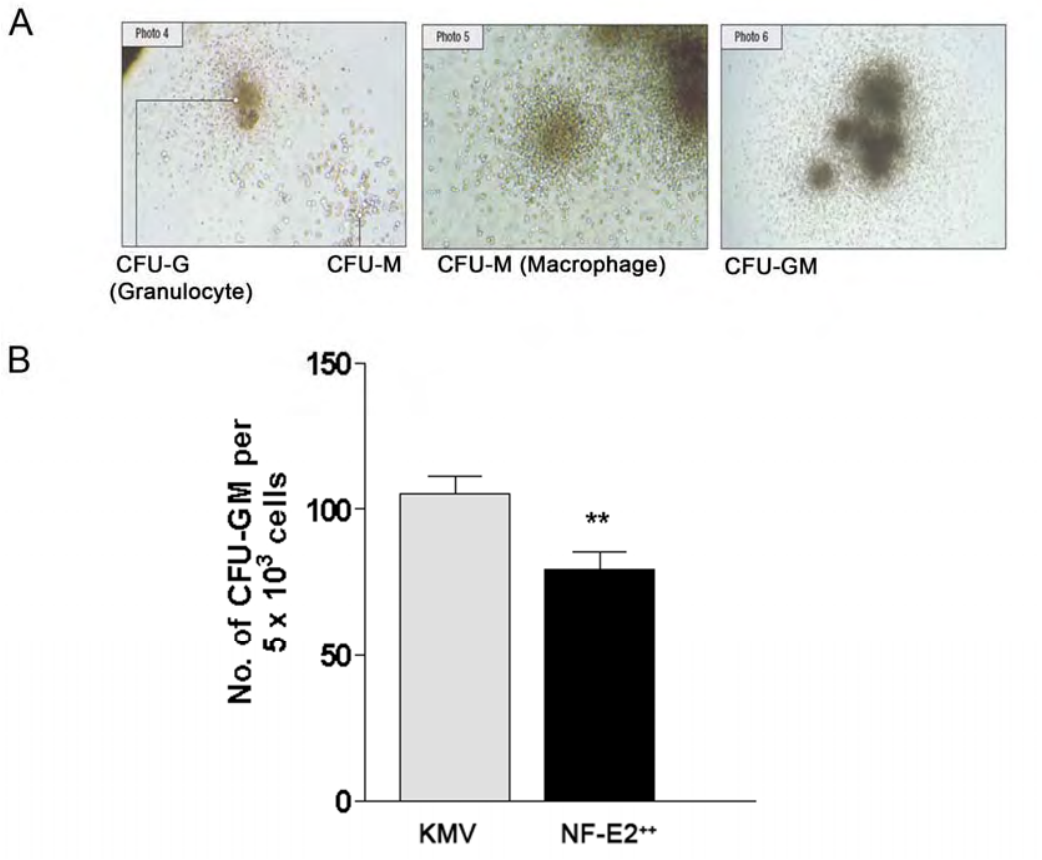


Figure 3.7 Enforced p45 NF-E2 expression inhibits myeloid (CFU-GM) colony formation. Sorted 2 day 5FU BMCs were cultured in methylcellulose media containing WEHI-3 conditioned medium to promote CFU-GM formation. (A) CFU-GM colonies (includes CFU-G, -M and GM) were scored after 7 days based on morphology. Examples of the colonies examined are shown. Pictures provided by Stem Cell Technologies. (B) CFU-GM frequencies were calculated by dividing their respective numbers by the total number of input cells (5 x 10^3 cells). Results are the mean ± SD of 3 independent experiments.

Upregulation of MK-Specific NF-E2 Target Genes by p45 NF-E2 Overexpression

To date, a number of NF-E2 target genes have been identified, the majority of which are directly involved in thrombopoiesis (Deveaux et al., 1997; Lecine et al., 2000; Tiwari et al., 2003; Kerrigan et al., 2004). Therefore, the effect of p45 NF-E2 overexpression on these target genes was examined. Quantitative real-time PCR was used to characterise expression levels of NF-E2 target genes in transduced 8 day 5FU BMCs that had differentiated under TPO conditions for 5 days.

Two target genes that are central to proplatelet formation and thrombopoiesis are β1-tubulin and Rab27b (Lecine et al., 2000; Tiwari et al., 2003). On day 5, the expression of both β1-tubulin and Rab27b was significantly increased (1.7-3.3 fold) compared with the KMV group (Figure 3.8). Among other genes known to be NF-E2 targets are caspase 12 and TXS. Caspase 12 being the most down-regulated gene product in the absence of NF-E2 (Kerrigan et al., 2004) had a remarkable 5.3 fold mean increase in expression in NF-E2^{++} cells on day 5 (Figure 3.8). To a lesser extent, the same trend was also observed in TXS gene expression (2 fold) compared to the control group (Figure 3.8). Real-time PCR was also carried out on RNA extracted from transduced BMCs on day 3. Day 3 samples demonstrated a similar upregulation of all tested target genes (data not shown).

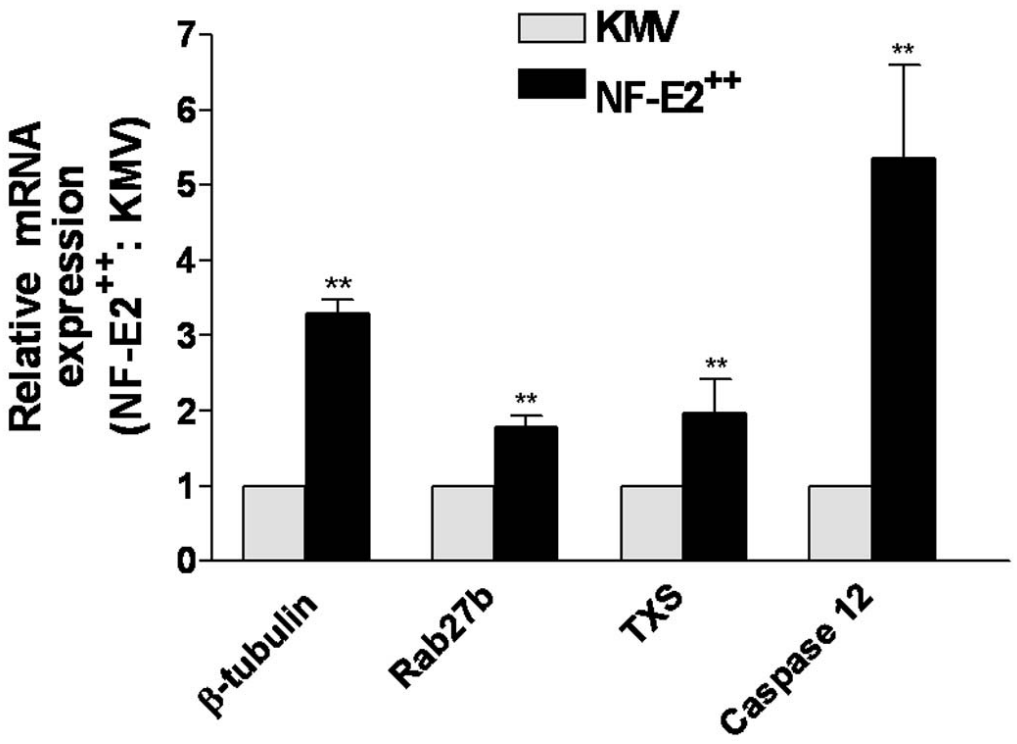


Figure 3.8 Overexpression of p45 NF-E2 increases NF-E2 target gene expression. Retrovirus-transduced 8 day 5FU Lin$^-$ BMCs were grown under TPO alone conditions for 5 days. NF-E2 target gene expression was studied using RNA extracted from pKMV or NF-E2^{++} transduced BMCs on day 5 and subjected to real-time RT-PCR for the messages indicated. Target quantities were normalised to GAPDH and presented relative to the KMV control. Each real-time PCR reaction was performed in triplicate and presented as the mean ± SD.

Enhanced Proplatelet and Functional Platelet Production In Vitro.

Having previously observed marked increases in both MK maturation and in the expression of NF-E2 targets involved in thrombopoiesis, we proceeded to determine if these results were indeed reflected in proplatelet and platelet development. Equal numbers of $CD41^+$ transduced Lin^- 8 day 5FU cells were cultured in media containing TPO alone for 5-7 days before the cells showed a dramatic morphological change (Figure 3.9A). Numerous cells exhibited long beaded projections, so-called proplatelets, from which platelets would eventually be produced (Italiano et al., 1999).

Proplatelets at various stages of development as previously described by Italiano et al, (1999) were observed (Figure 3.9A). When counted under a fluorescent microscope, there was a significant 1.7- to 2-fold increase in the number of proplatelets present in the NF-$E2^{++}$ cultures (Figure 3.9B). From our observations, proplatelet numbers peaked between days 4-6 of differentiation. However, after another 2-3 days in culture, proplatelet numbers appeared to have decreased and small dust-like particles were observed floating in the medium. Our findings indicate that p45 NF-E2 overexpression results in an increase in proplatelet-bearing MKs.

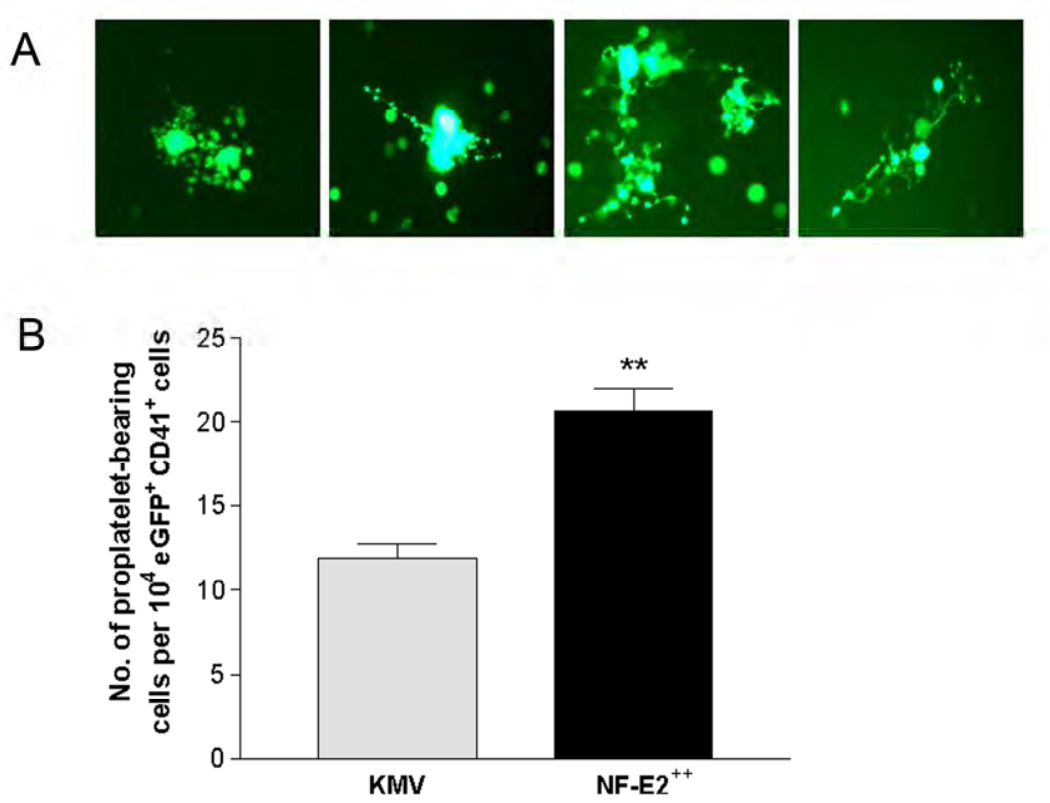


Figure 3.9 Proplatelet development enhanced by p45 NF-E2 overexpression. (A) Equal numbers of eGFP$^+$/ CD41$^+$ transduced cells were seeded and cultured for 5 days before proplatelets at various stages of development were counted using the fluorescent microscope (100x). (B) Proplatelet-bearing MK frequency was calculated by dividing the proplatelet number by the total number of eGFP$^+$/ CD41$^+$ input cells. Each experiment was done in triplicate and these data (mean ± SD) are representative of 3 independent experiments.

To determine whether platelets were eventually produced from the proplatelet-bearing MKs, culture medium was collected two days after proplatelets were observed, and any platelets produced in culture were enumerated by flow cytometry. A gate was fixed in the forward and side-scatter dotplots of peripheral blood platelets obtained from adult mice. Cultured particles that were $eGFP^{+}$ and had the same scatter properties as blood platelets were then gated out. The size of analysed particles obtained from the culture medium was within the platelet gate (Figure 3.10A), the majority of which were positive for CD41 and another platelet-specific antigen, CD42b (Figure 3.10B). This suggested that platelets were released into the culture medium from mature MKs, which first developed proplatelets. Accurate counts from flow cytometry analysis showed a significant 2.7 fold increase in the number of platelets present in the NF-$E2^{++}$ culture at day 7 (Figure 3.10C). These results provide evidence that overexpression of p45 NF-E2 enhances both proplatelet and platelet production *in vitro*.

Platelets produced from our cultures were then tested for function by carrying out standard P-selectin immunostaining and Annexin V binding assays. Surface expression of P-selectin was observed in platelet samples after stimulation by AYPGFK and ADP, but not in unstimulated platelets (Figure 3.11A), suggesting that a release reaction had occurred. Likewise, platelets that had been stimulated also bound Annexin V conjugated to PE (Figure 3.11B), indicating that platelet activation had taken place. The platelet activation profiles from both the KMV and NF-$E2^{++}$ groups were very similar; suggesting that overexpression of NF-E2 had no measurable effect on platelet function (Figure 3.11). These results were comparable to peripheral blood samples that had undergone similar treatment and imply that the *in vitro* cultured platelets were functional and phenotypically similar to platelets derived from peripheral blood.

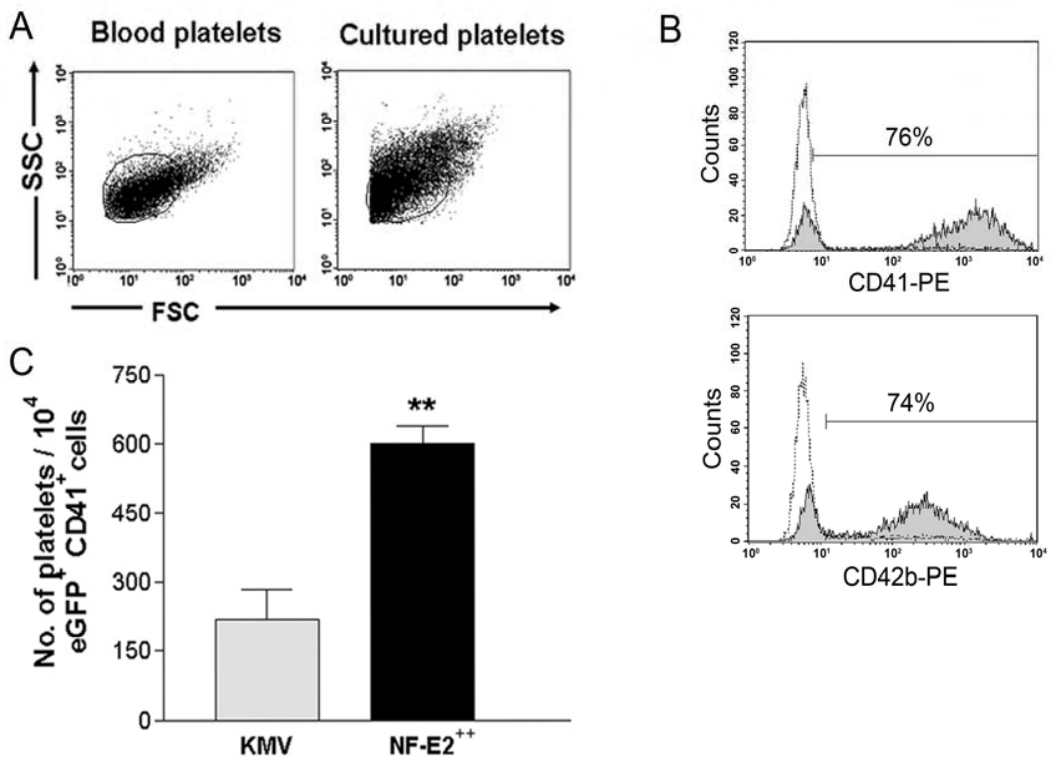


Figure 3.10 Overexpression of p45 NF-E2 increases functional platelet production *in vitro*. Equal numbers of transduced $eGFP^+$/ $CD41^+$ 8 day 5FU Lin^- BMCs were differentiated in TPO alone media. (A) Particles released into the culture medium were then analysed on day 7 by flow cytometry. A platelet gate was fixed in the forward- and side-scatter profiles of peripheral blood platelets from adult mice (left). Particles from our culture, which were within that gate, were analysed (right). (B) Cells collected from culture supernatant were labelled with platelet specific antibodies. The majority of cells were positive for CD41 (top) and CD42b (bottom), as shown by the gray histogram. Dotted line: control antibody. (C) Platelets collected from the culture medium were counted as platelet-sized and $eGFP^+$/ $CD42b^+$ cells by flow cytometry. The values shown are the mean of 2 independent experiments.

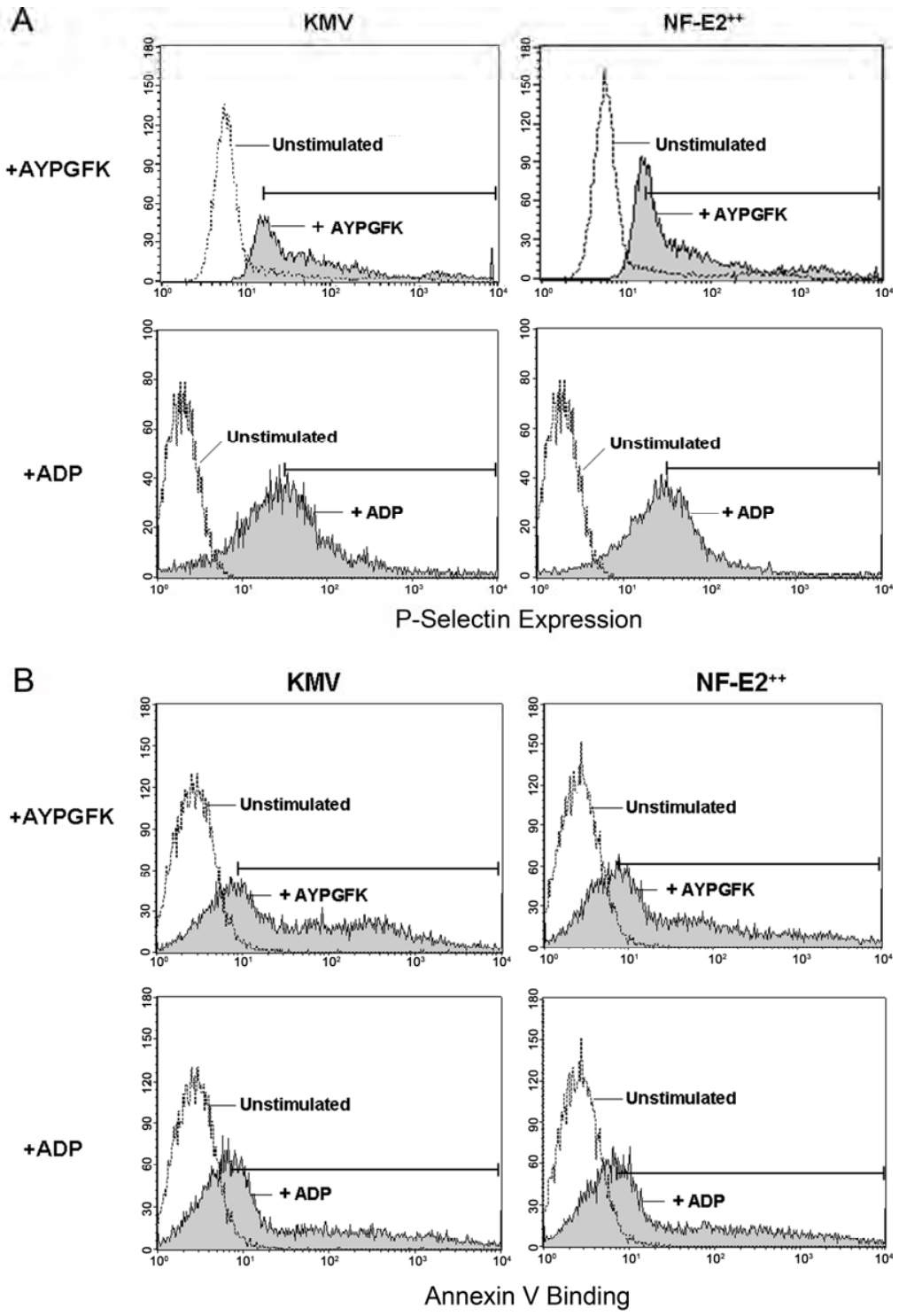


Figure 3.11 Functional assays of *in vitro* produced platelets derived from transduced haematopoietic BMCs. Platelets were obtained from the culture medium of both KMV (left panels) and NF-E2^{++} (right panels) groups on day 7. Unstimulated

and stimulated platelets by PAR4 thrombin receptor-activating peptide AYPGFK (top panels) or ADP (bottom panels) were subjected to (A) P-selectin immunostaining and (B) Annexin V binding assays before analysis by flow cytometry. Dotted line: experimental control using unstimulated platelets.

DISCUSSION

The focus of this chapter was to investigate the functional and biological consequences of p45 NF-E2 overexpression in the MK lineage. These experiments demonstrated that p45 NF-E2 is a limiting factor for megakaryopoiesis and thrombopoiesis and its significance in early and terminal MK maturation, proplatelet and functional platelet production were highlighted. In addition, p45 NF-E2 was identified as a previously unrecognised negative regulator of myeloid differentiation.

Contrary to our expectations, p45 NF-E2 overexpression appeared to only modestly increase total progenitor cell proliferation with no significant difference in the average colony size compared to the control. This modulation was predicted to be more dramatic since NF-E2 deficient MK progenitors have previously been shown to display reduced proliferation *in vitro* (Levin et al., 1999). In the knockout cells, only conservative attenuation of CFU-MK frequency was observed whilst significant reductions in average colony size were demonstrated. However, other NF-E2 knockout studies have shown megakaryocytosis *in vivo* and additional proliferation in response to pharmacologic administration of TPO (Shivdasani et al., 1995; Shivdasani et al., 1997a). These latter studies suggest that the replication potential of MK progenitors was intact and not influenced by NF-E2.

It is possible that *in vivo*, additional factors such as TPO-induced signalling in the presence of severe thrombocytopaenia, may operate to produce or maintain the baseline level of MKs present. Indeed, the expansion of individual cell lineages has been found to largely be regulated by extracellular growth factors (Metcalf, 1998). Therefore, in the current studies, the cells were cultured *in vitro* in the presence of controlled culture conditions and recombinant TPO and IL-3. This may have inadvertently exerted an external cap on the proliferative capacity of the cells. Alternatively, given that the MK lineage represents a small percentage of the total population, the difference noted in day 5 (Figure 3.5B) could be quite significant for the lineage in question. In addition, the present experiments were conducted in the presence of TPO and IL-3, which allows us to study the effects of p45 NF-E2 overexpression in all lineages. Thus, an increase in

MK numbers within the total culture may also have been diluted by the significant decrease in myeloid progenitors (Figure 3.7B).

