Gene Therapy of Mpl Deficiency

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meiner Familie

II. Abstract (deutsch)

MPL Defizienz beruht auf inaktivierenden Mutationen im Thrombopoietin (THPO) Rezeptor *c-mpl*, was zu der Erkrankung Kongenitale Amegakaryozytäre Thrombozytopenie (CAMT) führt. MPL spielt eine Schlüsselrolle in der Regulation von hämatopoetischen Stammzellen und Megakaryozyten. Mit dem Ziel eine Gentherapie für MPL Defizinz zu entwickeln, haben wir Mpl retroviral in einem murinen Knochenmark-Transplantationsmodell überexprimiert. Nur einige Wochen nach der Transplantation entwickelten die Tiere eine Chronische Myeloproliferative Erkrankung (CMPD), welche sich im weiteren Verlauf zu einer Panzytopenie mit einem Myelodysplastisch-ähnlichen Syndrom (MDS) entwickelte. Mit Hilfe eines verkürzten Mpl Rezeptors, bei welchem ein Teil der intrazellulären Signaldomäne fehlte, konnten wir zeigen, dass die CMPD auf einer supra-physiologischen Signalaktivierung beruht. Weiterhin konnten wir den Mechanismus der Panzytopenie und MDS als einen dominant negativen Effekt erklären. Ein kleiner Teil der transplantierten Mäuse entwickelte eine erythroide Leukämie (3/27) ausgelöst durch Insertionsmutagenese.

Um eine umfassende Risikoabschätzung durchzuführen, konstruierten wir eine konstitutiv aktive Mpl Rezeptor Mutante (caMpl), welche hoch leukämogen war. Nur drei Wochen nach der Transplantation entwickelten die Mäuse erythroide und myeloide Leukämien. Die Leukämieentstehung war unabhängig von der Bindung des Liganden Thpo und reduzierte Expression des caMpl Proteins führte zu einer verlängerten Latenzzeit. Eine weitere caMpl Mutante, bei welcher die letzten drei Tyrosinaminosäuren fehlten, die wichtig für die Aktivierung des Ras-Signalwegs sind, war nicht leukämisch.

Wir konstruierten optimale Vektoren für eine Gentherapie aus selbstinaktivierenden (SIN) Vektoren und dem niedrig und linienspezifisch exprimierenden Mpl Promotor (MplP). Tiere, die mit genkorrigierten $Mpl^{-/-}$ Zellen transplantiert wurden, zeigten keine Nebenwirkung bei normaler Teilnahme der korrigierten $Mpl^{-/-}$ Zellen an der Hämatopoese.

Wir konnten dosislimitierende Nebenwirkungen der Gentherapie für CAMT (CMPD, MDS, Panzytopenie und Leukämie) identifizieren und zeigen, dass physiologisch genkorrigierte $Mpl^{-/-}$ Zellen nach Transplantation langzeitlich anwachsen.

III. Abstract (englisch)

MPL deficiency is based on inactivating mutations in the thrombopoietin (THPO) receptor *c-mpl* resulting in a disorder called congenital amegakaryocytic thrombocytopenia (CAMT). MPL is a key regulator of hematopoietic stem cells and is responsible for megakaryocyte differentiation and platelet production. With the aim of developing a curative gene therapy, we retrovirally overexpressed Mpl in a murine bone marrow transplantation (BMT) model. Only a few weeks after transplantation, mice developed a chronic myeloproliferative disease (CMPD) that developed further into pancytopenia and myelodysplastic-like syndrome (MDS). With a truncated Mpl mutant lacking the signaling domain, we could show that CMPD was caused by supra-physiological signaling. In addition, we could describe the mechanism of pancytopenia and MDS-like disorder as a dominant negative effect resulting from Thpo trapping. Another serious adverse event of retroviral vector-mediated Mpl expression was erythroleukemia, which developed in a minority of mice (3/27) and was induced by insertional mutagenesis.

To perform an even broader risk assessment, we constructed a constitutively active Mpl receptor mutant (caMpl) that was highly leukemogenic. Animals developed erythro- or myeloid leukemias only three weeks after BMT. We could show that the leukemia development was independent of Thpo binding and that a reduced transgene expression level prolonged the latency. Interestingly, a truncated receptor mutant of caMpl lacking the last three tyrosine residues important for activation of Ras signaling did not induce leukemia.

We constructed optimized vectors for the gene therapy of CAMT with self inactivating (SIN) constructs. To reduce the expression level and to mediate differentiation-specific expression of the transgene, we used the endogenous Mpl promoter (MplP) to drive expression of wild type Mpl. Gene-corrected $Mpl^{-/-}$ BM cells transduced with the SIN.MplP.wtMpl vector showed engraftment after BMT and normal participation of these gene-corrected $Mpl^{-/-}$ cells in hematopoiesis without serious adverse events.

Taken together we identified the challenges of Mpl gene therapy (CMPD, MDS, pancytopenia and leukemia) and showed that gene-corrected $Mpl^{-/-}$ cells could be engrafted in a competitive *in vivo* assay after BMT.

Drei deutsche Schlagworte:

Megakaryopoese
Blutstammzellen
Anämie

Drei englische Schlagworte:

Megakaryopoiesis
Blood Stem Cells
Anemia

IV. Inhaltsverzeichnis

V. Abkürzungsverzeichnis

°C	degrees Celsius
µ	micro
Ψ	packaging signal
aa	amino acids
Ab	antibody
AKT	serine/threonine protein kinase
BCP	B-cell progenitor
BFU-E	burst-forming units erythroid
BM	bone marrow
BMT	bone marrow transplantation
bp	base pair
BSA	bovine serum albumin
caMpl	constitutively active Mpl
CAMT	congenital amegakaryocytic thrombocytopenia
canrMpl	ca-no Ras signaling-Mpl
cantMpl	ca-no THPO binding-Mpl
CD	cluster determinant
CGD	chronic granulomatous disease
CID	chemical inducers of dimerization
CIS	common insertion site
CLP	common lymphatic progenitor
CMP	common myeloid progenitor
CMPD	chronic myeloproliferative disease
c-mpl	Mpl gene
cnnMpl	ca-nt-nr-Mpl
CNS	central nervous system
CRM	cytokine receptor homology module
CSF	colony stimulating factor
d	days
D-MEM	Dulbecco-Vogt modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dnMpl	dominant negative Mpl
dpt	days post-transduction
E. coli	Escherichia coli
e. g.	exempli gratia, for example
ECD	extracellular domain
eco	ecotropic
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electromobility shift assay
Env	envelope

EP	erythroid precursor
EPO	erythropoietin
EPOR	erythropoietin receptor
ERK	extracellular response–stimulated kinase
et al.	et alii
etc.	et cetera
EtOH	ethanol
FA	Fanconi anemia
FACS	fluorescent associated cell sorting
FCS	fetal calf serum
FET	familial essential thrombocythemia
fig	figure
FOX	fork head
g	grams
GAB	GRB binding
Gag	group specific antigen
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage CSF
GMP	granulocyte monocyte progenitor
GP	granulocyte precursor
GRB2	growth factor receptor bound protein
HA	hemagglutinin
HCT	hematocrit
HEPES	N-(2-hydroxyethyl)piperazine-N‘-2-ethanesulfonic acid
HIV	human immunodeficiency virus
HSC	hematopoietic stem cell
ICD	intracellular domain
IDDb	insertional dominance database
IL	interleukin
IN	integrase
IP	internal promoter
IPTG	isopropyl β-D-thiogalactoside
IRES	internal ribosome entry site
IRS	insulin receptor substitute
JAK	Janus kinase
kb	kilo base pair
KO	knock-out
LacZ	β-galactosidase
LB	Luria Bertani
Lin-	lineage-negative BM cells
LK	lineage-negative ckit positive fraction
LL	lower left
LM-PCR	ligation-mediated PCR
LR	lower right

LSK	lineage-negative Sca pos ckit pos fraction
LT	long-term HSC
LTR	long terminal repeat
m	meter
M	Mol
MA	matrix
mAb	monoclonal antibody
MACS	magnetic associated cell sorting
MAPK	p38 mitogen-activated protein kinase
max	maximum
M-CSF	macrophage CSF
MDS	myelodysplastic disease
MEP	megakaryocyte erythroid progenitor
min	minute
Mk	megakaryocyte
MkP	megakaryocyte precursor
mL	milliliter
MLV	murine leukemia virus
mM	millimole
MP	monocyte precursor
MPD	myeloproliferative disease
MPL	thrombopoietin receptor
MplP	endogenous Mpl promoter
MPLV	myeloproliferative leukemia virus
NC	nucleocapsid
Nef	negative effector
neg	negative
neo	neomycin
NK	natural killer cells
NKP	natural killer cell progenitor
nm	nanometer
nM	nanomole
nrMpl	ca-no Ras signaling Mpl
ntMpl	ca-no Thpo binding Mpl
ORF	open reading frame
P	phosphorylation
p.	page
pA	poly A tail
PB	peripheral blood
PBC	peripheral blood cell count
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pen	penicillin
Pfu	Pyrococcus furiosus polymerase
PGK	phosphoglycerate kinase
pH	potentia hydrogenii
PI	propidium iodide

PI3K	phosphoinositide 3-kinase
PIC	pre-integration complex
PLT	platelets
Pol	polymerase
pos	positive
PR	protease
qPCR	quantitative PCR
Ras	small guanine nucleotide–binding protein
RBC	red blood cell count
Rev	regulator of viral gene expression
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RRE	rev responsive element
RT	reverse transcriptase
RTCGD	retroviral tagged cancer gene database
RT-PCR	reverse transcriptase PCR
SA	splice acceptor
SAP	shrimp alkaline phosphatase
SB	Southern blot
SCF	stem cell factor
SD	splice donor
sec	second
SF	SFFV, spleen focus forming virus enhancer-promoter
SHC	Src homology containing
SHP2	Shc homology domain containing phosphatase
SKY	spectral karyotyping
SOS	son of sevenless
ST	short-term HSC
STAT	signal transducer and activator of transcription 3 and 5
Strep	streptomycin
SU	surface protein(gp120)
SV40	simian virus 40
t	time
Tab.	table
TAE	tris-acetate
Taq	Thermus aquaticus
TAR	thrombocyte radius aplasia
Tat	transcriptional activator
TCP	T-cell progenitor
TE	tris-EDTA
Tgo	Thermococcus gorgonarius
THPO, Thpo	thrombopoietin
ThpoR	c-Mpl, THPO receptor
TM	transmembrane
TMD	transmembrane domain
TNK	T natural killer cells
TPO	thrombopoietin (outdated)

Tx	transplantation
U	enzyme activity
UL	upper left
UR	upper right
UV	ultraviolet
V	volt
Vif	viral infectivity factor
v-mpl	viral oncogene
Vpr	viral protein R
Vpu	viral protein U
w/o	without
WAS	Wiskott Aldrich Syndrome
WB	Western blot
WBC	white blood cell count
wks	weeks
wPRE	woodchuck hepatitis virus post transcriptional element
WT	wild-type
wtMpl	wild-type Mpl
X-Gal	5-bromo-4-chloro-3-indolyl β-galactopyranoside
Y	tyrosine

1. Introduction

1.1. Hematopoietic stem cells are a small population of pluripotent cells

Hematopoietic stem cells (HSC) represent the earliest and most undifferentiated blood cells. They are pluripotent stem cells, because they are able to give rise to all hematopoietic lineages the peripheral blood (PB) cells, but typically not to other tissue organs. HSC mostly reside in the bone marrow (BM) niche (Yin and Li, 2006); only small numbers circulate in the PB. Cytokine and chemokine-mediated interaction of HSC with their niche is required for hematopoiesis (Avecilla et al., 2004). It is estimated that about 1 in 100,000 BM cells is a true HSC. Despite their low frequency, HSC have the ability to regenerate hematopoiesis with their unique ability to differentiate and to self-renew. When undergoing the process of self-renewal, the HSC divide but remain in the undifferentiated state. HSC have a slow division rate and about 20% of all divisions are asymmetrical (Ho and Wagner, 2007) which means that one daughter cell retains the stem cell character whereby the other one differentiates (Giebel et al., 2006; Leary et al., 1985; Mayani et al., 1993). Since the development of monoclonal antibodies (mAb) against specific cell surface markers and fluorescence-activated cell sorting (FACS), it is possible to characterize and purify stem cell fractions. Murine HSC are enriched in the lineage-negative (Lin-) fraction which is defined as $Gr1^-$, $CD11b^-$ (myeloid markers), $TER119^-$ (erythroid marker), $CD5^-$ (B-cell marker) and $CD45R^-$ (naïve T-cell and leukocyte marker). To further purify HSC, they can be sorted in the Lin- fraction for $Sca1^+$ and $ckit^+$ cells which comprise the LSK fraction. The LSK fraction can again be further separated and contains the long-term (LT) and short-term (ST) repopulating HSC (Morrison and Weissman, 1994; Osawa et al., 1996; Smith et al., 1991; Uchida and Weissman, 1992).

Since HSC can reconstitute all blood lineages, their transfer is used in many therapeutic protocols. Disorders which are regularly treated by BM transplantation (BMT) are leukemia, lymphoma, inborn metabolic disorders and inherited anemias (Muraoka et al., 2005; Steward and Jarisch, 2005; Thomas et al., 1957).

1.2. Hematopoiesis is the production of all peripheral blood cells

The process of HSC giving rise to all hematopoietic lineages is described as hematopoiesis. The HSC thereby differentiate into two arms, common myeloid progenitors (CMP) and common lymphoid progenitors (CLP) (Akashi et al., 2000; Kaushansky, 2006b; Kondo et al., 1997). CMP have the potential to build the red cells, platelets and the white blood cells and thus develop into megakaryocyte erythroid progenitors (MEP) and granulocyte monocyte progenitors (GMP).

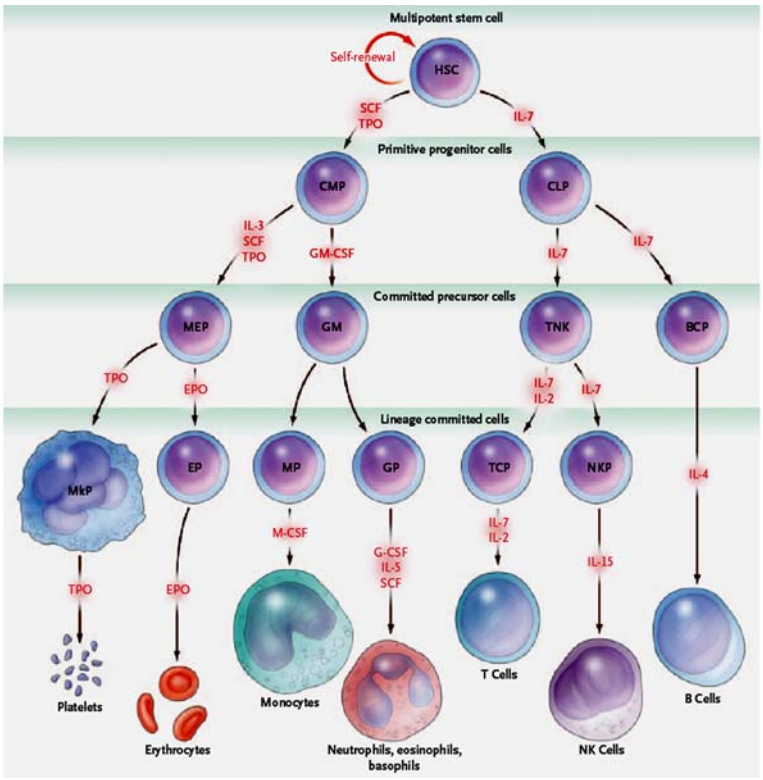

Figure 1.1: General model of hematopoiesis (Kaushansky, 2006b). The blood cell development is emanating from HSC and controlled by several factors. HSC have the potential to self-renew or differentiate into blood progenitors. These processes are mediated by several cytokines. Abbreviations: HSC hematopoietic stem cell, CMP common myeloid progenitor, CLP common lymphoid progenitor, MEP megakaryocyte erythroid progenitor, GM granulocyte progenitor, TNK T-cells and natural killer cells, BCP B-cell progenitor, MkP megakaryocyte progenitor, EP erythroid progenitor, MP monocyte progenitor, GP granulocyte progenitor, TCP T-cell progenitor, NKP natural killer cell progenitor, IL interleukin, TPO thrombopoietin, SCF stem-cell factor, M-CSF macrophage colony-stimulating factor, GM-CSF granulocyte-macrophage CSF, EPO erythropoietin.

GMP finally differentiate via other precursors (monocyte precursor, MP and granulocyte precursor, GP) into mature monocytes and granulocytes (divided into neutrophils, eosinophils and basophils). Further differentiation of MEP results in erythroid precursors (EP) and mature red blood cells (erythrocytes) and in megakaryocyte precursors (MkP) and megakaryocytes (Mk) which produce all blood platelets (PLT, thrombocytes) (Figure 1.1).

Megakaryocytes constitute less than 1% of the myeloid cell mass in the BM. However, their amount can increase during disease up to ten times. Mk are easily distinguished from other blood cells because of their increased size (~100 μm). The primary site for Mk is the BM. Lower amounts can also be found in the lung and in the PB. The structure of their nuclei is described as "horseshoe-shaped". Known surface markers are CD34, CD41, CD42, CD61, CD51 and CD110 (MPL) (Kirito and Kaushansky, 2006; Pang et al., 2005). The Mk progenitors (megakaryoblasts) lose their capacity to divide but retain their ability for DNA replication (endoreduplication) and cytoplasmic maturation. Mk undergo multiple rounds of endomitosis which amplifies their DNA content. The nuclear envelope breaks down and the mitotic cycle is arrested during anaphase B. When the nuclear envelope reforms, the Mk nucleus is polyploid (multilobed nucleus). Polyploidization can reach from 4N, 8N, 16N up to 256N whereby 16N is the most common state in half of the Mk population (Deutsch and Tomer, 2006; Patel et al., 2005). Mk have a high concentration of ribosomes which are needed for platelet production. Proplatelets are formed by fragmentation from the membrane of mature Mk which then mature to platelets. Human Mk produce about 10^{11} platelets per day in steady-state hematopoiesis (Kaushansky, 2008).

1.3. Hematopoietic cytokines and their receptors regulate hematopoiesis

Many hematopoietic processes like proliferation, differentiation, apoptosis and self-renewal are regulated by cytokines and their receptors. Cytokines are small protein molecules which float in the PB and are transferred to the hematopoietic organs. They are produced by cells of different organs such as the kidneys and the liver, by stromal cells or hematopoietic cells in the hematopoietic microenvironment. Cytokines can be produced

constitutively or inducibly. Inflammatory mediators or BM stromal cells can lead to inducible cytokine production (Kaushansky, 2006b) (Figure 1.2).

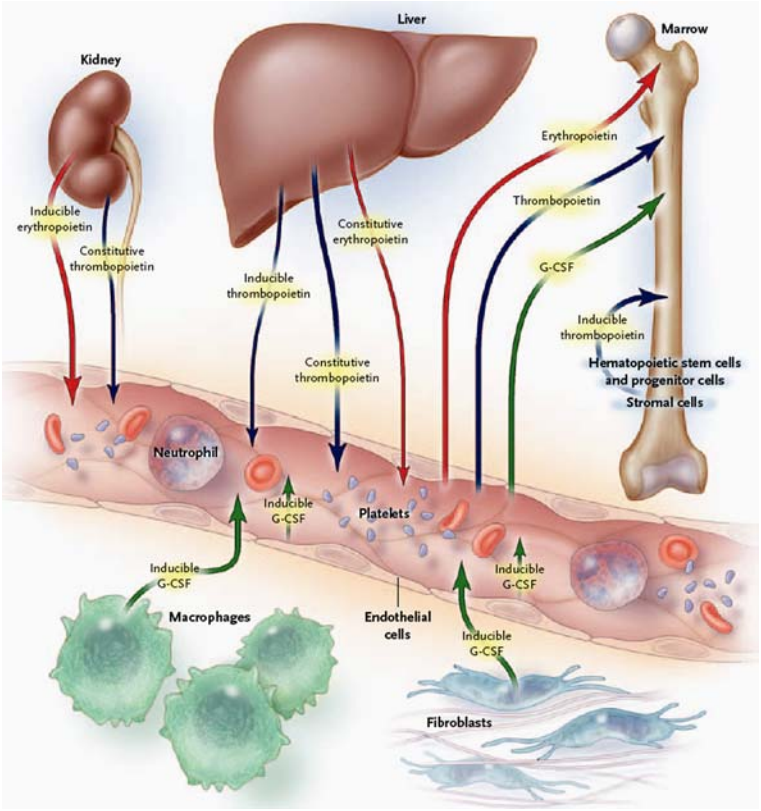

Figure 1.2: Production of hematopoietic growth factors (Kaushansky, 2006). Cytokines are produced by different organs like the kidneys and liver. Thrombopoietin (blue arrows) is produced constitutively by the liver (accounting for approximately 50% of baseline thrombopoietin levels). The kidneys produce only a minority of the total THPO amount. The production also is inducible in the liver by inflammatory mediators and from BM stromal cells by thrombocytopenia. Abbreviations: G-CSF granulocyte colony stimulating factor.

Hematopoietic growth factor–receptors of the hematopoietin cytokine receptor superfamily 1 are cell membrane-spanning protein molecules. Two receptor subunits can dimerize to one receptor. Ligand binding substantially changes the conformation of the receptor, bringing the cytoplasmic domains into close juxtaposition and allowing the tethered kinases to cross-phosphorylate (activate) each other. One of the first substrates for Janus kinase 2 (JAK2) are receptor tyrosine (Y) residues. Upon their phosphorylation (P), they serve as docking sites for adaptors, phosphatases, G-protein activators and lipid

kinases. Eventually, most of such signals affect nuclear transcription of antiapoptosis molecules, cell-cycle regulators, differentiation associated proteins and other growth factors and their receptors. With the binding to their receptor, cytokines are removed by internalization (THPO, G-CSF) or unused cytokines can be excreted in the urine like erythropoietin (EPO). Cytokines, their receptors and downstream signal cascades play a pivotal role in different human neoplasias (Steelman et al., 2004) (Figure 1.3).

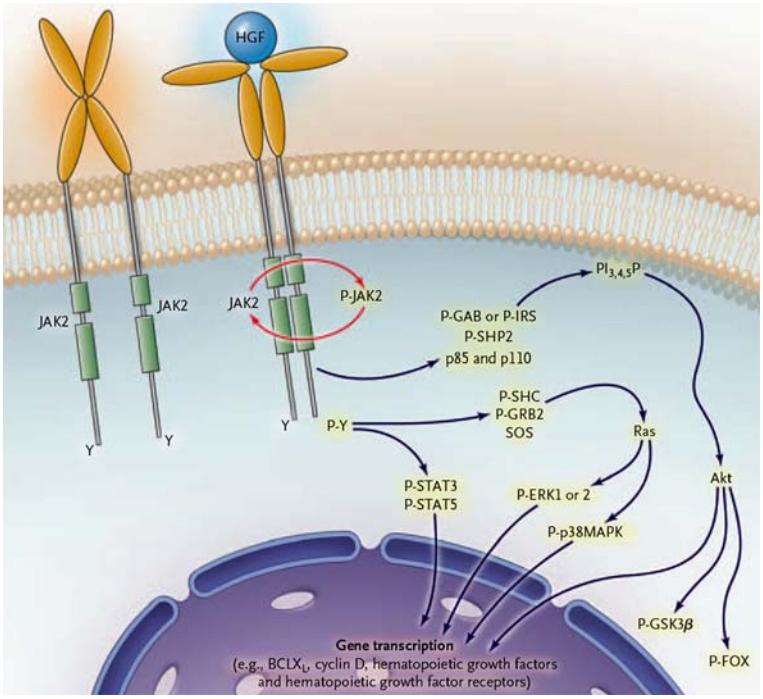

Figure 1.3: Hematopoietic growth factor receptor signaling (Kaushansky, 2006). Growth factor receptors of the hematopoietin cytokine receptor super family 1 are composed of two subunits (monomers). Ligand binding changes the conformation of the receptor and results in cross-phosphorylation (activation). Janus kinas 2 (JAK2) phosphorylates (P) tyrosine (Y) residues. Abbreviations: SHC Src homology containing, GRB2 growth factor receptor–bound protein 2, GAB Grb binding, IRS insulin-receptor substrate, SHP2 Shc homology domain containing phosphatase, SOS son of sevenless, PI3K phosphoinositol 3-kinase, STAT signal transducer and activator of transcription 3 and 5, PI3,4,5P phosphoinositol 3,4,5-phosphate, Ras small guanine nucleotide–binding protein, AKT serine/threonine protein kinase, MAPK p38 mitogen-activated protein kinase, ERK extracellular response–stimulated kinase, P-FOX phosphatase forkhead, P-GSK3β phosphatase glycogen synthase kinase 3β, BCLXL nuclear transcription of antiapoptosis molecules, cyclins cell-cycle regulators, HGF hematopoietic growth factor.

1.4. The cytokine receptor MPL is activated by its ligand thrombopoietin

The cytokine receptor MPL was identified in 1992 (Vigon et al., 1992) and its ligand THPO was found two years later (Bartley et al., 1994; Lok et al., 1994). MPL is named by the myeloproliferative leukemia virus (MPLV) (Wendling et al., 1986) which encodes the oncogene *v-mpl* (Souyri et al., 1990). THPO is produced by the liver and the kidneys (Qian et al., 1998). Therefore, an influence of THPO on hepatoblastoma cells is discussed (Romanelli et al., 2006). A steady-state amount of hepatic THPO is regulated by platelet MPL receptor–mediated uptake and destruction of the cytokine (Fielder et al., 1997). Upon binding to platelet's MPL receptors, THPO is removed from the circulation and destroyed, which reduces THPO levels in the PB. Thrombocytopenia or inflammation can enhance the THPO production of the liver (Kaushansky, 2005) (Figure 1.4).

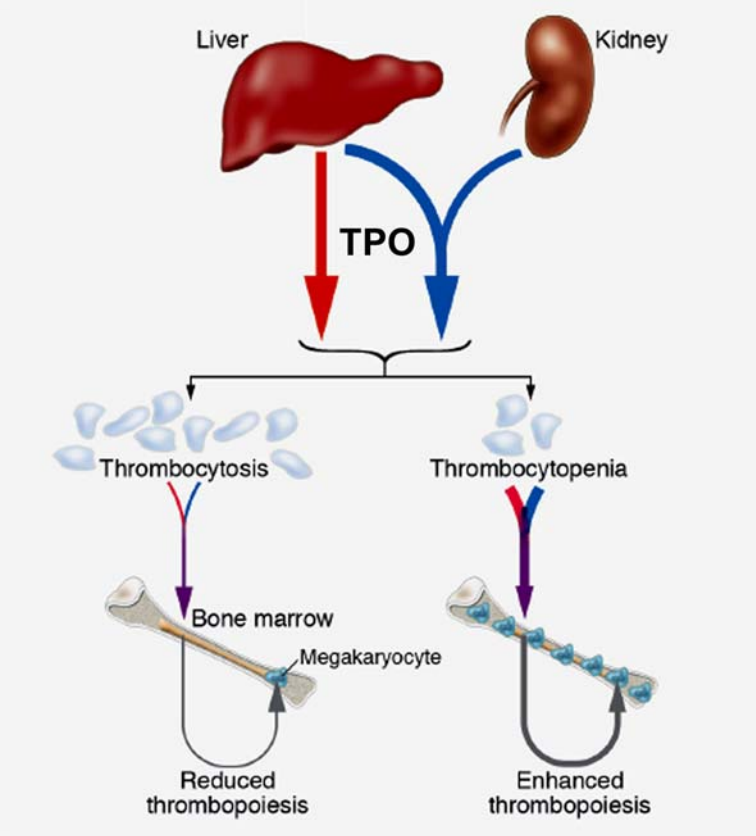

Figure 1.4: Regulation of thrombopoietin levels (Kaushansky, 2005). A steady-state amount of thrombopoietin (THPO) is produced by the liver and the kidneys. It is regulated by platelet MPL receptor–mediated uptake and destruction of the cytokine. In the presence of inflammation THPO production by the liver is enhanced. Additionally, thrombocytopenia leads to enhanced marrow stromal cell production of thrombopoietin.

THPO is the only ligand of the MPL receptor. Upon ligand binding, receptor conformation shifts bringing the cytoplasmic domains together (Luoh et al., 2000; Miyakawa et al., 2000). The membrane-proximal Box 1 and Box 2 cytoplasmic domains constitutively bind Janus kinase 2 (JAK2) leading to cross-activation after ligand binding which initiates signal transduction. THPO activates both JAK2 and TYK2 (tyrosine kinase 2) but only JAK2 is essential for signaling and is the predominant isoform activated in primary Mk (Drachman et al., 1999). The active JAK kinase phosphorylates the receptor's tyrosine residues and molecules that promote cell survival and proliferation, including the STATs (Signal transducer and activator of transcription 3 and 5), PI3K (phosphoinositide-3 kinase) and MAPKs (mitogen-activated protein kinase). Furthermore, JAK2 phosphorylates molecules that limit cell signaling like SHP1 and SHIP1 phosphatases and SOCS. PI3K activates its downstream effector Akt (serine-threonine protein kinase) (Bouscary et al., 2001; Miyakawa et al., 2001), and blocking this pathway inhibits THPO-induced cell survival and proliferation (Geddis et al., 2001). The pathways downstream of Akt in Mk and platelets include the transcription factor FOXO3a, the cell cycle inhibitor p27 and glycogen synthase kinase 3β (GSK3β). In addition to PI3K, THPO stimulates two of the MAPK pathways, p42/p44 (ERK1 and ERK2) and p38 MAPK (Drachman et al., 1997; Kirito et al., 2003). These events are mediated by binding and phosphorylation of Grb2, SHC and SOS and exchange of GDP for GTP on Ras (Rojnuckarin et al., 2001). The functional consequences of the events mediated by MAPK p38 include induction of the transcription factors HoxB4 and HoxA9 which affects HSC expansion (Kirito et al., 2003; Kirito et al., 2004). ERK1/2 induce proliferation and polyploidization of Mk (Kaushansky, 2005; Rojnuckarin et al., 1999) (Figure 1.5).