Absence of p45 NF-E2, associated with severe thrombocytopaenia in mice (Shivdasani et al., 1995), has been demonstrated to spare early MK differentiation including endomitosis, but imposes a profound block in proplatelet formation (Lecine et al., 1998b). Previous expression profiling of human MK cells have also shown that p45 NF-E2 is barely detected in uninduced progenitors, but accumulates further down the MK pathway during intermediate and advanced stages of differentiation (Terui et al., 2000). Furthermore, p45 NF-E2 null MKs were found to carry wildtype levels of MK progenitor markers, elevated levels of nearly every marker assigned to intermediate MKs and at least a 2-fold reduction of transcripts linked to terminally mature MKs on the verge of proplatelet formation (Chen et al., 2007). These results provide distinct molecular correlates for the arrested state of NF-E2$^{-/-}$ MKs (Shivdasani et al., 1995). Collectively, these studies have established a strong precedent for NF-E2's involvement in MK differentiation, but only at the terminal stages.

In contrast, this report has found that p45 NF-E2 not only enhances terminal differentiation of committed MK precursors, but also favours early MK differentiation with apparent increased direction of HSCs/ early HPCs toward MK commitment. It has been shown that NF-E2 is involved in erythroid lineage commitment (Sayer et al., 2000), however to date there has been no clear evidence of a role for NF-E2 in directing lineage fate towards MK commitment. Interestingly, an identified target of NF-E2 is 3β-hydroxysteroid dehydrogenase (Nagata et al., 2003), which controls the biosynthesis of estrogen. A recent study showed that high dose estrogen can enhance MK colony formation *in vitro* (Bord et al., 2004). More importantly, there is now a growing collection of data, which shows that commitment of HSCs into a particular lineage can be influenced by the overexpression or underexpression of particular transcription factors (Dahl et al., 2003; Iwasaki et al., 2003; Iwasaki et al., 2006; Taschner et al., 2007). Therefore, although HSCs/ early HPCs may express NF-E2 at a low level (Orlic et al., 1995; Terui et al., 2000), its enforcement by retroviral transduction into these cells may result in their specification into the MK lineage. As is the case with GATA-1

or GATA-2 overexpression, which mainly formed MK-erythroid or eosinophil colonies respectively (Iwasaki et al., 2006; Iwasaki et al., 2003).

The MK and erythroid lineages are closely related in ontogeny (Suda et al., 1983; Ogawa, 1989) suggesting that p45 NF-E2 when overexpressed, may also favour early MK development over myeloid maturation (Sayer et al., 2000). In fact, the significant decrease in CFU-GM colony formation (Figure 3.7B) observed in this study is compatible with the hypothesis that NF-E2 may be a negative regulator of myeloid differentiation, while promoting MK commitment over the myeloid lineage. Indeed, NF-E2 is found to be lacking in normal myeloid cells (Labbaye et al., 1995). On the one hand, overexpression of p45 NF-E2 induces commitment to the MK lineage and leads to increased production of MK appropriate proteins (Figure 3.8). On the other hand, this same process may also repress the expression of myeloid specific genes necessary for myeloid commitment and expansion (May, 2001). Alternatively, there is also evidence of cells exhibiting intolerance to the expression of lineage inappropriate genes (Chiang and Monroe, 1999). The introduction of p45 NF-E2 into primary cells that normally differentiate down the myeloid lineage will confirm whether p45 does indeed suppress and reprogram its differentiation.

The respective stages of megakaryopoiesis that are clearly augmented as a result of enforced p45 NF-E2 expression are briefly summarised in Figure 3.12. Of the unique aspects of MK differentiation looked at in this study, the process of endomitosis stood out as one which was not found to be affected by p45 NF-E2 overexpression. This is consistent with the normal increased DNA ploidy observed in the p45 knockout model (Shivdasani et al., 1995) and confirms that endomitosis is an NF-E2-independent stage of MK development, probably controlled by other factors such as GATA-1 (Vyas et al., 1999) or TPO (Italiano Jr and Shivdasani, 2003). This abortive event occurring in the late anaphase of mitosis has also been found to be regulated by G1/ S cell cycle factors, such as cyclins A, B, E and D3, none of which are targets of NF-E2 (Bermejo et al., 2002).

In contrast, enhanced late MK maturation by p45 NF-E2 overexpression was reflected in subsequent increases in both proplatelet formation and functional platelet production *in vitro* within NF-E2^{++} cultures. Arguably, these observations could be a result of enhanced terminal differentiation and proplatelet production of existing MK precursors or an increased generation of MK progenitor cells, but as explained below our findings support both hypotheses. Not only was an increase in CFU-MKs obtained from HSCs overexpressing p45 NF-E2 (Figure 3.6B), but when an equal number of transduced MKs was seeded, both proplatelet and platelet production were also enhanced ~2-3 fold (Figure 3.9B; Figure 3.10C)**.** These results indicate that p45 NF-E2 overexpression enhances MK differentiation at both the early stages of MK precursor development and the terminal stages of proplatelet formation and platelet release.

NF-E2 coordinates a broad transcriptional program to drive platelet biogenesis. Therefore, the upregulation of such transcriptional targets are likely to result in more efficient platelet assembly, inside-out signalling through the $\alpha_{IIb}\beta_3$ integrin, and intracellular organelle trafficking. Ultimately, resulting in the improved terminal MK differentiation and thrombopoiesis phenotype observed here in NF-E2^{++} MKs. Indeed, mRNA expression analysis of transduced BMCs revealed that NF-E2 target genes such as β1-tubulin, Rab27b, TXS and caspase 12 were found to be upregulated at day 3-5 of differentiation in NF-E2^{++} cells (Figure 3.8). These data allude to us having an increased number of MKs in transition toward platelet assembly and terminally mature MKs, characterised respectively by the upregulation of genes associated with the cytoskeleton, cell migration and G-protein signalling and an accumulation of haemostatic factors (Chen et al., 2007). Since the level of these target genes are normally only increased in intermediate-late megakaryopoiesis, in line with the initiation and subsequent elaboration of proplatelets en route to platelet release; the derepression of these genes in early megakaryopoiesis by p45 overexpression may accelerate terminal MK differentiation and thrombopoiesis.

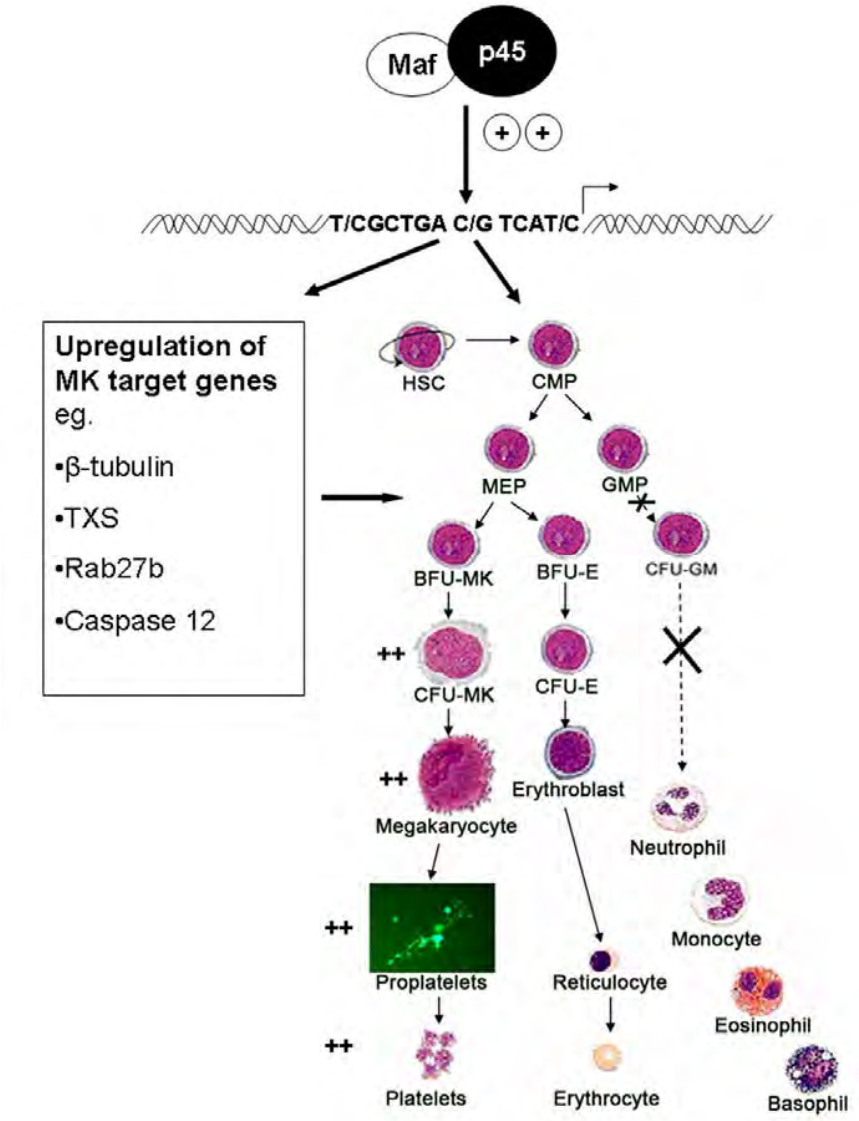


Figure 3.12 Schematic summary of the effects of p45 NF-E2 overexpression on various haematopoietic lineages. An increase in p45 levels results in the upregulation of NF-E2 target genes. Consequently, with the exception of endomitosis, multiple stages (indicated by ++) of MK differentiation are enhanced, as assessed by marker expression, AchE activity, proplatelet and functional platelet production. Myeloid differentiation was shown to be inhibited (indicated by crossed-out dotted line) *in vitro* at early stages of CFU-GM formation, resulting in reduced levels of WBCs *in vivo*. The erythroid lineage is also favoured by ectopic p45 expression (Sayer et al., 2000). CMP: common myeloid progenitor; MEP: megakaryocyte-erythroid progenitor.

In addition, there is at least one other layer of regulation of NF-E2 dependent genes. As illustrated in Figure 3.13A, when NF-E2's smaller p18 subunit is in abundance, Maf homodimers form and compete for the same binding sites to repress transcription (Motohashi et al., 1997; Motohashi et al., 2006). Only a 4-fold difference in the amount of transfected MafK is sufficient to turn activation to repression (Motohashi et al., 1997). Both proplatelet formation and TXS mRNA levels are also exquisitely sensitive to cellular doses of small-Maf proteins (Motohashi et al., 2000). On the flip side, if there exists a higher abundance of p45 relative to the Maf proteins (Figure 3.13B), p18-p45 heterodimers would be the dominant species in the cell, hence favouring the activation of its target genes. It has also been suggested that besides a simple competition with Maf homodimers, the promotion of p18-p45 activity is likely to also involve changes at the structural chromatin level, to generate a permissive chromatin environment (Motohashi et al., 2006). Both these scenarios were likely to be the case in this report.

In summary, we have successfully increased the number of morphologically mature MKs and platelets produced *in vitro*. Despite previous reports that NF-E2's function is primarily limited to terminal megakaryopoiesis, this chapter has now also demonstrated that the increment of a single factor, p45 NF-E2, is able to enhance early megakaryopiesis at the expense of myeloid precursor formation. This is the first report to provide such evidence. Commitment is therefore regulatable and although these experiments were done *in vitro*, the results were reproducible. It is therefore reasonable to conclude that under physiological conditions, the same events would occur *in vivo*.

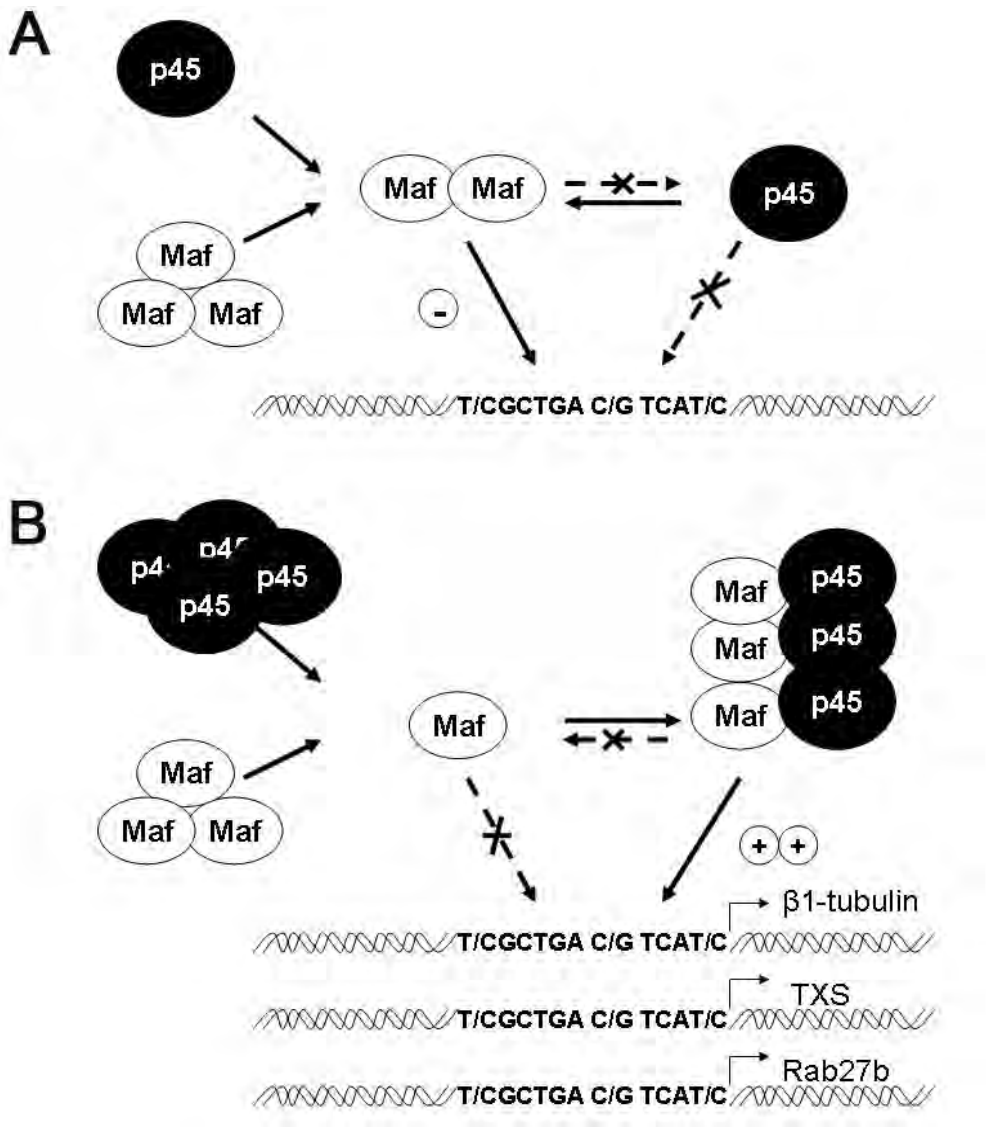


Figure 3.13 Quantitative balance between the p45 subunit and Maf proteins essential for transcriptional activation or repression of target genes. Since p45 cannot form functional homodimers, it requires the small p18 Mafs as obligatory binding partners to bind to AP-1 like consensus sites (bold DNA sequence). (A) If there exists a higher abundance of Maf relative to p45, small Maf homodimers form and suppress transcription by occupying the AP-1 like binding sites. (B) If the abundance of p45 exceeds that of the small Mafs, p18-p45 NF-E2 heterodimers form instead and activate transcription of its target genes via the AP-1 like binding sites.

CHAPTER 4

FUNCTIONAL STUDIES OF ENFORCED P45 NF-E2 EXPRESSION *IN VIVO*

INTRODUCTION

Many approaches have been explored experimentally to produce novel haemostatically active platelet products to treat thrombocytopaenia and other platelet disorders (Lee and Blajchman, 2001; Matsunaga et al., 2006). In general, these novel strategies are designed to either replace platelets with modified or artificial platelets or to augment the function of existing MKs and platelets to achieve primary haemostasis. Regardless of the approach taken, any new platelet product ultimately designed for clinical use should be tested *in vivo* for haemostatic efficacy. In doing so, the insights derived from such studies will also allow for a better understanding of the complex haematopoietic system.

Since the first reports of haematopoietic *in vivo* transplantation into lethally irradiated recipients (Ford et al., 1956; McCulloch and Till, 1960), assays of haematopoietic stem and progenitor cell potential have undergone substantial refinement while still remaining the foundation for defining haematopoietic stem cell biology and gene function. No satisfactory *in vitro* clonal-culture technique exists for HSCs and hence much of the biology of HSCs has had to be deduced from multi-potential cells that can be analysed *in vitro* but that are likely to be more mature than repopulating cells (Metcalf, 2007). How far these events can be extrapolated back to repopulating cells is necessarily speculative. However, with the advent of antibody technology, culture capability, transplantation and FACS-based techniques, a range of different methods for characterising stem and progenitor cell populations are now available for use in haematopoietic cell research.

To date, by using various *in vivo* transplant mice models, the biological influence of several transcription factors on differentiation potentials has been successfully elucidated. For example, competitive repopulation studies have clearly demonstrated that downregulation of GATA-2 expression is necessary for stem cells to contribute to haematopoiesis *in vivo* (Persons et al., 1999) and GATA-3 expression promotes lineage commitment and differentiation toward the erythroid/ MK lineage (Chen and Zhang, 2001). Such a study has yet to be carried out on p45 NF-E2 and would be useful in addressing the effects of p45 NF-E2^{++} expressing HSCs/ progenitors on differentiation under physiological conditions.

Arguably, long-term *in vivo* repopulating assays are instrumental in identifying critical regulators of development and for measuring HSCs. This assay measures the functional potential of the unknown source of HSCs to repopulate against a set known number of HSCs (usually whole BMCs). These studies provide qualitative or at best semi-quantitative information about the HSCs within a given population, but they cannot distinguish between the number of HSCs or their quality (progeny produced per HSC) (Purton and Scadden, 2007). It has been shown that the reliability of this assay is critically dependent on the number of HSCs present in the assessed populations. When too few or too many HSCs ($<1 \times 10^5$ or $>2 \times 10^7$) are introduced, the data may not be meaningful (Harrison et al., 1993). As a result, different methods involving FACS have been developed to purify populations of HSCs to maximise long-term repopulating potential.

The most commonly used conditions for FACS-purified populations of HSC/ progenitor cells is based on their expression of stem cell antigen-1 (Sca-1), *c-kit*, and lack of lineage expression markers (Lin^-) (Okada et al., 1992; Ikuta and Weissman, 1992; Spangrude et al., 1995). Based on numerous studies, it has been shown that a minimum of 16 weeks is required and an optimal 6 months is suggested for monitoring long-term reconstitution post-transplant (Purton and Scadden, 2007).

On the other hand, analysing haematopoietic cell content in mice using short-term *in vivo* assays provides a reasonably more efficient alternative. Such studies produce results within 1-2 weeks of initiating experiments and are are generally a reflection of the more mature progenitor cells as opposed to immature HSCs (Purton and Scadden, 2007). These early engraftment progenitors are more immature than colony forming cells, but are more mature than HSCs and hence provide radioprotection to irradiated mice for the first 2-3 weeks post-transplantation when pancytopenia usually occurs (Till, 1961). Investigators, however, need to be cautious about forming conclusions regarding HSC content from short-term assays. Nonetheless, such studies are often undertaken as the first steps toward analysing haematopoietic cell content and can be used to determine whether it is worthwhile proceeding with long-term repopulating

HSC assays, which are lengthy and costly. For now, such an assay is sufficient for our purposes.

As the work in Chapter 3 demonstrates, an increase in p45 NF-E2's expression successfully leads to an enhancement of MK differentiation and platelet production *in vitro*. To further investigate the functional relevance of p45 NF-E2 overexpression in an *in vivo* mouse model may provide further insight. In the field of haematopoiesis and stem cell research, *in vivo* imaging of labelled transplanted cells has become an invaluable tool for observing cell fate over time in an intact animal (Duda et al., 2007). Indeed, irradiated mice transplanted with NF-E2^{++} cells tagged with eGFP, were found to produce significantly more red blood cells (RBCs) and platelets than the control mice, but only over a 2 week period. The white blood cell (WBC) population of NF-E2^{++} recipients was also found to be reduced compared to the KMV control mice, which provides further evidence towards NF-E2 being a negative regulator of myeloid differentiation. These experiments set a solid precedent for future long-term repopulation assays involving p45 NF-E2^{++} mice, and may provide further understanding into the relevance of p45 NF-E2 as a potential therapeutic target.

RESULTS

Transplantation of NF-E2^{++} BMCs Display Increased Platelet and RBC Production, While Inhibiting WBC Differentiation In Vivo.

To determine whether p45 NF-E2 overexpression had the same effects *in vivo* as *in vitro*, we transplanted lethally irradiated mice with equivalent numbers of either pKMV or NF-E2^{++} BMCs and serially bled them over 1 month. Each peripheral blood sample underwent haematologic analysis and eGFP^{+} expression of platelet and cell populations were identified and quantified using FACS (Figure 4.1i). Immunostaining for Ter-119, CD41 and CD42a was carried out on blood samples to confirm the RBC and platelet gates respectively (Figure 4.1ii-iv).

No gross abnormalities in total blood counts and differentials were observed in all transplant recipients. Besides slight discolouration of their coat of hair, the mice appeared healthy throughout the study period. p45 NF-E2 is involved in erythroid cell development (Ney et al., 1990; Talbot and Grosveld, 1991) and overexpression in primary haematopoietic progenitors was found to increase erythroid colony formation expression (Sayer et al., 2000). We, therefore, first analysed the RBC levels of the recipient mice (Figure 4.2A). eGFP^{+} RBC numbers increased sharply from day 6 to day 18, with eGFP^{+} RBC levels 2-fold higher in the NF-E2^{++} mice at day 10 compared to the KMV group. However, after day 18, eGFP detection levels started to decline.

In a similar fashion, a steady increase in eGFP^{+} platelet production occurred from day 6-10, with the total eGFP^{+} platelet number found to be 3-fold higher in the NF-E2^{++} group compared to the control group (Figure 4.2B). However, kinetic studies also revealed a sharp decrease in eGFP^{+} platelets in NF-E2^{++} recipients from day 12 onwards. eGFP levels became undetectable past day 32 despite increasing total platelet numbers (Figure 4.3). In both cases, eGFP^{+} detection decreased at a faster rate in NF-E2^{++} recipients compared to KMV mice. These findings were consistent in 14 mice over 2 independent experiments and suggests that p45 NF-E2^{++} BMCs do result in increased RBC and platelet production *in vivo*, but only for a short period of time.

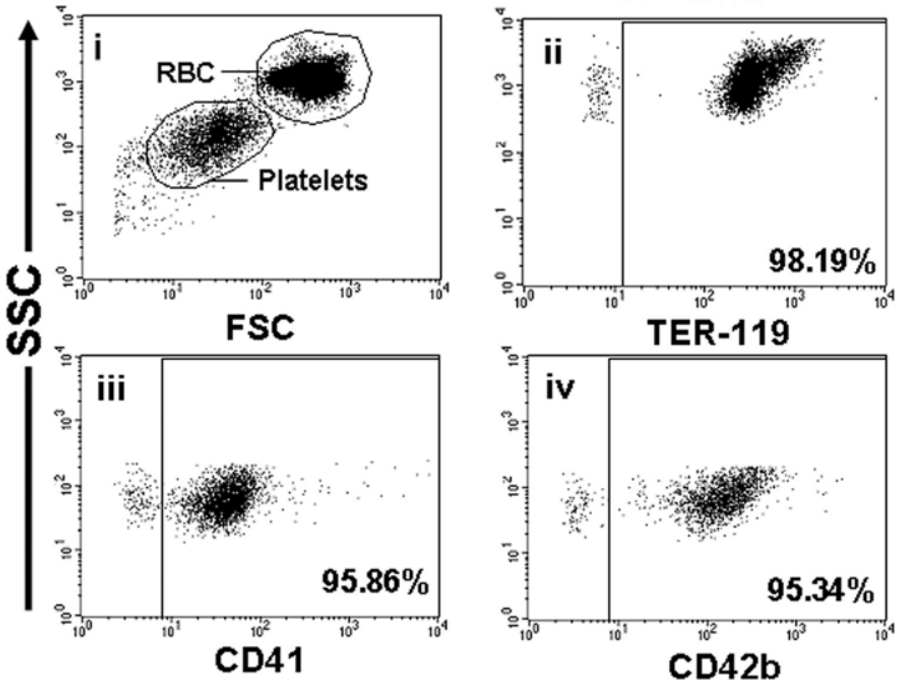


Figure 4.1 Determination of recipient mice platelet and RBC populations. Blood samples from normal and transplant recipient mice were obtained by venous puncture of the lower limb and analysed by flow cytometry. (A) (i) RBCs and platelets were gated on FSC and SSC profile and confirmed by (ii) Ter-119, (iii) CD41 and (iv) CD42b antibody staining respectively. In each case, a representative flow profile with the percentage of positively stained cells in each population is indicated.

The WBC populations of recipient blood samples were also analysed by flow cytometry following lysis of the RBCs and confirmation by Mac-1, Gr-1, CD5 and B220 immunostaining (data not shown). In contrast to the expression of eGFP/ p45 NF-E2^{++} in RBCs and platelets, donor cells continued to give rise to eGFP^{+} WBCs at day 32 (Figure 4.2C). NF-E2^{++} recipients showed a 39% lower level of eGFP^{+} WBCs compared to KMV mice, which is consistent with the CFU-GM inhibition observed in *in vitro* colony assays (Figure 3.7C).

Overall, these transplantation results demonstrate that when overexpressed, p45 NF-E2 has the potential to increase platelet and RBC levels at the expense of WBC production. However, given the transient nature of this elevation, we can only confirm enhanced platelet (and RBC) production from committed MK (erythroid) progenitors and not enhanced engraftment of donor HSCs.

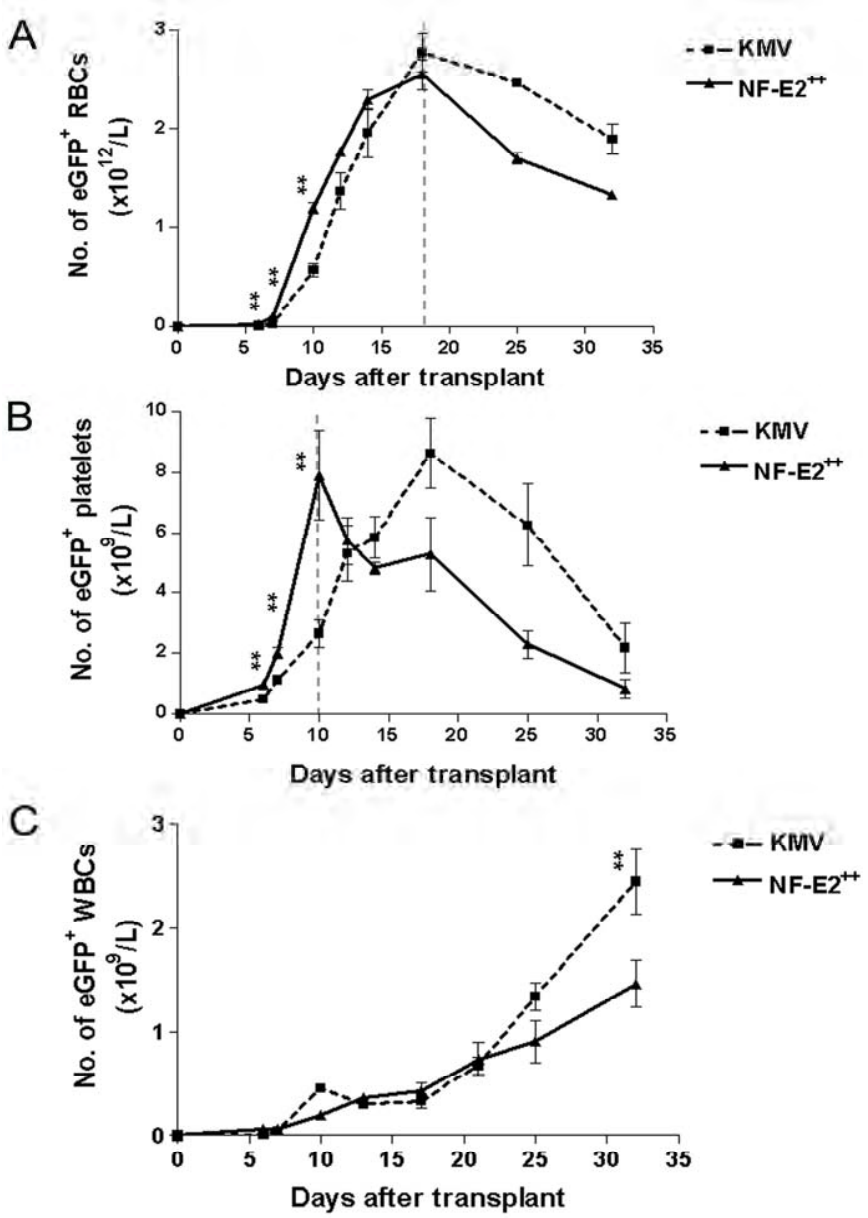


Figure 4.2 Overexpression of p45 NF-E2 increases platelet and RBC production while inhibiting WBC differentiation *in vivo*. pKMV (KMV group) or NF-E2^{++} transduced 8 day 5FU Lin$^-$ BMCs were injected into lethally irradiated recipients. Recipient blood samples were obtained at various time points and analysed by flow cytometry. Short-term reconstitution by transduced eGFP$^+$ cells was quantified in peripheral blood, including (A) RBCs, (B) platelets and (C) WBCs. Each time point represents mean data from 3-5 mice.

Analysis of Total Platelet Counts

Possible explanations for the decline in eGFP expression over time in the platelet population (Figure 4.2B) include: (1) the majority of donor cells were progenitor cells, which matured rapidly *in vivo* and only had a limited lifespan or (2) eGFP reporter expression was being lost over time. To clarify this, we analysed the total recovery of platelet levels within the recipients post-transplantation to ensure that reconstitution of platelets was taking place.

Despite observing a 3-fold increase in $eGFP^{+}$ platelet levels at day 10 (Figure 4.2B) in the NF-$E2^{++}$ mice, there was no significant difference in total platelet reconstitution between the KMV and NF-$E2^{++}$ recipients up to day 18 (Figure 4.3). The accelerated platelet recovery from $eGFP^{+}$/ NF-$E2^{++}$ donor cells compared to pKMV cells was not large enough to reflect a change in total platelet levels. However, total platelet counts of the NF-$E2^{++}$ recipients were found to be marginally higher ($p>0.05$) than those of the KMV mice at day 25 and 32. This may suggest that although eGFP levels were declining, platelet production in the NF-$E2^{++}$ group was still increasing past the point of eGFP detection. No noticeable difference in macroscopic bleeding tendency was observed between both groups of recipients.

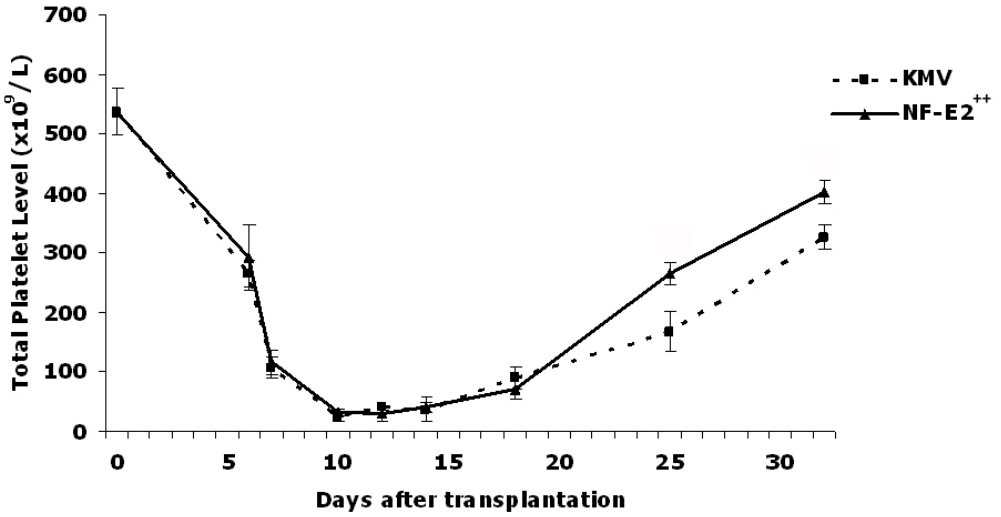


Figure 4.3 Recovery of total platelet counts following lethal irradiation and transplant. Groups of 3-5 recipient mice were lethally irradiated and transplanted with the equivalent of 3.8 x 10^5 transduced 8 day 5FU Lin^- pKMV or NF-$E2^{++}$ BMCs. Recipient blood samples were obtained at various time points and total platelet counts were analysed by an automated haematology analyser.

Transplantation of Purified Transduced BMCs

The transplantation experiments were repeated, but this time with purified $eGFP^+$ populations of donor cells. In the transplant experiments described previously, heterogeneous populations of unsorted cells were used as donors. Although this enabled us to transplant more cells, the donor population also contained cells not transduced with the viral vector. These cells may have eventually outgrown the $eGFP^+$ donor cells. In order to minimise this occurrence and transplant a higher frequency of transduced HSCs/ HPCs, only sorted $eGFP^+$ donor cells were transplanted. Analysis of the FACS-enriched cell populations before transplantation showed purities of 95% and 92% for control pKMV and NF-$E2^{++}$ donor cells respectively (data not shown). The kinetics of $eGFP^+$ platelet levels in individual recipients were monitored over time.

In the KMV group, only 2 out of 5 mice showed long-term repopulating potential with varying delays in the start of the second wave (Figure 4.4A- green and black). On the other hand, all the recipients in the NF-$E2^{++}$ group only exhibited increasing $eGFP^+$ platelet levels over one wave (Figure 4.4B). Similarly, compared to the first transplant study using a heterogeneous population of donor cells (Figure 4.2B), the percentage of $eGFP^+$ platelets was also low (~5%) at day 32 (Figure 4.4B).

Interestingly, it was noted that the NF-$E2^{++}$-1 recipient demonstrated increasing $eGFP^+$ platelet levels up to day 20 and appeared to be a likely candidate for longer-term reconstitution (Figure 4.4B-red). Although this was not the case and $eGFP^+$ levels did eventually decline, platelet levels at day 20 was ~2-4 fold higher than any of the KMV recipients (Figure 4.4A; B-red). This result suggests that the NF-$E2^{++}$-1 recipient had more CFU-MKs and hence was eventually able to produce more platelets in the lifespan of that population of MKs. Collectively, these results suggest that by using a purified population of donor cells and hence increasing the frequency of transduced HSCs being transplanted, long-term platelet reconstitution may be more likely (KMV group: 2/5 showed long-term repopulating potential). However, it also reflects that lost of exogenous gene expression is still probable.

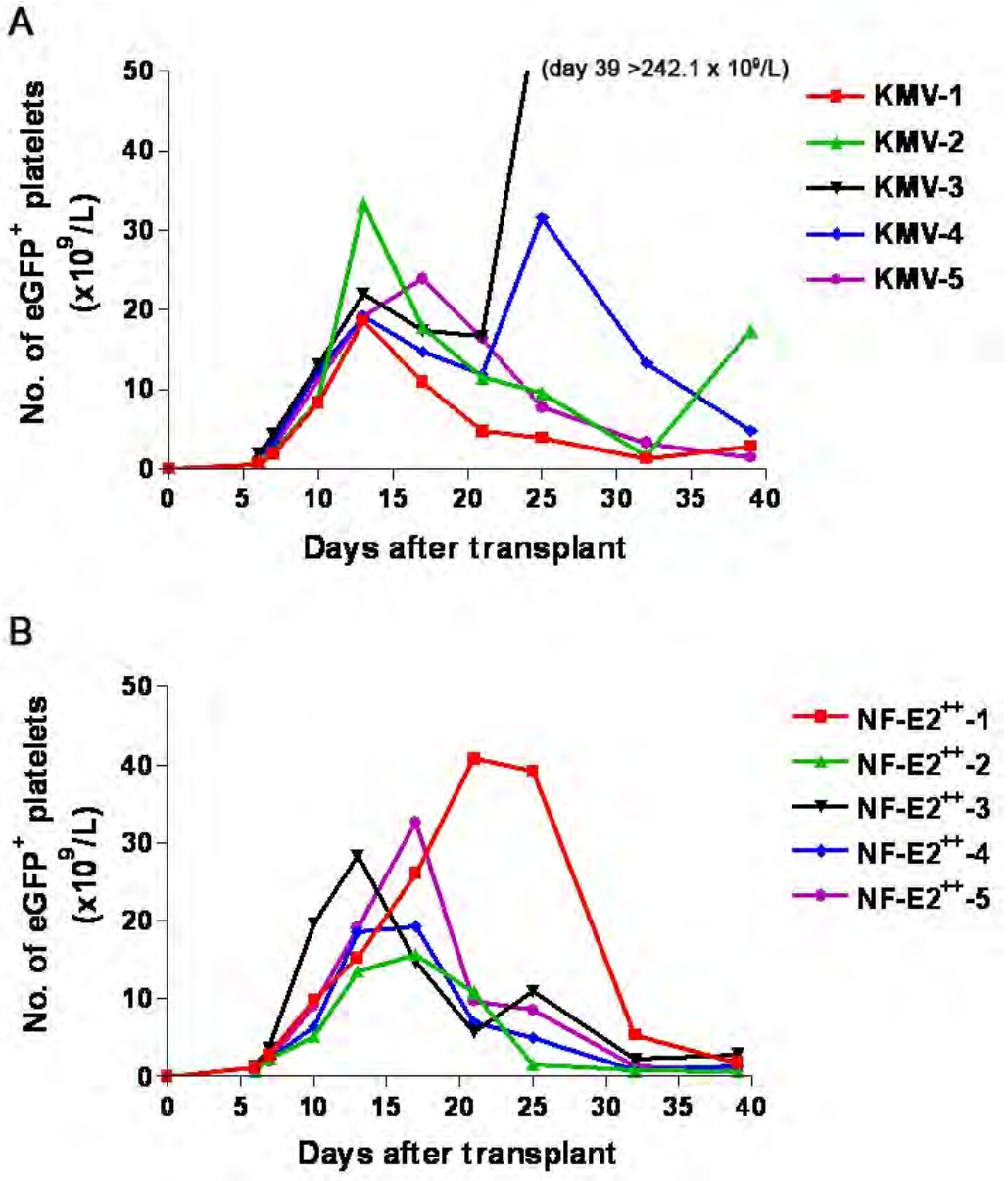


Figure 4.4 eGFP$^+$ platelet levels following transplant with purified transduced BMCs. Lethally irradiated mice were transplanted with 2 x 10^5 sorted 8 day 5FU Lin$^-$ (A) pKMV or (B) NF-E2^{++} BMCs. Recipient blood samples were obtained at various time points and analysed by flow cytometry as previously described (Figure 4.1). Individual kinetic data from 5 recipient mice/ group is shown.

Decreased eGFP Expression in Recipient Bone Marrow Cells

To confirm whether exogenous gene silencing was indeed occurring, we carried out quantitative real-time PCR to verify eGFP expression. Purified erythroid and leukocyte (WBC) populations were obtained from transplant recipients that either showed gradual loss of eGFP detection (-ve KMV-1, -4; Figure 4.4A-red and blue) or consistent increase in eGFP expression (+ve KMV-3; Figure 4.4A-black). This was done by immunostaining with either Ter-119 or WBC-specific markers respectively, followed by positive magnetic activated cell sorting (MACS). RNA was then extracted from these individual cell populations as described in Chapter 2 (pages 51-52).

As shown in Figure 4.5A, expression of eGFP in the erythroid population of KMV-3 was ~193 fold higher at the messenger level than the erythroid population of KMV-1/4. In the WBC population, there was no significant difference in eGFP expression between the 2 groups of mice (Figure 4.5B). These results are consistent with the declining $eGFP^+$ RBC, but high $eGFP^+$ WBC levels observed in previous transplants (Figure 4.2A; C). These results imply that selective exogenous gene silencing was taking place within the erythroid cells, but not in WBCs.

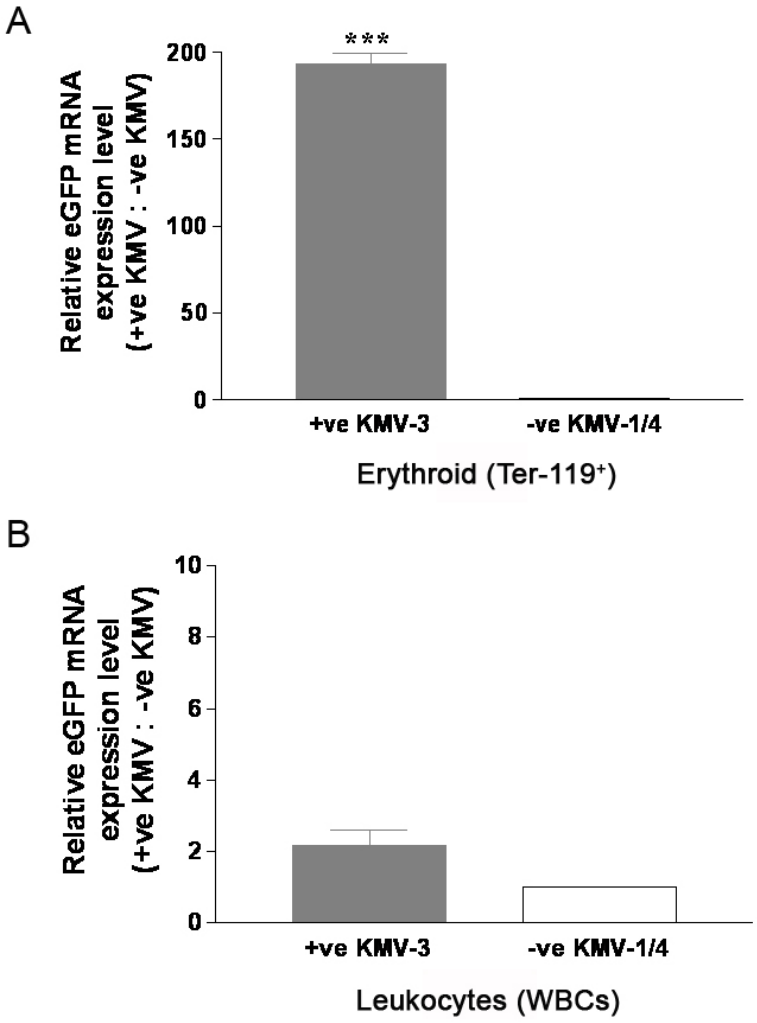


Figure 4.5 Significant decrease in eGFP expression at the messenger level in transplant recipients. Lethally irradiated mice were transplanted with pKMV transduced 8 day 5FU Lin$^-$ BMCs and their blood content were monitored over 39 days. The mice were subsequently sacrificed and their BMCs stained with either Ter-119 or a combination of Mac-1, Gr-1, CD5 and B220 antibodies to obtain purified erythroid and leukocyte populations respectively by positive MACS. eGFP gene expression was then studied using RNA extracted from the (A) erythroid cells and (B) leukocytes of a mouse still positive for eGFP expression (KMV-3) and mice that had low levels of eGFP platelet and RBC populations (KMV-1 and -4). Target quantities were normalised to GAPDH and presented relative to the eGFP negative control (-ve KMV-1/4). Each real-time PCR reaction was performed in triplicate and presented as the mean ± SD.

DISCUSSION

Previous work described in Chapter 3 and by others, clearly highlights p45 NF-E2's essential role in regulating distinct stages of MK differentiation, extending from the birth of early committed progenitors to the final step of platelet release. *In vitro* studies have now also demonstrated for the first time p45 NF-E2's ability to enhance MK differentiation and platelet production, making it a potential target for developing strategies toward treating thrombocytopaenia and bleeding disorders. The effects of enforcing p45 NF-E2 expression *in vivo* was, however, still unclear and hence was of particular scientific interest.

The short-term platelet reconstitution kinetics of irradiated mice transplanted with NF-E2^{++} BMCs supported our *in vitro* results. Over a single wave after transplantation, there was a significant 3-fold increase in platelet production observed in mice transplanted with NF-E2^{++} BMCs. Consistent with the previous *in vitro* data (Figure 3.3B), this accelerated platelet production from day 6-10 is likely a result of a larger number of terminally differentiated MKs present in the bone marrow during that period. However, our data suggest a lack of long-term repopulating potential and expansion of transduced donor cells as both eGFP^{+} platelet and RBC levels declined over time. With the exception of 2 mice from the control KMV group, a similar loss in eGFP expression was also obtained when purified transduced donor populations were transplanted.

A combination of factors was most likely responsible for this loss in eGFP detection. Firstly, the number of HSCs within the assessed population was almost certainly insufficient. Already, HSCs in mouse bone marrow are a rare population- 1 in 10^4-10^5 (Orlic et al., 1995). The donor populations in these experiments were derived from 8 day 5FU-treated mice; and while administration of 5FU treatment itself to mice 2-4 days prior to harvesting their bone marrow promotes cell cycling with the gradual loss in *c-kit* expression on Lin^{-} , Sca-1^{+} HSCs (Randall and Weissman, 1997), 8 day 5FU-treated mice have been found to be enriched for more mature CFU-MKs (Bradley et al., 1989). Such populations are ideal for studying NF-E2 effects, which were previously documented to be in the later stages of MK differentiation and platelet release (Shivdasani, 1996; Levin et al., 1999). Conversely, these 8 day 5FU cell populations

may not be as suitable for long-term studies requiring HSCs rather than HPCs. A minimum of 1 x 10^5 HSCs has previously been shown to produce reliable long-term reconstitution (Harrison et al., 1993). In future long-term reconstitution studies, it is probably best to carry out limiting dilution assays to determine the frequency of transduced HSCs being transplanted (Purton and Scadden, 2007).