THPO and MPL are the most potent regulators of megakaryopoiesis (de Sauvage et al., 1994). Thpo supports colony formation of megakaryocytes (Broudy et al., 1996; Majka et al., 2002). Antisense oligonucleotides against Mpl specifically inhibit megakaryopoiesis (Methia et al., 1993). The analysis of $Mpl^{-/-}$ mice (Alexander et al., 1996c) and $Thpo^{-/-}$ mice showed that these animals have a defective hematopoiesis with thrombocytopenia and drastically reduced numbers of HSC and progenitors (Carver-Moore et al., 1996; Murone et al., 1998). However, the maintenance of steady-state hematopoiesis is ensured

with only a few progenitor cells to populate and maintain the BM, spleen, and PB with nucleated cells in the mouse (Broxmeyer et al., 2005).

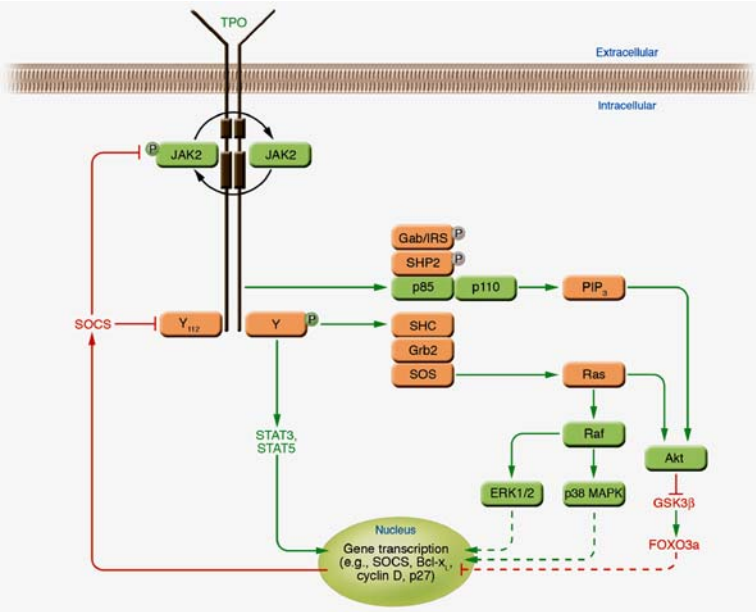

Figure 1.5: Downstream signaling pathways of MPL (Kaushansky, 2005). Signaling pathways are activated by thrombopoietin binding to MPL. Several pathways for cell cycling processes are induced. Abbreviations: P phosphorylation, Y tyrosine residue, JAK2 Janus kinase 2, STAT3 and STAT5 Signal transducer and activator of transcription, Bcl-xL anti-apoptotic signaling molecule, SHC Src-homology collagen protein, Grb2 growth factor receptor bound protein 2, SOS son of sevenless, Ras rat sarcoma, Raf protein kinase, MAPK mitogen activated protein kinase, ERK1/2 extracellular signal-regulated kinase, PI3K phosphoinositol-3-kinase, SHP2 SH2 domain containing phosphatase-2, Gab1 Grb2-associated binder, PIP2 membrane–bound phosphoinositol-4,5-biphosphate, PIP3 phosphoinositol-3,4,5-triphosphate, Akt serine/threonine protein kinase, GSK3β glycogen synthase kinase-3, FOXO3a transcription factor, p27 cell cycle inhibitor, SOCS suppressor of cytokine signaling.

MPL is found on HSC and can be used as a selective surface marker on human HSC (Ninos et al., 2006). Mpl/Thpo support the proliferation and maturation of primitive hematopoietic progenitors and the HSC self-renewal (Ku et al., 1996; Sitnicka et al., 1996; Tong et al., 2007). $Mpl^{+/+}$/$Thpo^{+/+}$ cells show a significantly better engraftment than $Mpl^{-/-}$/$Thpo^{+/+}$ cells in mice (Solar et al., 1998). Recent studies suggested that THPO signaling is also involved in the maintenance of HSC quiescence (Qian et al., 2007; Yoshihara et al., 2007). Mpl further is involved in the erythroid differentiation: Progeny of normal human burst-forming units-erythroid (BFU-E) contain Mpl receptor mRNA, and flow cytometric analysis shows the presence of Mpl receptor protein on the surface of

these cells (Broudy et al., 1997). Thpo was also found to expand erythroid progenitors, increase red cell production, and enhance erythroid recovery after myelosuppressive therapy (Kaushansky et al., 1995). The cross-reactivity of Thpo with the EpoR supports erythroid proliferation (Rouleau et al., 2004).

Deregulation of either MPL or THPO is connected with several human disorders. Recently, a constitutively activating mutation of MPL (MplW515LK) was found which results in human myeloproliferative disorder (MPD) (Pardanani et al., 2006). Expression of MplW515LK in mouse BM induced an MPD-like disorder (Pikman et al., 2006). MPL Baltimore is an MPL receptor polymorphism associated with thrombocytosis at amino acid (aa) position K39N resulting in chronic myeloproliferative disorder (CMPD). Patients suffer from significantly higher platelet counts and show reduced MPL expression in platelets (Moliterno et al., 2004). Familial essential thrombocythemia (FET) is associated with a dominant-positive activating mutation of the MPL gene (Ding et al., 2004). Absence of MPL is also discussed in negatively regulating the central nervous system (CNS) and the brain growth (Yang et al., 2004). A monogenous defect caused by mutations in the *c-mpl* gene is congenital amegakaryocytic thrombocytopenia (CAMT).

1.5. CAMT is a hematopoietic disorder based on MPL inactivation

Thrombocytopenias can be divided into inherited and acquired forms. Acquired thrombocytopenia results from infections, auto-immunity or medications. Inherited forms are very rare and often diagnosed in early childhood. Inherited thrombocytopenias are e.g. thrombocytopenia-radius-aplasia (TAR), wiskott-aldrich syndrome (WAS), fanconi anemia (FA) and congenital amegakaryocytic thrombocytopenia (CAMT).

CAMT was first characterized 1974 as constitutively aplastic anemia type III (Hughes, 1974). It is a very rare disease with about one to two new cases in Germany per year. CAMT is a recessive inherited BM failure syndrome starting with an isolated thrombocytopenia after birth in association with hypomegakaryocytic BM. Further progression of the disorder results in a pancytopenia affecting all hematopoietic lineages. The pancytopenia development points to a basic malfunction in hematopoiesis on the HSC level. Results of Freedman and Estrov showed that pancytopenia must originate from an intrinsic HSC defect (Freedman and Estrov, 1990). Patients suffer from

spontaneous bleeding which can become life-threatening and develop lethal aplastic anemia in the first years of their life. The only known therapy today is allogeneic BMT (Muraoka et al., 2005).

Ihara et al. found point mutations in exons 4 and 10 of the *c-mpl* gene which led to a translational stop and therefore truncations of the MPL receptors (Ihara et al., 1999). In the following years, many nonsense and missense mutations were detected in the whole *c-mpl* gene, which directly correlates with the development of CAMT (Ballmaier et al., 2001; Germeshausen et al., 2006; van den Oudenrijn et al., 2000) and aplastic anemia (Ballmaier et al., 2003). A genotype-phenotype correlation could be found which led to the identification of two CAMT subgroups, CAMT 1 and CAMT 2 (Germeshausen et al., 2006). Mutations underlying CAMT 1 lead to a complete inactivity of the MPL receptor. These patients have low platelet counts directly after birth and develop pancytopenia in a short time. CAMT 2 patients have missense mutations which provide residual receptor activity. These patients show a moderate phenotype progression and develop pancytopenia in a later stage of life after about three years (Gandhi et al., 2005). New mutations are still found in MPL which cause CAMT (Passos-Coelho et al., 2007). Besides hematologic phenotypes resulting from MPL deficiency, malformations in other organs like heart and brains could be observed.

1.6. Gene therapy is a possible treatment for CAMT

The term gene therapy was created in the 1980s. Gene therapy describes the medical intervention by which genes are introduced into patient cells to cure a disease (Mulligan, 1993). The approaches can be used to cure infectious diseases, to cure cancer and as a therapy for inherited disorders. The therapeutic strategy ranges from somatic gene therapy to germline therapy and from *in vivo* to *ex vivo* therapy. In somatic gene therapy, cells from patients are genetically altered to cure the disease but do not transmit the alterations to the offspring. With germline therapy, the alteration of the genome will also be transferred to the patient's offspring which, is not desired and thus ethically banned in most countries. *In vivo* therapy entails the direct application of the therapeutic genes into the patient which is connected to many risks like nonspecific targeting of cells. The *ex vivo* approach is safer because cells from the patient are harvested and the therapeutic

gene is introduced into this defined population only. Afterwards, these cells are returned to the patient.

The target for gene therapy in many hematopoietic disorders like CAMT are the HSC. The permanent introduction of a gene into an HSC means that all progeny cells arising from this cell carry the therapeutic gene which increases the chances for the therapy. HSC are harvested from patients and transduced with the recombinant gene vectors. The stem cell fraction has to be cultured or expanded *ex vivo* where the surface phenotype of the HSC changes from that of freshly isolated HSC. Depending on the cytokine cocktail used this does not necessarily alter their repopulation capability (Zhang and Lodish, 2005). The successful use of retroviral gene transfer has been shown in patients (Cavazzana-Calvo et al., 2004). Recently, about 20 patients with X-linked severe combined immunodeficiency (SCID-X1) have been treated with HSC that were genetically modified with retroviral vectors, and most of the patients showed evidence of the therapeutic benefit of gene therapy (Cavazzana-Calvo et al., 2000; Kohn, 2008). Furthermore, correction of X-linked chronic granulomatous disease (CGD) by gene therapy could be shown (Ott et al., 2006). To mediate the addition of a therapeutic gene into the host genome, a transfer vehicle is needed (Mann et al., 1983). These transfer vehicles can be bacterial, non-viral (like naked DNA) or viral.

1.7. Viral vectors for gene therapy

Viral transfer constructs are called vectors and are based on different viruses depending on the application needed. Vectors can be non-integrating or integrating and introduce the therapeutic gene temporarily or permanently into the host cell. Retro- and lentiviral vector backbones like murine leukemia virus (MLV), human immunodeficiency virus (HIV) or foamy virus-based vectors integrate permanently into the host genome. For non-integrating applications, adenoviral and adeno-associated viral vectors are very common (Flotte, 2007). Retroviral vectors have the advantage of providing efficient integration into the host genome with stable expression of the transgene in all progeny cells and the viral genes can be completely replaced by the recombinant transgene. A disadvantage for using retroviral vectors is their limited capacity for the therapeutic gene of about 8 kbp. Lentiviral vectors have the advantage of also infecting non-dividing cells compared to

retroviral vectors based on MLV (Baum et al., 2006; Vigna and Naldini, 2000). CAMT as a monogenous disorder resembles a classical target for a permanent somatic retroviral gene therapy of HSC.

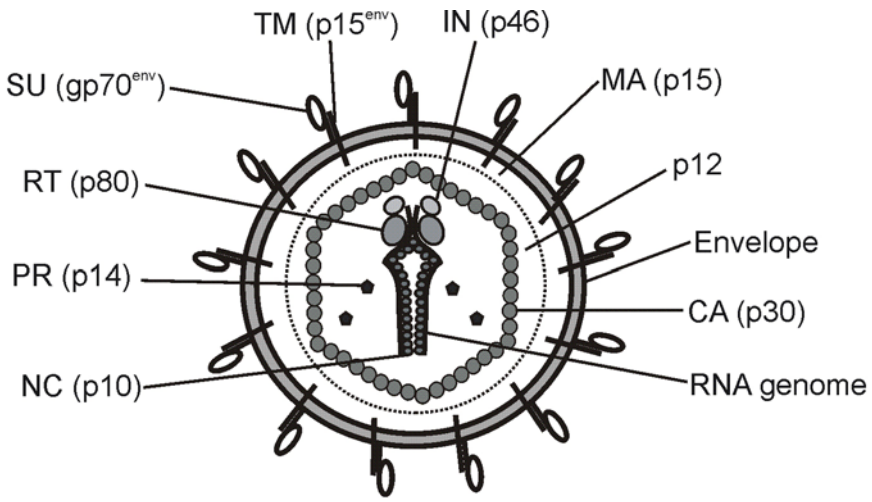

Figure 1.6: The gamma-retroviral particle (Galla, 2008). The scheme of a mature MLV (murine leukemia virus) retroviral particle shows several retroviral elements. Particles are surrounded by a membrane which includes the envelope surface proteins for binding to the host cell. The two RNA molecules are surrounded by the capsid. Several enzymes and accessory proteins are incorporated into the viral particle. Abbreviations: IN integrase, MA matrix, CA capsid, NC nucleocapsid, PR protease, RT reverse transcriptase, SU surface subunit, TM transmembrane subunit.

Retroviral particles are about 100 nm in size and are surrounded by a membrane. This membrane incorporates the viral surface proteins (envelope, env). The membrane surrounds the core which is made of the *gag* protein (group specific antigen). Inside the core are more viral proteins like the viral protease (pro), the reverse transcriptase (RT) and the integrase. The genome is made up of two identical plus stranded RNA molecules (Coffin et al., 1996) (Figure 1.6). Retroviruses can infect defined cell populations by receptor-mediated fusion or endocytotic uptake (Greene and Peterlin, 2002). After binding to the host cell membrane (attachment) and entry, the capsid protein with the viral genome and further proteins are released (uncoating). Viral reverse transcriptase catalyses the transcription of the viral genomic RNA to viral DNA, after which the pre-integration complex (PIC) is formed. The PIC may be transported into the nucleus. MLV lacks the ability to transduce non-dividing cells, therefore the nuclear membrane has to

break down during cell division. The viral integrase catalyses the process of DNA integration into the host genome. The viral DNA is transcribed and translated to build new virus particles and proteins. Viral RNA and proteins are transported to the host membrane and bud off the cell whereby the new virus is coated by the host membrane. Following maturation, new infectious virus particles are formed.

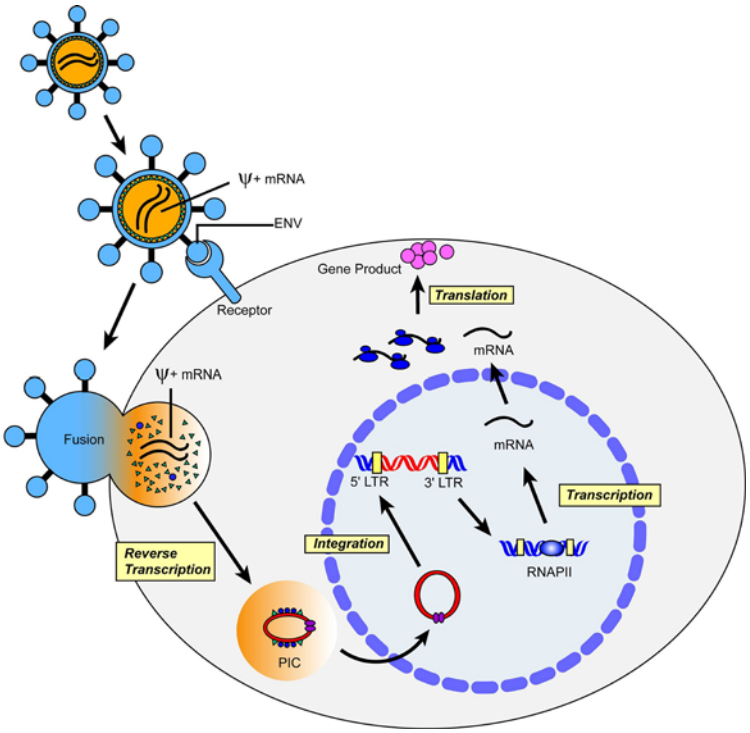

Figure 1.7: Gene therapy with retroviral vectors (Baum et al., 2006). The vector particle binds with the env protein to specific surface markers of the target cell. Membranes of host and virus fuse and after capsid degradation the viral RNA and accessory proteins are brought into the cytoplasm. Reverse transcription takes place and the PIC (pre integration complex) is formed. The viral DNA and the integrase gain access to the nucleus after the nucleus membrane breaks down during mitosis. Viral DNA is then integrated into the host genome. The therapeutic gene can be transcribed and translated and can fulfill its function. Abbreviations: mRNA messenger RNA, ENV envelope protein, LTR long terminal repeat, RNAPII RNA polymerase II.

The first retroviral vector was described in 1981 (Wei et al., 1981). This work was based on the harvey murine sarcoma virus and showed successful introduction of the thymidine kinase gene into thymidine kinase$^{-/-}$ cells. One year later, Tabin et al. reported the same experiment with the moloney murine leukemia virus (Tabin et al., 1982). Subsequently

positive selectable retroviral vectors containing the *neo*^{R} gene for selection (Beck-Engeser et al., 1991; Friel et al., 1987; Ostertag et al., 1986) were built on the basis of the myeloproliferative sarcoma virus (Ostertag et al., 1980; Stocking et al., 1986) and other variants adapted to better expression in multipotent HSC (Grez et al., 1990).

Gene therapy with retroviral vectors today mostly uses constructs based on gamma-retroviruses (Miller and Rosman, 1989) and lentiviruses (Naldini, 1998). Common for all viral vectors is that gene therapy makes use of the normal replication process of viruses by which they introduce their genome into the host cell (Figure 1.7). An important safety feature of retroviral vectors is that they are replication-incompetent. This avoids the uncontrolled spread of gene therapy vectors in patients. The risk of horizontal gene transfer (to other persons) or vertical gene transfer (to the patient's offspring) is thereby eliminated. The transcription and translation of the vector coding genes do not produce proteins to build new viruses but translate the therapeutic gene.

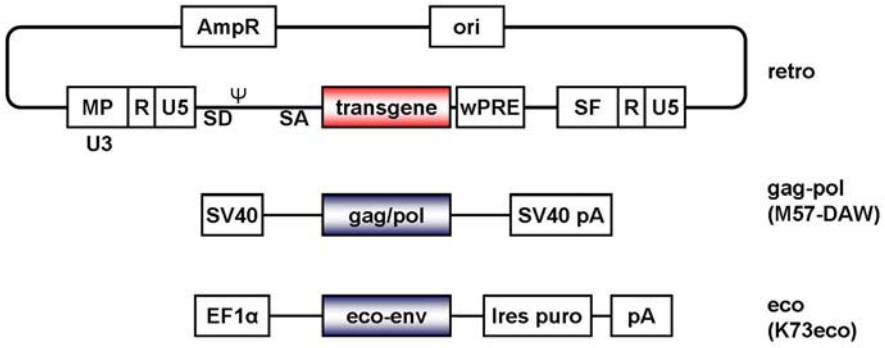

Figure 1.8: Viral vectors and packaging plasmids. Plasmids are used to produce retroviral particles for gene therapy harboring the gene of interest. For safety reasons proteins like integrase (IN), reverse transcriptase (RT), protease (PR) (gag-pol) and envelope proteins (env) are separated on different plasmids. Additionally the packaging signals (Ψ) on these constructs are removed, to avoid the packaging of the viral protein coding sequences into the new viral particles. Only the retroviral vector harboring the gene of interest has the packaging signal. Via co-transfection of those vectors (gag-pol, env) replication defective viral particles are produced. Abbreviations: AmpR ampicillin resistance, ori origin of replication, MP murine leukemia virus promoter, SD splice donor, SA splice acceptor, wPRE woodchuck hepatitis virus posttranscriptional regulatory element, SF spleen focus forming virus promoter, SV40 simian virus 40, gag/pol accessory proteins, env envelope, eco ecotroph, pA poly A signal, Ef1α Ef1α promoter, Ires internal ribosome entry site, puro puromycin.

The reason for vectors being replication-incompetent is based on the deletion of important viral genes like the *gag*, *pro*, *pol* and *env* genes. This is possible because the early steps of replication of a retrovirus (attachment, reverse transcription, integration, synthesis) are not dependent on the coding sequences of the vector. The elimination of the retroviral genes provides space for the therapeutic sequences (Mulligan, 1993). The gene of interest is thereby flanked with viral long terminal repeats (LTR). These are strong enhancer-promoter regions which provide high transgene expression (Figure 1.8).
The LTR U3 (unique 3) region (500 bp) provides the enhancer-promoter activity, the R region (60 bp) forms the cap and polyadenylation site of the LTR and the U5 region (75 bp) is essential for the reverse transcription and integration. Vectors are not able to produce new vector particles until viral regulatory enzymes and proteins are co-transfected. For safety reasons, these proteins are encoded on other plasmids lacking the packaging signal (Ψ) (Berkowitz et al., 1996). Gag with all of its components (matrix, capsid, nucleocapsid) provides the viral enzymes like the reverse transcriptase (RT), integrase (IN) and protease (PR) (Figure 1.8). Eco (ecotropic = mouse cells) encodes the envelope *env*-protein recognizing the murine ecotropic mCAT-1 receptor (Wang et al., 1991). Also, the use of packaging cell lines instead of co-transfection is very common (Markowitz et al., 1988a; Markowitz et al., 1988b). After the co-transfection or transfection of a packaging cell line, viral particles are produced similar to normal retroviral infection. But only the therapeutic vector harboring the packaging signal (Ψ) is packaged into the viral particles. They bud off the membrane and secrete into the surrounding media. After sterile filtrating the media supernatant, which also removes cells and cell debris, it can be used to transduce target cells.

1.8. Gene therapy bears the risk of side effects

Gene therapy with retroviral vectors uses the ability of viruses to introduce their viral genome permanently into the host genome. This mechanism bears the risk of insertional mutagenesis and toxicity of the transgene (Baum et al., 2003). The introduction of the viral DNA into the host genome is more or less random with preferences depending on the vector type (Laufs et al., 2003; Schroder et al., 2002; Wu et al., 2003). Viral DNA can insert into the coding region of a gene (exon insertion) in HSC and thereby destroy the

gene, which can have severe consequences for hematopoiesis. Furthermore, viral vectors can insert into the non-coding region of a gene (intron insertion) which can influence the splicing. However, the most common integration site found after natural selection for gamma-retroviruses are in front of a gene in the 5' untranslated or promoter region (Wu et al., 2003) or in other DNAse I hypersensitive sites that may overlap with regulatory regions (Lewinski et al., 2006). These integrations can influence the expression pattern via promoter interference or enhancer interactions. Vectors contain strong enhancer and promoter elements to ensure a good expression level of the transgene. However, the enhancer-promoter activity can also target neighboring host genes which can strongly deregulate their gene expression. Overexpression of proto-oncogenes can lead to an increased signaling or suppressed apoptosis, resulting in uncontrolled proliferation and dysplasia. With or without further additional insertions, the cell can became leukemogenic after gene therapy (Hacein-Bey-Abina et al., 2003; Li et al., 2002; Modlich et al., 2005) (Figure 1.9).

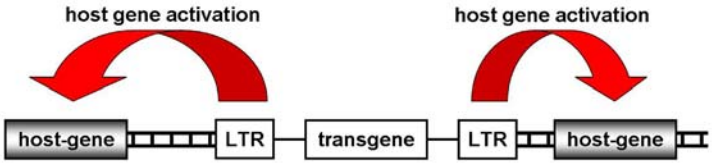

Figure 1.9: Side effects of retroviral insertion. Viral vectors integrate randomly into the host genome. Enhancer-promoter regions (LTR) of the vectors drive the transgene expression. The LTRs also induce activation of host genes in close proximity. This can lead to genotoxicity via insertional mutagenesis. Abbreviations: LTR long terminal repeat.

Another risk for mutations in gene therapy with retroviral vectors is found in the process of reverse transcription of the viral RNA. Point mutations or the sequence rearrangements and recombination of the viral genome are an evolutionary advantage for the virus but bear the risk of side effects in gene therapy. Following the transduction, the viral RNA is transcribed into a copy of viral DNA. The reverse transcriptase has no proofreading activity which can lead to point mutations. On average, RT introduces mistakes into 1 of every 10^4 to 10^5 base pairs (Mansky, 1998). For transgenes like cytokine receptors (such as MPL) this can be a risk because single point mutations and endangered regions are

known which can lead to constitutive activation of the receptor (Abe et al., 2002; Pardanani et al., 2006; Romanelli et al., 2006). These activating mutations can have similar consequences like the upregulation of genes via insertional mutagenesis. Aberrant signaling and the increase of downstream targets of the receptor can lead to immortalization and increased proliferation. However the risk of acquiring one of these activating mutations is statistically very low.

1.9. Safer vector constructs and transgene design can reduce side effects

Major research is ongoing to develop methods to reduce genotoxic side effects of gene therapy (Baum et al., 2006). One way is to design safer vector constructs by deleting the strong enhancer-promoter regions of the early generations of gamma-retroviral vectors. These vectors are called self inactivating vectors (SIN) (Schambach et al., 2006b; Yu et al., 1986) (Figure 1.10).

Following the deletion of the U3 region, an internal promoter (IP) is needed to drive transgene expression. It is thus possible to use promoters that show a more physiological transgene regulation and avoid the activation of surrounding host genes (Zychlinski et al., 2008). However, these promoters still need to produce high enough amounts of transgene product to reach therapeutically relevant levels (Schambach et al., 2006a).

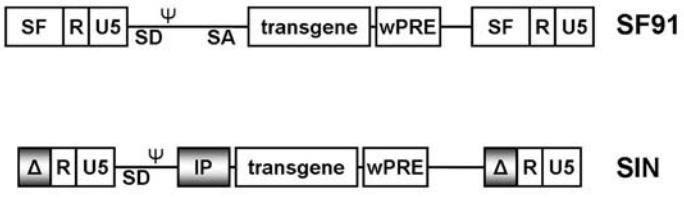

Figure 1.10: Self inactivating vector design. A standard retroviral vector (SF91) is flanked by the two LTR regions which are strong enhancer-promoter elements. After integration into the host genome the LTRs can induce transcription of surrounding genes and lead to insertional mutagenesis. To reduce the risk of side effects after gene therapy the enhancer-promoter activity of the LTRs is deleted from the U3 region. These vectors are called self inactivating (SIN) vectors. Now an internal promoter is needed for transgene expression. Abbreviations: SF spleen focus forming virus enhancer-promoter, SD splice donor, SA splice acceptor, Ψ packaging signal, wPRE woodchuck hepatitis virus posttranscriptional regulatory element, IP internal promoter.

Furthermore, promoters can be differentiation-specific (endogenous promoters) and mediate expression only in the specific tissue compartment. There are several more strategies for reducing side effects, like insulators, included sequences in the viral LTR, which reduce host gene activation from the internal promoter by shielding (Emery et al., 2000). Furthermore small molecular RNA target sequences (Brown et al., 2007) can be introduced into the vectors to restrict RNA translation in non-target cells.

1.10. Specific aims of this work

CAMT is an inherited BM failure syndrome resulting from inactivating mutations in the THPO receptor MPL. The only known therapy today is allogeneic BMT. In an experimental approach, we wanted to develop a curative gene therapy for CAMT. A gene therapy would be of great benefit for CAMT patients because it is very difficult to find a matching donor for allogeneic BMT and it still bears the risk of the rejection of the donor cells.

With our approach we wished to answer the following questions:
What is the consequence of ectopic Mpl expression?
What are the mechanisms of hematopoietic alterations caused by ectopic Mpl expression?
Can we define a therapeutic index for CAMT gene therapy?

Earlier, some groups described the expression of Mpl *in vitro* and *in vivo*. In most cases, the mechanisms for effects resulting from overexpression of Mpl could not be found or originated from the expression system, like the use of replicative competent virus. We wanted to address the above questions in our experimental setup by retrovirally overexpressing murine Mpl on HSC after BMT into lethally irradiated mice. CAMT patients need a lifelong expression of the therapeutic gene. To ensure this criterion, we decided to choose a gamma-retroviral vector system which permanently integrates into the host genome. Retroviruses bear the risk of malignant clonal dominance by insertional mutagenesis. Following that, we wanted to perform a broad risk management addressing the insertion sites and the ectopic overexpression. *In vitro* experiments should be performed to ensure the function of the transgene and vector and gain a first prediction for the *in vivo* experiments. Mice should be analyzed macroscopically, histopathologically and molecularly to clarify the mechanisms causing possible side effects. We also wanted to design safer and better vectors based on the results of the previous experiments to gain a therapeutic expression of Mpl.

2. Materials & Methods

2.1. Molecular biological techniques

If not stated otherwise, standard protocols by E.F. Fritsch, J. Sambrook, and T. Maniatis (1989) were used.

Bacteria

DH5α and XL1-Blue were used for transformation of plasmid DNA. These bacteria are derived from Escherichia coli K12. For cultivation of bacteria, LB (Becton Dickinson, Heidelberg, Germany) media including Ampicillin (final concentration 100 µg/mL) were used.