In addition, our data most likely also reflects selective silencing of exogenous genes within the MK and erythroid lineage. In all sets of transplantation experiments, platelets and RBCs that did not express eGFP/ NF-E2^{++}, markedly out competed eGFP^{+}/ NF-E2^{++} cells in the reconstitution of lethally irradiated mice. eGFP message was also significantly reduced in recipient erythroid cells as shown by quantitative real-time PCR (Figure 4.5). Achieving stable vector genomes from retroviral vectors has been a longstanding challenge, often resulting in only short-lived expression *in vivo* (Bestor, 2000; Challita and Kohn, 1994; Klug et al., 2000). Increased copy numbers of a transgene have been found to markedly reduce expression from a reporter gene as a result of hypermethylation and the adoption of a repressive local chromatin configuration (Forsberg et al., 1999). In addition to copy number, other factors influencing transgene silencing in mice include the integration site, the lineage in which expression occurs and the cis-acting control elements within the transgene (Martin and Whitelaw, 1996).

Our *in vivo* transplantation analysis also included the assessment of p45 overexpression on the myeloid lineage. Recipient mice surprisingly continued to give rise to eGFP^{+}/ NF-E2^{++} WBCs at day 32 with the majority (>90%) of the population being eGFP^{+}. Interestingly, NF-E2^{++} recipients also showed a significant decrease in reconstituted WBCs (Figure 4.2C). This further supports our *in vitro* data (Figure 3.7) and reiterates NF-E2's inhibitory role in myeloid differentiation. Contrary to the RBC and platelet populations, the WBCs did not experience loss of eGFP expression over time, suggesting that the insert could be under completely different silencing influences in different lineages. DNA that is actively transcribed in one lineage may be inactive and packaged as heterochromatin in another (Martin and Whitelaw, 1996). Most importantly, erythroid cells also represent an extreme example of a lineage with

extensive heterochromatinisation as the nucleus is condensed during terminal differentiation. This lack of nuclei represents a challenge for exogenous gene studies involving RBCs and platelets as both cell populations are likely to express reporter genes for only a short period of time. WBCs, on the other hand, are equipped with the organelles and machinery required to sustain prolonged gene and protein expression.

Interestingly, in clinical instances such as following chemotherapy where only short-term heightened platelet production is desirable, systems that increase p45 NF-E2 concentration temporarily may be advantageous. Patients undergoing HPC transplants to reconstitute haematopoiesis following myeloablative chemotherapy often suffer from severe thrombocytopaenia for as long as 2-3 weeks (Kessinger et al., 1988; Williams et al., 1990). Supplementation of conventional autografts with an *ex vivo* expanded NF-E2^{++} MK-rich product may enhance *in vivo* platelet production and shorten the period of thrombocytopaenia. Indeed, this chapter demonstrates that within 10 days, our NF-E2^{++} cells were capable of accelerated platelet production *in vivo*. Furthermore, anaemia can contribute significantly to the prolonged bleeding time seen in thrombocytopaenic animals or patients and RBC transfusions are capable of correcting this bleeding time (Blajchman et al., 1994; Ho, 1998). Thus, the resultant elevated RBC counts from NF-E2^{++} BMCs may be an added bonus in the management of a bleeding thrombocytopaenic patient who may also be anaemic.

In conclusion, from transduced progenitor cells, we have successfully increased the number of platelets *in vivo*. In addition to the clinical implications already mentioned, the short-term NF-E2 mediated enhancement of platelet production demonstrated here, suggests that NF-E2 could also be targeted by agents that either promote its expression or prolong the half-life of the protein. For instance, in cancer treatment, by targeting the Hdm2 E3 ubiquitin ligase-p53 interactions with synthetic peptides or monoclonal antibodies, E3 ligase-mediated targeted degradation of p53 is disrupted (Sun, 2006). Consequently, the levels of p53 are increased and cancer growth arrest and apoptosis are induced (Duncan et al., 2003). p45 NF-E2 interacts with ITCH E3 ubiquitin ligase, which is also a co-repressor of p45 (Chen et al., 2001), and hence a similar approach may be feasible to promote its accumulation within MKs to heighten platelet levels.

CHAPTER 5

REGULATION OF THROMBOPOIESIS BY THE NUCLEAR SHUTTLING MECHANISMS AND SUMOYLATION OF P45 NF-E2

INTRODUCTION

Loss of p45 NF-E2 function results in dramatic embryonic lethal thrombopoietic defects such as severely hampered platelet release and the lack of normal circulating platelets (Shivdasani et al., 1995; Shivdasani, 1996). In contrast, and as shown in Chapters 3 and 4, overexpression of p45 NF-E2 enhances MK differentiation, proplatelet development and platelet release *in vitro* and *in vivo*. Given p45 NF-E2's crucial role in MK development and function, the precise regulation of p45 NF-E2 concentration and activity is vital and a key mechanism by which this may occur is via nuclear shuttling.

After being synthesised and post-translationally modified in the cytoplasm, most macromolecules need an active, signal-mediated transport mechanism to reach the nucleus. Molecules containing classical nuclear localisation signals (NLSs) of one (monopartite) or two clusters (bipartite) of basic amino acids are transported into the nucleus through the nuclear pore complex (NPC) with the aid of nuclear import receptors (Pemberton and Paschal, 2005). A monopartite NLS, such as the prototypical simian virus 40 large T antigen NLS (PKKKRKV), is composed of several basic amino acids (Kalderon et al., 1984). Bipartite NLSs on the other hand, consist of two clusters of basic amino acids separated by a 10-12 amino acid linker. The nucleoplasmin NLS (KRPAATKKAGQAKKKK) is an example of a typical bipartite NLS (Robbins et al., 1991).

Nucleocytoplasmic transport (refer to Chapter 1, page 30) is a multi-step process beginning with the NLS-containing protein binding to a shuttling transport factor belonging to the karyopherin family (includes importins (IMPs), exportins (EXPs), tranportins). In the classical pathway, the cargo binds first to IMP α followed then by one of a number of IMP βs. In non-classical pathways, the cargo binds only to a member of the IMP β family or interacts directly with the nucleoporins (Nups) of the NPC. This is then followed by the docking of the complex at the NPC before being translocated into the nucleus via interactions with the Nups (Gorlich and Kutay, 1999). Nuclear export is analogous to nuclear import, except that exportins (EXPs) are recognised by hydrophobic nuclear export signals (NESs).

The complete repertoire of IMP/ EXP/ Nup regulators is now known from whole genome sequences. There are multiple homologs of both IMP α (eight in humans as opposed to one in yeast) (Mason et al., 2002) and IMP β (>20 in humans) (Poon and Jans, 2005). Different IMP αs have been found to demonstrate distinct properties in terms of NLS recognition/ nuclear transport efficiency for specific cargo and differential tissue/ cell-type expression (Jans et al., 2000; Mason et al., 2002; Sekimoto et al., 1997; Kohler et al., 1999; Miyamoto et al., 1997). Of the >20 IMP β family members, only IMP β1, IMP β2 (transportin), IMP β3 (Ran-binding protein 5), IMP7 (RanBP7), and EXP CRM1 have been functionally characterised in higher eukaryotes (Jakel and Gorlich, 1998; Poon and Jans, 2005). NPCs are composed of ~40-50 distinct Nups that have different affinities for distinct IMPs/ EXPs suggesting that changes in specific Nup expression may modulate nuclear transport efficiency (Allen et al., 2001). Different combinations of the above karyopherins together with specific mechanisms that regulate cargo-karyopherin recognition provide the complexity required to facilitate the nuclear transport of a relatively large number of protein cargo.

The domain structure of the p45 NF-E2 protein has typical features of bZip transcriptional regulators and indeed has been shown to function as a powerful transcriptional transactivator of MK-specific gene expression (Deveaux et al., 1997; Lecine et al., 2000; Kerrigan et al., 2004). In addition to its characteristic DNA-binding and leucine-rich bZip domain that enables the formation of alternative heterodimeric combinations with Maf proteins, p45 NF-E2 would also contain a nuclear transport signal. Both endogenous and transiently expressed p45 NF-E2 are detected mainly in the nucleus, where it is associated with nuclear bodies (Francastel et al., 2001; Shyu et al., 2005). However, like all other nuclear proteins, p45 NF-E2 is first synthesised in the cytoplasm and must be imported into the nucleus to perform its transcriptional function. The mechanisms and precise amino acid residues by which p45 NF-E2 facilitates nucleocytoplasmic transport remain unknown.

Based on its sequence, human p45 NF-E2 appears to contain a bipartite NLS (271RRRGKNKVAAQNCRKRKL285) and also a potential NES in its C-terminal leucine zipper. Indeed, when compared to other bipartite NLSs from NF-E2-related

factor 2 (Nrf-2) (Jain et al., 2005), nucleoplasmin (Robbins et al., 1991), p73 (Inoue et al., 2002) and p53 (Liang and Clarke, 1999; Liang and Clarke, 2001), amino acids 271-285 of p45 NF-E2 showed high sequence homology with these previously described NLSs. In addition, nuclear export signals from several proteins including Nrf-2, Iκ-Bα, TFIIA, p53, p73, hDM2 and PKI-α have similar hydrophobic, leucine rich regions that are also found in the C-terminal of p45 NF-E2 (Gorlich and Kutay, 1999).

Another key mechanism for achieving dynamic gene regulation is via post-translational modifications. Indeed, acetylation of the p18 subunit of NF-E2 has been found to augment its DNA binding and transcription activities (Hung et al., 2001), while phosphorylation of the p45 subunit was suggested to affect interaction(s) of its transactivation domain with other transcriptional regulatory protein(s) (Casteel et al., 1998). In addition, SUMOylation (refer to Chapter 1, page 32-36) of p45 NF-E2 at lysine 368 (K368) by SUMO-1 has recently been reported to enhance the transactivation and DNA-binding capabilities of NF-E2 in the erythroid context (Shyu et al., 2005). Furthermore, it was found that SUMOylated NF-E2 factors were concentrated together with RNAPII in nuclear bodies where the majority of the euchromatic β-globin loci were anchored in an erythroid cell-specific manner (Shyu et al., 2005).

It was suggested that SUMO-modified p45 NF-E2 is co-localised with its target gene loci in the same nuclear subcompartments in order to function. It has also been put forward that certain substrates may be SUMOylated and deSUMOylated upon their passage through the NPC (Seeler and Dejean, 2003; Pichler et al., 2002). In support of this hypothesis, nuclear transport of a number of proteins has been found to be dependent on SUMO modification (Rangasamy et al., 2000; Comerford et al., 2003; Wood et al., 2003). Studies (reviewed in Johnson, 2004) have clearly shown that SUMOylation can affect the biochemical and biological properties of its substrate, although the effects of p45 NF-E2 SUMOylation in a MK setting has yet to be elucidated.

In the current study, we set out to examine the nuclear import signals and mechanisms of p45 NF-E2. Toward this end, a monopartite NLS was identified in the DNA binding domain of p45 NF-E2. The identified NLS facilitated nuclear import via the non-classical pathway through interaction with IMP β, although the results suggest intramolecular masking of the NLS within the full length folded protein. Mutation of the NLS led to loss of exclusive nuclear localisation of p45 NF-E2 and diminished transcriptional activity of its downstream genes. In addition, in the absence of a functional NLS, p45 NF-E2's ability to enhance proplatelet and platelet development from primary murine MKs was significantly reduced. These results provide strong evidence that nuclear import of p45 NF-E2 plays a significant role in gene expression and is crucial for MK differentiation and thrombopoiesis.

In this chapter, we also present data that p45 NF-E2 can be SUMOylated by SUMO-2/3 in murine BMCs. Mutation of the SUMOylation site of p45 NF-E2 had no visible effect on nuclear localisation in both COS and murine BMCs when compared to the wildtype (WT). However, p45 NF-E2 SUMOylation was required to elevate the transcriptional activation of MK-specific genes and enhance platelet production. These results confirm that SUMOylation of p45 NF-E2 has important physiological consequences in the MK lineage, including regulation of MK-specific gene transcription and subsequent thrombopoietic processes.

RESULTS

Identification of the p45 NF-E2 NLS Within the DNA Binding Domain

p45 NF-E2 can be divided into four major domains (Figure 5.1A): the proline rich acidic transactivation domain located at the N-terminus (aa 1-209) (Gavva et al., 1997); the cap 'n collar (CNC) domain, which is distinguished by a conserved region of amino acids (aa 210-266) and is located immediately upstream of the bZip domain (Bean and Ney, 1997). This latter domain can be further divided into two regions: the basic DNA binding domain (aa 267-293); and the heptad leucine zipper domain located at the C-terminus, which is involved in p18-p45 heterodimerisation (Bean and Ney, 1997).

To locate the NLS in p45 NF-E2, we constructed full length (FL) p45 NF-E2 and various deletion fragments fused upstream or downstream to GFP (Figure 5.1B). GFP alone (27 kDa) does not contain a functional NLS and can travel freely between the nucleus and cytoplasm and was thus used as a negative control. These constructs were transiently transfected into COS-7 cells, and the intracellular localisation of the fusion proteins was analysed by confocal microscopy.

As expected, in the mock control, GFP was localised in both the nucleus and cytoplasm in the absence of an active NLS (Figure 5.2i). Full length p45 NF-E2 fused to GFP (Figure 5.2ii) was expressed and actively targeted to the nucleus, suggesting active nuclear transport facilitated by an intact NLS. Interestingly, ~20% of the transfected cells showed preferential accumulation of GFP in subnuclear speckles. In contrast, the N-terminal fragment of p45 NF-E2, aa 1-209, localised to both the nucleus and cytoplasm (Figure 5.2iii). This suggested that active transport was inhibited, but some protein could still enter the nucleus via passive diffusion. Similarly, constructs that included just the CNC domain (aa 210-266) or the leucine zipper C-terminal (aa 294-373), failed to accumulate solely in the nucleus (Figure 5.2iv; vi). Consequently, of the deletion fragments generated, only the basic DNA binding domain, aa 267-293, was targeted exclusively to the nucleus, similar to FL p45 NF-E2 (Figure 5.2v). Therefore, the minimal region that is able to facilitate active nuclear import of p45 NF-E2 was determined to be within aa 267-293.

In the absence of an NLS, GFP fusion proteins were detected in both the nucleus and cytoplasm. None of the fragments examined were excluded from the nucleus, suggesting that p45 NF-E2 does not contain a NES.

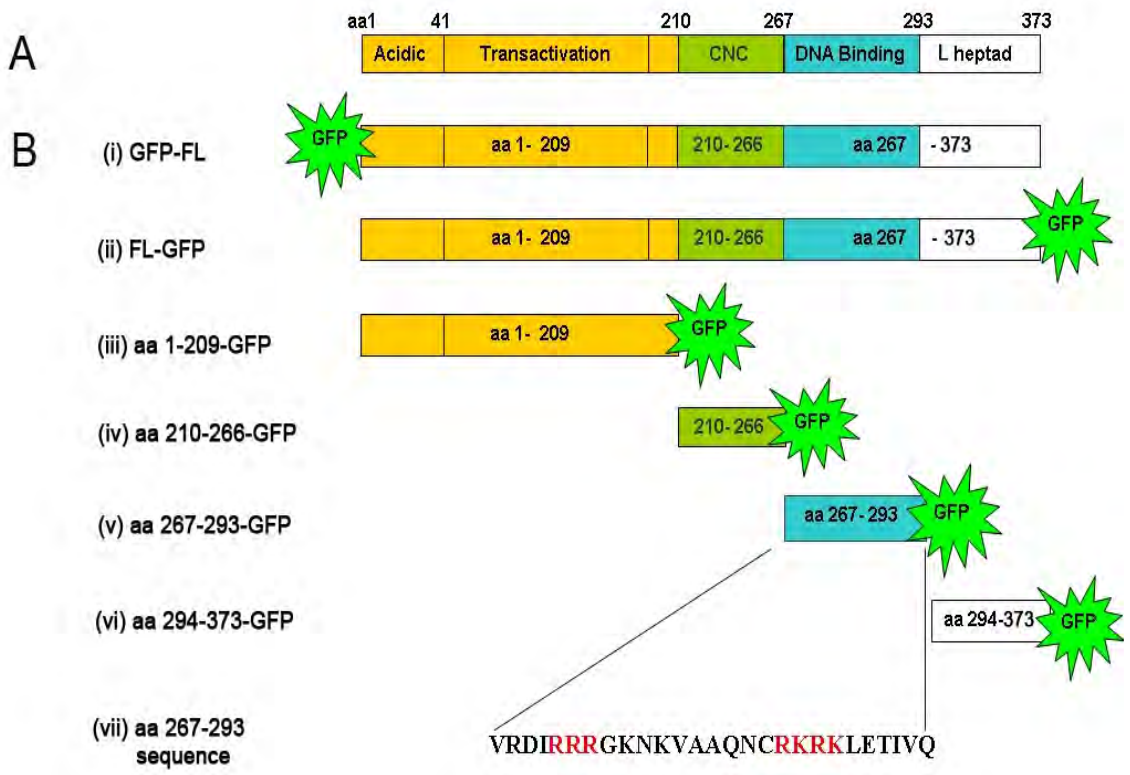


Figure 5.1 Schematic representation of the p45 NF-E2 NLS mapping process. (A) Full length (FL) p45 NF-E2 and its respective transactivation, cap 'n collar (CNC), DNA binding and heptad leucine (L) domains. (B) FL p45 NF-E2 (i-ii) and various deletion fragments (iii-vi) tagged to either an N-or C-terminal GFP were constructed. (vii) The amino acid sequence of the potential NLS region (aa 267-293) is shown with its positively charged amino acid residues highlighted in red.

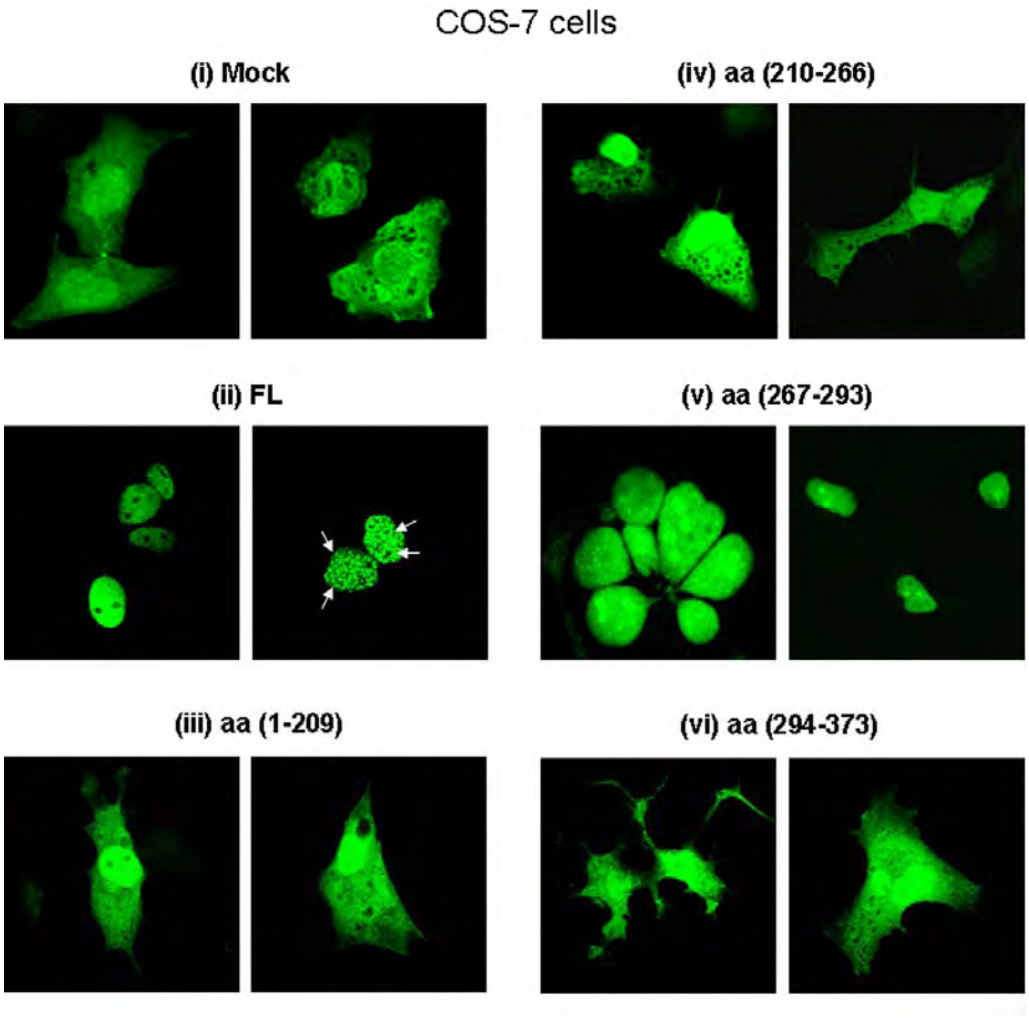


Figure 5.2 Intracellular localisation of GFP-tagged FL and deletion constructs. Constructs (i-vi) were transfected into COS-7 cells and their intracellular locations were analysed by confocal microscopy after 48 hours. A total of 50 cells were analysed in each slide and representative pictures of the majority localisation pattern are shown. (i) Right panel: preferential accumulation of GFP in subnuclear speckles (indicated by white arrows) were observed in some FL cells. N- and C-terminus GFP tagged constructs gave similar outcomes and hence only the results of the C-terminus GFP fragments are shown above.

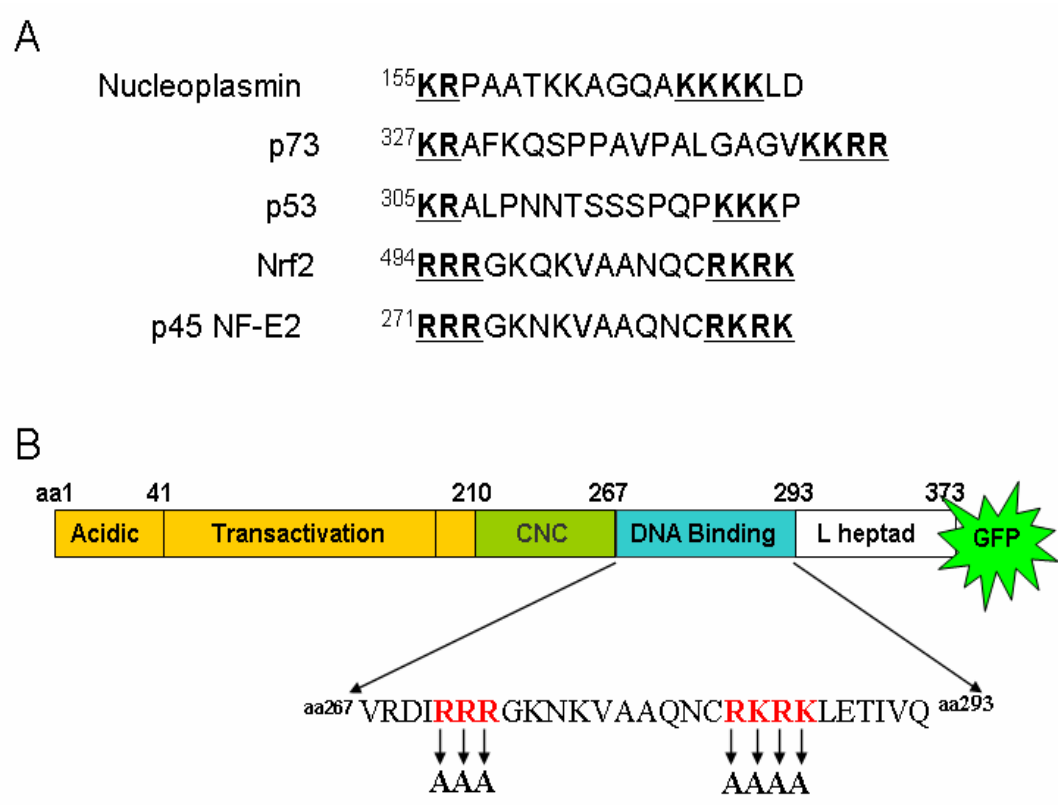
The Monopartite p45 NF-E2 NLS is Disrupted by Single Mutations of its Positively Charged Amino Acids

Alignment of the p45 NF-E2 sequence with previously characterised NLS sequences from nucleoplasmin, p73, p53 and Nrf2 (Figure 5.3A) suggested a potential NLS signal, which may also have a bipartite structure that includes the basic residues within aa 267-293. To test this possibility, mutations of the respective basic amino acid clusters were performed as illustrated in Figure 5.3B. The positively charged arginine (R) and lysine (K) residues were mutated to the neutral alanine (A) in the N-terminal GFP tagged FL construct, and transfected into COS-7 cells. The first three cluster mutants generated were: R271-273A, RK284-287A and R271-273A/ RK284-287A.

As shown in Figure 5.3Cii, mutation of the first cluster of basic residues, R271-273A, resulted in definitive loss of nuclear GFP accumulation. GFP was localised in both the cytoplasmic and nuclear fractions in more than 95% of the cells examined. This indicated that the NLS had been abolished and active nuclear import inhibited. This same observation was made in mutants where in addition to R271-273A, the second positively charged cluster, RK284-287A, was simultaneously mutated (Figure 5.3Civ). However, unexpectedly, mutations to the second cluster, RK284-287A, on its own had little effect on the NLS, with the majority (90%) of cells displaying nuclear accumulation of the GFP-fusion protein (Figure 5.3Ciii). In some cases, GFP could be weakly detected in the cytoplasm. The above results demonstrate that the four amino acids, R284, K285, R286 and K287 are not crucial for p45 NF-E2 nuclear localisation. Instead, the single NLS of p45 NF-E2 is of monopartite structure and includes amino acids R271-273.

To further fine map which amino acids within aa 271-273 were crucial for nuclear import of p45 NF-E2, single amino acid mutations were carried out. The following mutants in p45 NF-E2 were generated and transfected into COS-7 cells: R271A, R272A and R273A. Mutation of R271 resulted in weak cytoplasmic staining with GFP, although accumulation of the protein was substantially more intense in the nucleus (Figure 5.3Cv). This phenotype was observed in all the cells examined. It was however noted that the R271A mutants did exhibit a higher occurrence (50-70%) of speckling compared to the WT or other mutants (20-27%).

In comparison, mutation of the second amino acid, R272 had no effect on the NLS, with the same nuclear GFP accumulation as the WT (Figure 5.3Cvi). Of the three point mutations carried out, only R273A was able to hinder definite nuclear targeting of p45 NF-E2, as observed by distribution of GFP in both the cytoplasm and nucleus (Figure 5.3Cvii). Disruption of the NLS was detected in ~75% of cells examined for the R273A mutant. These results suggest that although not as effective as mutating all three residues between R271-273, a single mutation is sufficient to inhibit the NLS of p45 NF-E2. In particular, amino acids R271 and R273 are crucial for facilitating active nuclear import.



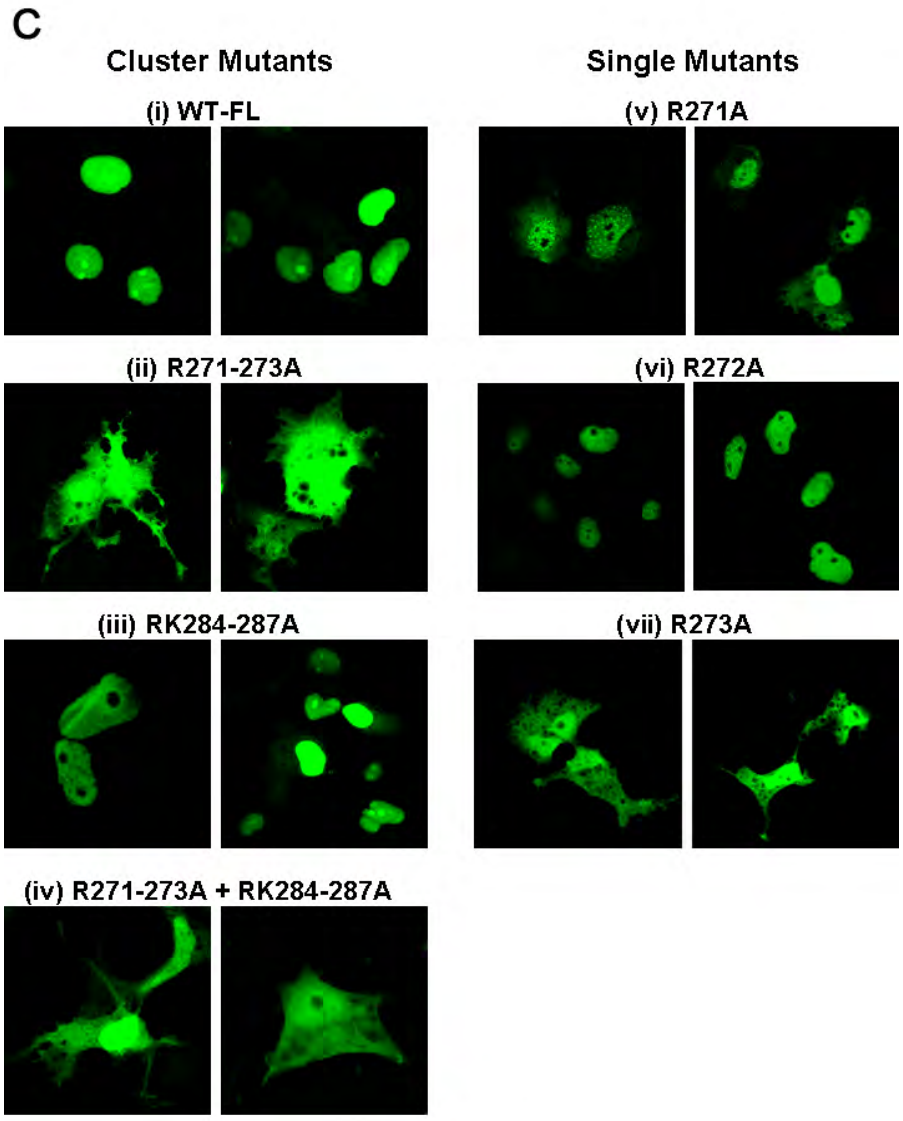


Figure 5.3 Identification of a monopartite NLS in p45 NF-E2 using site-directed mutagenesis and confocal microscopy. (A) Alignment of p45 NF-E2 with other protein NLSs revealed a potential bipartite NLS within aa 271-287. Homologous amino acids are shown in boldface type and underlined. (B) Sequence of the putative NLS region is shown. Positively charged arginine (R) and lysine (K) residues (red font) were mutated into alanine (A) using site-directed mutagenesis (indicated by the arrows). (C) Mutant constructs were transfected into COS-7 cells and their nuclear localisation compared to the WT full length (WT-FL) by confocal microscopy. One hundred transfected cells were counted in each case.

Nuclear Import of p45 NF-E2 in Primary Bone Marrow Cells

The above nuclear localisation studies were conducted in COS-7 cells due to their high transfection efficiency and absence of endogenous p45 NF-E2 expression (Andrews et al., 1993). However, since NF-E2 is involved in haematopoiesis and MK function, we verified our results using a more biologically relevant cell type that expresses endogenous p45 NF-E2, namely haematopoietic bone marrow cells (BMCs). The murine BMCs were transduced with V5-tagged p45 NF-E2 WT and mutants and their nucleocytoplasmic distribution was determined using anti-V5 immunofluorescence staining and confocal microscopy. The pKMV retroviral vector used for delivering the V5 constructs into the cells also encodes for the eGFP cDNA in a bicistronic manner (refer to Figure 3.1A). eGFP alone is small and distributes evenly throughout the entire cell and hence suitably marks out the cell boundary (Figure 5.4-left panel).

As expected, the small, diffusible V5 tag, which was used as our negative control resulted in both nuclear and cytoplasmic localisation (Figure 5.4i). Similar to COS-7 cells, WT p45 NF-E2 and RK284-287A were only detected in the nuclei of murine BMCs (Figure 5.4ii and iv). Simultaneous mutations to amino acids R271, R272 and R273 once again appeared to have disrupted the p45 NF-E2 NLS, as observed by both nuclear and cytoplasmic V5 (Figure 5.4iii). V5 fused to p45 NF-E2 is still only ~50 kDa and hence can enter the nucleus via passive diffusion in the absence of active nuclear transport. None of the cells examined demonstrated exclusive cytoplasmic V5 accumulation, confirming that the presence of an NES is unlikely. These results, using murine BMCs as a relevant haematopoietic cell type, confirmed that the basic residues within aa 271-273 and not 284-287 were responsible for the nuclear import of p45 NF-E2. In addition, p45 NF-E2 does not contain a functional NES.

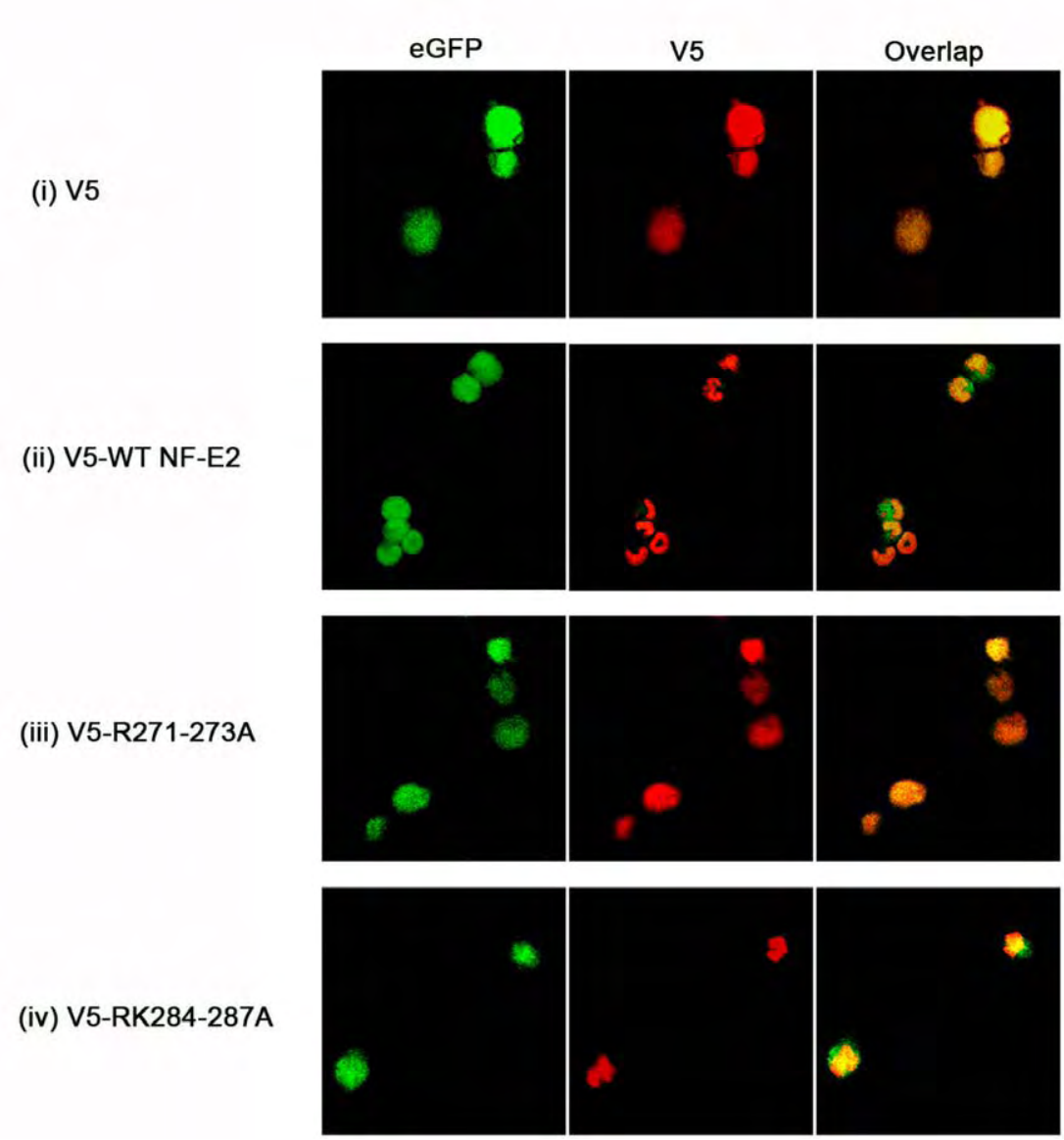


Figure 5.4 Intracellular localisation of p45 NF-E2 constructs and mutants tagged to V5 in haematopoietic BMCs. Murine BMCs were transduced with (i) pKMV-V5; (ii) pKMV-V5NF-E2 (wildtype: WT) and its respective NLS mutants; (iii) pKMV-V5R271-273A and (iv) pKMV-V5RK284-287A as described in the Materials and Methods. Transduced cells were identified by eGFP expression (green) as detected by confocal microscopy and V5 expression (red) as examined by immunofluorescence staining. The two images were digitally overlapped to observe co-localisation (yellow).

Nuclear Import Mechanisms of p45 NF-E2

Typically, nuclear import is facilitated by the importins, via the classical or non-classical pathway. In the first, the protein is recognised and bound via its NLS to IMP α, followed by the binding of IMP β before transiting through the NPC. In the non-classical pathway, IMP β alone binds and transports the substrate into the nucleus. To elucidate the mechanisms of nuclear import, GST pull-down assays were carried out. In particular, IMP β1 has been observed to be commonly used by many proteins (Philips et al., 2007; Kwok et al., 2007) for nuclear transport and was hence chosen as the prime IMP β candidate for investigation.

The ^{35}S-labelled DNA binding domain of p45 NF-E2 (aa 267-293), which contains the minimal NLS region, was allowed to interact with IMP α and IMP β1 GST-fusion proteins or with GST alone as a negative control. As shown in Figure 5.5i, ^{35}S-aa 267-293 failed to bind GST alone and IMP α (lanes 1 and 3), but bound strongly to IMP β1 (lane 2). Lane 4 shows 20% input of the ^{35}S protein alone, suggesting that almost all of the ^{35}S-aa 267-293 protein had bound to IMP β1 in lane 2. This indicates that p45 NF-E2 nuclear import is mediated via the non-classical pathway through the direct interaction of IMP β1 and the NLS region.

To further elucidate the role of the amino acids found to be involved in p45 NF-E2 nuclear import, interaction of the NLS mutant with IMP β was investigated. The p45 NF-E2 fragment, aa 267-293, with the minimal NLS region mutated (R271-273A), greatly reduced IMP β1 binding (Figure 5.5ii- lane 4). GST alone failed to interact with aa 267-293 or its mutant (lanes 1 and 3). The generation of TNT ^{35}S-labelled product is demonstrated in Figure 5.5ii (lanes 5 and 6), showing the respective 20% input of proteins. These results confirm that the amino acids R271-273 of p45 NF-E2 mediate nuclear import by interaction with IMP β1.

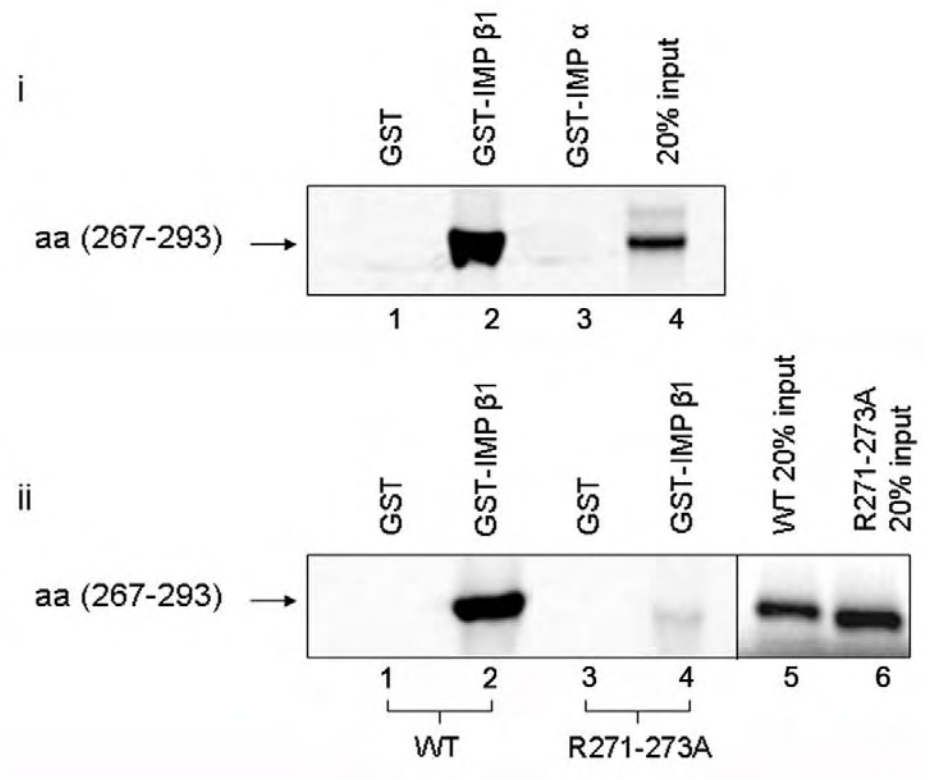


Figure 5.5 Interaction of the p45 NF-E2 NLS sequence to importins. (i) TNT synthesised ^{35}S-aa (267-293) of p45 NF-E2 was generated and its interaction with GST-IMP α or β1 was determined by pull-down assays (lanes 1-3). Lane 4 is representative of 20% of the ^{35}S-labelled input. (ii) Comparison of IMP β1 interaction with either ^{35}S-aa (267-293) WT (lanes 1 and 2) or the R271-273A NLS mutant (lanes 3 and 4). Lanes 5 and 6 contain 20% input of the respective proteins.

The initial experiments were performed using the NLS fragment of p45 NF-E2, aa 267-293. Therefore, to confirm these results in the context of the FL protein, the previous pull-downs were repeated using ^{35}S-labelled FL p45 NF-E2 (FL-NF-E2). Similar to the previous observations, wildtype FL-NF-E2 only bound to IMP β1, albeit weakly in comparison to the input (Figure 5.6A- lanes 1-3 and 7). This weak binding was consistently observed with all FL-NF-E2 products and mutants. It is possible that some inhibitory or regulatory element may be located outside of aa 267-293, or the FL protein may provide some steric hindrance to NLS recognition, as documented for other

proteins (Riviere et al., 1991; Craig et al., 2002). Alternatively, the FL protein was not folded properly *in vitro* in the TNT system.

Despite localisation studies clearly demonstrating that the R271-273A mutant inhibited nuclear accumulation (Figure 5.3Cii), there was no significant difference in IMP β1 binding between the wildtype (WT) and R271-273A mutant (Figure 5.6A- lanes 2 and 5). This finding suggests that alternative amino acids in the FL protein could possibly bind IMP β1 independently of R217-273, but may not act as a functional NLS.

The C-terminal leucine zipper of p45 NF-E2 binds other p18 Maf proteins, which may be able to interact with importins and facilitate nuclear import of the heterodimer by means of a "piggyback" mechanism (Waldmann et al., 2007). To test for this possibility, the C-terminal of p45 NF-E2 (aa 1-294) was deleted and GST pull-down assays were repeated to investigate interaction with IMP β1. Both the WT and R271-273A mutant of aa 1-294 still bound IMP β1 to the same degree (Figure 5.6B-lanes 1 and 3). This confirms that the C-terminus of FL-NF-E2 does not interact with IMP β1 *in vitro*.

We then tested the remaining N-terminal domain of p45-NF-E2 (aa 1-209) to ascertain its interaction with IMP β1. The aa 1-209 fragment still bound IMP β1 to a similar extent as the WT-FL NF-E2 (Figure 5.6C- lane 1). Upon re-examining the p45 NF-E2 sequence, a region in the N-terminal activation domain that contains three arginine residues resembling a possible NLS consensus was identified. These three arginines were simultaneously mutated to alanines (R167-169A) in the N-terminal fragment of p45 NF-E2. As seen in Figure 5.6C, the R167-169A mutant completely abolished IMP β1 binding (lane 3). However, comparing this observation to previous localisation studies (Figure 5.2iii), the N-terminal transactivation domain was not likely to play a crucial role in nuclear import, as shown by both the nuclear and cytoplasmic distribution of GFP. Taken together, these results identify a secondary site by which p45-NF-E2 recognises IMP β1 *in vitro*, but does not act as a functional NLS.