Retroviral vectors and DNA work

For transgene expression *in vitro* and *in vivo,* an LTR-driven gamma-retroviral vector (SF91) containing the spleen focus-forming virus (SF) enhancer-promoter element (Hildinger et al., 1999; Schambach et al., 2000) and gamma-retroviral self-inactivating vector constructs (SIN), which have a deletion in the U3 region to remove the enhancer-promoter elements were used (Schambach et al., 2006b). Because cloning of the vectors was a major part of this work, transgenes, their modifications and the vector constructs are described in "Results". Plasmid DNA isolation and purification methods are based on alkaline lysis (Birnboim and Doly, 1979). Depending on the amount of plasmid DNA, the QIAprep Miniprep Kit or Qiagen Plasmid Purification Maxi Kit (Qiagen GmbH, Hilden, Germany) was used following the manufacturer's instructions. When purification by phenol extraction and ethanol precipitation was performed, the protocol of (Chomczynski and Sacchi, 1987) was used. Enzymatic digestion of plasmid was performed with nucleases from Fermentas GmbH, St. Leon-Rot, Germany, following the manufacturer's instructions. DNA fragments were electrophoretically analyzed in a 1.0 to 1.2% agarose gel (Seakem LE Agarose, Lonza Group Ltd, Basel, Switzerland) and DNA was cut out of

the gel and purified with the QIAquick Gel Extraction Kit (Qiagen). Phosphorylation with T4 polynucleotide kinase of PCR products or dephosphorylation with calf intestine alkaline phosphatase of vector backbones was carried out with enzymes from Fermentas. Ligation of DNA fragments was performed with ligase (Fermentas) and transformation of ligated constructs into competent DH5α or XL1-Blue was done following standard protocols.

Table 2.1: Primer for Mpl mutants

#	Oligo	Function
1	TTG GGC GTA GTC GGG CAC GTC GTA GGG GTA TTG GCT GGT GAC TTG TGC CTG GTT TGG	HA-tag
2	GAG GTC TGC AGT GGA AGG CTG GAG	caMpl
3	GGA GAT GCA GTG ACA ATT GGA CTT CAG	ntMpl-fw
4	TTG GGC GTA GTC GGG CAC GTC G	ntMpl-rev
5	GGA ATT CTC ACA GAG GGA AAG GAG TGC TCT CTG	nrMpl

Preparation of Genomic DNA

DNA of peripheral blood leukocytes, BM or spleen was purified using QIAmp Blood DNA Preparation Kit (Qiagen) following the manufacturer's instructions. Briefly, cells were lysed with Qiagen lysis buffer and protease K at 56°C for 10 min. Suspension was precipitated with Qiagen buffer and genomic DNA was purified with separation columns. The concentration of genomic DNA was determined in a photometer and DNA was stored at 4°C until use.

Preparation of RNA

BM cells were washed with PBS and pellet was lysed after centrifugation in 1 mL RNAzole (WAK Chemie GmbH, Steinbach, Germany). 200 µL chloroform was added to the suspension, vortexed and centrifuged afterwards for 15 min at 4°C. The hydrophilic part was collected and precipitated with isopropanol (16,000 g, 30 min, 4°C). Pellet was washed with 75% EtOH and resuspended in water including RNAase inhibitors (Fermentas) and stored at -20°C.

qPCR (quantitative PCR)

For quantification of vector copy numbers from BM, spleen or PB, genomic DNA was used with a primer detecting a 94 bp wPRE-specific sequence (wPRE-specific primers forward 5'-GAG GAG TTG TGG CCC GTT GT-3' and reverse 5'-TGA CAG GTG GTG GCA ATG CC-3'). The wPRE signal was normalized by the signal of a housekeeping gene *flk-1* intron enhancer (AF061804, bases 352-459, forward 5'-GTG AAT TGC AGA GCT GTG TGT TG-3' and reverse 5'-ATT CAT TGT ATA AAG GTG GGA TTG-3') using the Sybr Green (Qiagen) reagent on a 7300 Real Time PCR System (Applied Biosystems, Foster City, USA). Results were quantified using the comparative C_T method.

Titer of the SIN.MplP.wtMpl expressing SC1 cells was alternatively determined to flow cytometry via qPCR. C_T values for MplP-driven constructs were compared to the SF promoter-driven constructs as detected by flow cytometry for titer determination.

Reverse Transcriptase PCR and Real Time PCR

RNA from BM cells was reverse transcribed with the Qiagen QuantiTect Kit following the manufacturer's instructions (Qiagen). Briefly, RNA, reverse transcriptase, oligo nucleotides and Qiagen buffer were mixed in appropriate amounts and incubated 30 min at 37°C. cDNA content was measured on a photometer. cDNA was then used for real time PCR (7300 Real Time PCR System, Applied Biosystems) which was performed after the following cycling protocol for Sybr Green (Qiagen): 95°C 15 min; 40x: 60°C 0.5 min, 72°C 0.5 min, 95°C 0.25 min; 60°C 0.5 min, 95°C 0.25 min. Primers for the Mpl sequence were used from Qiagen. Results were quantified using the comparative C_T method.

LM-PCR (Ligation-Mediated PCR)

LM-PCR was performed as described previously (Schmidt et al., 2001). Briefly, 100 ng – 500 ng DNA were digested with 2.5 U *Tsp509*I (New England BioLabs GmbH, Frankfurt, Germany). Primer extension was performed following the first PCR (95°C 5 min; 30x: 95°C 1 min, 55°C 0.5 min, 68°C 2 min; 68°C 10 min) using Extensor Hi-

Fidelity PCR Master Mix (ABgene, Hamburg, Germany). The nested PCR was performed under identical conditions. PCR products were isolated by gel electrophoresis, purified using QIA Quick Gel Extraction Kit (Qiagen) and sequenced (Beckman Coulter GmbH, Krefeld, Germany) after subcloning into TA cloning vector (Invitrogen GmbH, Karlsruhe, Germany). Recovered sequences were screened using the NCBI mouse genome database (NCBI37, accessed April 2008).

Sequencing

Sequencing was performed with the DTCS Quick Start Kit on the Beckman Coulter CEQ 8000 sequencer following the manufacturer's instructions (Beckman Coulter). Briefly, DNA was incubated with appropriate primers, polymerase and fluorescent labeled oligo-nucleotides provided in a master mix by Beckman Coulter. Sequencing reaction was done under following conditions: 96°C 1 min, 50°C 20 sec, 60°C 4 min 33 cycles. DNA was precipitated with isopropanol (17,000 g, 30 min, 4°C) and pellet dissolved in the manufacturer's buffer. Mix was then sequenced and analyzed with CEQ Systems software and DNAStar.

Table 2.2: Primer for Mpl cDNA sequencing

#	Oligo	Function
1	ATG CCC TCT TGG GCC CTC TTC ATG	sequencing
2	GAG GAC CTC ACC TGC TTC TGG	sequencing
3	GCT GGA TCA AAG TCT GGT TGA G	sequencing
4	GAA ACC TGC TGC CCC ACT TTG	sequencing
5	GAA CCG CGT CCA GGGA TCA CAG	sequencing
6	GTG ACT GCT CTG CTC CTG GTG	sequencing

Table 2.3: Primer for MplP (promoter) cDNA sequencing

#	Oligo	Function
1	GAA GCT TGC TTG TAG AAC AGG CTG	sequencing
2	GCT TCC ACC TTC TGT GAG CTG	sequencing
3	GAG GTT AGA GGG CAT TTA AAG	sequencing
4	GTC CAG GAC AGC TAG GAG GAG	sequencing
5	GAG CAC ACA CCA AGG TTG CTG	sequencing
6	GTG TCT CCA TTT GAT TCA CAG	sequencing
7	CTT CTC CGG CAC TGT GTG CCT GCC TTA G	sequencing

Southern Blots

10 µg of genomic DNA from wPRE transgenic cells was digested with *Bgl*II or *Nhe*I (Fermentas) and electrophoretically (0.8% agarose gel, 24 V, over night) separated. DNA was blotted on a nitro cellulose membrane (PALL Corporation, Pensacola, USA) by diffusion. DNA was fixed by heat at 80°C for 2 h and hybridized with the 700 bp 32P αdCTP radioactively labeled probe for the wPRE. In the case of SF91.RRE.caMpl vectors which do not inherit the wPRE element, the probe was generated from the RRE element by digestion with *Spe*I and *Not*I (Fermentas).

Preparation of Protein Lysates

32D cells were transduced with viral supernatant and cells were grown to reasonable numbers. Cells were starved overnight in minimal media (RPMI, 4% BSA) and stimulated on the following day with different cytokines (Thpo, IL-3) for 10 min (PeproTech GmbH, Hamburg, Germany). Cells were then washed in ice-cold PBS with phosphatase inhibitors (sodium orthovanadate) and pelleted (17,000 g, 10 min, 4°C). Afterwards pellet was applied to lysis buffer (50 mM HEPES, 150 mM NaCl, 50 mM NaF, 10 mM $Na_4P_2O_7$, 10% glycerin, 1% IGPAL) including phosphatase and protein inhibitors (leupeptin, pepstatin, EDTA, phenylmethylsulfonyl fluoride [PMSF], pefablock, aprotinin) and frozen to -80°C (Meyer et al., 1998). To determine the protein concentration, lysates were diluted and mixed with the Bradford reagent (BioRad Laboratories GmbH, München, Germany). Suspension was applied to a photometer measuring at 595 nm and concentration of proteins calculated compared to standard controls (dilution serious of BSA).

Western Blots

10 - 20 µg of protein samples were separated by electrophoresis as described previously (Meyer et al., 1998). Briefly, protein lysates were loaded on a 5% collection acryl amid gel and 9% separation acryl amid gel, 1 – 2 h at 40 mA in running buffer (0.1% SDS, 15 µg/mL glycine, 3 µg/mL Tris). Blotting was performed (1 - 2 h, 300 – 400 mA, 4°C, transfer buffer: 1% SDS, 20% methanol) on a nitrocellulose membrane (PALL

Corporation) which was afterwards blocked in 5% milk (Carl Roth, Karlsruhe, Germany) and incubated with one of the following antibodies: anti-pERK (p44/42 MAPK, T202/Y204) and anti-pAKT (Ser473). For loading controls, blots were stripped and reprobed using anti-ERK and anti-AKT antibodies (Cell Signaling Technology, Danvers, USA). Appropriate secondary antibodies were used coupled to horseradish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany) using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, USA).

EMSA (Electromobility Shift Assay)

EMSA and Supershift were performed to detect STAT3 and STAT5 activation (Meyer et al., 2002). The same protein lysates were used as for Western blots. Briefly, DNA substrate β-casein for STAT5 and USTE-oligo for STAT3 were P32 γ-ATP radioactively labeled and incubated (30 min, RT) with the protein lysates (1 - 2 µg). Afterwards, DNA/protein mixture was separated by electrophoresis (20% acryl amid gel, 2 - 3 h, 300 volt) and analyzed by autoradiography. For Supershift protein/DNA mixture was incubated additionally 30 min with STAT-specific antibodies c-terminal anti-STAT3 (C-20), n-terminal anti-STAT5N (N-20) or c-terminal anti-STAT5C (C-17) (Santa Cruz Biotechnology) following the same separation by electrophoresis.

Thpo ELISA

Plasma was collected from peripheral blood samples by centrifugation 10 min at 400 g. Supernatant was collected and frozen at -20 °C. Thpo ELISA on 96-well plates was performed following the manufacturer's protocols (Quantikine M, R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) using precoated 96-well antibody plates.

2.2. Cell culture techniques and analysis

Cells and Cell Lines

All cells were cultivated in an incubator (Heraeus Holding GmbH, Hanau, Germany) at 37 °C, 5% CO_2 and H_2O saturated air.

SC1 is an adherently growing fibroblast cell line obtained from a mouse embryo. Cells are susceptible to murine leukemia virus (MLV) and were cultivated in DMEM (PAA Laboratories GmbH, Cölbe, Germany, 10% FCS, 100 µg/mL penicillin and 50 µg/mL streptavidin, 1% L-glutamine) (Hartley and Rowe, 1975).

293T is an adherently growing epithelial cell line derived from human kidney. 293T is a highly transfectable derivative of the 293 cell line into which the temperature-sensitive gene for SV40 T-antigen was inserted (Pear et al., 1993). Cultivation was performed in DMEM media (PAA Laboratories, 10% FCS, 100 µg/mL penicillin and 50 µg/mL streptavidin, 1% L-glutamine).

32D mouse BM cell line was established from long-term BM cultures of C3H/HeJ mice infected with the friend murine leukemia virus and are constitutively growth factor-dependent (Greenberger et al., 1983). Cells were cultivated in RPMI media (PAA Laboratories) containing 10 ng/mL IL-3, 10% FCS, 100 µg/mL penicillin, 50 µg/mL streptavidin and 1% L-glutamine.

BM Lin- (WT, KO) were derived from freshly sacrificed animals. Bones were flushed or crushed depending on the application and mononuclear cells were enriched via histopaque (Sigma-Aldrich, St. Louis, USA) gradient separation or directly used for MACS (magnetic associated cell sorting) separation. Cells were incubated with lineage-depleting antibodies and beads (MACS Lineage Cell Depletion Kit, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The cell suspension was then separated via MACS and lineage-negative cells were frozen alive (10% DMSO, 90% FCS) or cultivated in StemSpan media (StemCell Technologies, Grenoble, France) with 100 µg/mL penicillin, 50 µg/mL streptavidin, 1% L-glutamine and cytokines (10 ng/mL IL-3, 25 ng/mL SCF, 100 ng/mL IL-11, 100 ng/mL Flt-3L, PeproTech).

Spleen cells were singularized by pressing the spleen through a cell strainer (70 µm, Sarstedt GmbH, Sartstedt, Germany). Afterwards cells were frozen alive (10% DMSO,

90% FCS) or kept in StemSpan media (StemCell Technologies) with 100 µg/mL penicillin, 50 µg/mL streptavidin, 1% L-glutamine and cytokines (IL-3, SCF, IL-11, Flt-3L, PeproTech).

Virus Production (transfection)

293T cells were plated one day before transfection in a concentration of 5 x 10^6 cells in a 10 cm culture dish in 10 mL media. On the following day, co-transfection of the transgene-expressing vector with viral-gag-pol (M57-DAW) and viral-env (K73eco)-expressing vectors (Figure 1.8) using the calciumphosphat ($Ca_3(PO_4)_2$) transfection method was performed. Supernatant was harvested 36 h, 48 h, 60 h and 72 h post-transfection, sterile filtered (70 µm) and frozen at -20°C. An aliquot was taken for estimation of the viral titer.

Virus Titration (transduction)

SC1 cells were plated one day before transduction in a concentration of 1 x 10^5 cells/mL in a 24-well plate. On the following day, cells were transduced with the virus supernatant with different amounts (1 µL, 5 µL and 20 µL). Titer was measured two days afterwards by flow cytometry and was determined using the following formula:

$$titer = \frac{(pos.cells \cdot cell\,number)}{virus\,volume}$$

Growth Kinetic Assays (32D)

32D cells were transduced with different vector constructs expressing either wtMpl, mutated receptor forms (caMpl, RRE.caMpl, ntMpl, nrMpl, cnnMpl) or fluorescence control proteins (GFP) and cultivated in different cytokine conditions (IL-3, Thpo, w/o). Growth was measured up to 14 d and cultures were splitted when necessary. Transgene expression was regularly checked by flow cytometry. Surviving cells were harvested for final analysis by flow cytometry.

Megakaryocyte Differentiation Assay

Lin- BM cells were harvested from C57Bl6/J mice WT or $Mpl^{-/-}$ and transduced by the retronectin method with Mpl-expressing vector constructs or GFP as a control. One day after transduction cells were switched to media supporting megakaryocyte growth including StemSpan (StemCell Technologies), 100 µg/mL penicillin, 50 µg/mL streptavidin, 1% L-glutamine (PAA Laboratories), 25 ng/mL SCF, 100 ng/mL IL-6, and 50 ng/mL Thpo (PeproTech). Cells were further expanded in this media for 12 to 14 d and cytospins were generated by centrifugation 100 g for 8 min for microscopic analysis. Megakaryocytes were counted after May-Grünwald/ Giemsa staining.

Colony-Forming Assays (CFU)

Myeloid colony–forming assays were performed in methyl cellulose-based medium (M3434) containing 3 U/mL erythropoietin, 10 ng/mL IL-3, 10 ng/mL IL-6, and 50 ng/mL SCF (full media), and in M3234 medium containing 50 ng/mL murine Thpo w/o additional cytokines (Thpo media), as per the manufacturer's protocols (StemCell Technologies). Cells were plated at 2.5 x 10^5 cells per dish for BM cells and 2.5 x 10^4 cells per dish for Lin- BM cells, in duplicates. Plates were incubated at 37 °C for 7 d and colonies were counted.

Spectral Karyotype (SKY) Analysis

Leukemogenic cells from singularized spleen cells were cultivated two to four days in StemSpan media. Cells were then incubated with colcemid for 5 h and metaphase-chromosomes were prepared. The samples were dehydrated in 22°C and 48% H_2O saturated air in an increasing ethanol gradient and dried over night at 37°C. Hybridization was done with SKY-Paint-Kit DNA-M10 (Applied Spectral Imaging, Migdal HaEmek, Israel) for 2 d. After counterstaining with DAPI, samples were analyzed using a fluorescence microscope. 10 - 15 metaphases were analyzed per sample.

2.3. Mouse experiments and analysis

Animals (C57Bl6/J, CD45.1 (Ly5.1), WT and Mpl KO)

C57Bl6/J mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). All mice were bred and kept in the pathogen-free animal facilities of the Hannover Medical School, Germany. Ly5.1 and Mpl KO animals were derived for our own breeding. Animal experiments were approved by the local ethics committee and performed according to their guidelines.

Murine Bone Marrow Transplantation Model (BMT)

Lin- BM cells of C57Bl6/J mice were transduced and transplanted as previously described (Li et al., 2003; Modlich et al., 2005). Briefly, Lin- cell population was cultured for two days in StemSpan media (StemCell Technologies), containing 25 ng/mL SCF, 100 ng/mL Flt-3L, 100 ng/mL IL-11, 10 ng/mL IL-3, 100 µg/mL penicillin, 50 µg/mL streptavidin and 2 mM L-glutamine. Lin- cells were infected on two following days (day 3 and 4) using Retronectin-coated plates (TaKaRa, Otsu, Japan) and vector. For preparation of plates, the surface of the wells was coated with 24 ng/mL Retronectin and virus-loaded by centrifugation of viral supernatants at 900 - 1000 g for 30 minutes at 4°C. Virus preloading was repeated up to two times. Transgene expression was measured on day 5 just prior to transplantation. 5 - 10 x 10^5 cells were transplanted into lethally irradiated mice (gamma irradiation, 0.45 Gy/min, 6MV Photons).

Mouse Analysis

Mice were sacrificed for final analysis or when they were moribund and showed clear symptomatic and macroscopically pathological abnormalities. Enlarged organs were weighed. A panel of tissues (spleen, kidney, liver, heart, lung, sternum) was collected, fixed in 4% formalin, and paraffin-embedded for histological examination. Cells from BM, spleen and PB were subjected to flow cytometry (FACS). Blood was collected from the armpit and analyzed by cell counter (Scil ABC Vet Blood Counter, ABX Diagnostics,

Montpellier, France) and blood smears. Plasma was purified by centrifugation 400 g for 10 min and platelets and Thpo levels were analyzed. Genomic DNA was used for copy number determination, LM-PCR and Southern blot analysis.

Flow Cytometry (FACS Calibur, Aria)

Cells from BM, spleen and PB were subjected to flow cytometry (Becton Dickinson) and analyzed with CellQuest and Diva software (BD Biosciences, Heidelberg, Germany). After RBC lysis, PB cells were stained with lineage-specific antibodies against Gr1, CD11b, TER119, CD71, CD19, CD3, Sca1, ckit, lineage marker cocktail, CD34, CD16/32 or HA-Tag (Becton Dickinson; eBioscience, San Diego, USA; Roche Diagnostics, Mannheim, Germany). Antibodies were usually linked to FITC, PE, APC, Alexa488, Alexa700, PerCP-Cy5.5, PE-Cy7 fluorochromes or a secondary antibody via biotin-streptavidin binding was used. Dead cells were excluded by propidium iodide (PI) or 4', 6-diamidino-2- phenylindole dihydrochloride (DAPI) staining.

Blood Samples and Marking of Animals

Peripheral blood was collected periodically from the retro-orbital cavity using EDTA-treated glass capillary tubes (BD Bioscience) or by final analysis after narcotization with diethyl ether from the armpit. RBC lysis was performed from PB when using for flow cytometry (RBC lysis buffer: 10 mM $KHCO_3$, 0.15 mM NH_4Cl, 0.1 mM Na_2EDTA, pH7.3). Samples were analyzed by automated complete differential blood cell counts (Scil ABC Vet Blood Counter, ABX Diagnostics). Blood smears were performed and leukocyte morphology was evaluated by May-Grünwald/Giemsa staining. For further analysis, genomic DNA was prepared from PB. After centrifugation 10 min by 400 g plasma was purified from blood samples and platelets were analyzed by flow cytometry and Thpo level was measured by ELISA. Animals were marked with ear clip tongs.

Cytospins

Cells from spleen or BM were singularized and 1 to 2 x 10^5 cells in 200 µL PBS were spun (10 min, 150 g) on glass slides. Leukocyte morphology was evaluated by May-Grünwald/Giemsa staining and cytospins were fixed with fixation and a cover glass.

Histopathology

Organs of mice (sternum, spleen, liver, kidney, heart, and lung) were taken for histology. Bones were decalcified and organs were trimmed for paraffin embedding. All necropsies were fixed in buffered methanol-formol (BM) or formalin solution (specimens from the other organs) for at least 24 hours and embedded in paraffin (WAX) after decalcification of the BM specimens by ethylenediaminetetraacetic acid. From all necropsies, 3 µm thick sections were cut and stained with hematoxylin-eosin (HE), Giemsa, PAS (Periodic Acid Schiff), Prussian blue and Gomori silver impregnation. Micrographs were taken with an Olympus Microscope BX51TF (Olympus, Japan).

Statistical Analysis

Student's T-Test: With independent groups of observation one is interested in the mean difference between two groups, but the variability between subjects becomes important. Both the confidence interval and the two sample t tests are based on the assumption that each set of observations is sampled from a population with a Normal distribution, and that the variances of the two populations are the same. So the t test with unequal variations (which means that we do not have information requiring us to assume equal variation) and two tailed (which means that the mean can be higher or lower as the control group) is an appropriate method (Altman, 1999).

Wilcoxon Signed Rank Test: is a non-parametric test for 2 continuous variables. It is similar to the Student's T-test in that it also is a test for 2 continuous variables, but unlike the t-test the Wilcoxon signed rank test (also named Mann-Whitney U test) it does not require assumptions about normality of the distribution. It can therefore be used on data that is not normally distributed (Altman, 1999).

Log Rank Test: Survival data is usually tested with a log rank test. The log rank test is a non-parametric method for testing the null hypothesis that the groups being compared are samples from the same population as regards to survival experience (Altman, 1999).

3. Results

3.1.

Murine bone marrow transplantation reveals the narrow therapeutic window for CAMT gene therapy

3.1.1. Mpl receptor and vector construction

The *c-mpl* cDNA contains the signal peptide (SP), the extracellular domain (ECD), the transmembrane domain (TMD) and the intracellular domain (ICD) (Figure 3.1A). The SP is cleaved off by proteases after processing to the cell membrane. To detect the receptor via antibodies by flow cytometry and to distinguish it from endogenous Mpl receptor molecules, a hemagglutinine tag (HA-tag) was added to Mpl at position bp 78 between the SP and the ECD via PCR (Figure 3.1A, Appendix 6.2). HA is a nine-amino-acid-long peptide which is recognized by specific monoclonal antibodies (mAb). The mAb are coupled to fluochromes like FITC or specific binding sites like biotin for secondary mAb. The complete cDNA containing the HA-tag was introduced into the pcDNA3.1 cloning vector backbone from Invitrogen via *Not*I and *Bam*HI restriction sites (Figure 3.1B). The expressing plasmid was used for transfection of 293T cells by the calcium phosphate method. Two days after transfection, transgene-positive cells could be detected by flow cytometry analysis after HA-tag-FITC mAb staining (Figure 3.1C). The HA-tagged Mpl construct was then introduced via *Not*I and *EcoR*I into the viral gamma-retroviral vector backbone based on the murine leukemia virus (MLV) (Figure 3.2A). The LTR-driven vector (SF91) contained the spleen focus-forming virus (SF) enhancer-promoter element and the MESV-derived gag-frame leader 91 (Hildinger et al., 1999; Schambach et al., 2000). Viral supernatants were produced by transfecting 293T cells with the LTR driven vectors. 32D cells were transduced with the titered viral supernatants. The microscopic picture shows a representative 32D cell, when using fluorescence light green color after HA-tag FITC staining appeared (Figure 3.1D). 32D cells showed transgene-positive cells by flow cytometry two days after transduction compared to the isotype control (Figure 3.1E). To conclude, the HA-tag cloning to the ECD of the Mpl receptor is sufficient to detect 293T transgene-positive cells after transfection via flow cytometry. Furthermore, gamma-retroviral vectors were cloned and showed expression of Mpl via mAb detection after retroviral transduction of 32D cells. Transgenic Mpl can now be distinguished from the endogenous Mpl receptor for *in vivo* application.

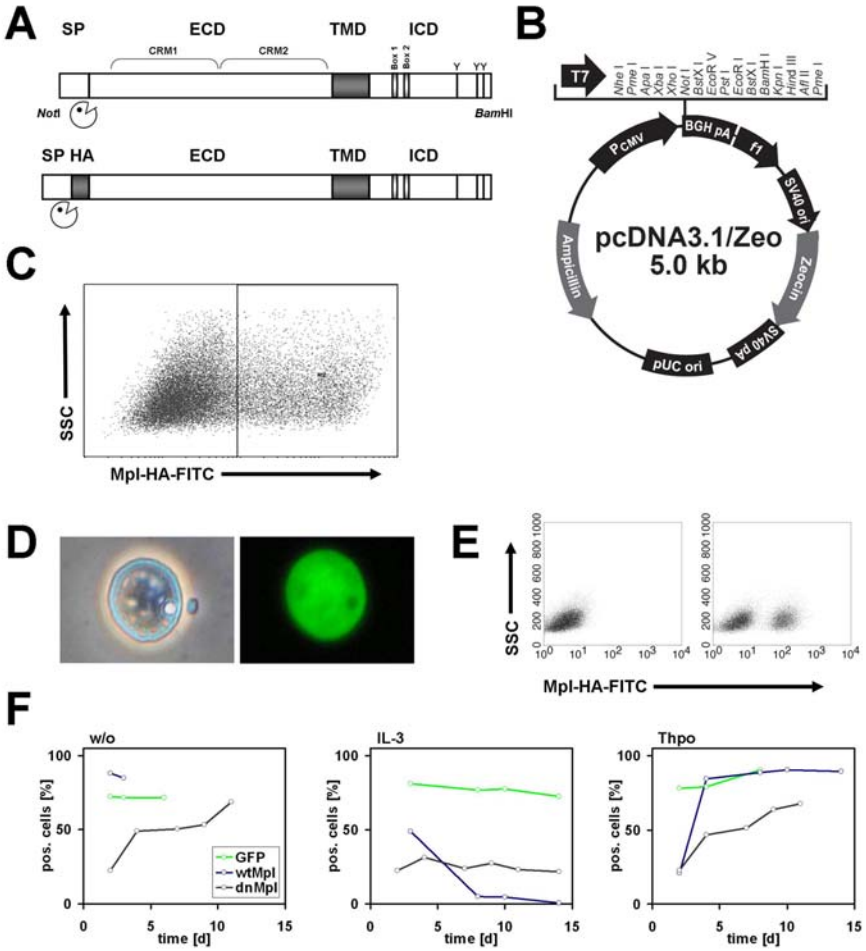

Figure 3.1: Mpl cloning and expression analysis. (A) Schematic structure of the Mpl receptor with signal peptide (SP), extracellular domain (ECD, with CRM1 and CRM2 [cytokine receptor homology module]), transmembrane domain (TMD) and intracellular domain (ICD) is shown, see also Appendix 6.2. The SP is cleaved off by proteases. The nine-amino-acid-long HA-tag (hemagglutinine-tag) was introduced between SP and ECD. (B) The Mpl cDNA was introduced into the pcDNA3.1 cloning vector from Invitrogen via *Not*I and *BamH*I restriction sites. (C) The expressing plasmid was used for transfection of 293T cells by calcium phosphate method. Two days after transfection transgene positive cells could be seen by flow cytometry analysis after HA-tag-FITC mAb staining. (D) 32D cells were transduced with the viral supernatants gained from transfected 293T cells with retroviral vectors. The light microscopic picture shows a representative cell, when using fluorescence light green color after HA-tag FITC staining could be seen. (E) 32D cells showed a population of transgene positive cells two days after transduction with the retroviral vector as detected by flow cytometry compared to isotype control. (F) Transduced IL-3 dependent 32D cells were cultured in different cytokine conditions. Shown are transgene positive cells in percent as detected by flow cytometry against time of observation in days. Without (w/o) any cytokine substitution cells died only a few days after shifting to the new condition. The difference in survival time of the SF91.dnMpl (dominant negative) transduced cells to the SF91.wtMpl (wild type Mpl) and SF91.GFP (green fluorescence protein) transduced cells may result from residual IL-3 in the new condition because cells were not washed before applying them to the new condition media. In the IL-3 condition all cells survived. Applying Thpo conditions only SF91.GFP and SF91.dnMpl transduced cells died after switching to the new conditions. Interestingly, SF91.wtMpl transduced 32D cells were now able to proliferate with Thpo substitution only.

3.1.2. Mpl expression confers a selective growth advantage to murine bone marrow cells

To assess the potential of a gene therapy for Mpl deficiency we used the gamma-retroviral vectors expressing murine wild-type Mpl (wtMpl) receptor or green fluorescent protein (GFP) as a control. IL-3 dependent 32D cells were transduced with the retroviral vectors and cultured in different cytokine conditions (Figure 3.1F).