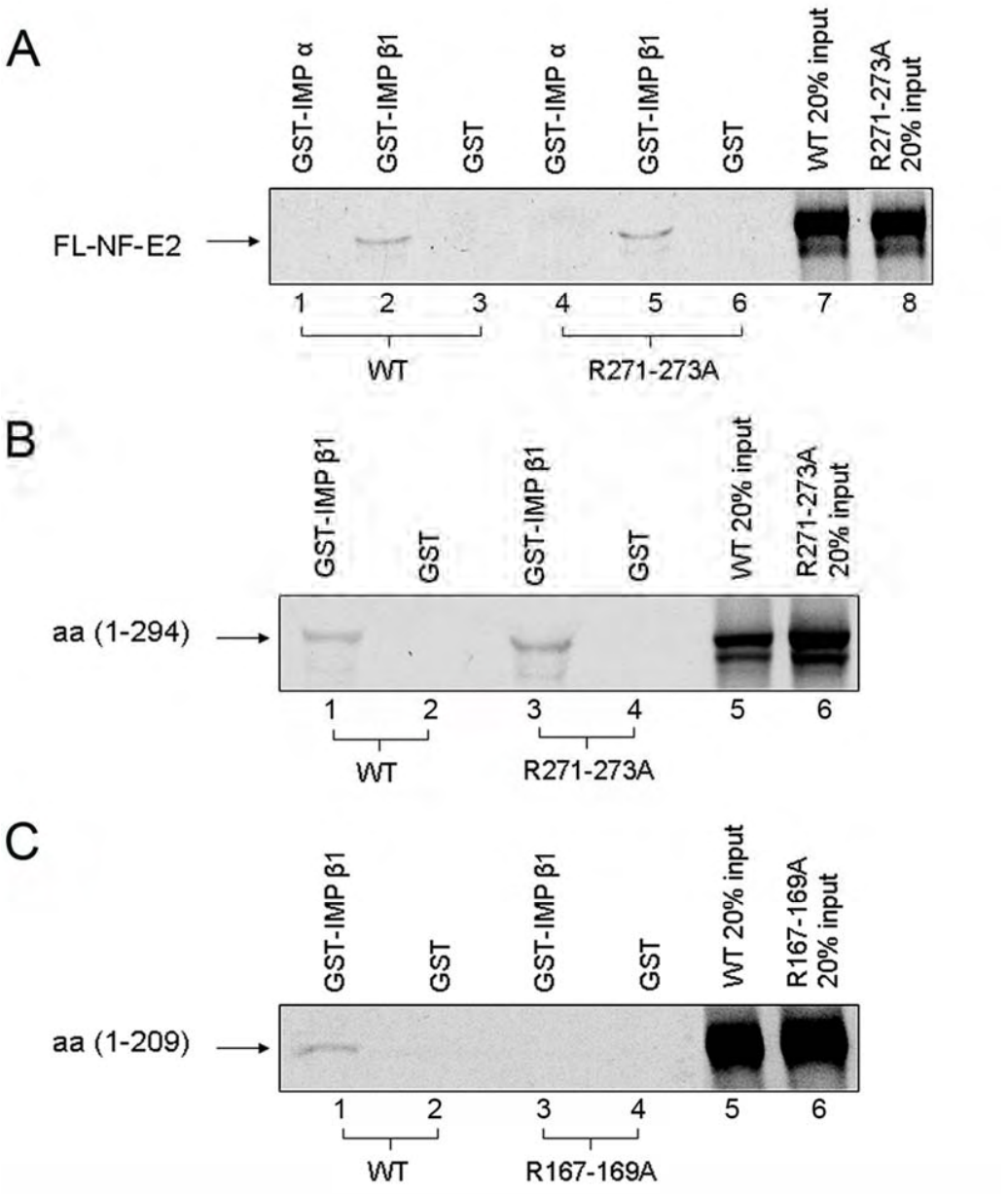


Figure 5.6 Identification of a secondary IMP β recognition site in p45 NF-E2. TNT synthesised ^{35}S- p45 NF-E2 constructs and mutants were generated and their interaction with GST-IMP α or β1 were determined by pull-down assays. (A) Wildtype (WT) (lanes 1-3, 7) and mutant (lanes 4-6, 8) FL p45 NF-E2 interactions with IMP α or β1. (B) IMP β1 interactions with WT (lanes 1-2, 5) or mutant (lanes 3, 4, 6) aa 1-294 fragment are shown. (C) IMP β1 interactions with WT (lanes 1-2, 5) or R167-169A mutant (lanes 3, 4, 6) of the aa 1-209 fragment.

Effect of Importin β Knockdown on p45 NF-E2 Nuclear Localisation

To further establish the role of the non-classical pathway in p45 NF-E2 nuclear import, IMP β1 expression was selectively knocked down using siRNA targeting exons 3 and 9 of IMP β1. The cells were then transfected with GFP-tagged p45 NF-E2 after 48 hours and nuclear localisation was examined at 72 hours.

As determined by western blot and densitometry, siRNA targeting of both exon 3 and 9 significantly decreased IMP β1 expression by 58% and 74% respectively, compared with the control lysates (Figure 5.7A). This reduction in expression was sustained up to 72 hours. The negative control siRNA had no effect on IMP β1 expression compared with untreated control cells (Figure 5.7A). Equal protein loading was measured by Ponceau S staining (Figure 5.7B).

Unexpectedly, despite significant suppression of IMP β1 expression (Figure 5.7A), p45 NF-E2 was still actively targeted to the nucleus (Figure 5.7C). This was interesting considering that our previous experiments suggested an IMP β1-mediated import mechanism (Figure 5.5; 5.6A). This suggests that in the event that the IMP β1 non-classical pathway is inhibited, alternative IMP βs or rescue mechanisms may take over to maintain p45 NF-E2 levels.

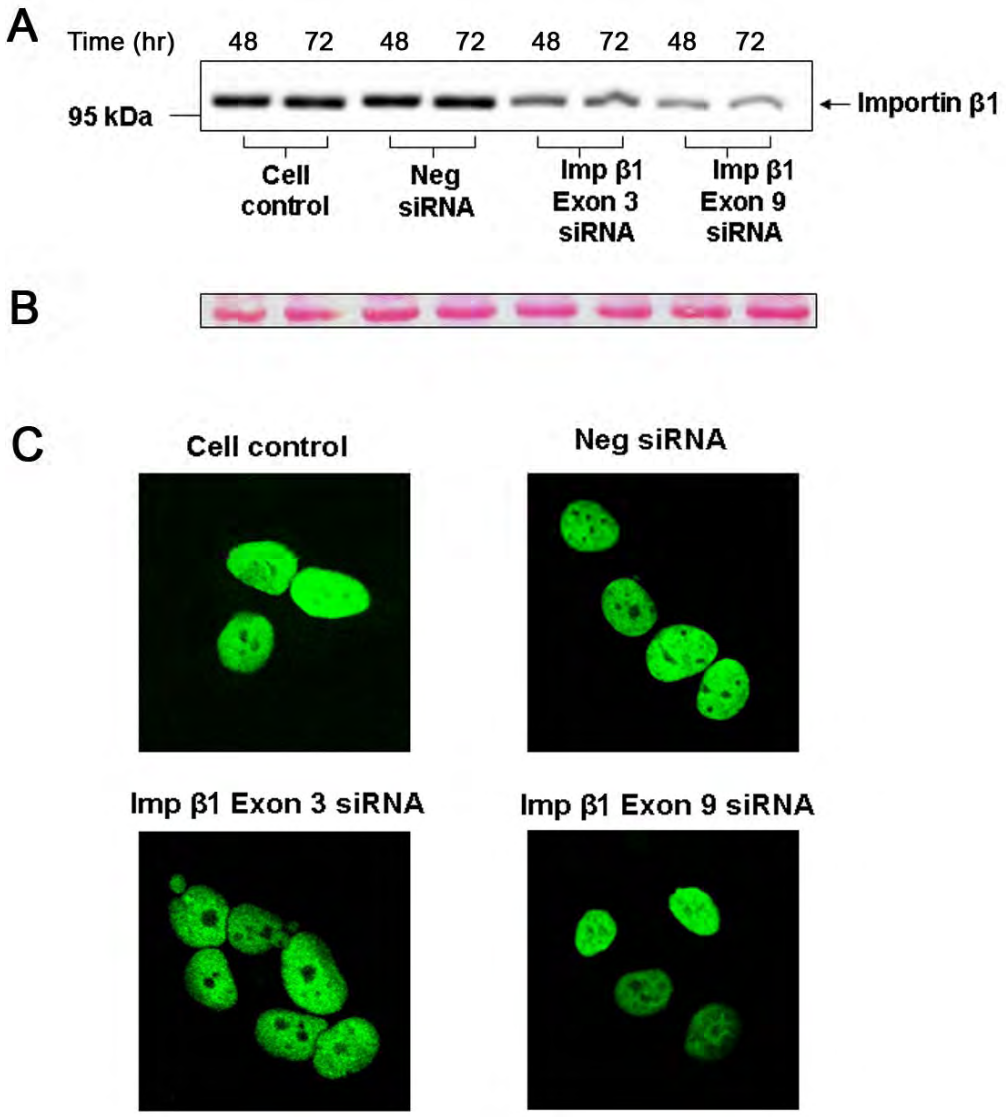


Figure 5.7 Effect of IMP β1 knockdown on p45-NF-E2 nuclear localisation. Cells were transfected with 100 pmol siRNA targeted towards exon 3 and 9 of IMP β1. After 48 hours, the cells were transfected with GFP-p45 NF-E2. (A) Cell lysate samples were obtained 48 and 72 hours after siRNA treatment. Immunoblot of IMP β1 expression in non-transfected cells, cells transfected with negative (Neg) control siRNA, or siRNA targeted to exon 3 and 9 of IMP β are shown. (B) Equal loading was determined by Ponceau S staining. (C) GFP-p45 NF-E2 localisation was determined by confocal microscopy at 72 hours.

DNA Binding Activity of p45 NF-E2 Mutants

The residues found to be involved in nuclear import are located in the overlapping NLS and DNA binding domain of p45 NF-E2. Therefore, to investigate whether these amino acids affect the DNA binding activity of p45 NF-E2, electromobility shift assays (EMSAs) were performed using the porphobilinogen deaminase (PBGD) promoter probe containing NF-E2-AP-1 binding sites (Mignotte et al., 1989). In order to enable clearer visualisation of the protein-DNA complex, all the samples were supershifted using an anti-p45 NF-E2 antibody (Figure 5.8i).

As expected, WT p45 NF-E2 (Figure 5.8i- lane 3) demonstrated significant binding to the probe (Shyu et al., 2005) and the fragment lacking the bZip DNA binding domain, aa 1-209, failed to bind DNA (lane 9). All the p45 NF-E2 mutants analysed in this study including the NLS mutant, R271-273A, RK284-287A, R271A, R272A and R273A were still able to bind DNA (Figure 5.8i- lanes 4-8). Although RK284-287A and R271A showed slightly reduced DNA binding compared to the WT, despite equal protein loading, this difference would at most reflect little effect on DNA binding (Figure 5.8i- lanes 5 and 6). All proteins were equally translated *in vitro* as shown in Figure 5.8ii.

The above results indicate that the residues found to be vital for nuclear import, R271-273, even when mutated simultaneously, are not crucial for DNA binding. Thus, any subsequent effects on transcriptional activity observed with these mutants can be attributed to its nuclear import rather than DNA binding activity.

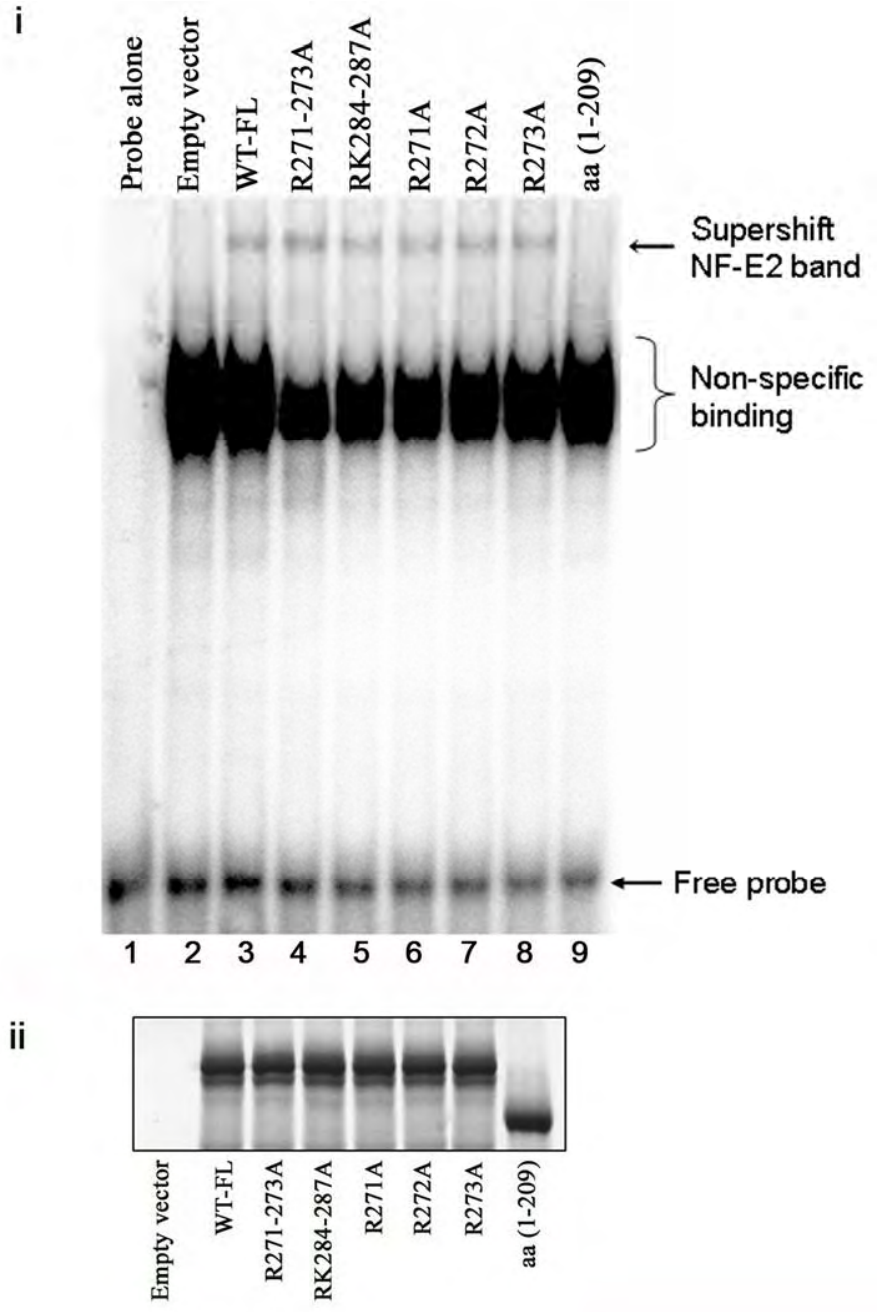


Figure 5.8 Effects of p45 NF-E2 mutations on DNA binding activity. (i) EMSA was carried out using a ^{32}P-labelled probe containing an NF-E2 binding site. Supershift was performed using an anti-p45 NF-E2 antibody. Typical outcome of three experiments shown. (ii) Wildtype p45 NF-E2 (WT-FL) and its mutants in the pDEST 53 vector were *in vitro* translated.

Transcriptional Activation by p45 NF-E2 is Inhibited by Disrupted Nuclear Localisation

Given that NF-E2 is an important transcription factor involved in erythroid and MK gene expression, we considered that nuclear import might affect p45 NF-E2 transactivation function. To determine this, the pRBGP2-luciferase (Luc) reporter (Igarashi et al., 1995), which contains tandemly arranged NF-E2/AP1 binding sites linked to the TATA box of the β-globin promoter (Figure 5.9i), was co-transfected with p45 NF-E2 cDNA (WT and mutants) into COS-7 cells. Transfection efficiency was normalised with the control vector, phRG-Tk and the luciferase activity assessed. Western blot analysis on transfected cells demonstrated that WT p45 NF-E2 and all mutant constructs were translated into proteins (Figure 5.9ii inset).

As previously reported (Shyu et al., 2005), WT p45 NF-E2 transactivated the reporter up to ~6.5 fold (Figure 5.9ii). Only mutants that earlier presented with inhibited nuclear import (R271-273A and R273A) showed marked reductions in luciferase activity compared to the WT and mutants, which showed minimal effect on nuclear localisation (RK284-287A, R271A and R272A) (Figure 5.9ii). These results imply that residues crucial for nuclear import are consequently also essential for transcriptional activity of p45 NF-E2. Mutation of these residues results in the protein being unable to accumulate exclusively in the nucleus and transcription being effectively suppressed.

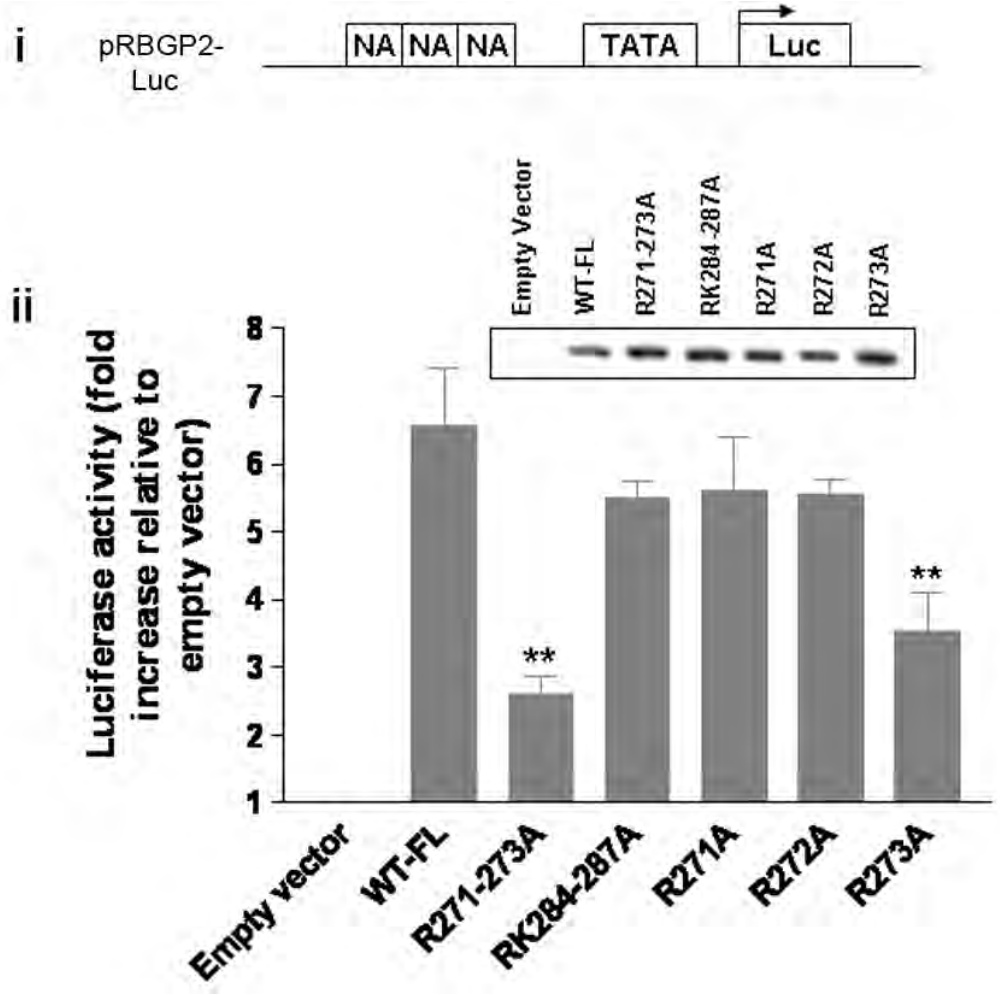


Figure 5.9 Transactivation activity of p45 NF-E2 and its mutants. COS-7 cells were transiently transfected with 15 ng phRG-Tk renilla control reporter vector, 600 ng of the (i) pRBGP2-Luc reporter plasmid and 1.4 μg of WT or mutant p45 NF-E2 expression vectors. NA: NF-E2/AP1 binding sites. (ii) The luciferase activities were measured and calculated after 48 hours. Results were normalised against the renilla luciferase control readings and are presented as average fold increases in firefly luciferase, compared to the empty pDEST53 vector. Data shown is the mean and standard errors of three independent assays, each carried out in duplicate with its significance compared to WT. The amounts of p45 NF-E2 and its mutants in different transfectants were compared by immunoblotting (inset of ii).

Proplatelet and Platelet Development Inhibited by Impaired Nuclear Import of p45 NF-E2

In previous chapters, it was clearly shown that when overexpressed, p45 NF-E2 has the ability to enhance proplatelet and platelet development (refer to chapters 3 and 4). Therefore, to further elucidate the biological importance of p45 NF-E2 nuclear import, we proceeded to examine the effects of the p45 NF-E2 NLS mutant on normal proplatelet and platelet production *in vitro*. Lin^- BMCs were transduced with WT and mutant p45 NF-E2 retroviral vectors and differentiated under TPO conditions for 5-7 days. On the fifth day, proplatelet numbers from $CD41^+$ MKs were enumerated and subsequent platelet levels *in vitro* were analysed on day 7, as described in the Material and Methods (page 53).

As formerly described (Figure 3.9B; Figure 3.10C), overexpression of WT p45 NF-E2 was able to increase proplatelet and platelet levels 1.5 and 2.5 fold respectively compared to the pKMV control (Figure 5.10). However, when the NLS is disrupted by mutations to R271-273, p45 NF-E2 was no longer able to heighten proplatelet and platelet production, achieving only the same levels as the negative control (Figure 5.10). In contrast, mutations in the same region, which do not affect nuclear localisation (RK284-287A), were still able to achieve augmented proplatelet and platelet levels compared to the control (Figure 5.10). These results further establish that residues R271-273 are vital for p45 NF-E2 function as it regulates nuclear import, transcriptional activity as well as proplatelet and platelet development.

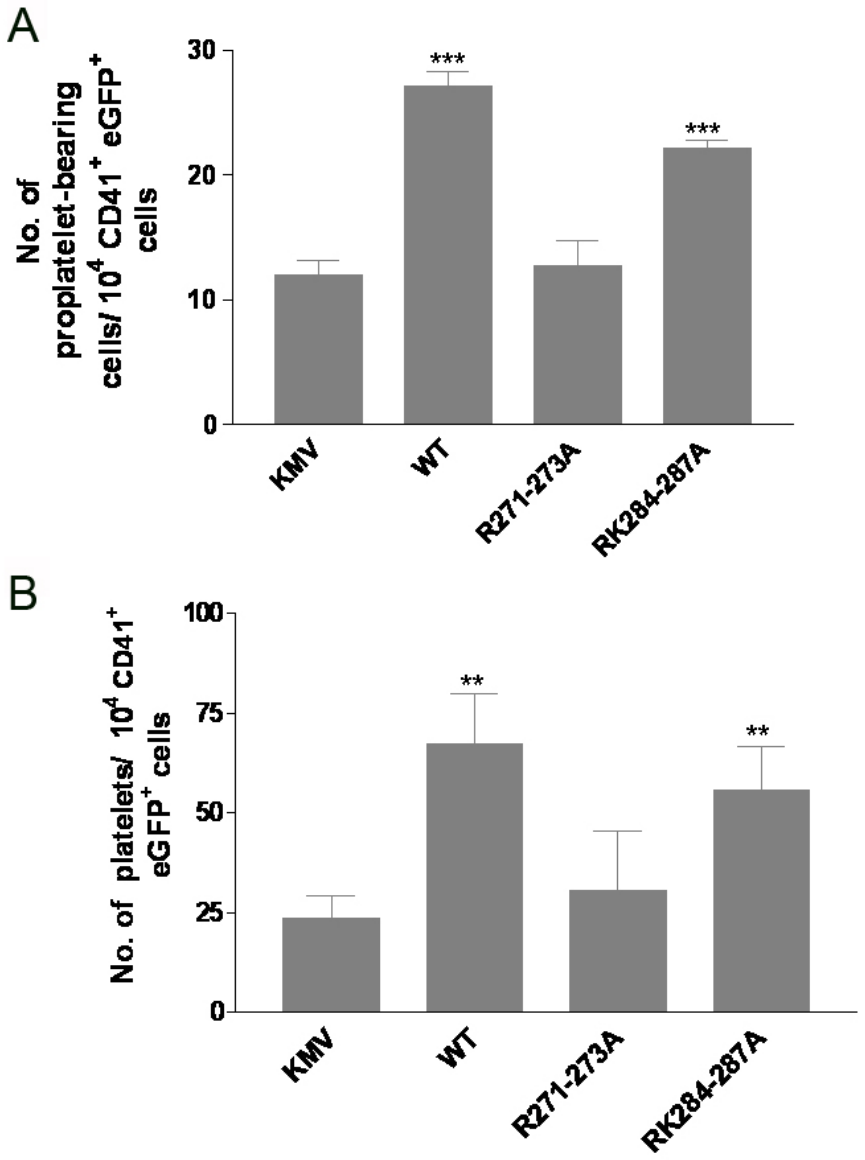


Figure 5.10 The effects of p45 NF-E2 nuclear import on *in vitro* proplatelet and platelet development. Equal numbers of $eGFP^+$/ $CD41^+$ transduced cells were seeded and cultured for 5-7 days before proplatelets were counted using the fluorescent microscope and platelet numbers were analysed by flow cytometry. (A) Proplatelet-bearing MK frequency was calculated by dividing the proplatelet number by the total number of $eGFP^+$/ $CD41^+$ input cells. Each experiment was done in triplicate and this data (mean ± SD) is representative of 2 independent experiments. (B) Platelets collected from the culture medium were counted as platelet-sized and $eGFP^+$/ $CD42b^+$ cells by flow cytometry. The values shown are the mean of 2 independent experiments.

In Vivo SUMOylation of p45 NF-E2 by SUMO-2/3

Apart from nuclear trafficking, post-translational modifications such as SUMOylation have also been found to play vital roles in gene regulation and in the modulation of protein function (Johnson, 2004). It is known that the p45 subunit of NF-E2 possesses a single SUMOylation site at amino acid K368 (Shyu et al., 2005). This site was shown to be modified by SUMO-1 *in vitro* and *in vivo* in K562 and foetal liver cells (Shyu et al., 2005). We sought to confirm and further expand upon these observations by determining whether p45 NF-E2 could also be SUMOylated in BMCs and to establish the biological significance of SUMOylation in MKs.

p45 NF-E2 SUMOylation was examined in murine BMCs, which is a physiologically relevant cell type. The previously identified SUMOylation site of p45 NF-E2 (K368) was also confirmed by transduction of BMCs with the K368R mutant, which can no longer be SUMOylated. As shown by western blot in Figure 5.11, a band of a molecular weight corresponding to p45 NF-E2 (~45-50 kDa), was clearly detected (Figure 5.11-panel 1). The band directly above p45, running at about 55 kDa is a non-specific band, which often appears in BMC lysates when probed with the primary antibody. A slower migrating upper band, running at about 70 kDa, was also observed to be significantly more prominent in the WT lysate compared to the K368R sample (Figure 5.11 –panel 1). The predicted molecular weight of SUMO is 11 kDa, but it runs at an apparent molecular weight of ~20 kDa (Kamitani et al., 1997). Thus, it appeared that this upper band was SUMOylated p45 NF-E2 present either as exogenous or endogenous protein in lanes 1 and 2 respectively. Indeed, when the membrane was stripped and re-probed this band could also be detected using an anti-SUMO-2/3 antibody (Figure 5.11-panel 2). In contrast, no bands were observed when the membrane was stripped and re-probed with the SUMO-1 antibody (Figure 5.11-panel 3). A final band, running at about ~90 kDa, was also detected when probed with anti-p45, -SUMO-1 or –SUMO-2/3 (Figure 5.11-panel 1-3). This upper band was likely to be p45 homodimers that were not denatured properly. Taken together, these results suggest that p45 NF-E2 is SUMOylated *in vivo* by SUMO-2/3, but is not modified by SUMO-1 at a detectable level in murine BMCs.

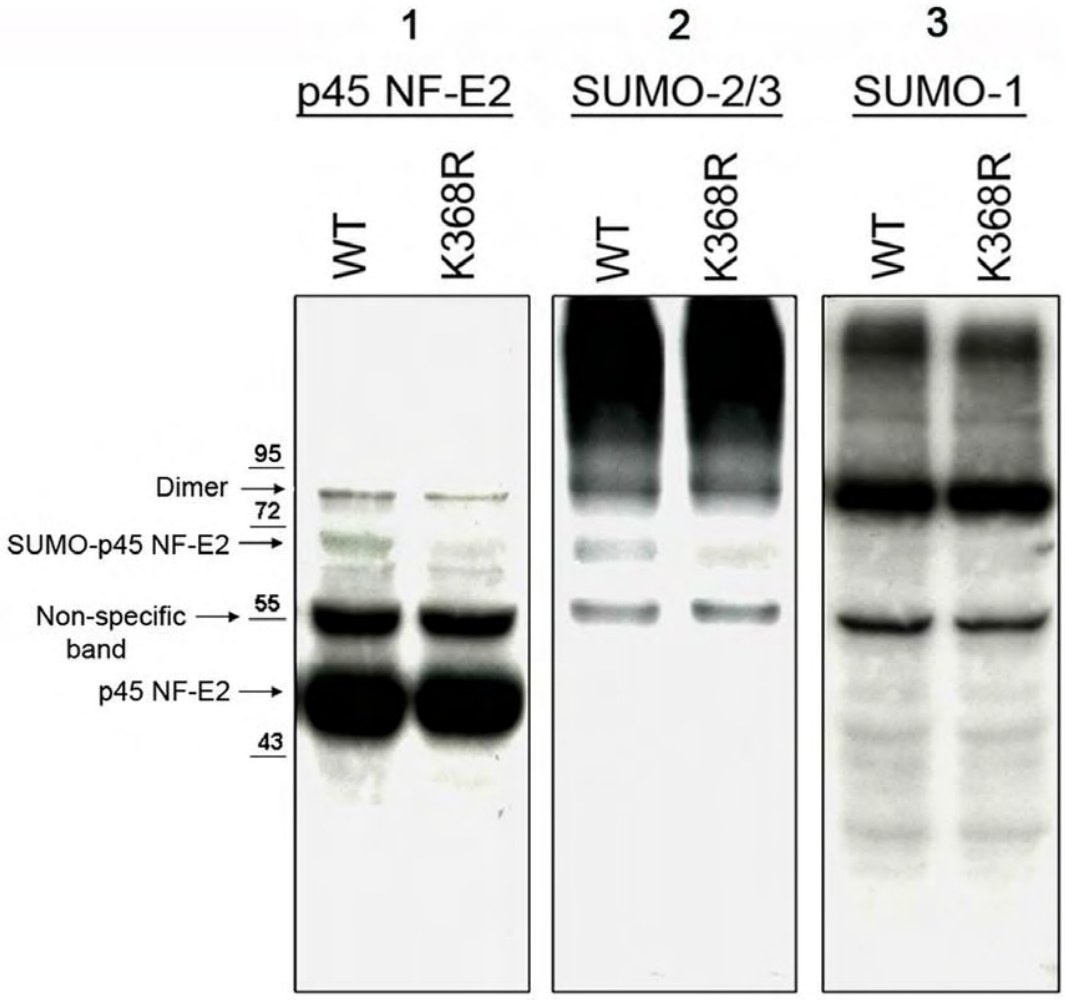


Figure 5.11 SUMOylation of p45 NF-E2 *in vivo*. 8 day 5 FU Lin$^-$ murine BMCs were transduced with either pKMV-NFE2 (p45) or pKMV-K368R as described in the Materials and Methods. The cells were allowed to grow and differentiate for 7 days under TPO and IL-3 conditions. Whole cell extracts were then prepared and analysed by western blotting using anti-p45 (panel 1), anti SUMO-2/3 (panel 2) or anti-SUMO-1 (panel 3) as the probe. The underlined numbers represent the marker position in kDa.

Effect of SUMOylation on p45 NF-E2 Nuclear Localisation

SUMO modification has been shown to play an integral role in the nuclear transport of several proteins (Pichler and Melchior, 2002). In particular, SUMOylatable p45 NF-E2 in K562 cells has been shown to be co-localised with the nuclear bodies promyelocytic leukaemia protein (PML) oncogenic domains (PODs) that are enriched with PML, SUMO-1 and RNAPII (Shyu et al., 2005). Therefore, in order to resolve whether intact SUMOylation sites were required for nuclear localisation of p45 NF-E2, WT p45 NF-E2 or its SUMO mutant (K368R) were fused downstream of GFP and transfected into COS-7 cells. The intracellular localisation of the fusion proteins were determined by confocal microscopy.

As shown previously, WT p45 NF-E2 was found exclusively in the nucleus of 100% of the cells examined (Figure 5.12A). No change was observed for the SUMO mutant (Figure 5.12A), with even a similar percentage of cells demonstrating preferential accumulation in nuclear speckles (~27%) compared to the WT (~23%). These results indicate that SUMOylation plays no role in the cellular distribution of p45 NF-E2 in COS-7 cells.

Similar observations were made in the more biologically relevant cells, primary BMCs. In Figure 5.12B, murine BMCs were transduced with V5-tagged WT p45 NF-E2 or its SUMO mutant (K368R) and V5 nucleocytoplasmic distribution was examined. This was carried out using an anti-V5 antibody followed by a red Alexa Fluor® 594 secondary. Both WT p45 NF-E2 and the K368R mutant were found exclusively in the nucleus of haematopoietic bone marrow cells (98-100%) (Figure 5.12B). No difference in sub-nuclear localisation was observed in mutant cells compared to the WT. These findings demonstrate that p45 NF-E2 nuclear transport in primary BMCs is not dependent on SUMOylation.

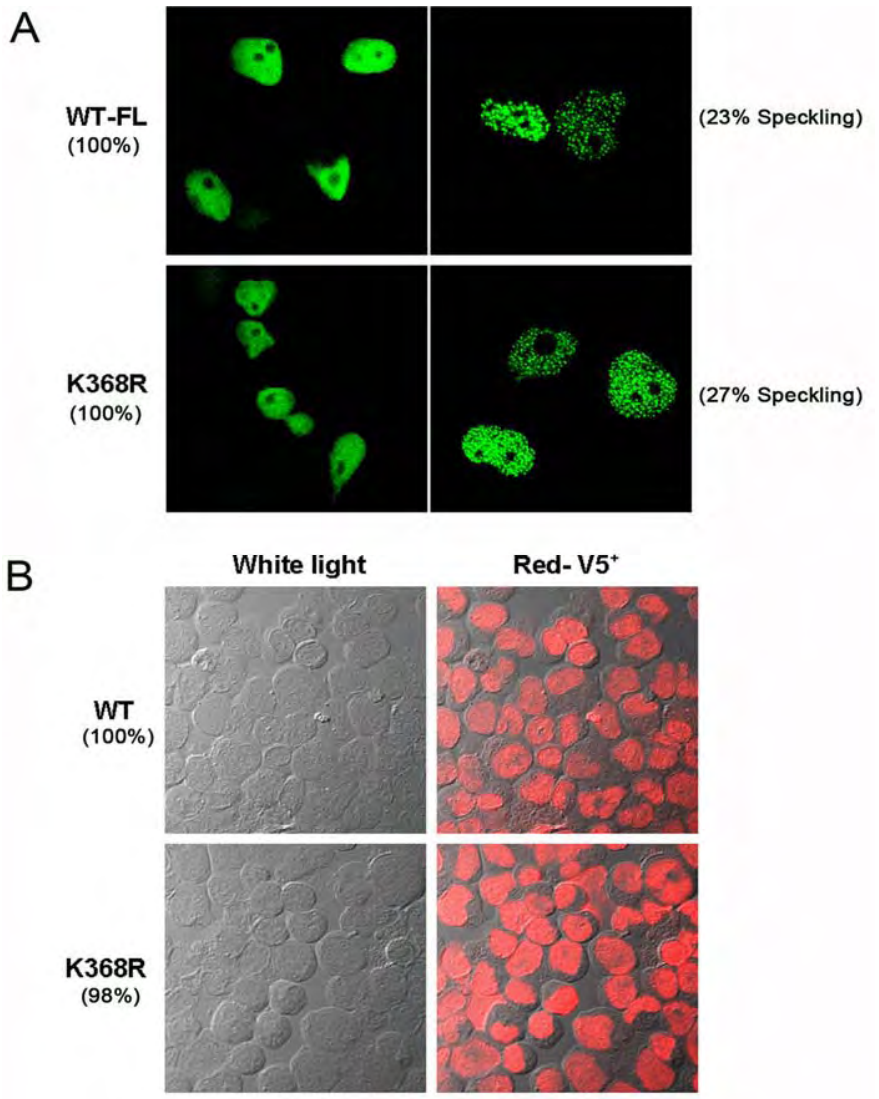


Figure 5.12 Intracellular localisation of p45 NF-E2 and its SUMO mutant. Upon transfection or transduction of p45 NF-E2 and the K368R mutant expression vectors into (A) COS-7 or (B) murine BMCs, distribution of the reporter tag expression was analysed via confocal microscopy. (A) p45 NF-E2 and its SUMO mutant were fused downstream of GFP in pDEST53 and transfected into COS-7 cells. The percentages of cells displayed exclusively in the nucleus and in nuclear speckles are indicated in brackets. A hundred cells were counted for each slide. (B) 8 day 5FU Lin^- BMCs were transduced with pKMV-V5NFE2 or pKMV-V5K368R and localisation of the V5 protein was examined using anti-V5 (red). One hundred transduced cells were examined in each slide.

SUMO Modification of p45 NF-E2 Increases Transactivation of MK-Specific Genes and Platelet Production

Shyu et al. (2005) have shown that p45 SUMOylation enhances the transactivation capability and DNA binding of NF-E2 in erythroid cells. The effects of SUMO modification may vary under different cellular or tissue contexts. Therefore, to clarify the effects of SUMOylation on p45 function in MKs, we transduced haematopoietic progenitor cells with WT p45 NF-E2 or the K368R SUMO mutant and enabled them to differentiate down the MK lineage via treatment with TPO. Transactivation of NF-E2 target genes, β-tubulin, thromboxane synthethase (TXS) and caspase 12 were then evaluated by quantitative real-time PCR and platelet production from mature MKs was analysed by flow cytometry.

As expected, p45 NF-E2 was shown to be successfully overexpressed at the RNA level in both the WT and mutant cells compared to the empty vector control (KMV) (Figure 5.13A). Overexpression of WT p45 NF-E2 resulted in a 1.5-2 fold upregulation in expression for all the various MK genes tested compared to the KMV control (Figure 5.13A). The K368R mutant on the other hand failed to significantly increase the expression of any of the target genes tested (Figure 5.13A). These results indicate that intact SUMO sites of p45 are essential for increasing the transactivation capability of NF-E2 on its target genes.

Interestingly, platelet production was also observed to be significantly reduced when the SUMO site of p45 NF-E2 was mutated (Figure 5.13B). Enforced expression of the WT protein resulted in a 1.5 fold increase in platelet production *in vitro*, while the SUMO mutant demonstrated a significant reduction in platelet production compared to the WT (Figure 5.13B). Taken together, these results suggest that SUMOylated p45 NF-E2 is crucial for maintaining normal thrombopoiesis, possibly by the SUMO-mediated regulation of NF-E2 target genes involved in megakaryopoiesis and thrombopoiesis.

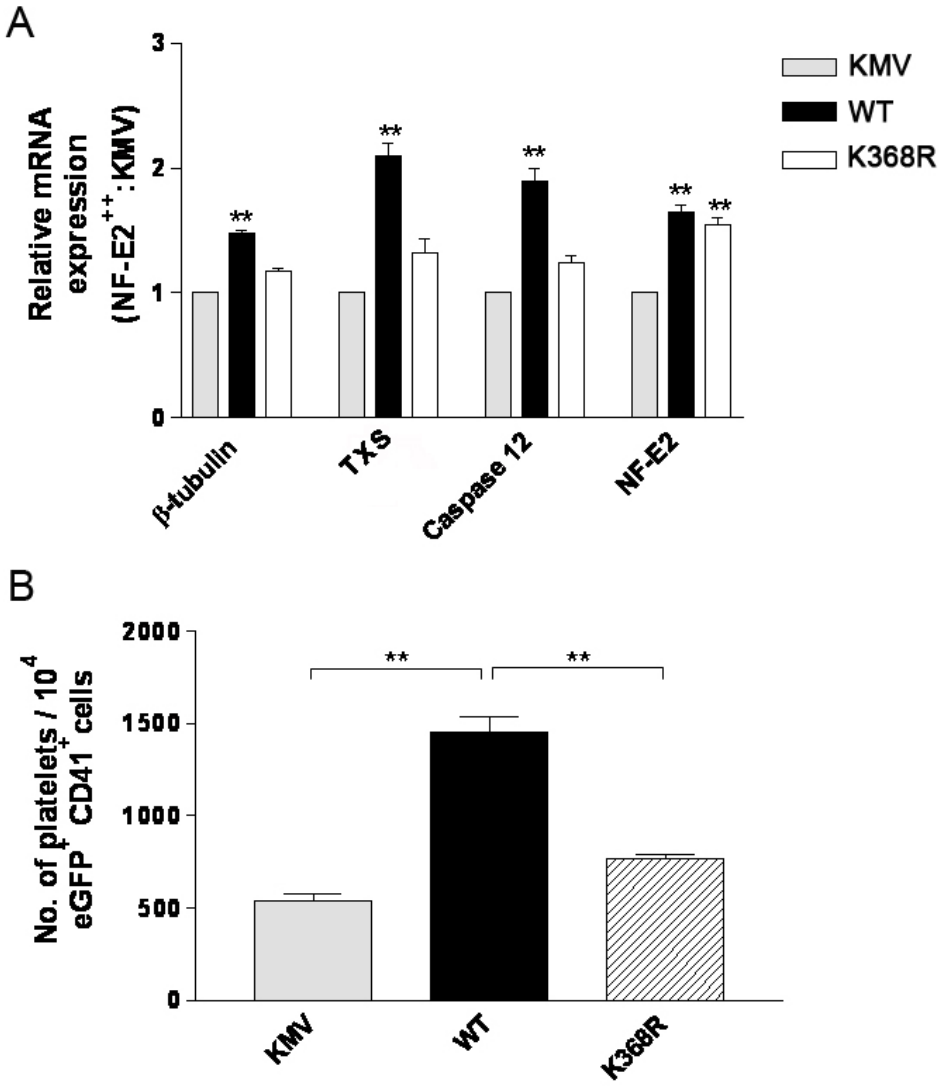


Figure 5.13 Biological effects of p45 NF-E2 SUMOylation on transactivation of MK-specific genes and platelet production. An enriched population of MK progenitor cells derived from 8 day 5FU Lin$^-$ BMCs were transduced with pKMV-NFE2 or pKMV-K368R and allowed to differentiate under TPO conditions for 5-7 days. (A) Quantitative real-time PCR was performed on cDNA samples obtained from the RNA of transduced cells harvested at day 5. Target quantities were normalised to GAPDH and presented relative to the KMV control. Each real-time PCR reaction was performed in triplicate and presented as the mean ± SD. (B) At day 7, platelets collected from the culture medium were counted as platelet-sized and eGFP$^+$/ CD42b$^+$ cells by flow cytometry. The values shown are the mean of 2 independent experiments.

DISCUSSION

The p45 subunit of the bZip transcription factor, NF-E2, is crucial in regulating the expression of genes involved in the development and normal cell function of MKs. Indeed, p45 NF-E2 has been linked to diseased states such as leukaemogenesis (Lu et al., 1994), essential thrombocytopaenia (Catani et al., 2002) and polycythaemia vera (Goerttler et al., 2005). As such, the mechanisms involved in regulating p45 NF-E2 levels and function must be precisely controlled. Two such processes include transport into the nucleus where gene transcription occurs, and post-translational modifications such as SUMOylation. These two mechanisms are not fully understood.

The current study is the first to investigate the detailed mechanisms of p45 NF-E2 nuclear transport in both COS-7 and murine BMCs. It was demonstrated that p45 NF-E2 can enter the nucleus via passive diffusion as well as active transport, independent of its leucine zipper. A non-classical monopartite NLS was also identified and fine mapped to include only arginine residues between R271-273 that interact with IMP β1 for nuclear import. Furthermore, the essential role of these residues in p45 NF-E2 function was further exemplified by their importance in transcriptional gene regulation and thrombopoiesis.

Initial sequence analysis of p45 NF-E2 identified a potential NLS region within the DNA binding domain between amino acids 271-285, which aligned perfectly well with previously characterised bipartite NLS sequences from nucleoplasmin (Robbins et al., 1991), p73 (Inoue et al., 2002), p53 (Liang and Clarke, 1999; Liang and Clarke, 2001) and Nrf-2 (Jain et al., 2005). However, site-directed mutagenesis confirmed that p45 NF-E2's NLS was in fact monopartite, with the second positively charged cluster (RK284-287) apparently redundant for nuclear import. This result may be surprising especially compared to Nrf-2, which shares high sequence homology with p45 NF-E2 and is also a bZip protein. It is however not clear whether the bipartite clusters in the Nrf-2 study were analysed individually by site-directed mutagenesis or just assumed to be bipartite by sequence analysis (Jain et al., 2005). Similarly, when the amino acid sequence of the autoimmune regulator, AIRE, was analysed, it was first thought to contain a bipartite NLS (110RKGRKPPAVPKALVPPPRLPTKRK133). However, like

p45 NF-E2, only one of these basic clusters of residues was found to facilitate nuclear import (Ilmarinen et al., 2006).

It is conceivable that although the RK284-287 cluster of residues may not directly function as an NLS, the basic nature of these amino acids may serve to confer full function to this region by supporting the minimal NLS domain. An example of this would be by promoting the exposure of the NLS to the protein surface to facilitate binding of the NLS receptor. Undeniably, this secondary amino acid sequence is highly conserved in other bZip proteins, indicating important functional roles for these residues and in some cases, they have been found to be involved in nuclear transport (Jain et al., 2005; Waldmann et al., 2007).

In general, larger proteins (>40 kDa), such as the constructs used in this study, are excluded from the nucleus in the absence of an NLS (Paine et al., 1975). However, it is possible that the conformation of p45 NF-E2 allows it to passively diffuse through the diameter of the NPC, as has been observed with other larger proteins (Hu et al., 2005; Philips et al., 2007; Kwok et al., 2007). This would explain the presence of GFP-p45 NF-E2 in both the nucleus and cytoplasm even in the absence of an intact NLS. Alternatively, it is plausible that a small subset of p45 NF-E2 is heterodimerised with the p18 subunit in the cytoplasm and imported into the nucleus at least to some extent, by means of a 'piggyback' mechanism as described for the bZip protein c-Jun (Waldmann et al., 2007).

On the other hand, active nuclear import of p45 NF-E2 was found to be facilitated predominantly by the non-classical import pathway with the direct interaction of p45's NLS with IMP β1. Interestingly, this interaction was found to be consistently stronger with the aa 267-293 DNA binding fragment (Figure 5.5) than the FL p45 NF-E2 (Figure 5.6). This could be a result of inter- or intramolecular masking of the NLS within the FL protein *in vitro* as commonly observed in other proteins (Riviere et al., 1991); (Stommel et al., 1999; Poon and Jans, 2005). As p45 NF-E2's leucine zipper enables heterodimerisation with other bZip proteins, it was hypothesised that this new dimeric complex could have resulted in intermolecular masking of the p45 NLS, as is the case

for p53 (Stommel et al., 1999). However, deletion of the leucine zipper of p45 NF-E2 did not result in stronger binding to IMP β1 (Figure 5.6B). Instead, a post-translational modification, such as phosphorylation/ dephosphorylation, may be required to change the charge or conformation of the protein in order to unmask the NLS (Riviere et al., 1991; Beals et al., 1997). In addition, it has been reported that phosphorylation sites close to the target signal may even enhance the NLS-IMP binding affinity (Xiao et al., 1997; Hubner et al., 1997). Incidentally, a secondary IMP β binding site (R167-169), which was unlikely to be involved in p45 NF-E2 nuclear import by itself, was located immediately adjacent to the major kinase A phosphorylation site of p45 NF-E2 (Serine170) (Casteel et al., 1998) (Figure 5.6C).