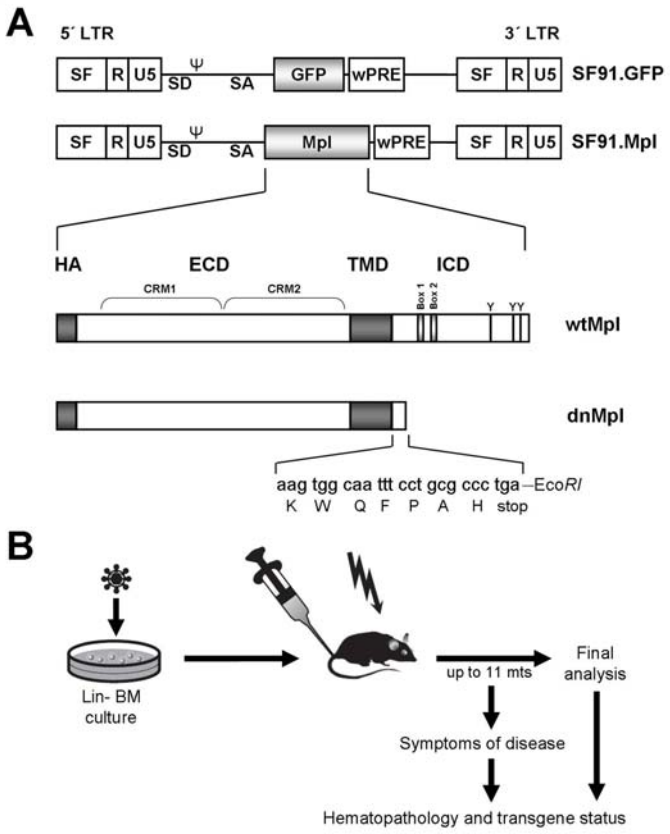

Figure 3.2: Vector constructs and experimental setup. (A) The gammaretroviral vectors used in this study are shown. Transgenes, either Mpl or GFP, are flanked by the two LTRs. The dnMpl is lacking the ICD with Box 1 and Box 2 for kinase binding and the tyrosine residues which are phosphorylated by the kinases. Abbreviations: LTR (long terminal repeat), SF (spleen focus forming virus enhancer-promoter), Ψ packaging signal, HA (hemagglutinine tag), ECD (extracellular domain), CRM (cytokine receptor homology module), TMD (transmembrane domain), ICD (intracellular domain), GFP (green fluorescent protein), wtMpl (wild type Mpl) and dnMpl (dominant negative Mpl). dnMpl is a truncated form of Mpl missing the intracellular signaling domain (ICD). (B) The schema of BMT is shown. Lineage negative cells were isolated from BM of untreated C57Bl6/J mice. Cells were transduced with retroviral supernatant on two following days and transplanted into lethally irradiated recipient mice. Animals were observed for symptoms of disease up to eleven months post transplantation or sacrificed when moribund.

Transgene expression was regularly measured by flow cytometry. Without (w/o) any cytokine substitution SF91.wtMpl (blue) and SF91.GFP (green) transduced cells died only a few days after culturing in the new media condition. The difference in survival time of the SF91.wtMpl transduced cells to the SF91.GFP transduced cells may result from residual IL-3 in the new condition because cells were not washed before applying them to the new condition media. In the IL-3 condition all cells survived (Figure 3.1F second graph).

#	mice number	vector and transgene	transgene [%] before BMT	observation time [d]	diagnosis
Exp. 1	18	wtMpl-GFP-mix*	59 - 69 (wtMpl) 62 - 64 (GFP)	34 - 341 225 311	15 pancytopenia/MDS 1 leukemia (#1) 2 d.o.u.c.
Exp. 2	4	SF91.wtMpl	46	180, 196 62, 102	2 MDS 2 leukemia (#2, #3)
Exp. 3	5	SF91.wtMpl	26	66 - 145	5 CMPD
	5	SF91.dnMpl	69	61 - 115	5 pancytopenia/MDS
	4	SF91.GFP	74	145	4 healthy
Exp. 4	5	SF91.dnMpl	73	54 - 188	5 pancytopenia/MDS
	6	SF91.GFP	58	19 - 207	6 healthy

* SF91.wtMpl and SF91.GFP were mixed 1:1 before transplantation

Table 3.1: Performed *in vivo* experiments. # running number of experiment, mice numbers, vectors and transgenes, transgene in percent before BMT, observation time after BMT and diagnosis are listed. Each experiment was an individual transplantation experiment using the protocol described in the Materials and Methods section. MDS, myelodysplastic like syndrome, d.o.u.c. death of unknown cause, CMPD chronic myeloproliferative disease.

Furthermore, SF91.wtMpl transduced cells were lost or lost the transgene during time. Applying Thpo conditions only SF91.GFP transduced cells died after switching to the new conditions. Interestingly, SF91.wtMpl transduced 32D cells were able to proliferate with Thpo substitution only and selected for transgene positive cells (Figure 3.1F third graph). *In vitro* expression of SF91.wtMpl in the IL-3 dependent myeloid cell line 32D rendered these cells responsive to Thpo.

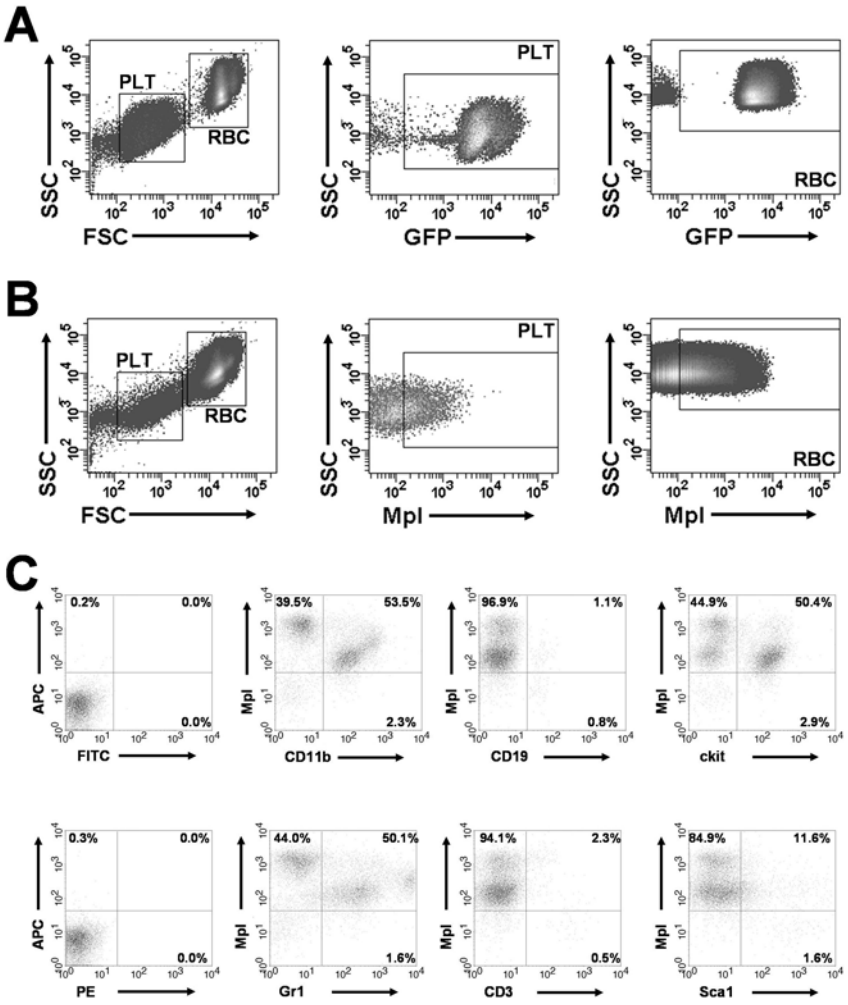

Figure 3.3: Retroviral transgene expression in all hematopoietic lineages. (A) The first panel shows the gating strategy for platelets (PLT) and erythrocytes (RBC) using logarithmic scale in forward scatter (FSC) and side scatter (SSC). Mice transplanted with retrovirally (SF91.GFP) transduced BM cells showed relevant gene marking of GFP in the PB on PLT (second panel) and RBC (third panel). (B) Shows the gene marking in the PB of mice receiving BM cells transduced with SF91.wtMpl. Mpl expression on PLT (second panel) and RBC (third panel) can be seen. (C) Mice transplanted with retrovirally transduced BM cells expressing Mpl showed transgene expression in different lineages of the spleen. The first panels show the isotype controls, the second panels represent CD11b and Gr1 and Mpl double positive cells, the third panels show the staining for CD19 and CD3 and Mpl double positive cells and the last panels display ckit and Sca1 and Mpl double positive cells. Percentages of populations are as indicated. The mouse showed symptoms of disease which may explain altered levels of hematopoiesis.

Secondly we transplanted lethally irradiated C57Bl6/J mice with SF91.wtMpl and SF91.GFP transduced BM cells and analyzed transgene expression by flow cytometry in the hematopoietic lineages (Figure 3.2B). Using the logarithmic scale in the forward scatter (FSC) and the side scatter (SSC) we gated for platelets (PLT) and red blood cells (RBC) (Figure 3.3A, B first panel). Either mice receiving SF91.GFP (Figure 3.3A) or SF91.wtMpl (Figure 3.3B) transduced BM cells showed transgene expression in PLT and RBC from PB samples. Analyzing the different hematopoietic lineages like myeloid cells (CD11b, Gr1 positive), lymphocytes (CD19, CD3 positive) or HSC markers (ckit, Sca1 positive) transgene expression after transplantation of retrovirally transduced cells could be found in all lineages of the spleen by flow cytometry (Figure 3.3C). The analyzed mouse showed symptoms of disease which may explain altered levels of the hematopoietic lineages in the spleen to steady state hematopoiesis.

To evaluate the possible competitive advantage of cells ectopically expressing wtMpl, SF91.wtMpl-transduced BM cells were transplanted into recipient mice (Exp. 1, 2, 3, n=27, Figure 3.2A, B, Table 3.1). Only in Exp. 1 were SF91.wtMpl transduced BM cells mixed in a competition assay with SF91.GFP transduced cells at a ratio of 1:1 before transplantation (n=18). The percentage of wtMpl and GFP-expressing cells as detected by flow cytometry was similar before mixing (Table 3.1) (transgene-positive cells after mixing: 18% - 36% wtMpl and 19% - 44% GFP, Figure 3.4A).

We regularly monitored transgene expression in PB cells after BMT. In all cases of Exp. 1 except two mice, the percentage of wtMpl-expressing cells increased while GFP-expressing cells decreased (Figure 3.4A, Wilcoxon test *$P=5.7 \times 10^{-5}$ at six weeks post-transplantation including the two GFP exceptions). From weeks 6 to 13, wtMpl-positive cells rose up to 80% in the PB whereas GFP-positive cells further decreased. It can be concluded that BM cells ectopically expressing wtMpl gain a multi-lineage growth advantage, probably via enhanced wtMpl signaling.

According to the growth advantage of SF91.wtMpl-transduced cells, all mice (Exp. 1, 2, 3) developed erythrocytosis, thrombocytosis and leukocytosis in the first weeks after transplantation (Figure 3.4B, C), resulting in a striking reddish appearance of nose and paws. Those mice showed an increase of erythropoiesis in the spleen and BM.

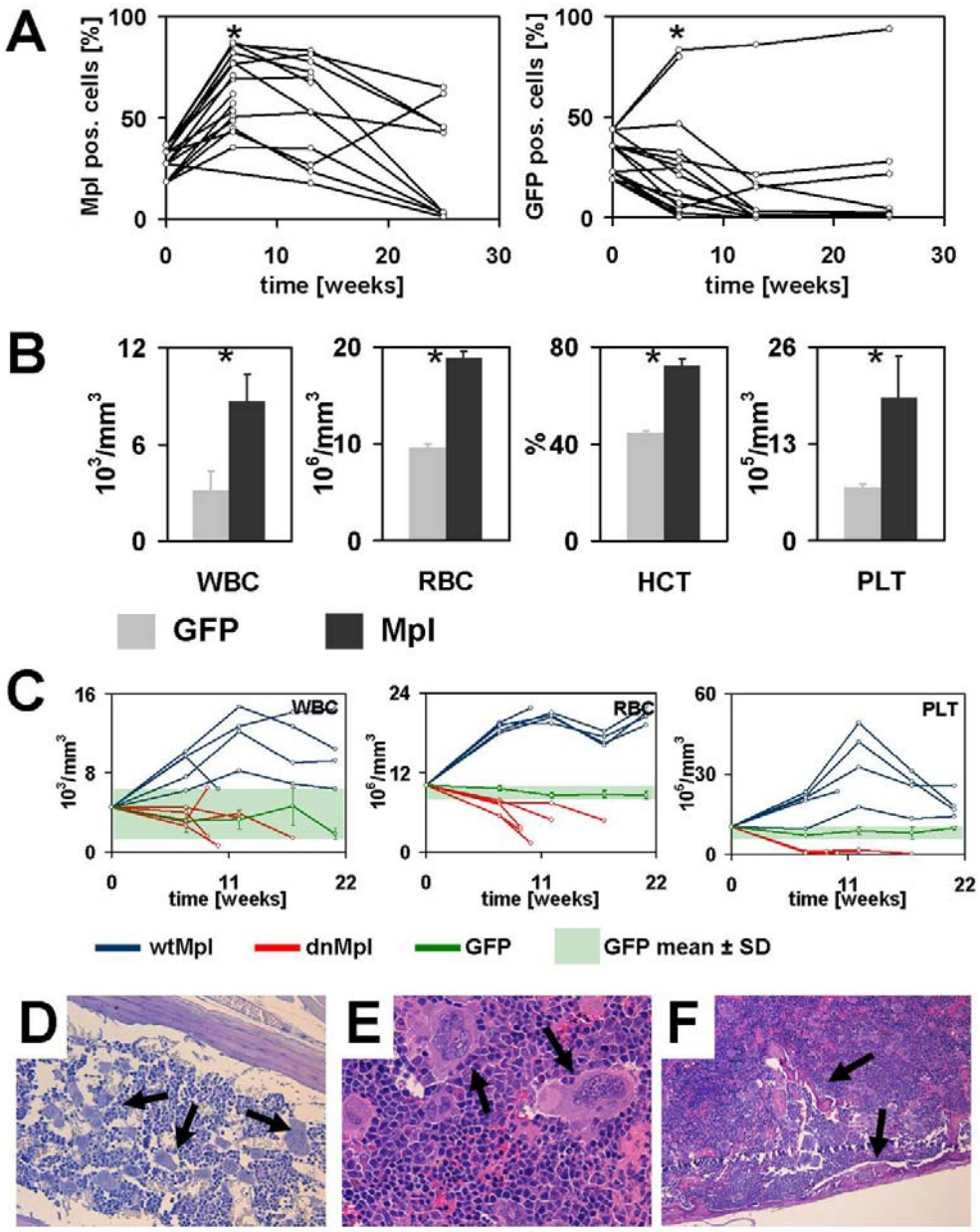

Figure 3.4: Kinetics and histopathology of wtMpl expressing animals. (A) Percentage (positive cells, pos. cells) of transduced PB cells over time after BMT is shown. Mice received a 1:1 mix of SF91.wtMpl and SF91.GFP transduced cells in a competition assay. Initially SF91.wtMpl transduced cells showed a growth advantage compared to SF91.GFP expressing cells (week 6, *P = 5.7 x 10^{-5}, Wilcoxon test). Subsequently SF91.wtMpl transduced cells and co-existing unmodified cells (data not shown) decreased (Exp. 1 and 2, Table 1). (B) PB counts of SF91.wtMpl (dark grey, n=5) transplanted animals at 7 weeks post transplantation (Exp. 2, Table 1). All hematopoietic values (WBC, RBC, HCT and PLT) were strongly increased compared to SF91.GFP control mice (light grey, n=4, arrow bars indicate SD) (all measurements: *P ≤ 0.009, t-test, two tailed, unequal variations). (C) Serial PB counts (SF91 vectors, Exp. 3). WBC, RBC

and PLT counts showed strong perturbation of multi-lineage hematopoiesis. SF91.wtMpl transduced BM cells receiving animals (blue lines) showed an early increase of hematopoiesis compared to GFP control group (green line, mean of n=4) with subsequent decrease. SF91.dnMpl transduced BM cells receiving animals (red lines) had cell reduction in all main lineages without initial increase. Mean of GFP group ± SD is indicated as control (light green, n=4). Zero time points originate from the analysis of wild-type C57Bl6/J mock mice (n=4): WBC 4.5±1.1 10^3/µL, RBC 10.1±0.5 10^6/µL, PLT 10±54 10^5/µL, respectively. (D, E, F) Histopathology of SF91.wtMpl expressing mice 20 weeks post transplantation is shown. Increase of atypical megakaryocytes in the BM (D), and spleen (E) and atypical neoformation of bone in the spleen (F), indicates CMPD. Magnification and staining: (D) 200x, PAS; (E) 400x, HE; (F) 100x, HE.

In some of the animals, the number of mature Mk was elevated within BM and spleen, with slightly atypical features in Mk (Figure 3.4D, E). In two mice an excessive increase of Mk with numerous giant forms could be detected. One animal presented atypical neoformation of the bone in the spleen (Figure 3.4F). The summary of these pathologic features in mice with increased multilineage hematopoiesis allows the diagnosis of a chronic myeloproliferative disorder (CMPD). For the development of gene therapy, these results indicate that the expression of ectopic wtMpl has to be tightly regulated to remain normal hematopoiesis.

3.1.3. Leukemias developing in wtMpl expressing mice require insertional mutagenesis

Three out of 27 SF91.wtMpl transplanted mice developed leukemia within a latency of 2, 3.5 and 7.5 months, respectively. They presented with up to ten times enlarged spleens (680 mg to 980 mg) and livers that had a typical cherry red color. Nucleated erythroid precursors were found in the blood (Figure 3.5E), compatible with erythroid leukemias. The leukemic cells in the spleen were CD71 and TER119 and wtMpl positive as detected by flow cytometry (Figure 3.5A). In the spleen, all three animals showed diffuse infiltrations of blasts which amounted to 80 – 90% of cells (Figure 3.5C, D). Erythroid precursors were also detectable within the lumen of the blood vessels (Figure 3.5E). The BM of these animals was infiltrated by a significantly lower amount of blasts which exceeded 20% of cells in two of the mice, but amounted to less than 20% in the third. In this animal, the BM showed morphologic features of a chronic myeloproliferative disorder (CMPD) with marked increase of mature giant Mk (Figure 3.5D).The liver of this animal showed an extensive infiltration mainly by immature erythroid precursors with only a low amount of myeloid blasts.

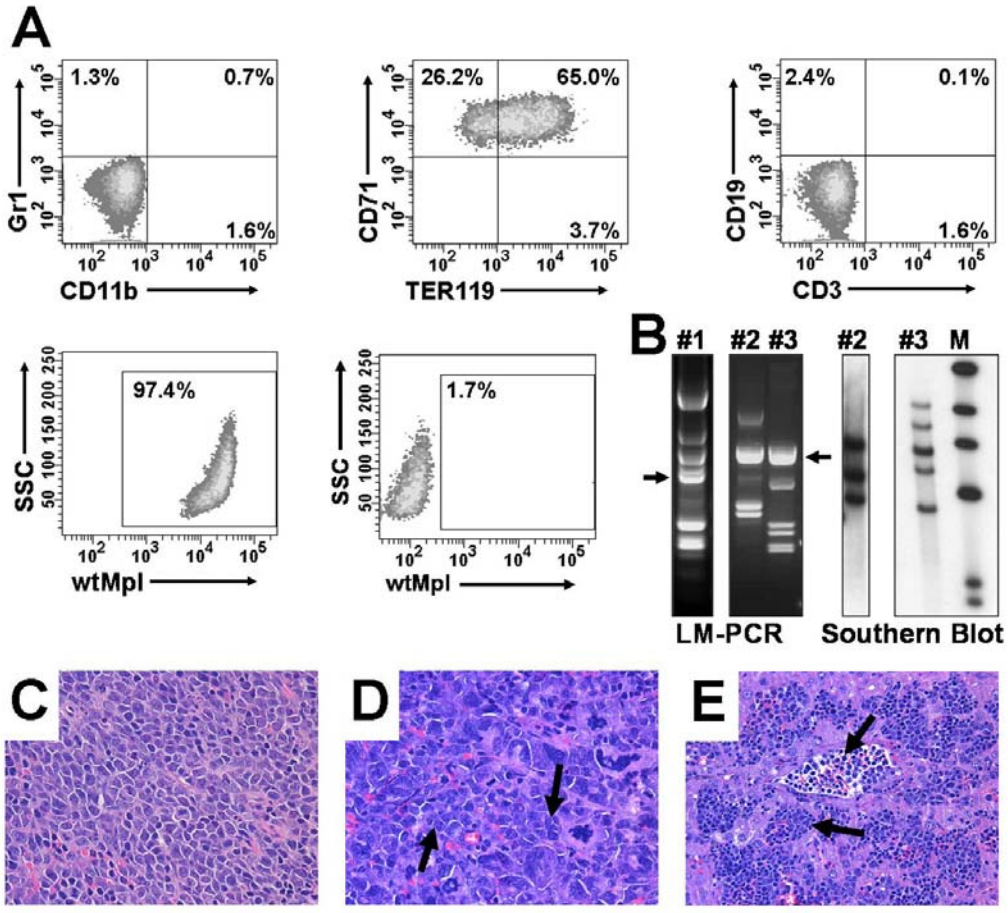

Figure 3.5: Phenotypic and molecular analysis of erythroid leukemias. (A) Three (#1, 2, 3) out of 27 animals transplanted with SF91.wtMpl transduced cells developed erythroid leukemia. Splenocytes were negative for CD11b, Gr1, CD3 and CD19 but expressed CD71, TER119 and transgene as compared to the isotype control (Exp. 3, case #3). (B) LM-PCR and Southern blot analysis showed monoclonality of leukemias. The number of insertion sites ranged from three to six. Insertion sites are listed in Table 3.2. Arrows in LM-PCR indicate internal control bands (marker, M). (C, D, E) The Histopathology of leukemias is shown. Diffuse infiltration of blasts and atypical megakaryocytes (arrows) in the spleen (C, D), infiltration of the liver by erythroid precursors (arrow) and detection of erythroid leukemia within the lumen of the blood vessels (arrow) can be observed (E). Magnification and staining: (C, D) 400x, HE; (E) 200x, HE.

Leukemias were clonal as indicated by Southern blot analysis and LM-PCR and contained three to six retroviral insertions (Figure 3.5B). In each leukemia one of the insertion sites was located in proto-oncogenic common insertion sites (CIS) like *Pu.1, Fli-1* and *Klf-3* (Table 3.2) which are known proto-oncogenes leading to erythroid leukemia (Funnell et al., 2007; Kirberg et al., 2005; Schuetze et al., 1993). As a

representative sample the whole cDNA of the HA-Mpl construct of mouse #1 was sequenced for mutations. No activating mutations responsible for the leukemogenic alterations could be found. However, the latency until the onset of leukemia was short compared to other reported cases of leukemias induced by insertional mutagenesis taking into account that the transgene may influence integration (Modlich et al., 2005; Modlich et al., 2008). In addition, all leukemias had an erythroid phenotype, which were rarely observed using vectors expressing other transgenes (Modlich et al., 2005). Furthermore, erythroid leukemias were also induced by vectors expressing constitutively active Mpl mutants, as shown later (Chapter 3.2). This indicates that ectopic wtMpl expression was a necessary additional factor for leukemia development. Because wtMpl expression alone did not trigger leukemogenic outgrowth in most of the mice (24/27), it can be concluded that insertional mutagenesis was an essential cooperating event for induction of leukemia.

mouse #	gene name	CIS (IDDb)	CIS (RTCGD)	gene ID	distance	orientation	name and function
1	**Gphn**			268566	-124234	F	gephyrin/catalytic activity, protein binding
1	**Itgb8**			320910	166737	F	integrin beta 8/protein binding
1	**Pck2**			74551	-469	F	phosphoenolpyruvate carboxykinase 2/kinase activity
1	**Sfpi1 (Pu.1)**		**6**	20375	-773	R	SFFV proviral integration 1/transcription factor
2	**Fmn1**			14260	69609	R	Formin 1/DNA, protein binding
2	**Gcc1**			74375	tbd	tbd	golgi coiled coil 1/Golgi apparatus
2	**Klf-3**		**6**	16599	tbd	tbd	Kruppel-like factor 3/DNA binding, transcription
3	**Dusp22**			105352	37701	R	dual specific protein 22/phophatase activity
3	**Fli-1**	**2**	**9**	14247	-4905	R	Friend leukemia integration 1/transcription factor activity
3	**Serping1**			12258	-24129	R	serine peptidase inhibitor/endopeptidase inhibitor
3	**Spflc2**			20773	tbd	tbd	serine palmitoyltransferase, long chain base subunit 2
3	**Thsd3**			217738	tbd	tbd	thrombospondin, type I, domain containing 3
3	**Ube2l6**			56791	681	F	ubiquitin-conjugating enzyme E2L 6/ligase activity

Table 3.2: Retroviral insertion sites (RIS) of leukemogenic animals. Insertions sites identified by LM-PCR in three leukemogenic animals transplanted with SF91.wtMpl transduced BM cells are listed. Insertion sites were amplified in genomic DNA of the spleen which was the major site of leukemia manifestation. RIS are related to the closest genes. Abbreviations: CIS common insertion site, IDDb insertional dominance database, RTCGD retroviral tagged cancer gene database, F forward, R reverse, tbd to be determined.

3.1.4. Long term ectopic overexpression of wtMpl leads to pancytopenia

The 24 mice that did not develop leukemia allowed us to address hematopoietic consequences of long-term ectopic wtMpl expression. Despite the initial growth advantage of SF91.wtMpl transduced cells and increase in multilineage hematopoiesis by wtMpl expression on non-target cells (Figure 3.3A, B, C), many animals gradually

developed lethal pancytopenia (n=15) (Figure 3.6A and Figure 3.4A). WBC, RBC, HCT and PLT counts steadily decreased until symptoms in transplanted mice became so severe (hunched posture, anemic, lethargic) that they had to be sacrificed. WBC counts of pancytopenic mice, in comparison to GFP-transplanted control animals, dropped to 50% at final analysis. RBC counts and HCT were as low as 20 - 30%. Platelets were more than tenfold reduced (Figure 3.6B). Flow cytometry of several lineage markers (Gr1, CD11b, TER119, CD71, CD19, CD3, ckit, Sca1, CD41, CD45) showed no alterations towards a specific lineage (data not shown). In conclusion, ectopic overexpression resulted in pancytopenia in most of the animals (17/22) observed long-term, following the initial increase of hematopoiesis (CMPD). The cell loss even involved co-existing untransduced hematopoietic cells, or GFP-transduced cells (Exp. 1) indicating consumption of an essential niche factor.

3.1.5. Pancytopenia after transplantation is induced by a dominant negative effect

As the development of pancytopenia even involved unmodified and GFP-expressing control cells, we addressed whether this effect was due to wtMpl signaling. We therefore constructed a truncated, dominant negative Mpl receptor (dnMpl) that could bind Thpo but lacked the intracellular signal transduction domain (Figure 3.2A, Appendix 6.2). The dnMpl receptor contained an artificial stop-codon at position bp 1563 resulting in a truncated cDNA. This should result in an early stop of transcription and deletion of the active signaling parts in the intracellular domain. dnMpl was shown not to induce any Thpo mediated signals (Alexander et al., 1996a). We confirmed that SF91.dnMpl transduced 32D cells could not grow Thpo-dependently while SF91.wtMpl-transduced 32D cells could (Figure 3.1F third panel). Lin- BM cells ($Mpl^{+/+}$) were transduced with SF91.dnMpl and transgene-expressing cells, sorted by magnetic beads (MACS) were plated into methyl cellulose. dnMpl expression inhibited Thpo-dependent growth of $Mpl^{+/+}$ cells, despite their endogenous Mpl receptor, confirming previously reported results with a similar truncated mutant Mpl receptor (Millot et al., 2002) (Figure 3.6C).

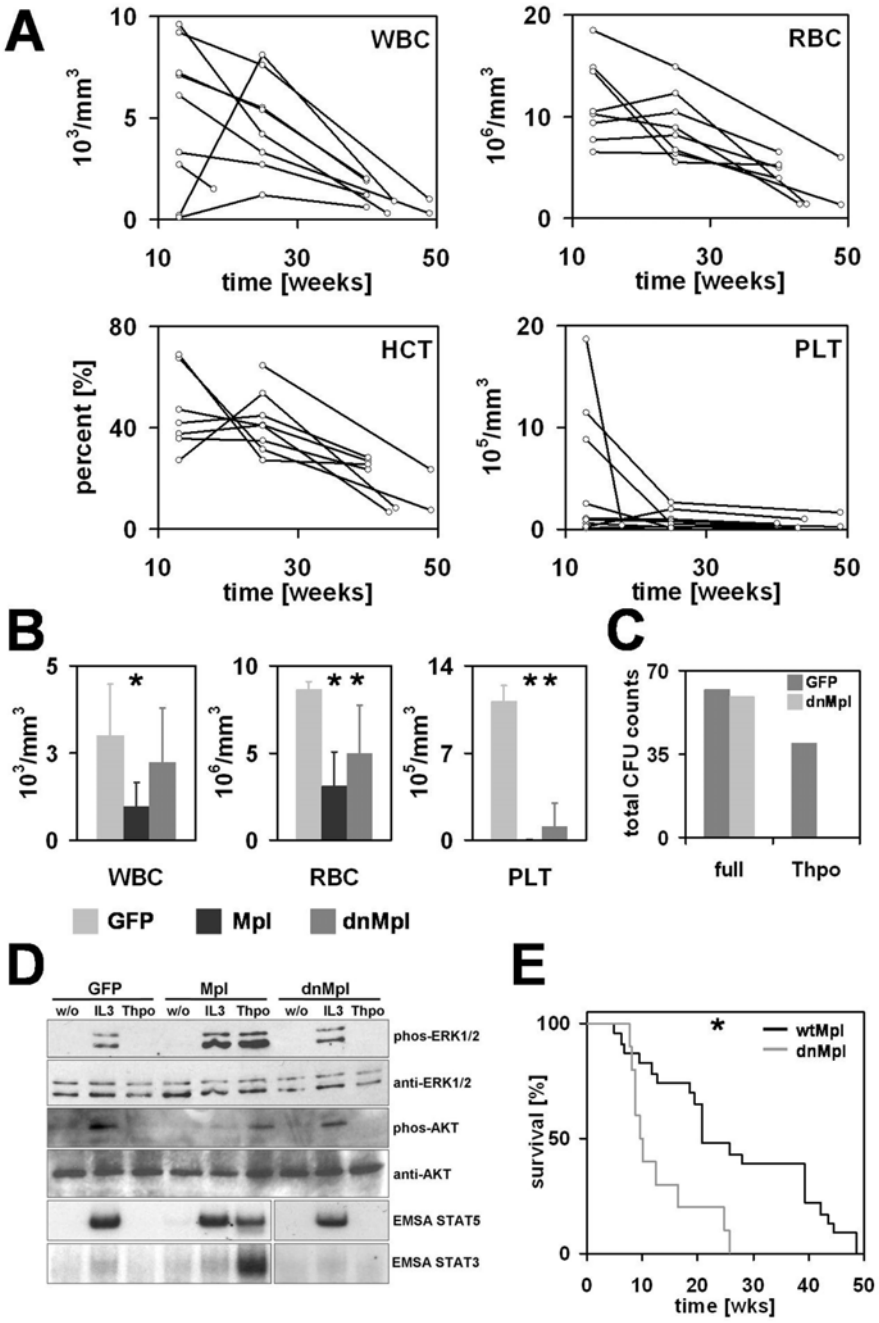

Figure 3.6: Long-term analysis of mice transplanted with cells ectopically expressing wtMpl or dnMpl. (A) After the initial increase of most PB cell counts, cells in all lineages decreased over time and pancytopenia developed (Exp. 1, n=9). (B) Blood counts of pancytopenic animals from SF91.wtMpl and SF91.dnMpl groups with SF91.GFP control are shown. Groups are represented by: SF91.GFP (light grey, n=11), SF91.wtMpl (dark grey, n=15, two animals died unexpected and escaped detailed analysis) and SF91.dnMpl (medium grey, n=10) (summary of all experiments). Error bars represent SD (*P ≤ 0.02, t-test, two tailed, unequal variations). (C) Colony forming capacity of transduced Lin- $Mpl^{+/+}$ BM cells. Cells

were sorted for transgene expression by MACS (magnetic associated cell sorting) and plated into full methyl cellulose conditions or Thpo methyl cellulose only. SF91.GFP transduced cells were able to form colonies under Thpo conditions whereas SF91.dnMpl transduced BM cells could not form any colonies. The experiment was performed in duplicates. (D) Western blot and EMSA of protein lysates from 32D transduced cells with SF91.wtMpl, SF91.dnMpl and SF91.GFP control are shown. GFP expressing cells presented no activation by Thpo stimulation, whereas wtMpl expressing cells showed to be stimulated with Thpo only. In SF91.dnMpl transduced cells no signal could be detected after Thpo stimulation (phos: Ab for phosphorylated protein). (E) Survival chart of mice transplanted with SF91.wtMpl (n=23, Exp. 1, 2 and 3) or SF91.dnMpl (n=10, Exp. 3 and 4) transduced cells which became cytopenic is displayed. dnMpl expressing animals had a statistically significant shorter latency to develop pancytopenia compared to wtMpl expressing animals (Log Rank test: *P ≤ 0.0006). Four SF91.wMpl animals were sacrificed for final analysis without symptoms of cytopenia (but CMPD, Exp. 3) at 20 weeks post transplantation.

Thpo stimulation of SF91.dnMpl-transduced 32D cells showed no STAT3, STAT5, ERK1/2 and AKT activation in contrast to wtMpl-transduced cells, as analyzed by Western blot and EMSA (Figure 3.6D). It can be concluded that dnMpl does not confer survival or proliferation signals after Thpo stimulation and that it also inhibits the signaling of the endogenous Mpl receptor.

Interestingly, animals receiving SF91.dnMpl-transduced BM cells developed the same pancytopenic phenotype as mice that received SF91.wtMpl-transduced cells (Exp. 3, 4). Blood counts were severely reduced (Figure 3.6B). However, dnMpl mice did not develop the initial CMPD as was observed in mice transplanted with SF91.wtMpl-transduced cells (Figure 3.4C, red lines). Accordingly, the mortality of SF91.dnMpl transplanted mice was more rapid compared to SF91.wtMpl-transplanted mice (survival 7 weeks to 6 months, *P ≤ 0.0006, Log Rank test Figure 3.6E). This strongly suggests that the lethal pancytopenia was not due to increased Thpo signaling but caused by a dominant negative effect of Thpo binding to the ectopically expressed wtMpl. However, the initial increase of erythropoiesis and thrombopoiesis was not observed in dnMpl-expressing mice and was therefore a specific effect of signaling via the ectopically expressed wtMpl.

Together, these data suggest that ectopic expression of Mpl induced an initial increase of multi-lineage hematopoiesis (CMPD) that depleted the hematopoietic niche from Thpo that is essential for survival of HSC, including the unmodified population.

3.1.6. Pancytopenic mice develop an MDS-like bone marrow failure syndrome

Mice that were sacrificed due to pancytopenia were subjected to detailed pathological analysis. There were no macroscopic alterations observed and spleen weights ranged between 70 mg and 218 mg. The BM was normo- to slightly hypocellular. All mice showed a dysmyelopoiesis (dysmegakaryopoiesis and dysgranulopoiesis) with reduction of hematopoiesis within the spleen. Also, a variable degree of reduction as well as incomplete maturation of erythroid cells and Mk within the BM could be observed. In all animals, mature Mk were reduced to a variable extent with relative increase of atypical micromegakaryocytes with a hypolobulated round nucleus (Figure 3.7A, B). Dysplastic features were rarely detected in erythroid and granulocytic precursors, and they amounted to less than 5% of cells. However, in 15 animals, more than 5% myeloblasts were detected within the spleen or the BM. Furthermore, a few histiocytes with phagocytosis of erythrocytes or nucleated myeloid cells were detectable within the BM or spleen in the majority of animals (Figure 3.7B). In seven animals, the number of mast cells was moderately to extensively increased within the BM (Figure 3.7C). There was no difference in the degree of dysmyelopoiesis between the wtMpl and dnMpl-expressing groups (Figure 3.7D, E, F). In contrast to human MDS that arises from a single MDS clone (Tiu et al., 2007), the BM of pancytopenic wtMpl-expressing mice appeared oligoclonal as analyzed by LM-PCR (Figure 3.7G). The clonality was similar to the GFP-expressing control group (5 months post transplantation, Exp. 3). It can be concluded that selection for an MDS clone did not occur in our experimental system. Although the morphologic alterations of hematopoiesis did not exactly match those detectable in human MDS, a lot of similarities were observed that allow the description of an “MDS-like” disorder.

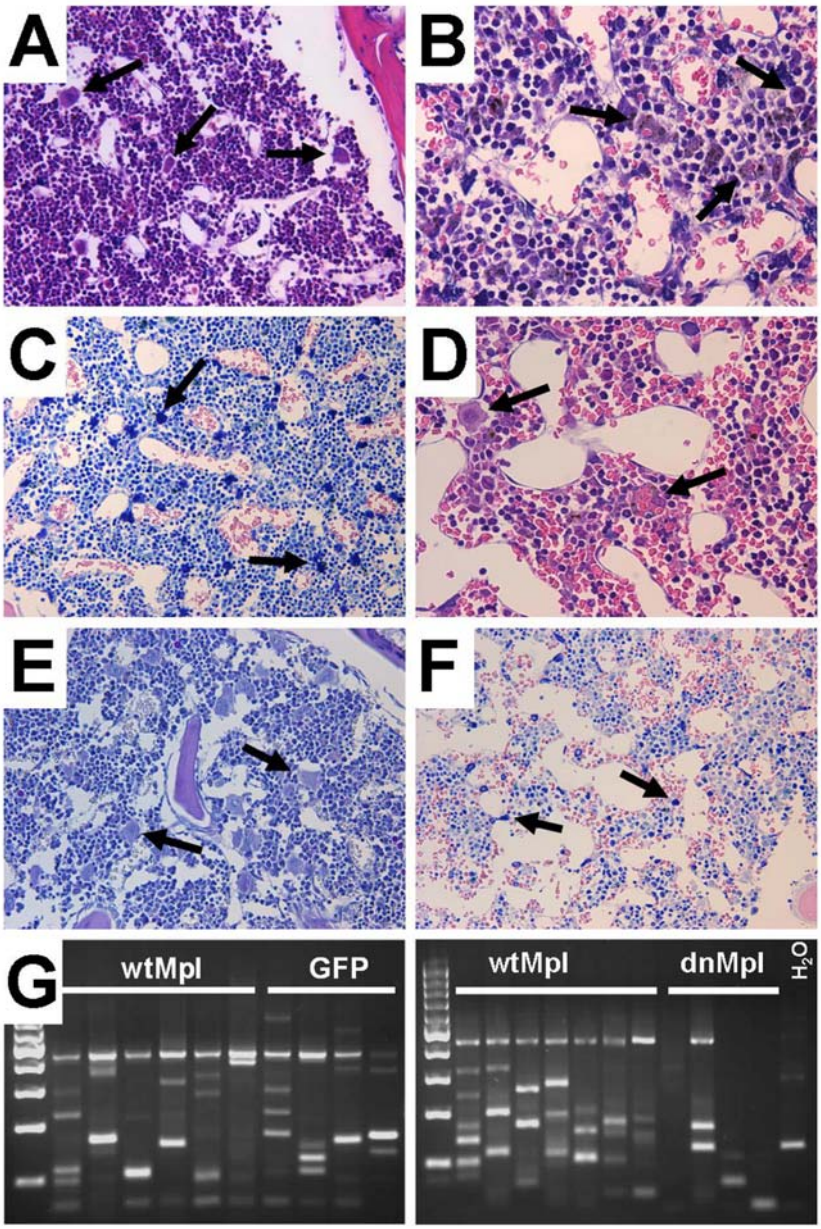

Figure 3.7: Histopathology of MDS-like disease. BM samples from the sternum of SF91.wtMpl (A, B, C), and SF91.dnMpl (D, E, F) transplanted mice are displayed (detected cases from all experiments). (A) Dysmegakaryopoiesis with increased number of atypical micro-megakaryocytes (arrows), (B) histiocytes with erythrocytophagocytosis and one atypical micro-megakaryocyte, (C) atypical mast cell proliferation (arrows), (D, E) dysmegakaryopoiesis with atypical micro-megakaryocytes and one histiocyte with erythrocytophagocytosis and (F) slight increase of mast cells indicate MDS like syndrome. Magnification and staining: (A) 200x, HE; (B) 400x, HE; (C) 200x, Giemsa; (D) 400x, HE; (E) 200x, PAS; (F) 200x, Giemsa. (G) LM-PCR data of pancytopenic SF91.wtMpl and SF91.dnMpl transduced BM cells receiving mice with GFP control group. Pictures suggest oligoclonal repopulation of MDS animals. Lanes of LM PCRs have not been identified. Bands appearing in water control can be due to primer dimerization.

3.1.7. Pancytopenic mice have reduced numbers of LSK, CMP and MEP cells

We next asked whether the dysplastic features in the BM altered the HSC compartment. We therefore analyzed the lineage-negative, Sca1 and ckit-positive faction (LSK) in the BM of pancytopenic animals in which the majority of HSC resides. The LSK fraction in the BM of wtMpl and dnMpl-expressing pancytopenic mice was remarkably reduced (Figure 3.8A, C). However, mice transplanted with SF91.wtMpl-transduced BM and analyzed after 20 weeks (proliferation state, CMPD) showed a normal to slightly increased number of LSK cells compared to mice transplanted with GFP-transduced control cells (Figure 3.8A, C).

We then addressed the question whether the decrease of LSK cells resulted in the decrease of the progenitor cell populations. Therefore, the Lin^-, $Sca1^-$, $ckit^+$ (LK) fraction of the BM was analyzed in detail. Alterations in the percentages of the common myeloid progenitors (CMP), granulocyte macrophage progenitors (GMP) and megakaryocyte erythroid progenitors (MEP) were found. Pancytopenic animals (wtMpl and dnMpl) showed reduction in all progenitor fractions with the strongest decrease in CMP (0.5% ± 0.2%, n=3 vs. GFP 6.5% ± 3.8%, n=3) and MEP (0.9% ± 0.9% vs. GFP 6.0% ± 1.6%) (Figure 3.8B, D). Those progenitor fractions are the main site of Mpl signaling under physiological conditions and therefore expected to suffer most from Thpo depletion.

During the stage of increased hematopoiesis (CMPD) in mice transplanted with SF91.wtMpl-transduced cells GMP counts were significantly higher compared to the GFP control group (Figure 3.8B, D). Alterations of CMP and MEP populations were more moderate (Figure 3.8D). Percentage of wtMpl-positive cells in CMPD animals in the LK fraction could be detected with a mean of 65 ± 35% (n=4) in contrast to pancytopenic animals with a mean of 32 ± 38% (n=3). The early increase in hematopoiesis in the wtMpl groups after transplantation was therefore most likely based on an increased proliferation of HSC and progenitor fractions, whereas animals that developed pancytopenia (wtMpl and dnMpl) showed a massive decrease in all of these fractions. Further analysis of BM cells from transplanted animals in CFU colony-forming assays in cytokines containing methyl cellulose revealed a growth disadvantage by reduced colony numbers for cells obtained from SF91.wtMpl-transplanted mice (n=4)

versus those obtained from SF91.GFP-transplanted mice (n=4) 20 weeks post transplantation (Figure 3.8E).

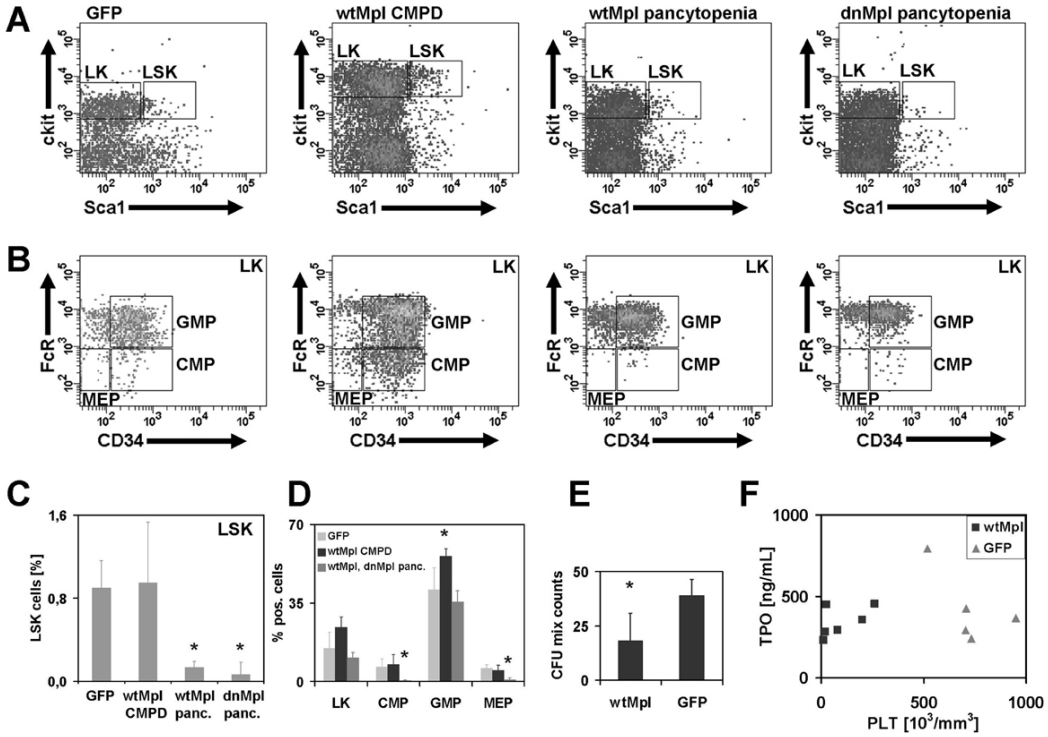

Figure 3.8: Phenotypic analysis and mechanism of pancytopenia. (A, C) LSK (Lin-, Sca1+, ckit+) BM cells from transplanted animals were analyzed. SF91.wtMpl vector treated mice 20 weeks post transplantation (Tx) in CMPD state (n=4) had a similar proportion of LSK cells compared to the GFP controls (n=3, 20 weeks post Tx). Pancytopenic animals transplanted with SF91.wtMpl (n=3, at 11, 19, 28 weeks post Tx) and SF91.dnMpl (n=3, at 8, 10, 24 weeks post Tx) transduced cells showed a reduced proportion of LSK cells (Exp. 1, 2, 4). (A) FACS blots show one example of each group, and (C) graph shows FACS data of mean ± SD of LSK fractions (*P ≤ 0.03, t-test, two tailed, unequal variations). (B, D) Cells from the LK (Lin-, ckit+, Sca1-) fraction were analyzed for common myeloid progenitors (CMP), granulocyte monocyte progenitors (GMP) and megakaryocyte erythrocyte progenitors (MEP). (B) The GMP fraction in CMPD mice (n=4) was significantly higher compared to the GFP control (n=3). CMP and MEP in pancytopenic animals (wtMpl n=1, dnMpl n=2) were reduced. (D) FACS data of progenitor fractions are shown as mean ± SD. GFP n=3, CMPD n=4, panc. n=3 (*P ≤ 0.03, t-test, two tailed, unequal variations). (E) CFU of whole BM samples from SF91.wtMpl expressing BM cells receiving animals (n=4) at CMPD state and SF91.GFP expressing BM cells receiving control animals (n=4, mean ± SD) (20 weeks post Tx, Exp. 3). wtMpl group has a significant disadvantage in colony forming capability in full media compared to the GFP control animals (*P ≤ 0.04, t-test, two tailed, unequal variation). (F) Thpo plasma levels from PB in transplanted animals were determined. The SF91.GFP (n=5, Exp. 3 and 4) group had normal platelet counts and Thpo levels from 300 pg/mL to 800 pg/mL. Mice receiving SF91.wtMpl (n=6, Exp. 1) expressing cells showed severely reduced platelet counts but animals had similar Thpo levels compared to the SF91.GFP group. Samples were taken from wtMpl group at 24 weeks post Tx and GFP group at 7 and 12 weeks post Tx.

3.1.8. Transplanted animals show alterations in serum Thpo levels

Physiologic THPO levels are regulated by MPL expression on PB thrombocytes (Kaushansky, 2005). We therefore investigated the effects of ectopic overexpression of wtMpl Thpo plasma levels. The retroviral expression resulted in high wtMpl levels on almost all leukocytes and red cells in addition to the platelet wtMpl (Figure 3.3A, B, C). A down regulation of Thpo plasma levels would be expected. SF91.wtMpl-transplanted animals showed reduced numbers of platelets compared to SF91.GFP-transplanted animals (Figure 3.8F). Low platelet numbers would be expected to result in high Thpo levels. However, wtMpl-expressing mice with low platelet counts showed Thpo levels similar to those of GFP-expressing mice with regular platelet levels. This indicates binding of the ligand on non-Thpo signaling target cells, e.g. erythrocytes expressing ectopic wtMpl. These findings support the hypothesis that the ectopic wtMpl competes with the endogenous Mpl for the ligand.

3.2.

Mpl receptor design can be an alternative therapeutic approach for CAMT to increase the HSC compartment

3.2.1. Constitutively active Mpl receptor mutants induce acute leukemia

Addressing a possible gene therapy for CAMT, we applied an extensive risk assessment. Therefore, we asked the question which effects an Mpl receptor gaining constitutively active mutations induces in hematopoiesis. Point mutations can occur during reverse transcription. To mimic such an event we constructed a constitutively active Mpl (caMpl) mutant as previously described by (Alexander et al., 1995).

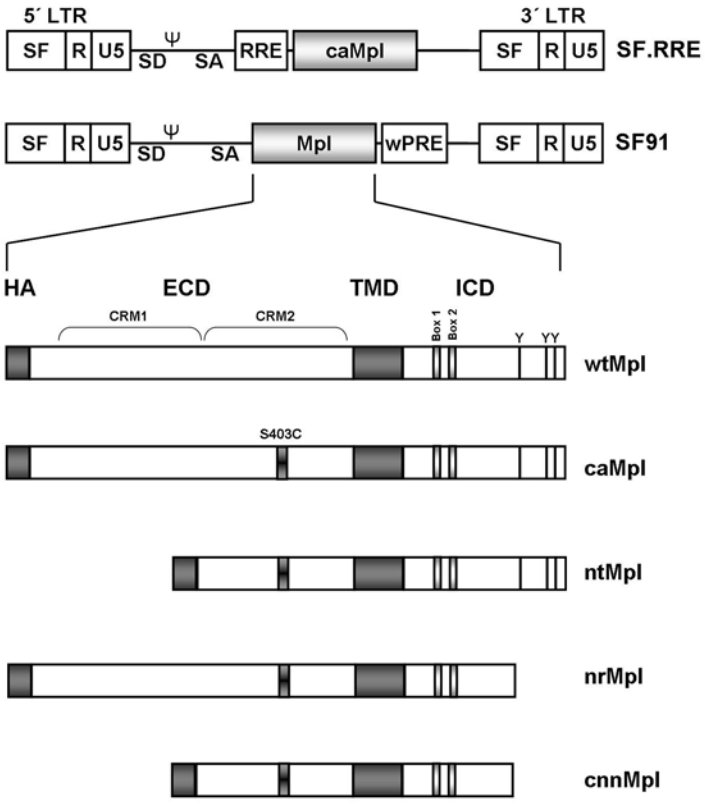

Figure 3.9: Vector and transgene constructs. LTR driven vector backbones were used in this part of the study to express several truncated Mpl receptors. RRE describes the rev responsive element from the human immunodeficiency virus. Different truncated versions of the constitutive active Mpl (caMpl) receptor were cloned into the SF91 backbone and further analyzed *in vitro* and *in vivo*. Abbreviations: wtMpl wild type Mpl, caMpl constitutive active Mpl, ntMpl constitutive active with no Thpo binding Mpl, nrMpl constitutive active with no Ras signaling Mpl, cnnMpl constitutive active with no Thpo binding and no Ras signaling Mpl, LTR long terminal repeat, SF spleen focus forming virus enhancer-promoter, Ψ packaging signal, HA hemagglutinine tag, ECD extracellular domain, CRM cytokine receptor homology module, TMD transmembrane domain and ICD intracellular domain, SD splice donor, SA splice acceptor, RRE rev responsive element.

One single point mutation in the serine codon t-c-a changes the aa configuration to cystein t-*g*-a. Thereby a permanent disulfide bond at aa position 403 is introduced which allows the constant homodimerization of two Mpl monomers without Thpo binding and results in constitutive receptor activity (Appendix 6.2). The LTR-driven gamma-retroviral vector SF91 was used as a backbone to express the protein (SF91.caMpl) (Figure 3.9). 32D cells transduced with SF91.caMpl render these cells factor-independent *in vitro* (Figure 3.10A). caMpl expressing cells were able to proliferate w/o any cytokine substitution, whereas GFP-expressing control cells died after some days.

#	mice number	vector and transgene	transgene [%] before BMT	observation time [d]	spleen weight [mg]	diagnosis
Exp. 5	3	SF91.caMpl	79	20 - 22	721 - 831	3 non lymphatic neoplasia
Exp. 6	5	SF91.caMpl	74	19 - 21	485 - 872	5 non lymphatic neoplasia
	6	SF.RRE.caMpl	> 50	42 - 112	414 - 498	4 non lymphatic neoplasia
				139	233	1 B-cell phenotype
				209	101	1 hypoplasia/MDS
	6	SF91.GFP	47	21 and 207	67 - 100	6 healthy
Exp. 7	2	SF91.caMpl	60	12 - 14	248 - 472	2 non lymphatic neoplasia
	5	SF91.cantMpl	48	15 - 21	422 - 792	5 non lymphatic neoplasia
	5	SF91.canrMpl	43	12 - 224	59 - 162	4 hypoplysia (no neoplasia)
						1 d.o.u.c.
Exp. 8	5	SF91.canrMpl		26 - 133	189	5 hypoplasia (no neaplasia)
	5	SF91.cnnMpl		37 - 201	100	4 prolonged engraftment
				156		1 irr. leukemia
	5	SF91.cantMpl		23	588	1 non lymphatic neoplasia
				13 - 33	83 - 133	4 engraftment failure

Table 3.3: Performed *in vivo* experiments. # running number of experiment, mice numbers, vectors and transgenes, transgene in percent before BMT, observation time of mice after BMT, spleen weights and diagnosis are listed. Each experiment was an individual transplantation experiment using the protocol described in the Materials and Methods section. MDS myelodysplastic-like syndrome, irr. leukemia irradiation derived leukemia, d.o.u.c. death of unknown cause.

Downstream pathway signaling studies of the Mpl receptor by Western blot and EMSA revealed that signaling pathways were active even without cytokine stimulation. We could also detect a hypersensitivity of caMpl to Thpo stimulation.

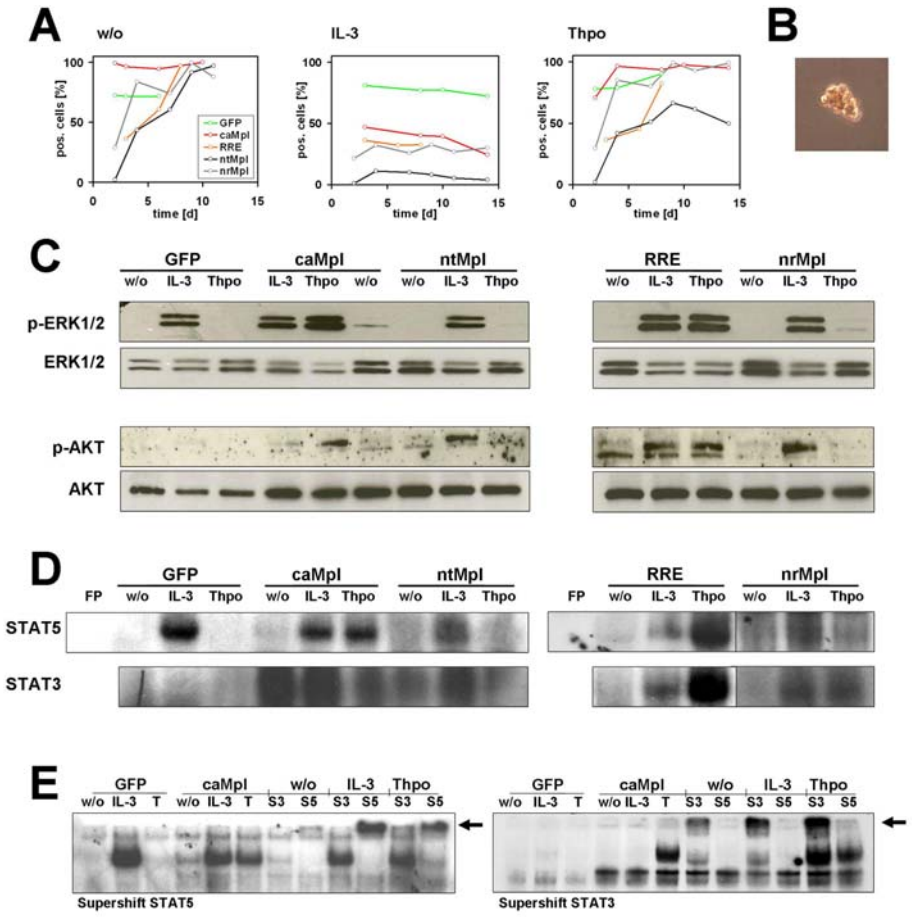

Figure 3.10: ***In vitro*** **and molecular analysis of caMpl mutants.** (A) IL-3 dependent 32D cells were transduced with the retroviral vector and cultured in different cytokine media conditions. Shown are transgene positive cells in percent as detected by flow cytometry against time of observation in days. Without (w/o) any cytokine substitution SF91.GFP transduced control cells were not able to survive. All other constitutive active mutants could proliferate and selected for transgene expressing cells. Using IL-3 substitution all cells expressing the different transgenes were able to proliferate. The shorter line for SF91.RRE.caMpl (RRE) transduced cells is due to a shorter observation time. Under Thpo conditions all cells receiving one of the constitutive active Mpl constructs could proliferate. Only the GFP expressing control cells died. The shorter line for SF91.RRE.caMpl (RRE) transduced cells is due to a shorter observation time. (B) CFU-E colonies were derived from the caMpl mutant without any cytokine substitution after 48 hours. caMpl transduced Lin- BM cells were able to form colonies even without EPO. (C) Western blot data of different Mpl mutants are displayed. 32D cells were transduced with the vector and cultivated to suitable amount of cells. Protein lysates were prepared and analyzed by Western blot analysis. caMpl transduced 32D cells showed weak activation even w/o cytokine stimulation compared to the GFP control cells. After Thpo stimulation caMpl expressing cells displayed even stronger activation than the GFP control. The ntMpl mutant could not be stimulated with Thpo. RRE showed the same activation profile as caMpl. The nrMpl mutant had strongly reduced Thpo stimulation activity in ERK. (D) Electromobility shift assays (EMSA) were performed from the same protein lysates to analyze the activation of STAT3 and STAT5. SF91.caMpl transduced cells showed a slight activation w/o cytokines and a strong signal after stimulation with Thpo. The SF91.ntMpl transduced cells showed no signal after Thpo stimulation. Cells transduced with the SF91.RRE.caMpl vector behaved similar as caMpl expressing cells. The SF91.nrMpl transduced cells showed moderate activation after Thpo stimulation in STAT5 and strong activation

in STAT3 compared to IL-3 stimulation. Bands in STAT5 from the nrMpl construct ran slightly higher in the gel. Supershift can confirm if these are the real STAT5 activated bands or artifacts. (E) Blots represent EMSA Supershift controls for specificity of STAT3 and STAT5 activation. Arrows indicate supershift. Abbreviations: T Thpo, w/o without any cytokine, IL-3 or Thpo stimulation. The second lower band in p-AKT is due to unspecific binding to IgG as per manufacturer, p = Ab against phosphorylated protein, FP free probe.