In the event that the IMP β1-mediated import pathway is inhibited, other rescue mechanisms may become the dominant pathway to maintain normal p45 NF-E2 import as shown in Figure 5.7. Several proteins (Yokoya et al., 1999; Ten Dijke and Hill, 2004; Marg et al., 2004) have been shown to interact directly with nucleoporins for transport and this may also be possible for p45 NF-E2. There are more than 40 nucleoporins making up the NPC and it cannot be ruled out that some of these nucleoporins may be regulators for p45 NF-E2 entry. In addition, *in vivo*, other importins may also interact with p45 NF-E2 to facilitate nuclear transport. Alternatively, the amount of IMP β present after siRNA knockdown could still be sufficient to transport p45 into the nucleus.

The leucine rich C-terminal of p45 NF-E2 also prompted the presence of a NES. However, in this study, nuclear export was not observed. Notably, many proteins that have previously been found to have a functional NES, are involved in stress responses often leading to detrimental effects if overexpressed (Liang and Clarke, 2001; Inoue et al., 2002; Jain et al., 2005). They hence require efficient import and export mechanisms to maintain balanced levels of the protein depending on its requirement. In contrast, NF-E2 is involved in normal MK development and its expression is already limited by the specificity of the p45 subunit (Andrews et al., 1993). Therefore, active nuclear export of p45 NF-E2 may not be necessary.

The overlap between the NLS and the basic DNA binding region of p45 NF-E2 has been well documented for other numerous transcription factors (Fernandez-Martinez et al., 2003; Nikolaev et al., 2003). About 90% of identified NLSs belonging to DNA binding proteins have been found to coincide with the DNA binding domain (Cokol et al., 2000) and may suggest evolutionary clustering of these domains to facilitate the execution of nuclear activities. That said, it can be argued that nuclear accumulation of p45 NF-E2 may be a consequence of nuclear retention as a result of DNA binding rather than active NLS-mediated transport. However, this is unlikely, as mutants that still bound DNA (eg. R271-273A, R273A), did not show restricted localisation to the nucleus of either COS-7 or primary BMCs (Figure 5.3Cii, vii**;** Figure 5.4iii). This indicates that the nuclear import function of p45 NF-E2 is distinct from its DNA binding activity.

Given that p45 NF-E2 is a transcription factor involved in erythroid and MK differentiation, it is clear that nuclear import would affect its function. Not surprisingly, a nuclear import-deficient form of p45 NF-E2 (R271-273A) was less able to activate gene expression in transfected cells (Figure 5.9). A single point mutation in the NLS (R273A) was not only sufficient to suppress nuclear localisation (Figure 5.3Cvii), but also significantly reduced gene expression by half, indicating that this process is stringently controlled. However, some transcriptional activity was still present in these NLS mutants (R271-273A and R273A) showing 2.5-3.5 fold activation in gene expression compared to the negative control. This was most likely due to passive diffusion or interaction with nucleoporins, which are independent of the NLS residues and able to facilitate nuclear import to a certain degree. These mutants were also found to still bind DNA (Figure 5.8i) and hence less likely to inhibit transcription once in the nucleus.

The control of nuclear transport is paramount for normal cell function, with the change of subcellular localisation of various molecules being associated with disease phenotypes including cancers and developmental abnormalities (El Ghouzzi et al., 2000; Fan et al., 2003; Kaiser et al., 2004). Indeed, disrupting the NLS of p45 NF-E2 was found to significantly reduce subsequent proplatelet and platelet production from

mature MKs (Figure 5.10), which may result in disease phenotypes in a clinical setting. Interestingly, an elegant study by Francastel et al. (2001) determined that prior to differentiation of MEL cells, the majority of p18 and p45 NF-E2 subunits are localised to different sub-nuclear compartments and only upon differentiation does the p18 subunit relocate to the p45 compartment to activate transcription (Francastel et al., 2001). It will be interesting to investigate the ordering of these events (heterodimerisation, nuclear import, sub-nuclear localisation) in the future.

In this study, we also demonstrated that p45 NF-E2 is SUMOylated *in viv*o, via a single SUMO acceptor site at residue K368. This is in agreement with a previous study by Shyu et al. (2005). However, it was further demonstrated that in haematopoietic BMCs, SUMO modification of p45 NF-E2 was carried out by SUMO-2/3, with no SUMOylation by SUMO-1 being detected (Figure 5.11). In other cell types, SUMO-1 may be involved in p45 NF-E2 SUMOylation as formerly shown in K562 and foetal liver cells (Shyu et al., 2005). In this latter study, it is not clear whether any experiments were performed examining the role of SUMO-2/3. However, this is not the first time a bZip target has been shown to be modified specifically by SUMO-2/3 in the bone marrow. Interestingly, it is the p45 obligate partner, MafG, which requires SUMOylation by SUMO-2/3 in the bone marrow for active transcriptional repression (Motohashi et al., 2006).

Although SUMO-2/3 might function in a capacity similar to that of SUMO-1, important distinctions between these isoforms are beginning to emerge. For example, SUMO-1 is ubiquitously expressed in many tissues (Xu and Au, 2005), whereas SUMO-2 and SUMO-3 are tissue-specific (Lapenta et al., 1997; Mannen et al., 1996). While the majority of SUMO-1 is conjugated to other proteins, there is a free pool of SUMO-2/3 that is readily available for conjugation (Saitoh and Hinchey, 2000). SUMO-2/3 also appears to be preferentially conjugated to proteins under stress conditions (Saitoh and Hinchey, 2000), although there have been examples of substrates that are specifically modified by SUMO-2/3 under normal physiological conditions (Eaton and Sealy, 2003; Azuma et al., 2003). In addition, SUMO-1 and SUMO-2/3 have been found to either target different substrates or common proteins with different extents of modification (Li

et al., 2006) while a SUMO protease that discriminates between SUMO-1 and SUMO-2/3 has also been recently identified (Gong and Yeh, 2006). Collectively, the evidence suggests that SUMO-1 and SUMO-2/3 perform non-redundant functions in a tissue specific manner, which may result in specific biological roles for the different SUMO family members.

SUMOylation has also previously been involved in nucleocytoplasmic trafficking (Comerford et al., 2003; Wood et al., 2003) and protein targeting to subnuclear structures (Duprez et al., 1999; Sternsdorf et al., 1997). In this study however, no obvious difference in localisation was observed between WT p45 NF-E2 and the K368R SUMO mutant. In both cases, the protein was still localised entirely to the nucleus and a similar occurrence of sub-nuclear accumulation in discrete dots was observed in COS-7 cells (Figure 5.12A). This speckled localisation of p45 NF-E2 is well documented as euchromatin distribution in MEL and K562 cells with the exceptional occurrence of relatively larger concentrated p45 bodies (Francastel et al., 2001; Shyu et al., 2005). Interestingly, an intact SUMOylation site of p45 was also found to be essential for the nuclear localisation of p45 bodies with SUMO-1 in PODs (Shyu et al., 2005). Further experiments to elucidate the intranuclear fate of mutant transcription factors and the mechanisms involved would provide additional novel understanding of gene transcription and regulation.

Despite the general trend that SUMOylation correlates with reduced transcriptional activity, there have been examples where SUMO modification increases transcription factor activity (Hong et al., 2001; Hietakangas et al., 2003; Yamamoto et al., 2003). Indeed, the lack of an intact SUMO site in p45 NF-E2, led to a significant decrease in transcriptional activation of its MK-specific target genes (Figure 5.13A). This is in agreement with Shyu et al. (2005) who also showed that p45 SUMOylation significantly enhanced the transactivation capability of NF-E2 in erythroid cells, by increasing its DNA binding affinity. Perhaps the most biologically significant finding of these experiments is that functional SUMOylation of p45 NF-E2 is essential for thrombopoiesis, as the mutant lacking an intact SUMO site (K368R) could only produce half the number of platelets as the WT (Figure 5.13B). This result is likely to be closely

tied to the transactivation of p45 NF-E2 target genes involved in proplatelet and platelet development.

How SUMOylation regulates transcription on a molecular level is poorly understood. However, since the SUMO recognition site in p45 NF-E2 is located within the leucine zipper region of p45 NF-E2, SUMOylation may alter the surface of the protein, causing either general conformational changes or specific changes at critical interfaces, which promote interaction with its p18 partner. SUMOylation might also block other lysine-targeted modifications such as acetylation or ubiquitination, which has been shown for Sp3 (Sapetschnig et al., 2002), p300 (Bouras et al., 2005) and Iκ-Bα (Desterro et al., 1998). Although the effects of such modifications have still not been characterised for p45 NF-E2, one could imagine a scenario in which p45 NF-E2 could be regulated by different modifications at the same lysine.

In summary, the current study demonstrated that both nuclear import and SUMOylation of p45 NF-E2 are crucial for thrombopoiesis. In particular, the novel mechanisms and residues involved in p45 NF-E2 nuclear transport were identified and found to be essential in effective proplatelet and platelet development. p45 NF-E2 was also found to be modified by SUMO-2/3 in the bone marrow and this SUMOylation was shown to play a critical role in the transcriptional regulation of MK-specific, NF-E2 target genes and consequently platelet production. Indeed, the knowledge gained in this study sheds light on the different regulatory aspects of p45 NF-E2, which may prove essential for the development of novel NF-E2-mediated clinical strategies for the management of severe bleeding disorders. For instance, peptides that block the NLS of p45 NF-E2 may be useful in the treatment of polycythaemia vera while agents that promote p45 SUMOylation may be employed to treat thrombocytopaenia.

CHAPTER 6

FINAL SUMMARY AND FUTURE DIRECTIONS

FINAL SUMMARY

An improved understanding of the mechanisms governing thrombopoiesis are of central importance to the field of haematology and cell biology. Not only because of the variety of human thrombocytopaenia syndromes prevalent today, but also due to MK differentiation encompassing so many unusual attributes. In particular, by obtaining a greater understanding of the transcription factors that control and co-ordinate MK-specific gene expression and differentiation, improvements to current therapeutic options and novel treatment strategies for MK disorders may be discovered.

A crucial regulator of MK differentiation and thrombopoiesis is p45 NF-E2. This thesis set out to further characterise this essential thrombopoietic regulator and elucidate its potential as a prospective target for treating platelet disorders. The first approach taken was to stably express exogenous p45 NF-E2 in murine bone marrow cells via a retroviral gene delivery system and study its functional effects on various stages of MK development. It was hypothesised that by increasing the concentration of p45 NF-E2, haematopoietic cells may have the propensity to enhance MK maturation and platelet production.

Using numerous *in vitro* cellular assays, enforced p45 NF-E2 expression was found to favour MK differentiation by firstly increasing the number of $AchE^{+}$ mature MKs expressing MK-specific markers such as CD41, CD42a and CD42b. The processes of endomitosis and progenitor cell proliferation were however found to be minimally affected by p45 overexpression. Interestingly, a key finding of this thesis was that the enhanced MK phenotype was not only a result of selective differentiation of already committed MK progenitors, but also a consequence of an increased generation of HSC-derived MK progenitor cells. NF-E2 has only previously been documented to be involved in the terminal stages of MK differentiation and platelet development (Lecine et al., 1998b; Lecine et al., 1998a). In contrast, we found that increasing p45 NF-E2 levels resulted in a significant rise in CFU-MK progenitor development. Therefore, it is attractive to hypothesise that if p45 NF-E2 expression is not repressed during early haematopoietic development, its expression may possibly also regulate early haematopoietic development and drive lineage determination.

The latter result prompted us to further investigate the underlying reason behind this unexpected increase in MK commitment and to see if other lineages were compromised as a result of this shift. Thus, myeloid colony assays were conducted on NF-E2^{++} HSCs/ early progenitor cells. A novel finding of this assay was that in the presence of exogenous p45 NF-E2, myeloid colony formation was inhibited *in vitro*. This identifies p45 NF-E2 as a previously unrecognised regulator of myeloid differentiation.

Of clinical interest and pertinent to this thesis, *in vitro* proplatelet and functional platelet development were also shown to be enhanced by p45 NF-E2 overexpression. The accompanying upregulation of NF-E2's target genes involved in proplatelet development and platelet release was likely in part to be responsible for this. In light of the increasing need for platelet transfusions to treat a variety of platelet disorders, p45 NF-E2 mediated enhancement of *ex vivo* MK expansion and platelet production (Fujimoto et al., 2003; Matsunaga et al., 2006) may be useful in early post-transplant support of patients and in the production of functional platelets for transfusions. This approach may safely overcome concerns involved in donor-derived platelet transfusions, such as platelet shortages and risk of pathogenic contamination (Blajchman, 2003).

It is becoming clear that functional studies of transcription factor involvement in haematopoiesis should be examined in the context of living animals where all the physiological components required for haematopoiesis are present (Purton and Scadden, 2007). Therefore, after demonstrating that p45 NF-E2 overexpression was able to enhance multiple stages of MK differentiation and platelet release *in vitro*, the next aim was to study the effects of enforced p45 NF-E2 expression *in vivo*. As described in Chapter 4, RBC and platelet production were both increased 2-3 fold in NF-E2^{++} recipients compared to the control KMV mice. These results corroborate with previous findings by Sayer et al. (2000) and our *in vitro* results (Chapter 3) respectively. Furthermore, the WBC population of NF-E2^{++} mice were also found to be suppressed *in vivo*, which further substantiates p45 NF-E2 as a negative regulator of myeloid differentiation. Although out of the scope of this thesis, it would be interesting to further

examine the molecular mechanisms through which NF-E2 modulates myeloid differentiation.

Another specific aim of this thesis was to gain insight into the molecular events required for normal p45 NF-E2 function. In Chapter 5, we proceeded to identify the nuclear localisation signal (NLS) and mechanisms involved in the nuclear transport of p45 NF-E2 and its importance in maintaining normal thrombopoiesis. To achieve this, we first carried out 5' and 3' deletions and site-directed mutagenesis to identify the NLS region of p45 NF-E2. It was determined that the region R271-273, and more specifically amino acids R271 and R273, were responsible for nuclear import. Equally important, p45 NF-E2 was not found to contain a functional nuclear export signal.

In addition, it was demonstrated that p45 NF-E2 nuclear import was facilitated by the IMP β non-classical pathway, although the interaction between the full length protein and IMP β appeared to be masked either by an inhibitory element or steric hindrance to IMP binding. We hypothesise that a post-translational modification, such as phosphorylation, may be required to change the charge or conformation of the protein in order to unmask the NLS (Riviere et al., 1991; Beals et al., 1997). Elucidating the 3-dimensional structure or p45 NF-E2 may help to explore these hypotheses. Nonetheless, important evidence that an intact NLS is imperative for maintaining normal p45 function is provided. Exogenous expression of the p45 NF-E2 NLS mutant failed to mediate the enhancement of proplatelet and platelet production compared to the wildtype.

One final aim was to investigate the biological significance of p45 NF-E2 SUMOylation on MK differentiation and thrombopoiesis. p45 NF-E2 was first shown to be specifically modified by SUMO-2/3 in primary BMCs, which was later demonstrated to be required for the activation of its MK-specific target genes. Importantly, it was also established that an intact SUMOylation site is required to achieve full p45 NF-E2 function and modulation of platelet development.

In conclusion, based on the findings presented here and those recently published, I have proposed a model (Figure 6.1) in which p45 NF-E2 is conjugated to SUMO-2/3, further modified (possibly by phosphorylation) to unmask its NLS, and imported into the nucleus via IMP β interactions. Increasing concentrations of p45 shifts the equilibrium to favour the formation of p18-p45 NF-E2 complexes that then proceed to activate its MK target genes. Consequently, MK-specific genes that are normally silenced during early MK differentiation are activated, turning on a molecular switch that promotes HSCs down the MK progression instead of the myeloid lineage. This effectively results in an increased number of mature MKs and enhanced proplatelet and platelet production. This thesis not only deepens our understanding of the molecular mechanisms involved in p45 NF-E2 function, but also defines the precise functional outcomes of its regulation in MK development, which may potentially be harnessed toward therapeutic ends.

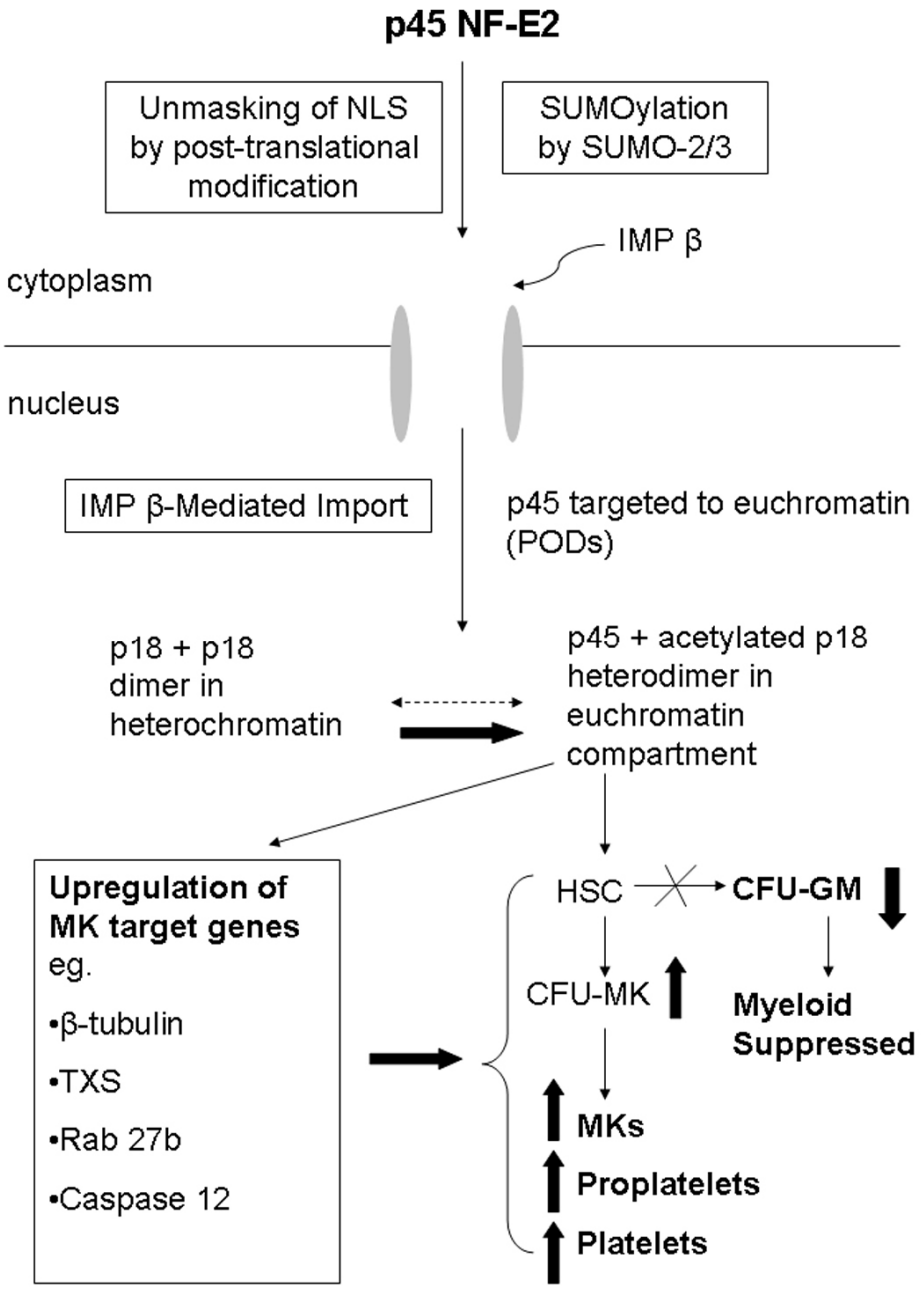


Figure 6.1 A model of possible mechanisms involved in p45 NF-E2 mediated enhancement of MK differentiation and platelet development. A simplified schematic representation of the major findings from this thesis put together with previously published data to form a proposed model, as described in the text.

FUTURE DIRECTIONS

This study provides comprehensive information about the implications of p45 NF-E2 overexpression on MK differentiation and thrombopoiesis as well as the nuclear import and SUMOylation mechanisms involved. However, a number of questions still remain.

p45 NF-E2 was identified as a previously unrecognised regulator of myeloid differentiation. However, the mechanisms involved in this regulation remain elusive. The introduction of p45 NF-E2 into cells that normally differentiate down the myeloid lineage will confirm whether p45 does indeed suppress its differentiation. In addition, by doing quantitative real-time PCR on these cells to compare the expression of myeloid–specific genes in the presence and absence of p45 NF-E2, novel myeloid target genes that are repressed by p45 NF-E2 may be identified.

In order to confirm p45 NF-E2's instructive role on MK commitment, it may also be useful to introduce exogenous p45 NF-E2 into common lymphoid progenitors (CLPs). CLPs are one of the most useful cell types for testing myeloid instructive signals. They do not have any myeloid potential nor express any major myeloid genes even at a genome-wide level (Akashi et al., 2000). Whereas, they do possess plasticity for all myeloerythroid lineages, which could be triggered by ectopic transcription factors or cytokine signalling (Iwasaki et al., 2003; Iwasaki-Arai et al., 2003; Iwasaki et al., 2006; Hsu et al., 2006). This approach would therefore provide clear-cut evidence for the lineage instructive action of p45 NF-E2 to reprogram cells into the MK lineage.

For the purpose of this study, short-term transplant studies were carried out to confirm the effects of p45 NF-E2 overexpression *in vivo*. The next step would be to study the role of p45 NF-E2 on the long-term repopulation of MKs and platelets post-transplant. Numerous studies have shown that a minimum of 16 weeks is required and an optimal 6 months is suggested for monitoring long-term reconstitution (Purton and Scadden, 2007).

The non-classical IMP β-mediated pathway was demonstrated to be responsible for p45 NF-E2 nuclear import. IMP β1 was however found to bind to the NLS weakly in the

full length protein, suggesting that the NLS may be subjected to intramolecular masking. Post-translational modifications such as phosphorylation/ dephosphorylation are the best understood mechanism to unmask a NLS within a protein (Poon and Jans, 2005). Repeating the pull-down assays with the p45 phosphorylation site mutated, may help validate this hypothesis.

To investigate the role of p45 SUMOylation in MKs, the target lysine residue was mutated in p45 and introduced into BMCs. Inevitably, SUMOylatable endogenous p45 NF-E2 is still present, hence to some extent masking the effects of the mutant. Instead, by utilising a p45 NF-E2 knockout cell line or mouse, rescue experiments with either the wildtype or the K368R mutant may be performed. In this way, the true effects of SUMOylation may be more accurately assigned. Current work in our laboratory is underway in exploring this approach for the GATA-1 transcription factor.

Finally, in this thesis (Chapter 4) we have suggested that in the future, NF-E2 could be targeted by agents that either promote its expression or prolong the half-life of the protein. A feasible approach modelled after current cancer treatments (Duncan et al., 2003; Sun, 2006) is to target a co-repressor of p45 NF-E2 such as ITCH E3 ubiquitin ligase (Chen et al., 2001) with synthetic peptides or monoclonal antibodies. This would hopefully promote p45 accumulation within MKs and as a result heighten platelet levels.

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