Besides hypersensitivity, there is also a second possibility that caMpl expression mediates stronger transgene signals due to the additional supra-physiological receptor molecules per cell. In addition to the Akt-specific signal (top band) a nonspecific product was detected by the antibody (lower band) as reported by the manufacturer (Figure 3.10C, D). Specificity of STAT3 and STAT5 activation in the EMSA was shown by supershifts (arrows indicate supershift) (Figure 3.10E). By plating caMpl-transduced BM cells into methyl cellulose, CFU-E colonies could be detected after 48 h without any cytokine substitution (even without EPO) whereas the wtMpl showed no such colony formation (Figure 3.10B).

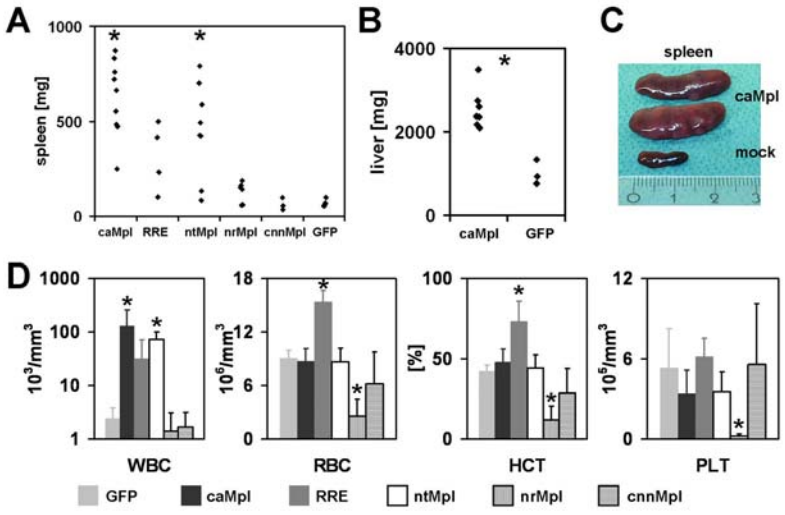

Figure 3.11: Macroscopic data and PB data of mice transplanted with the Mpl mutants. (A) Spleen weights in mg of different Mpl mutants expressing BM cells transplanted into mice were measured by final analysis. Spleens from caMpl, RRE and ntMpl expressing animals were increased (*P < 0.004 t-test, two tailed, unequal variations, in comparison to the GFP control). (B) Liver size of caMpl transplanted animals in mg by final analysis. Livers of animals were increased. Livers of other Mpl mutant expressing BM cells receiving animals were not weightened (*P < 0.0007 t-test, two tailed, unequal variations). (C) The picture shows two enlarged spleens from caMpl transplanted animals by final analysis. Mock spleen represents one untreated animal. (D) PB data of mice receiving different vector transduced BM cells were analyzed by final analysis. Shown are WBC (log10), RBC, HCT and PLT counts. Arrow bars represent SD (*P < 0.01 t-test, two tailed, unequal variations, compared to GFP controls). (GFP n=6, caMpl n=10, RRE n=4, ntMpl n=5 ,nrMpl n=6, cnnMpl n=3).

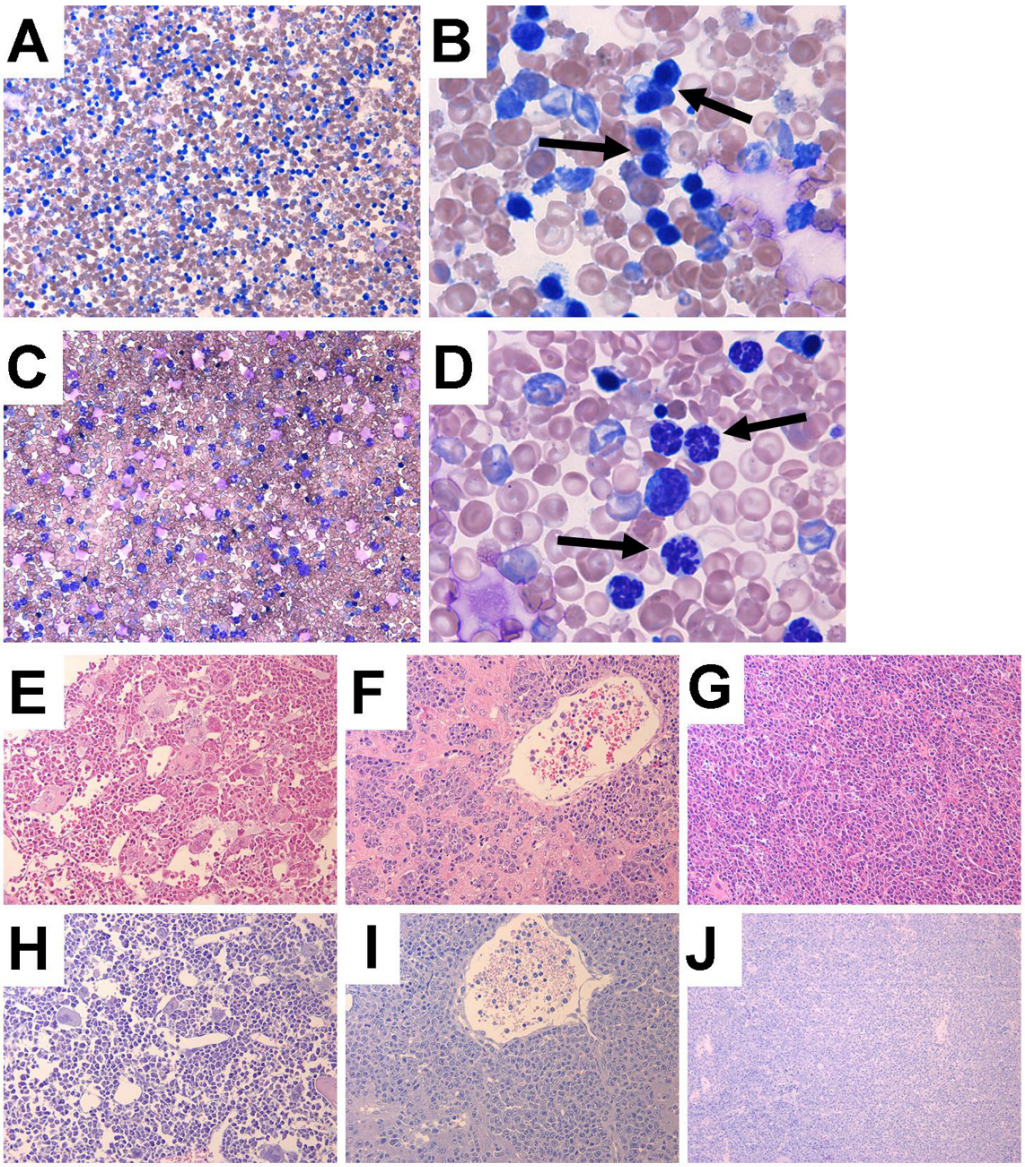

Figure 3.12: Histopathology data of caMpl expressing BM cells transplanted mice. (A, B) The pictures show PB smears of the caMpl expressing group developing erythroid leukemia after BMT. Dysplastic erythroid precursors are marked by arrows. (C, D) Micrographs show PB smears from caMpl expressing group developing myeloid leukemias. Dysplastic granulocytes are marked by arrows. (E, F, G) Data show massive infiltration of the BM, liver and spleen by erythroid progenitors of caMpl expressing group. Blood vessels in the liver show leukemogenic cells is the PB (F). Spleen architecture is completely disrupted (G). (H, I, J) Represents strong infiltration of the BM, liver and spleen by dysplastic granulocytes and precursors. Leukemogenic cells are present in the blood (I) and spleen architecture is disrupted (J). Magnification and staining: (A, C) 200x, May-Grünwald/Giemsa, (B, D) 1000x, May-Grünwald/Giemsa, (E, F, G, H, I) 200x, Giemsa, (J) 100x, Giemsa.

To study the effects of caMpl expression *in vivo,* Lin- BM cells were transduced with the SF91.caMpl constructs and transplanted into lethally irradiated mice (Exp. 5, 6, 7, Table 3.3). All mice showed clear signs of leukemia after only two to three weeks and had to be sacrificed. Mice showed massive hepatosplenomegaly (spleen: 248 mg to 872 mg, liver: 2090 mg to 3484) with a typical cherry red color of infiltrated organs (Figure 3.11A, B, C). Blood counts revealed a strong increase of WBC with up to 352 x 10^3 WBC/µL (Figure 3.11D). RBC, HCT and PLT were not significantly altered in caMpl-expressing animals. Two subgroups of leukemias could be defined. Both groups showed increased, dysplastic myeloid and erythroid cells and progenitors in the PB smears (Figure 3.12A, B, C, D). However, one group had significantly more erythroid cells and progenitors as detected by flow cytometry in the spleen (Figure 3.13A, B, n=7). TER119 and CD71 single and double positive fractions were increased, whereas myeloid markers like CD11b and Gr1 single and double-positive cells and CD45-positive cells were reduced. Blood smears showed more erythroid blasts compared to the smear of leukemogenic mice of the other group (Figure 3.12A, B, arrows). Histology slides of BM showed dysplastic megakaryocytes, infiltration of the liver by leukemogenic blasts and structural disruption of the spleen (Figure 3.12E, F, G). This group was therefore named “erythroid leukemia”. The second group had increased myeloid cells and precursors in the spleen as detected by flow cytometry (Figure 3.13C, n=3). Gr1 and Gr1/CD11b double-positive cells were significantly increased compared to the GFP-expressing group. Interestingly, TER119 and CD71 single and double-positive fractions showed no statistically relevant difference. In the PB smears, increased numbers of dysplastic myeloid cells and precursors could be seen (Figure 3.12C, D, arrows). Histopathology of the animals presented infiltration of leukemogenic blasts in the BM, liver and spleen (Figure 3.12H, I, J). This group was therefore named “myeloid leukemia”. To conclude, constitutive activation of Mpl receptor signaling is highly leukemogenic and results in hepatosplenomegaly only a few weeks after BMT, presenting as either erythroid or myeloid leukemia.

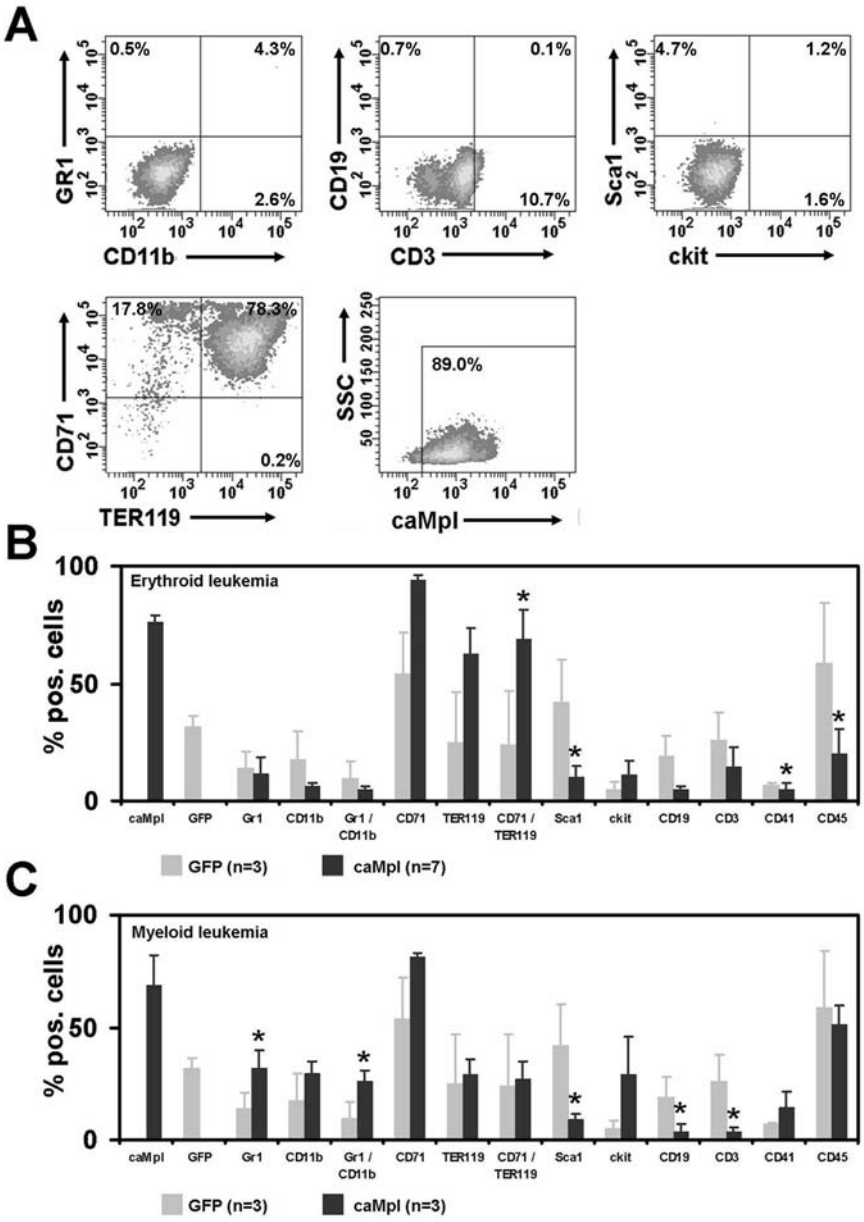

Figure 3.13: Flow data analysis of mice transplanted with caMpl expressing BM cells. (A) FACS blots from the spleen show that cells are negative for myeloid markers (Gr1, CD11b), B and T cell markers (CD19, CD3) as well as stem cell markers (Sca1, ckit). Cells were strongly positive for erythroid markers CD71 and TER119 and transgene (wtMpl). (B) Hematopoietic markers in the spleen were analyzed in the caMpl expressing group. Mice showed strong increase of erythroid markers (CD71, TER119 single and double positive) why this group was named erythroid leukemia. Arrow bars represent SD (*P ≤ 0.04, t-test, two tailed, unequal variations, compared to the GFP control group). Following to name this group as "erythroid leukemia". (C) FACS graphs show the same markers as in B. In this group erythroid markers were normal but myeloid markers were increased (Gr1, CD11b single and double positive). Arrow bars represent SD (*P ≤ 0.05, t-test, two tailed, unequal variations, compared to GFP control). This group was therefore named as "myeloid leukemia".

3.2.2. caMpl-induced leukemia selects for clones with retroviral vector insertions in proto-oncogenes

Leukemias resulting from caMpl expression in the BM were characterized by different molecular biological techniques. Genomic DNA from the infiltrated spleen was purified and analyzed by Southern blot. Strikingly, leukemias of SF91.caMpl-transplanted animals were found to be oligoclonal and contained 2 to 7 insertion sites (n=9) after only three weeks (Figure 3.14A).

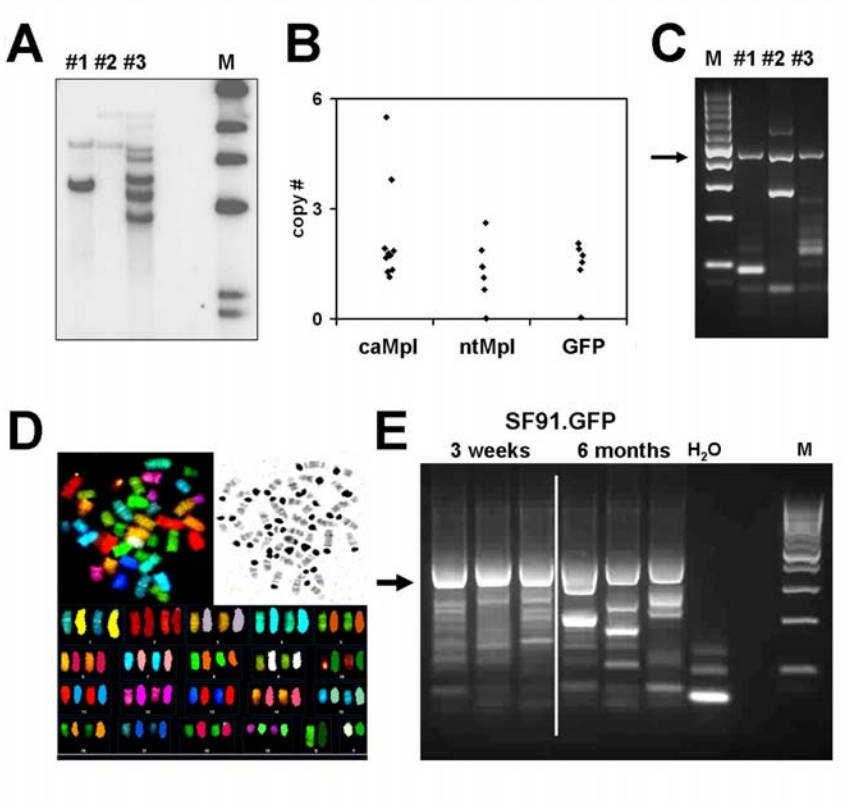

Figure 3.14: Molecular analysis of SF91.caMpl induced leukemias. (A) Southern blot analysis shows retroviral vector integrations in the genomic DNA of the spleen of three animals which received SF91.caMpl transduced BM cells. Two to seven insertions could be seen for animals #1, #2 and #3. (B) Graph shows vector copy numbers in the spleens of leukemic and control animals. (C) Same animals as seen in A are shown for LM-PCR analysis. Bands represent number of retroviral insertions. The internal control is marked by an arrow. M marker. (D) SKY analysis of cultured spleen cells from three leukemogenic animals was performed to test for chromosomal aberrations. Analysis revealed no chromosomal instability. Shown is one representative example. (E) LM-PCR analysis of animals transplanted with SF91.GFP transduced BM cells in a time dependent manner is shown. First three mice were sacrificed after 3 weeks and showed no clonality after that time. Latter three animals where sacrificed after six months and presented oligoclonal dominance. Bands in the H_2O control lane can be explained by primer dimerization. The internal control is marked by an arrow.

The mean retroviral vector marking in the BM quantified by qPCR in genomic DNA ranged from 1.14 to 5.49 and thereby resembles the data obtained by SB (Figure 3.14B). LM-PCR showed a similar picture of clonality as SB and qPCR (Figure 3.14C). To exclude leukemia development resulting from chromosomal aberrations, we performed Spectral Karyotype (SKY) analysis on three leukemogenic animals. No chromosomal aberrations could be detected in these analyzed samples (Figure 3.14D). Because mutations in the karyotype of the animals were obviously not the reason for the leukemia development, we did no further karyotype analysis on the other animals. To examine whether the selection of dominant clones was due to caMpl- expression, we analyzed BM samples of mice transplanted with SF91.GFP control vector-transduced BM cells, sacrificed at two different time points. GFP-expressing control animals analyzed for clonality by LM-PCR after three weeks showed polyclonality. However, GFP-expressing mice after six months post-transplantation showed clonal selection (Figure 3.14E). It can be concluded that clonality in leukemogenic animals after three weeks latency resulted from the expression of the mutated Mpl receptor. Comparison of insertion sites recovered from leukemic caMpl expressing mice with insertion sites of the RTCGD (retroviral tagged cancer gene database) (Akagi et al., 2004) and IDDb (insertional dominance database) (Kustikova et al., 2007) revealed a preference for insertions in possible proto-oncogenes (Table 3.4, Appendix 6.3). Table 3.4 shows a list of insertion sites with surrounding genes in 150 kbp distance. Orientation of vector insertion is marked F (forward) and R (reverse). Distance of LTR to transcription start site is displayed in negative numbers (5’ site of gene transcription start site) and positive numbers (3’ site of gene transcription start site). 77 insertions were found with 23 common integration sites (30%, CIS) as analyzed via RTCGD and IDDb. Whereas it has to be considered that CIS with a match of only 1 or 2 hits in one of the databases is not directly an oncogene. Most of the insertion sites detected are positioned closely to the transcription start site of genes, assuming a regulatory effect of vector enhancer-promoter elements on the expression of the host gene (Wu et al., 2003). Because of the fast leukemia development, insertional mutagenesis seems not to be the only reason for oncogenicity. However, it could have an influence on the clonal dominance or the phenotype of the leukemia. The other reason for the leukemia development must be the constitutively active Mpl mutation.

3.2.3. Reduced expression level of caMpl prolongs the latency of phenotype development (RRE.caMpl)

To address the question whether caMpl expression levels correlate with leukemia development, we constructed an SF91.caMpl-expressing vector that conferred a ten-fold lower expression level (SF91.RRE.caMpl). The reduced expression is based on the introduction of the rev responsive element (RRE) from the human immunodeficiency virus HIV-1 at 5' position of the transgene into the vector (Figure 3.9). The RRE element in HIV binds to the rev protein and thereby mediates nuclear export of the viral RNA. The reduced expression level in our vector system is most likely due to the longer 5'UTR region, reduced splicing capacity (Kraunus et al., 2006) and an own upstream open reading frame (ORF) in the RRE element before the transgene ORF. Reduced expression levels were analyzed by flow cytometry on SC1 cells (Figure 3.15A). 32D cells transduced with the low expressing mutant still grew factor-independently (Figure 3.10A). Activation of Mpl downstream pathways in Western blot and EMSA was similar to that observed in SF91.caMpl-transduced 32D cells (Figure 3.10C, D).

Animals transplanted with BM cells transduced with SF91.RRE.caMpl had a prolonged disease latency compared to animals receiving caMpl-transduced cells. The first animal developed leukemia after 6 weeks, while other animals survived as long as 7 months (Figure 3.15B). Mice which developed leukemia in the earlier timeframe (up to 20 weeks) displayed the same phenotypes as animals receiving cells with high expression of caMpl with hepatosplenomegaly (spleen: 101 mg to 498 mg) and cherry red color of infiltrated organs (Figure 3.11A), whereby the increase of PB cells was even more severe compared to SF91.caMpl-transplanted mice. Not only were WBC strongly increased but also RBC and HCT showed a statistical significant increase (Figure 3.11D). One animal developed a B-cell proliferation with a major fraction of CD19-positive cells as detected by flow cytometry. BM, liver and spleen were severely infiltrated by erythroid and myeloid blasts like the caMpl-expressing animals (Figure 3.15C, D, E). The BM was hypercellular (Figure 3.15C), infiltration of blasts in the blood vessels could be seen in the liver (Figure 3.14D) and the architecture of the spleen was totally disrupted (Figure 3.15E).

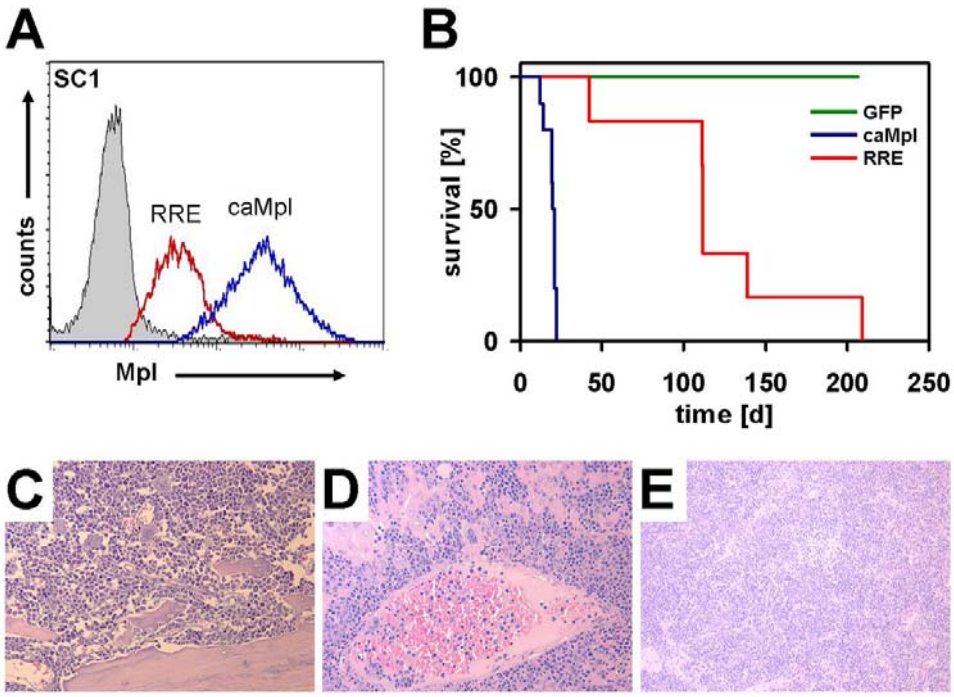

Figure 3.15: Analysis of RRE vector mutant. (A) Flow data of rev responsive element (RRE) vector (SF91.RRE.caMpl) compared to normal SF91 vector backbone (SF91.caMpl) in SC1 cells are shown. Histogram presents one log10 reduction of fluorescence intensity by introducing the RRE element into the vector backbone. (B) Graph represents the survival of caMpl-expressing mice compared to RRE construct and GFP control. The caMpl group died after only three weeks post transplantation. The RRE group prolonged the latency until 209 days. The GFP group survived long term. (C, D, E) Infiltration of the BM, liver and spleen from animals transplanted with cells transduced with the RRE vector construct showed dysplastic cells, erythroid progenitors and myeloid cells. (C) BM is hypercellular, (D) blood vessels in the liver show infiltration of blasts in the PB and (E) spleen architecture was disrupted. Magnification and staining: (C, D) 200x, Giemsa, (E) 100x, Giemsa.

Interestingly, one animal which had a strongly prolonged latency, showed signs of pancytopenia as was seen from the expression of wtMpl and dnMpl receptor (Chapter 3.1). PB counts were as low as: WBC 0.5 x 10^3/µL, RBC 1.86 x 10^6/µL, HCT 9.5% and PLT 18 x 10^3/µL. Here the constitutively active signaling of caMpl seemed to be so weak that the dominant negative effect of the Mpl receptor, derived from Thpo trapping, became the dominant effect compared to the constitutively active signaling. Taken together, leukemia development was dependent on transgene expression and lower expression levels resulted in prolonged latency or even no leukemogenic outcome in one animal.

3.2.4. Leukemia development is independent of Thpo binding (ntMpl)

As a next step, we addressed the influence of the dominant negative effect of ectopic Mpl expression seen in the wtMpl and dnMpl-transplanted animals on leukemia induction by caMpl (Chapter 3.1). We constructed a constitutively active mutant that lacked the Thpo binding domain (Sabath et al., 1999) (Figure 3.9). This was expected to render ntMpl signaling independent of the Thpo hypersensitivity seen by caMpl in Western blots and EMSA and eliminate the dominant negative effect of ectopic Mpl expression via Thpo trapping.

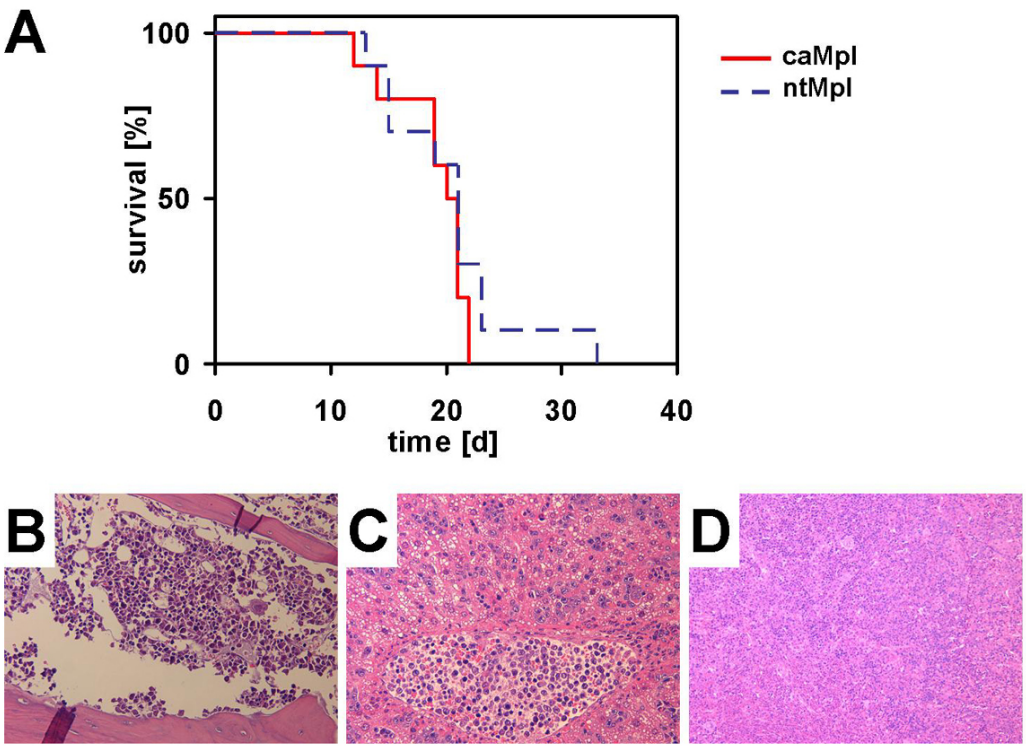

Figure 3.16: caMpl induced leukemias do not require the Thpo binding domain. (A) Survival chart of caMpl-expressing animals versus ntMpl-expressing mice is displayed. There was no change in time of survival resulting from the lack of the Thpo binding domain. (B, C, D) Histopathology of ntMpl group showed BM, liver and spleen. All organs had massive infiltrations of leukemic cells. (B) BM showed an increased amount of erythroid progenitors, (C) liver and blood vessels were infiltrated by erythroleukemic cells, (D) spleen architecture was disrupted and showed infiltration of leukemogenic cells. Magnification and Staining: (B, C) 200x, Giemsa, (D) 100x, Giemsa.

Two fragments of the caMpl receptor were derived by PCR, one fragment containing the signal peptide and the HA-tag from aa 1 (Met) to 36 (Gln) and the second one coding for the truncated extracellular domain, transmembrane region and intracellular domain from aa 284 (Gly) to 635 (Pro). The serine to cystein mutation at position aa 403 (a → c) resulting in the constitutive activation remained (Figure 3.9, Appendix 6.2). 32D cells transduced with the mutant constitutively active receptor that is unable to bind Thpo (ntMpl) showed a similar factor independent proliferation as 32D cells transduced with caMpl (Figure 3.10A). The signal activation in EMSA and Western blots without cytokine stimulation was hardly detectable, but the hypersensitivity seen before with normal caMpl was clearly missing (Figure 3.10C, D). Mice receiving BM cells transduced with the ntMpl mutant showed the same hepatosplenomegaly as mice receiving caMpl-expressing BM (Figure 3.11A). PB counts showed a strong increase in WBC and no significant alterations in the RBC, HCT or PLT (Figure 3.11D) similar to the caMpl that is still able to bind Thpo. Animals receiving BM cells transduced with the ntMpl mutant showed the same latency as mice transplanted with caMpl transduced BM cells (Figure 3.16A). Histopathology revealed infiltration of leukemogenic blasts into the BM, spleen and liver (Figure 3.16B, C, D). Blood vessels in the liver were severely infiltrated by precursor cells (Figure 3.16C) and the spleen architecture was completely disrupted (Figure 3.16D). It can be concluded that the leukemia development is independent of the Thpo binding.

3.2.5. Deletion mutants (nrMpl, cnnMpl) of caMpl lack the oncogenic potential but retain the positive proliferation effect

We then asked whether it was possible to design receptor mutants which retain the proliferation effect of caMpl but lack the oncogenic potential. Such receptor mutants could be used to expand the HSC population for CAMT gene therapy. They should submit proliferation signals ligand-independently and do not have any oncogenic signals by shutting off these distinct pathways. We therefore constructed constitutively active Mpl receptor mutants that lack the 52 C-terminal aa. This mutant was expected not to induce signaling via Ras activation (no Ras Mpl, nrMpl). A further nrMpl mutant was constructed by combining the nrMpl mutation with the ntMpl mutation (ca-nr-nt-Mpl,

cnnMpl) (Figure 3.9). Both mutants were derived via PCR products of the caMpl receptor. An artificial stop codon was introduced at aa position 566 t-g-t (c) to t-g-*a* (stop) resulting in truncated cDNAs (Drachman and Kaushansky, 1997). This leads to a truncated intracellular domain that lacks three tyrosine residues important for the activation of Ras signaling (Appendix 6.2). 32D cells transduced with either one of the caMpl mutants (nrMpl, cnnMpl) showed the factor-independent growth observed before with the caMpl receptor (Figure 3.10A). This proves that the truncated receptors still mediate a proliferation advantage. In Western blot and EMSA analysis, the specific defect in activating the desirable pathways (for Ras, MAPK, ERK1/2) was shown by the undetectable ERK1/2 phosphorylation compared to the signaling by caMpl after stimulation with Thpo (Figure 3.10C, D). However, Akt stimulation was hardly to be seen but activation of STAT3 and STAT5 in the nrMpl mutant was similar the activation by IL-3 stimulation.

Mice were transplanted with Lin- BM cells transduced with nrMpl or cnnMpl vectors as performed with the caMpl vector. Interestingly, none of the mice developed leukemia and they survived up to six months post-BMT (Figure 3.17A). None of the animals had signs of hepatosplenomegaly (Figure 3.11A). However, PB in the nrMpl group showed a severe reduction in WBC, RBC, HCT and PLT (Figure 3.11D) most likely resulting from the dominant negative effect of Thpo trapping. Subsequently, some of the nrMpl-transplanted animals died due to pancytopenia as observed in the wtMpl and dnMpl-transplanted animals (Chapter 3.1). In contrast, mice that engrafted with cnnMpl-expressing BM cells did not develop pancytopenia as seen in PB counts by finial analysis (Figure 3.11D). Spleen sizes were also normal (Figure 3.11A). One cnnMpl-expressing animal developed a donor-derived thymus leukemia after five months post-transplantation. The leukemia was most likely due to the irradiation and thymus cells analyzed by flow cytometry were not positive for the transgene cnnMpl. However, most of the cnnMpl-expressing animals did not survive long-term, which was probably due to insufficient engraftment seen in this specific experiment (Exp. 8). Some animals in this experiment had low gene marking and died early of engraftment failure. Therefore the experiment should be repeated.

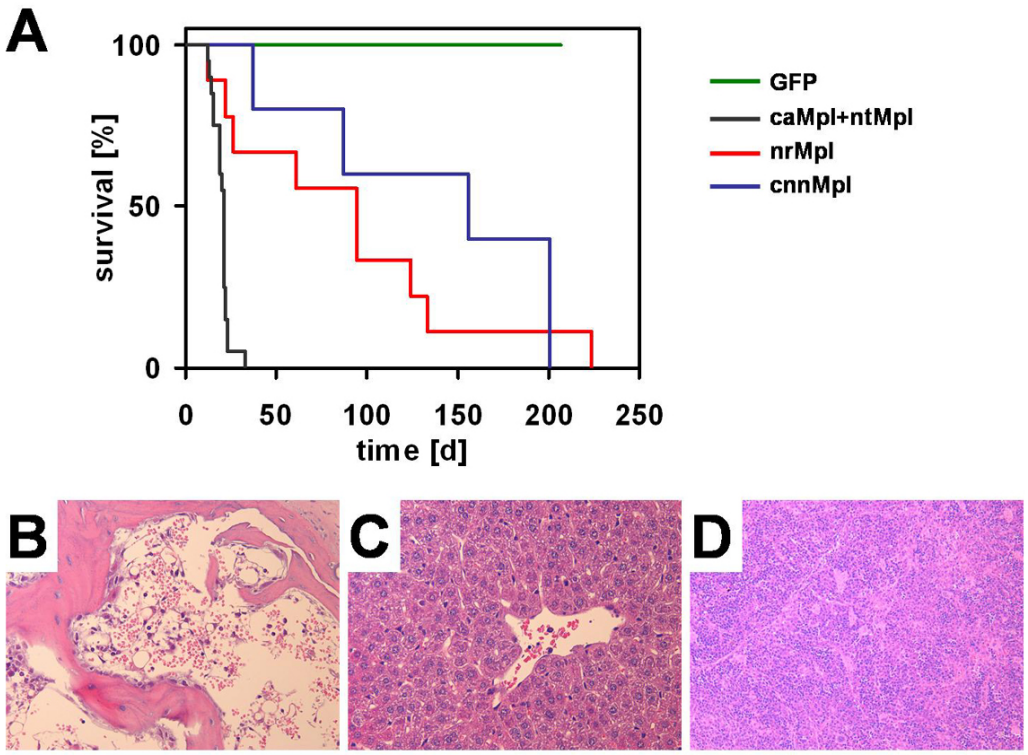

Figure 3.17: nrMpl mutant and cnnMpl mutant behave similar. (A) Graph shows survival chart of Ras deletion mutants compared to GFP and caMpl group. Animals receiving transduced BM cells with the Ras deletion mutants (SF91.nrMpl, SF91.cnnMpl) survived significantly longer than the caMpl expressing mice. (B) BM of the nrMpl group was hypocellular and showed no infiltration by leukemogenic progenitors. (C) Liver also showed no signs of infiltration. (D) Spleen had normal architecture and showed no sign of infiltration. Magnification and staining: (B, C) 200x, Giemsa, (D) 100x, Giemsa. (Pictures represent an nrMpl expressing animal).

In summary, it can be concluded that the Ras pathway is one or even the most important pathway for oncogenicity in Mpl signaling. Shutting off this pathway could avoid potential leukemogenic side effects of aberrant Mpl signaling. The cnnMpl mutant may possibly be used to expand the HSC fraction in a transcriptionally regulated promoter vector system or by transient expression (Gossen and Bujard, 1992). However, further transplantation experiments should be performed to rule out additional possible side effects.

3.3.
Gene therapy with improved vector constructs shows promising results for a curative approach to treat CAMT

3.3.1. Gamma-retroviral vectors containing the cellular Mpl promoter fragment show reduced transgene expression levels

In our previous experiments we observed that high expression of the Mpl receptor induced strong side effects like pancytopenia and MDS-like disorders (Chapter 3.1). To overcome these side effects, we used a 512 bp fragment of the ubiquitous low expressing promoter of the phosphoglycerate kinase gene (PGK) (Schambach et al., 2006a) and the 2 kb promoter fragment of the endogenous Mpl receptor (MplP) and compared the expression by those promoters with the expression by the SF promoter. All promoters were introduced into self-inactivating (SIN) gamma-retroviral vector constructs (Figure 3.18). The MplP should restrict transgene expression to Mk, HSC and MEP cells (Ziegler et al., 2002).

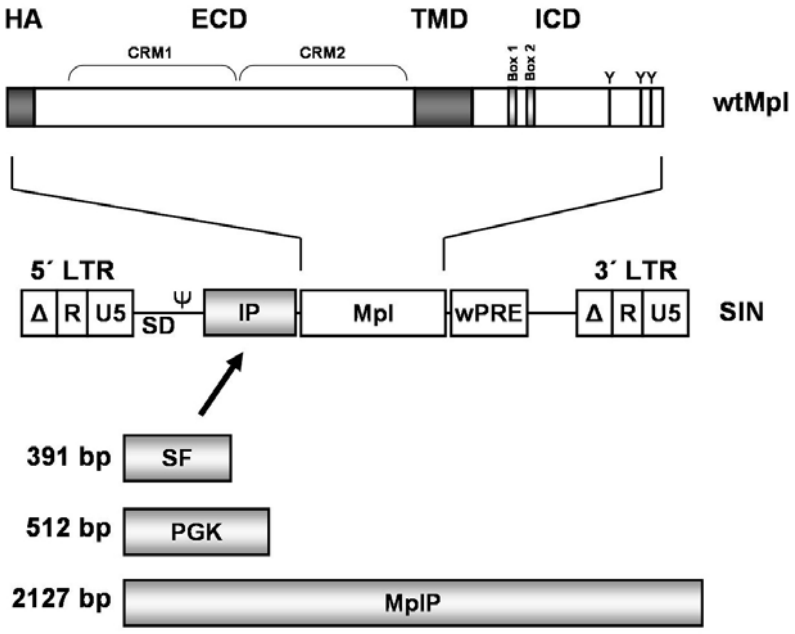

Figure 3.18: Vector constructs and promoters. Self inactivating (SIN) vectors have a deletion in the U3 region which eliminates the strong enhancer-promoter activity. Therefore internal promoters are used to drive the transgene expression. Promoters used in this experiment were SF (spleen focus forming virus), PGK (phosphoglycerate kinase) and the endogenous Mpl promoter fragment (MplP). Abbreviations: LTR long terminal repeat, Ψ packaging signal, HA hemagglutinine tag, ECD extracellular domain, TMD transmembrane domain and ICD intracellular domain, SD splice donor, CRM cytokine receptor homology module, wPRE woodchuck hepatitis virus post transcriptional element.

When company the GFP-expressing constructs (SIN.SF.GFP, SIN.PGK.GFP and SIN.MplP.GFP), a reduced fluorescence intensity was detected via fluorescence microscopy (Figure 3.19A) in 293T cells that expressed GFP by MplP promoter compared to the PGK and SF promoter. GFP expression levels were then analyzed in retrovirally transduced SC1 cells by flow cytometry (Figure 3.19B). The mean fluorescence intensity of SC1 cells expressing GFP under the control of the PGK promoter was reduced 10-fold compared to the expression levels from the SF promoter and the expression by the MplP promoter was further reduced compared to the PGK promoter. In conclusion, gamma-retroviral SIN vectors with the internal promoters PGK and MplP conferred reduced expression levels in murine fibroblast cells and MplP was the lowest expressing promoter, possibly because of its cell type-specific restriction of transgene expression.

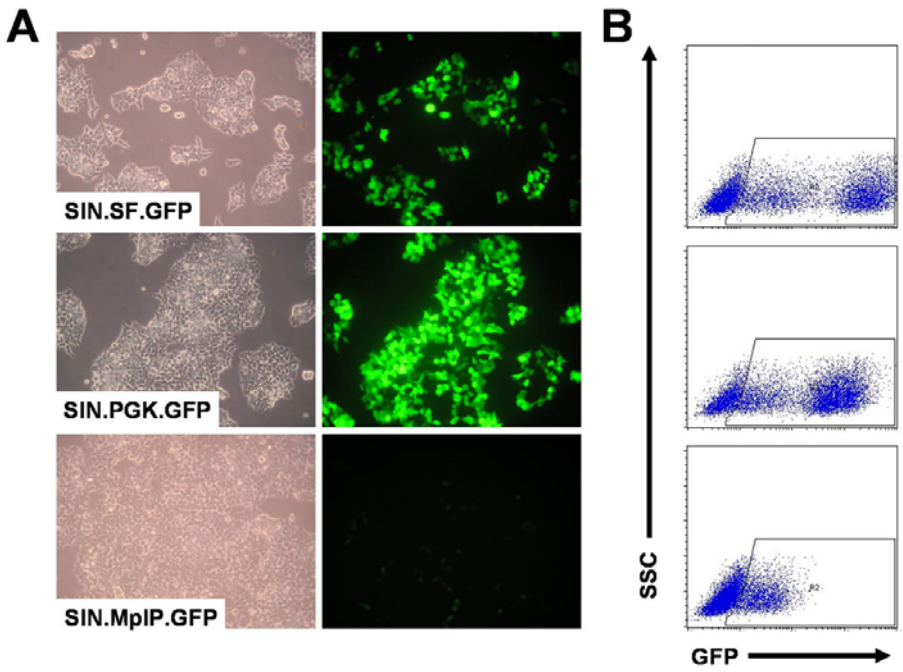

Figure 3.19: Virus production of the new vector constructs. (A) SIN vector constructs were transfected into 293T cells. Pictures show cell layer in cultures 2 days after transfection. First panel represents light microscope and second panel shows same cell layer via fluorescence activation of GFP expressing cells. (B) SC1 cells were transduced for titer determination with the supernatants of 293T transfected cells. Flow cytometry blots show GFP positive cells 2 days after transduction. Mean fluorescence intensity was reduced of about one log10 from SF to PGK promoter and again from PGK to MplP promoter.

3.3.2. The Mpl promoter shows a tropism for CD41$^+$ cells

To address the potential of the differentiation-specific expression by retroviral vectors that use the Mpl promoter (SIN.MplP.GFP), we transduced Lin- BM cells of wild-type C57Bl6/J mice and compared the expression of GFP to the expression driven by the SF promoter in gamma-retroviral SIN vectors (SIN.SF.GFP) in the CD41$^+$ fraction resembling Mk and their precursors. The ubiquitously expressing SF promoter was expected to confer the same amount of transgene positive cells in every cell lineage. However, expression by the MplP should be more specific for CD41$^+$ cells. In both cultures, the same numbers of CD41$^+$ cells (29%) were detected in the BM suspension four days post-transduction stimulated by the cytokines SCF, IL-3, IL-11, Flt-3L and cultivated in StemSpan media (Figure 3.20A, B, first panel). Cells transduced with the SF-driven retroviral vector showed high expression of the transgene on 50% of all BM cells (Figure 3.20A, second panel). The MplP promoter-driven vector had an about 100-fold reduced expression level with 10% positive cells using the same MOI (Figure 3.20B, second panel). Interestingly, in the GFP-expressing cell population, the MplP construct had twice the amount of CD41$^+$ cells compared to the SF promoter (55% to 29%) (Figure 3.20A, B, third panel). This observation indicated the cell type restricted expression by the Mpl promoter compared to the SF promoter.

We performed further analysis of the differentiation-specific expression by the MplP in a second experiment under the same conditions. We determined the ratio of specificity of MplP compared to the SF promoter. To calculate the specificity of GFP expression in the CD41$^+$ fraction we compared the CD41$^+$/GFP$^+$ cells with the CD41$^+$/GFP$^+$ + CD41$^+$/GFP$^-$ fraction (Figure 3.20C, D). By dividing this number by the number gained from the CD41$^-$/GFP$^+$ fraction compared to CD41$^-$/GFP$^+$ + CD41$^-$/GFP$^-$ fraction we determined a "differentiation-specific expression factor".

$$specificity = \frac{\left(\frac{UR}{UR+LR}\right)}{\left(\frac{UL}{UL+LL}\right)}$$

UR = upper right
LR = lower right
UL = upper left
LL = lower left

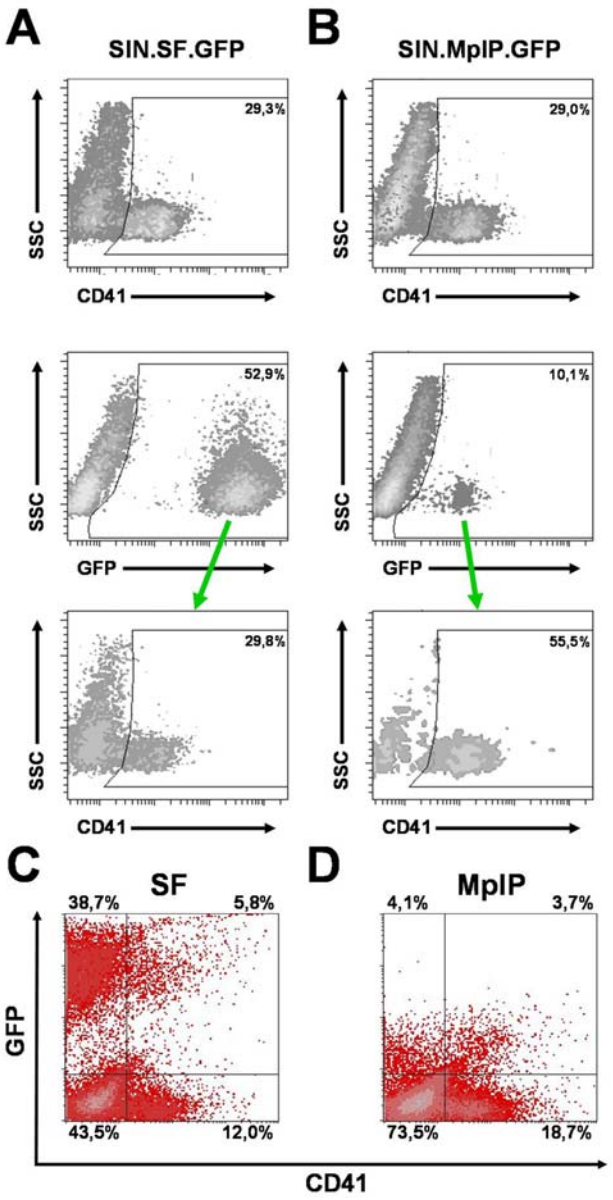

Figure 3.20: Transduction of BM cells with the MplP construct. (A, B) Lin- BM cells harvested from C57Bl6/J wild type animals were transduced by same MOI with the viral supernatants of SIN vectors containing the GFP reporter protein driven by either SF (A) or MplP (B) promoter. Cells were cultivated for 4 days in StemSpan media containing IL-3, IL-11, SCF and Flt-3L. The first row shows flow cytometry data of CD41 positive cells from the whole BM fraction. The second row represents the GFP expressing fraction in the whole BM sample, which is significantly reduced in number and fluorescence intensity for the MplP expressing construct compared to the SF construct. The third row shows the CD41 positive cells in the GFP expressing fraction. For the SF promoter it was the same amount whereby it was about twice as much in the MplP expressing vector constructs. (C, D) Lin- BM cells were transduced and cultivated in the same way as in experiment A. GFP expression is shown against CD41 positive cells. The number of CD41/GFP double positive cells was similar in both experiments whereas the total amount of GFP expressing cells was different (see explanation Results part for details).

The differentiation-specific expression factor for ubiquitous promoters would be expected to be one because they should mediate the same probability of transgene expression in every lineage. For the MplP promoter this factor is expected to be higher than one because the amount of GFP-expressing cells in the $CD41^+$ cell population should be higher. Interestingly, the differentiation-specific expression factor for the SF promoter was even a little bit lower in $CD41^+$ cells with 0.7, whereby the MplP showed a significantly higher factor for the expression in $CD41^+$ cells with 3.1. To determine how much more specific the MplP in $CD41^+$ cells is compared to the SF promoter, we calculated the ratio of MplP specificity to SF specificity, which was 4.5. This means that the MplP is about 4 - 5 times more specifically active in $CD41^+$ cells than the SF promoter.

To conclude, in two independent experiments the 2 kb MplP fragment introduced into a gamma-retroviral SIN vectors conferred differentiation-specific expression. However, expression levels were very low compared to SF promoter.

3.3.3. Differentiation-specific and low Mpl expression reduce the risk of side effects in mice after BMT

In previous experiments we found that an unregulated high expression of the wtMpl receptor induced pathologic side effect like CMPD, pancytopenia and MDS (Chapter 3.1). To further evaluate our hypothesis that the side effects are expression level dependent we used the gamma-retroviral SIN vector constructs in a BMT experiment. In the next serious of our studies we transplanted SIN.PGK.wtMpl, SIN.PGK.GFP and SIN.MplP.wtMpl, SIN.MplP.GFP transduced Lin- BM cells into lethally irradiated wild-type mice.

The MplP restricts wtMpl expression to megakaryopoiesis and the early HSC compartment whereas the PGK-driven expression is ubiquitous. Both promoters mediated greatly decreased transgene expression levels per BM cell as studied in vectors expressing GFP (Figure 3.21A). wtMpl expression by the Mpl promoter could hardly be detected on primary Lin- cells by flow cytometry. Efficient gene marking in the BM was shown by detection of the vector copy number by qPCR (Figure 3.21B). When using the PGK promoter, alterations in hematopoiesis could still be observed.

#	mice number	vector and transgene	donor / chimerism	recipient / chimerism	transgene [%] before BMT	observation time [d]	diagnosis
Exp. 9	4	SIN.MplP.Mpl	WT / -	WT / -	6	213	4 healthy
	4	SIN.MplP.GFP	WT / -	WT / -	10	213	4 healthy
	4	SIN.PGK.Mpl	WT / -	WT / -	45	84 - 213	4 cytopenic/MDS
	4	SIN.PGK.GFP	WT / -	WT / -	50	213	3 healthy
						193	1 irr. leukemia
Exp. 10	4	SIN.SF.wtMpl	KO / 45.2	WT / 45.1	36	295 - 355	4 cytopenic/MDS
	4		KO / 45.2	WT / 45.1		322 - 355	4 healthy
	2		WT / 45.2	WT / 45.1		288, 355	2 healthy
Exp. 11	5	SIN.MplP.wtMpl	KO / 45.2	WT / 45.1	3	152	2 healthy
						20 - 29	3 no engrfatment
	5	SIN.PGK.wtMpl	KO / 45.2	WT / 45.1	39	152	1 healthy
						62	1 cytopenic
						20 - 26	3 no engrfatment
	5*	SIN.SF.wtMpl	KO 45.2	WT / 45.1	33	19 - 29	5 no engraftment
	5*	SIN.SF.GFP	KO 45.2	WT / 45.1	49	8 - 29	5 no engraftment
Exp. 12	5**	SIN.MplP.wtMpl	KO / 45.2	WT / 45.1	4		5 healthy
	5**	SIN.PGK.wtMpl	KO / 45.2	WT / 45.1	51		5 healthy
	5**	SIN.SF.wtMpl	KO / 45.2	WT / 45.1	36		4 healthy
						10	1 no engraftment
	5**	SIN.SF.GFP	KO / 45.2	WT / 45.1	56		4 healthy
						7	1 no engraftment
	2		KO / 45.2	WT / 45.1			2 healthy

* SIN.SF.wtMpl (n=5) and SIN.SF.GFP (n=5) died of engraftment failure in the first four weeks
** 200,000 KO BM competitors

Table 3.5: Performed *in vivo* experiments. # running number of experiment, mice numbers, vectors and transgenes, donors, recipients and its chimerism, transgene in percent before BMT, observation time after BMT and diagnosis are listed. Each experiment was an individual transplantation experiment using the protocol described in the Materials and Methods section. Modifications are described in Chapter 3.3. Abbreviations: MDS myelodysplastic like syndrome, irr. leukemia irradiation derived donor leukemia. Exp. 12 histology has to be analyzed by a pathologist to confirm that animals have no abnormalities in hematopoiesis.

However, the changes were moderate compared to the alteration observed in mice that received LTR vector-transduced BM (Chapter 3.1). WBC, RBC and PLT counts were significantly altered compared to the GFP controls (Figure 3.21C). Interestingly, expression under the Mpl promoter fragment left PB cell counts almost unchanged (Figure 3.21C). Cell counts of mice transplanted with SIN.MplP.wtMpl-transduced BM cells showed only a moderate increase in WBC counts and no change in RBC and PLT counts compared to the animals receiving SIN.SF.GFP transduced BM cells. An MDS-like feature with high amounts of mast cells could be observed in mice receiving SIN.PGK.wtMpl transduced BM cells in histopathology (Figure 3.21D, E) but was absent

in mice transplanted with SIN.MplP.wtMpl transduced BM cells. This may result from a direct effect of the Mpl overexpression or by an indirect effect of Mpl upregulating other factors like SCF or IL-4 which also results in increased mast cell proliferation (Bischoff et al., 1999). It can thus be concluded that the restriction of Mpl expression by MplP will be of great value for the development of gene therapy for CAMT.

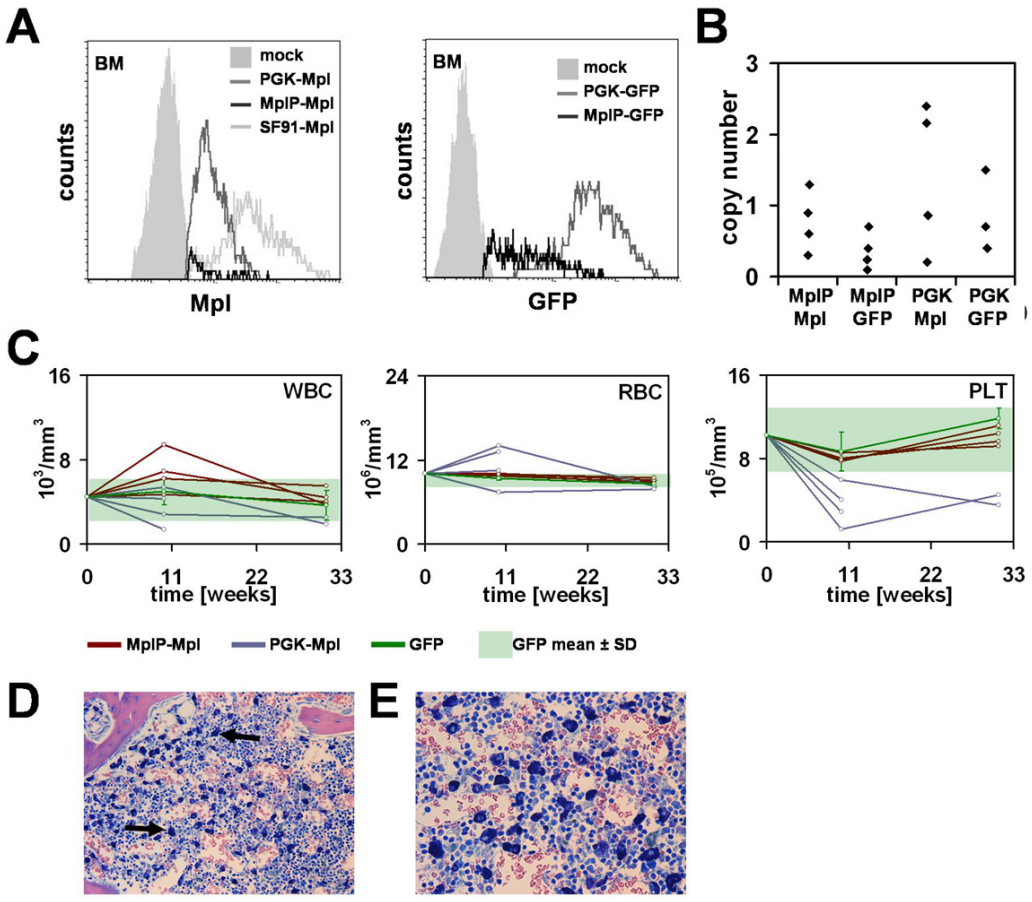

Figure 3.21: Transgene expression level correlates with side effects. (A) The transgene expression levels by different promoters and vector constructs are shown. Expression of two gamma-retroviral self inactivating (SIN) vectors compared to SF91.wtMpl transduced Lin- BM cells before transplantation (Exp. 9) are shown. Counts are presented in linear scale and transgene expression in logarithmic scale (log10). Constructs expressed either wtMpl or GFP under the control of the PGK promoter or the endogenous Mpl promoter (MplP). wtMpl expression driven by MplP was strongly reduced compared to SF enhancer-promoter driven vector (SF91) and PGK promoter. (B) Graph shows copy numbers of mice five months post transplantation from BM cells. All animals had relevant gene marking. (C) Diagram of the development of PB parameters over an observation time of 30 weeks. WBC, RBC and PLT counts were normal for SIN.MplP.Mpl (brown lines) transplanted animals compared to GFP control group (mean ± SD, green with light green bar for area of SD, n=8). In

contrast, mice receiving cells expressing wtMpl from the PGK promoter (blue lines) showed severe alterations especially in PLT counts (zero time points in both graphs originate from the analysis of wild type C57Bl6/J mock mice (n=4): WBC 4.5±1.1 10^3/µL, RBC 10.1±0.5 10^6/µL, PLT 10±54 10^5/µL). (D, E) BM of PGK animals by final analysis showed increased mast cell proliferation as seen in MDS-like state in wtMpl and dnMpl transplanted animals (Chapter 3.1). Magnification and staining: (D) 200x, Giemsa, (E) 400x, Giemsa.

3.3.4. $Mpl^{-/-}$ cells can differentiate into megakaryocytes after transduction with Mpl-expressing retroviral vectors

To show that the new gamma-retroviral SIN vector constructs are able to mediate therapeutic benefit, we developed a Mk differentiation assay. Lin- BM cells from wild-type mice were stimulated for 14 days in StemSpan media containing Thpo, SCF, and IL-6. Cells were then spun on glass slides and stained for histological analysis by May-Grünwald/Giemsa staining. Lin- BM cells produced a huge amount of Mk using the differentiation media (Figure 3.22B, D, E) compared to cells stimulated with the normal BM expansion media (StemSpan, IL-3, SCF, IL-11, Flt-3L) (Figure 3.22A, C). Furthermore, the Mk-forming capacity of $Mpl^{-/-}$ cells derived from knock-out mice was tested. $Mpl^{-/-}$ mice have a disruption of the *c-mpl* locus by homologous recombination of the first five exons resulting in a complete abrogation of Mpl function. $Mpl^{-/-}$ mice have unchanged WBC (3.2 ± 0.9 x 10^3/µL) and RBC (9.0 ± 1.2 x 10^6/µL) counts but strongly reduced PLT counts (71.5 ± 25.8 x 10^3/µL, n=6) in the PB and about 10 times less Mk and HSC in the BM (Alexander et al., 1996b). We transduced $Mpl^{-/-}$ Lin- BM cells with the SIN.SF.wtMpl vector construct and cultivated the cells in the Mk differentiation media. Cells were found to form Mk 12 days after stimulation (Figure 3.22F, G, H). The May-Grünwald/Giemsa staining revealed the multilobed nucleus that forms after polyploidization. The enormous size gain of Mk compared to other WBC is clearly seen. To conclude, the new vectors could mediate sufficient expression of the transgene to drive differentiation in transduced $Mpl^{-/-}$ to Mk as shown in Mk differentiation media.

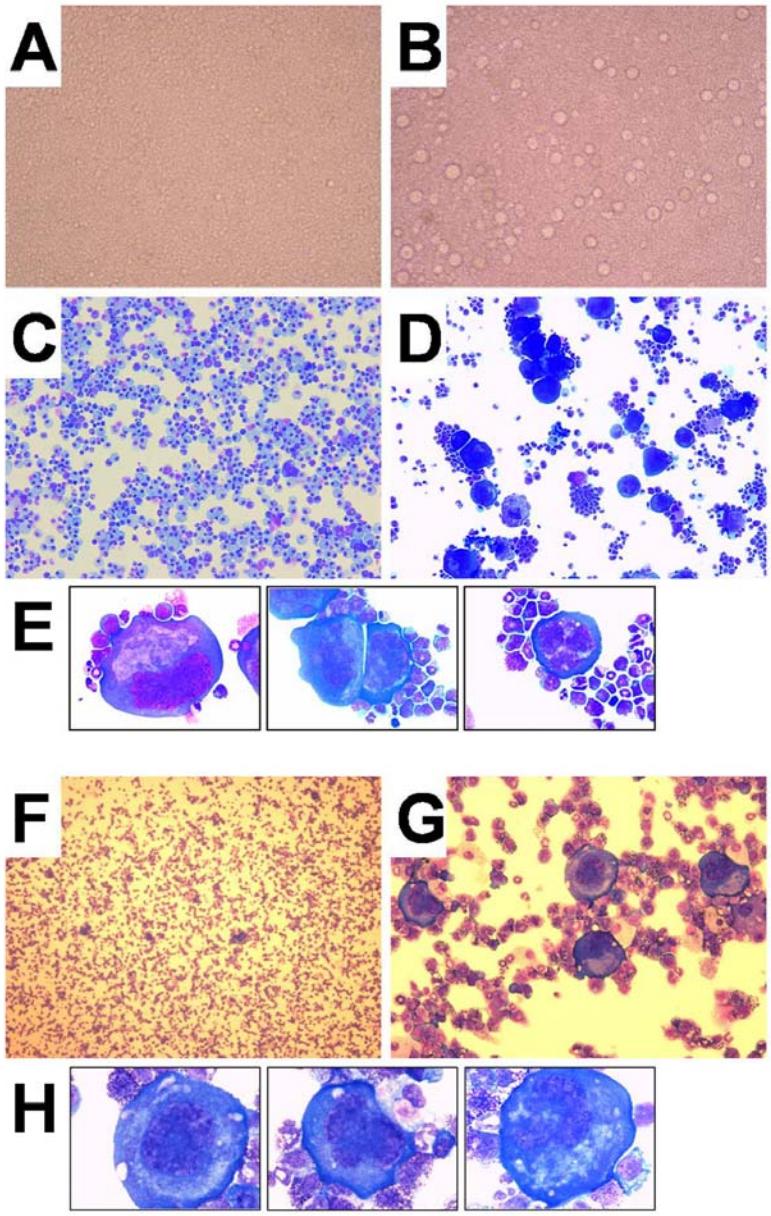

Figure 3.22: Megakaryocyte differentiation assay. (A, C) Lin- BM cells were cultured in StemSpan media containing IL-3, IL-11, SCF and Flt-3L for 14 days without transduction. (A) Suspension culture and cytospins (C) show no megakaryocytes. (B, D, E) Lin- BM cells were cultured in StemSpan containing Thpo, SCF and IL-6 for 14 days without transduction. Megakaryocytes can be seen in suspension culture (B) and cytospins (D). Cytokine conditions drove megakaryocyte development. (F, G, H) Lin- BM cells from $Mpl^{-/-}$ (knock out) animals were cultured in the megakaryocyte differentiation media (Thpo, SCF, IL-6) for 12 days after transduction with the SIN.SF.wtMpl construct. Megakaryocytes can be seen on cytospins after May-Grünwald/Giemsa staining. Magnification: (A, C, F) 200x, (B, D) 400x, (G) 600x, (E, H) 1000x.

3.3.5. Mpl gene-corrected $Mpl^{-/-}$ cells engraft long-term and are detected in the peripheral blood after BMT

After the promising *in vitro* results we further tested the new gamma-retroviral SIN vector constructs in $Mpl^{-/-}$ BMT experiments. Initial experiments in which gene-corrected $Mpl^{-/-}$ BM cells were transplanted into 10 Gy-irradiated $Mpl^{-/-}$ mice did not show long-term engraftment of BM cells and transplanted recipient mice died soon after BMT. It became obvious that $Mpl^{-/-}$ mice were more sensitive to condition regimes than the syngenic C57Bl6/J $Mpl^{+/+}$ mice. To overcome these transplantation problems, we transplanted gene-corrected $Mpl^{-/-}$ CD45.2 BM cells into lethally irradiated (10 Gy) CD45.1 $Mpl^{+/+}$ recipient mice. The first group of mice received $Mpl^{-/-}$ Lin- BM cells transduced with the SIN.SF.wtMpl vector construct (n=4), the second group received untransduced $Mpl^{-/-}$ Lin- BM cells as a negative control (n=4) and finally the last group received $Mpl^{+/+}$ Lin- BM cells as a positive control (n=2) (Exp. 10, Table 3.5). Following this strategy, all animals survived the BMT. The Mpl transgene was detectable in the PB. We also distinguished between donor (CD45.2) and recipient (CD45.1) cells. 6, 16, 25 and 39 weeks after BMT PB samples were taken. We measured the CD45.2 (donor) fraction and 6 weeks after BMT we found up to 90% CD45.2-positive cells which were then decreasing over time until a steady state was reached between 10% and 35% positive cells at 39 weeks post transplantation (Figure 3.23A). Interestingly, the Mpl-positive fraction in the $CD45.2^{+}$ cells first increased up to 50% and finally ranged between 10% and 40% (Figure 3.23B). PB counts showed no severe alterations for WBC and RBC. Interestingly, PLT counts were statistically significantly reduced compared to the control group (untransduced $Mpl^{+/+}$ BM) (Figure 3.23C). Similar to that seen for the LTR-driven vectors in Chapter 3.1, Mpl expression was still too high in SIN.SF.wtMpl vectors resulting in decreased PLT counts may have prevented a selective advantage in HSC.

In a subsequent experiment involving the vectors driven by the MplP, PGK and SF promoter-expressing wtMpl, most of the animals died shortly after BMT of graft failure (Exp. 11). Only two animals in the MplP group and one in the PGK group survived until final analysis after five months.

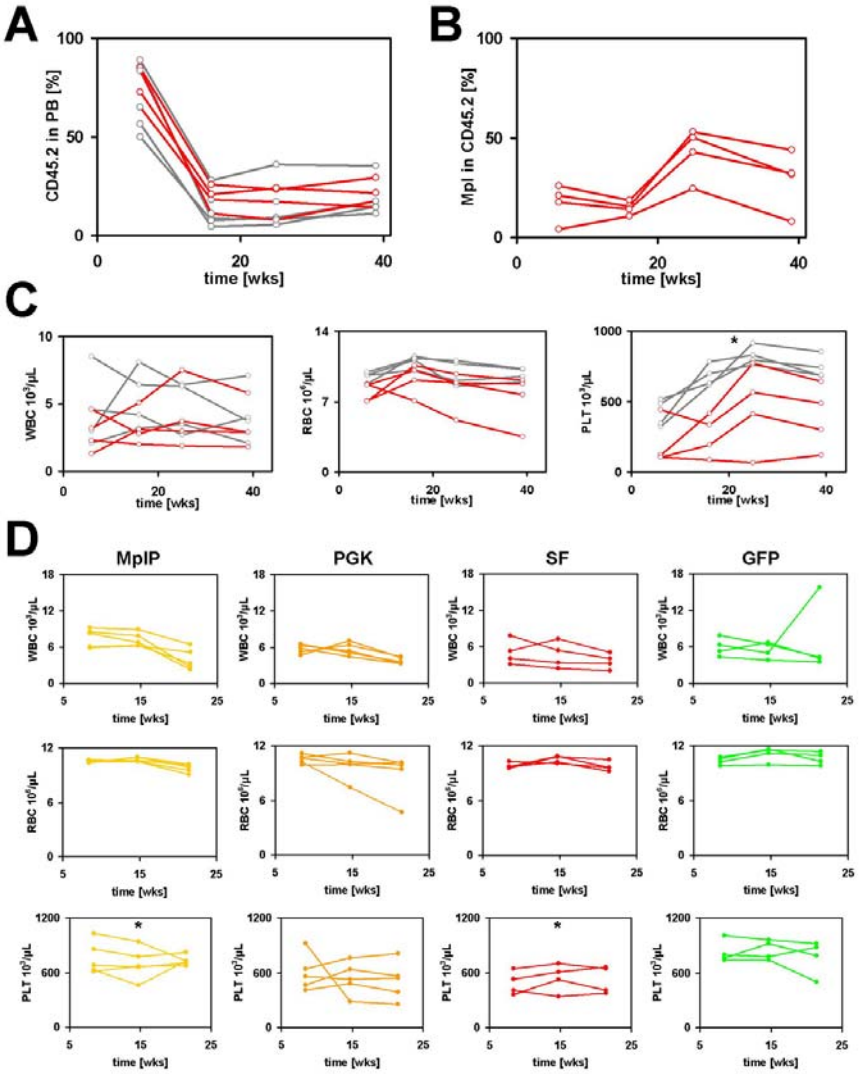

Figure 3.23: PB analysis of mice transplanted with wtMpl transduced Mpl$^{-/-}$ cells. (A) Lethally irradiated Mpl$^{+/+}$ mice received either transduced (red, SIN.SF.wtMpl) or untransduced (grey) Lin- Mpl$^{-/-}$ BM cells in a BMT experiment. The graph shows the donor fraction (CD45.2 positive cells) in the PB at different time points of observation. Donor cells decreased over time until they reached a steady state after about 18 weeks. (B) Graph represents the same PB data as seen in A. Stained are the Mpl positive cells via HA-tag in the CD45.2 positive fraction. Despite the decrease of the donor fraction in the periphery wtMpl positive Mpl$^{-/-}$ cells increased until they reached a steady state. (C) WBC, RBC and PLT from Exp. 10 are displayed (SIN.SF.wtMpl in red, untransduced Mpl$^{+/+}$ BM cells in grey). WBC and RBC showed no alterations whereas PLT counts from wtMpl expressing animals were decreased compared to the control animals (*P ≤ 0.03, t-test, two tailed, unequal variations). (D) PB data are shown from Exp. 12. WT mice were transplanted receiving Mpl$^{-/-}$ competitors. SIN vectors expressing either wtMpl or GFP driven by the SF, PGK or the MplP are shown. WBC and RBC data displayed no difference during observation time. PLT data showed a significant difference of PLT counts in the PB of MplP and SF promoter wtMpl driven groups. MplP animals had normal PLT counts compared to the GFP control group whereas the SF group had lower PLT counts during whole observation time (*P < 0.0004, t-test, two tailed, unequal variations). The MplP promoter avoids side effects of ectopic Mpl expression.

Interestingly, these were the groups with the low expressing promoters, leading to the assumption of graft failure from too high expression of Mpl as in the SF group or from lacking Mpl expression as in the SIN.SF.GFP control group.

We then performed the same BMT experiment, but the recipient mice received 200,000 $Mpl^{-/-}$ competitor cells. We compared the potential of the three gamma-retroviral SIN vectors containing the internal promoters SF, PGK and MplP: SIN.MplP.wtMpl (n=5), SIN.PGK.wtMpl (n=5), SIN.SF.wtMpl (n=5) and as a control the SIN.SF.GFP (n=5) vector construct (Exp. 12). Lin- $Mpl^{-/-}$ BM cells were transduced and transplanted with the competitors to ensure efficient short-term engraftment. Two animals died after only two weeks of engraftment failure (SIN.SF.wtMpl, SIN.SF.GFP). However, all other animals (18/20) survived long-term. Regular measurements of the PB cell parameters showed that there were no alterations in hematopoiesis over time for all constructs in WBC and RBC (Figure 3.23D). Interestingly, PLT counts of the SF group were statistically significantly lower (*$P < 0.0004$, t-test, two tailed, unequal variations) during the whole observation time compared to the MplP group which stayed in the normal range for PLT counts compared to the GFP group (Figure 3.23D). Transgene-positive cells of either construct driven by the SF, PGK or MplP promoter were hardly detected in the PB by flow cytometry. To conclude, transduced $Mpl^{-/-}$ cells could engraft $Mpl^{+/+}$ mice after BMT. The Mpl-transduced cells had a slight growth advantage compared to untransduced $Mpl^{-/-}$ cells (Figure 3.23B), by decreasing CD45.2 cells, similar to that seen in Chapter 3.1. Mpl expression by MplP left hematopoiesis unchanged whereas the SF-driven Mpl expression showed reduced PLT counts most likely due to ectopic overexpression of the transgene.

3.3.6. Mpl gene-corrected $Mpl^{-/-}$ bone marrow cells contribute to the LSK fraction after BMT

CD45.2 (donor) positive cells were found in the PB and in the BM fraction (Figure 3.24A, Exp. 12). To confirm the vector integration we determined the vector copy number via qPCR in the genomic DNA of the whole BM. After correction for CD45.2 positive cells in the BM (black) when available or alternatively in the PB (red) we found relevant gene marking in all animals (Figure 3.24B).

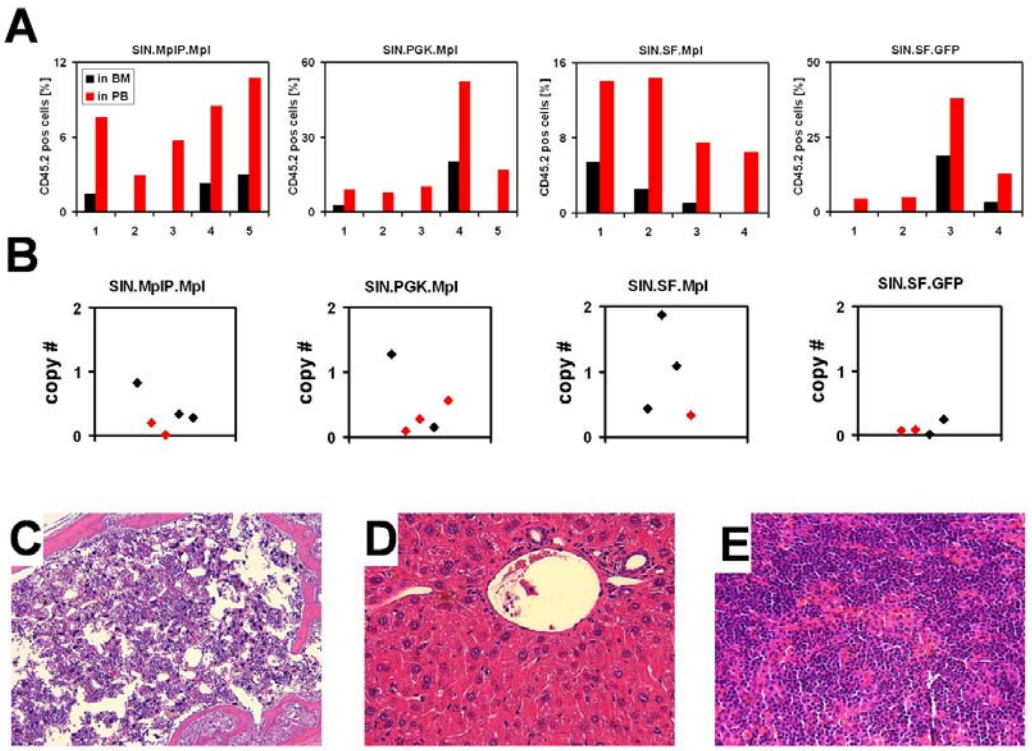

Figure 3.24: Gene marking and histology. (A) Flow cytometry data of BM (black) and PB (red) measurements of CD45.2 (donor) positive cells in $Mpl^{+/+}$ animals transplanted with SIN transduced $Mpl^{-/-}$ BM cells by final analysis. The animals showed reduced CD45.2 positive cells in the BM compared to the CD45.2 positive cells in the PB. The CD45.2 fraction was not analyzed in the BM in the following animals: SIN.MplP.Mpl 2, 3, SIN.PGK.Mpl 2, 3, 5, SIN.SF.Mpl 4 and SIN.SF.GFP 1, 2. (B) The vector copy number in the BM of animals receiving retrovirally transduced BM cells was determined by qPCR. Values from whole BM were corrected for the CD45.2 positive fraction in the BM (black) when available or in the PB (red). All animals showed relevant gene marking in the transduced $Mpl^{-/-}$ cells. (C, D, E) Histopathology analysis of SIN.SF.wtMpl transplanted mice from Exp. 10 shows dysplasia in the histopathology. The BM (C) shows increased mast cell proliferation. The liver (D) and the spleen (E) have no abnormalities. Magnification and staining: (C) Giemsa, 200x, (D, E) Giemsa, 100x.

Interestingly the gene marking for the SIN.SF.GFP transduced cells was the lowest compared to the other constructs which might be due to the absence of $Mpl^{-/-}$ correction. The expression of Mpl by the MplP was difficult to measure in the PB due to the differentiation-specificity or to the low expression. However, Mpl expression will be higher in the HSC compartment therefore we determined the RNA expression level of endogenous Mpl in different cell fractions of the BM of WT mice. GMP, CMP, MEP, MPP (multipotential progenitor), LT (long-term HSC), ST (short-term HSC) fractions

were analyzed via qPCR in one experiment and Mpl expression in the GMP fraction was set to one (Figure 3.25A). We found the highest expression of Mpl in LT cells with nearly 1900 times increase compared to GMP. Also, MPP (760-fold) and ST cells (116-fold) had higher amounts of Mpl RNA than the GMP fraction. All together the highest expression of Mpl in steady state hematopoiesis was in the LSK fraction resembling MPP, LT and ST fractions where we decided to measure the transgene expression mediated by the MplP.

Flow cytometry analysis of Exp. 10 showed that untransduced $Mpl^{+/+}$ cells can fully engraft $Mpl^{+/+}$ mice (n=2) after BMT. The LSK compartment (Figure 3.25B, first blot) was mainly comprised of CD45.2 donor cells (Figure 3.25B, second blot). However, untransduced $Mpl^{-/-}$ cells were hardly detected at final analysis after 12 months in the LSK fraction (n=4) (Figure 3.25B, third blot). Here, most of the cells in the LSK fraction were derived from the recipient (CD45.1). Importantly, SIN.SF.wtMpl gene-corrected $Mpl^{-/-}$ cells could be detected in the LSK fraction after BMT into $Mpl^{+/+}$ mice (Figure 3.25B, fourth blot, Exp. 10). Here most of the cells were derived from the donor animal and were CD45.2-positive (82.3%) and also wtMpl-positive (13.5%). Not all $CD45.2^{+}$ cells seemed to be transgene-positive. It may be possible that the expression of the Mpl transgene in the CD45.2 cells was so low that it could not be efficiently detected by FACS staining and therefore CD45.2 cells may appear false-negative.

In another experiment (Exp. 12), following the same setup, $Mpl^{+/+}$ animals were transplanted with SIN.MplP.wtMpl, SIN.PGK.wtMpl, SIN.SF.wtMpl and SIN.SF.GFP Lin- $Mpl^{-/-}$ BM-transduced cells. Again, wtMpl-positive cells were detected for SIN.MplP.wtMpl (14.4%) transduced cells in the LSK fraction by final analysis after five months observation time (Figure 3.25C). Also, transgene-positive cells for the other promoter-driven constructs were found in the LSK fractions with the SF (8.1%) promoter (Figure 3.25D) and the PGK (9.3%) promoter (Figure 3.25E). Surprisingly for the GFP-expressing group, GFP (10.2%) positive CD45.2 positive $Mpl^{-/-}$ cells were also found (Figure 3.25F). This is most likely due to a shorter observation time (5 months) compared to the Exp. 10 (12 months) where no CD45.2 cells could be seen without Mpl transduction.

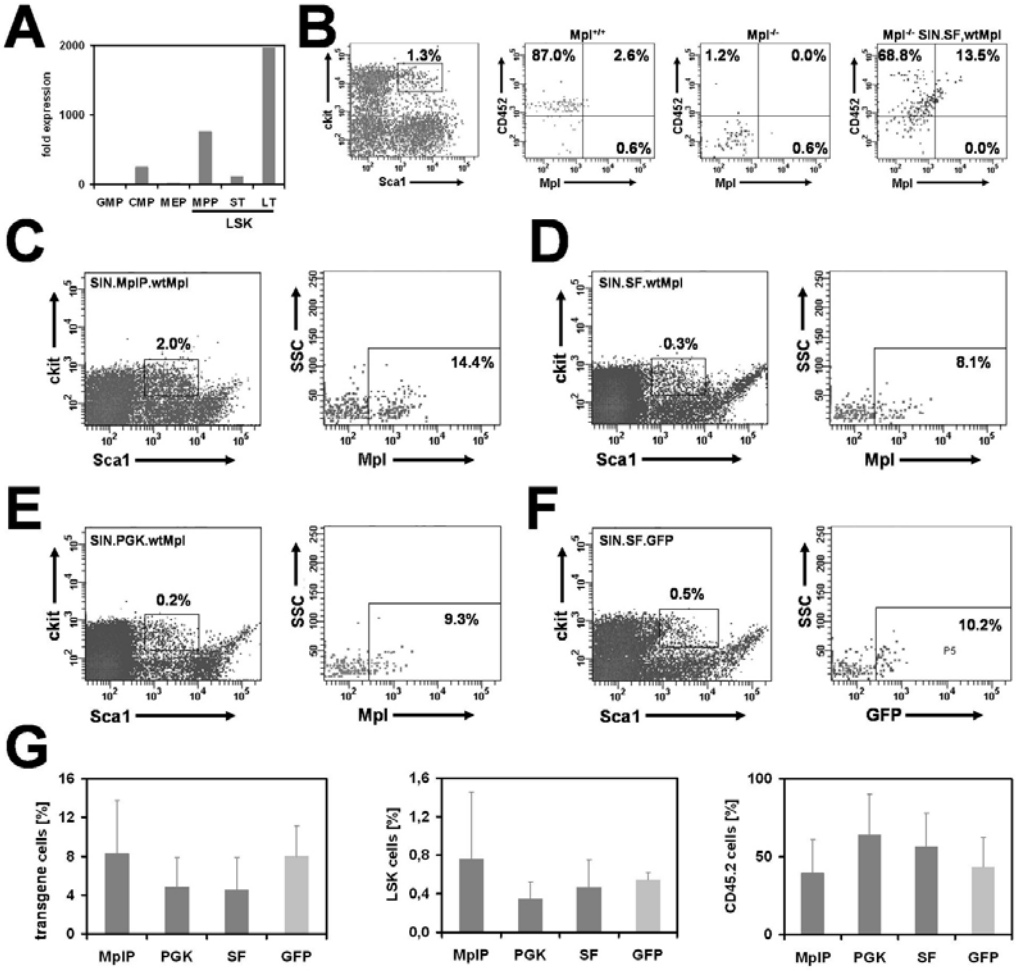

Figure 3.25: LSK fraction analysis. (A) RNA was prepared from sorted cell fractions of wild type mice and analyzed for endogenous Mpl expression after RT-PCR in the qPCR. Values were calculated after the comparative C_T method. Fold expression of fractions is shown compared to GMP (granulocyte monocyte progenitor) cells which was set to one. CMP (common myeloid progenitor), MEP (megakaryocyte erythroid progenitor), MPP (multipotential progenitors), ST (short term HSC), LT (long term SHC) built the analyzed fractions. The last three fractions (MPP, LT and ST) belong to the LSK (Lin-, Sca1+, ckit+) fraction which represents the highest amount of Mpl RNA in the BM of steady state hematopoiesis in wild type mice (one experiment measured in triplicates). (B) FACS blots from CD45.1 WT mice transplanted with transduced or untransduced CD45.2 $Mpl^{-/-}$ and WT CD45.2 BM cells from Exp. 10 are shown. First blot represents the LSK fraction. Second blot is a WT CD45.1 mouse which received WT untransduced BM cells. All LSK cells originated from the donor. The third blot shows one animal which received untransduced $Mpl^{-/-}$ BM cells for transplantation. Almost all LSK cells originated from the recipient mouse. The last blot shows one animals which received SIN.SF.wtMpl transduced $Mpl^{-/-}$ BM cells. Here most of the cells in the LSK fraction originated from the donor and some were transgene positive. (C) Mpl transgene positive cells could also be found with the MplP expressing construct. All the other promoter driven constructs, SF (D), PGK (E) and the GFP expressing control group (F) showed transgene positive cells in the LSK fraction. (G) The first graph shows the mean ± SD of transgene positive cells in the LSK fraction. The second graph represents the mean of all animals for the LSK fraction in the whole BM sample. The third graph displays the donor positive fraction CD45.2 in the LSK fraction. MplP n=5, PGK n=4, SF n=3, GFP n=2.

Taking all mice from Exp. 11 and 12 together, we found the highest number of wtMpl-expression in $Mpl^{-/-}$ cells transduced with the SIN.MplP.wtMpl (mean n=5: 8.3%) vector (Figure 3.25G, first graph). This leads to the conclusion that Mpl expression under the control of the MplP was close to the physiological expression level compared to SF (mean n=3: 4.6%) and PGK (mean n=4: 4.8%) promoter-driven vectors. This may have resulted in a positive selection advantage of MplP-transduced cells compared to the Mpl expression before transplantation (MplP: 4.0%, PGK: 51.0%, SF: 36.0%) seen in Table 3.5, Exp. 12. The MplP-driven group received about 10 time less Mpl positive cells as detected by flow cytometry. However, after BMT the MplP-driven group presented with the highest amount of transgene positive cells in the LSK fraction (Figure 3.25G, first graph). The MplP-driven group also showed the highest amount of LSK (mean, MplP: 0.8%, PGK: 0.4%, SF: 0.5%)-positive cells (Figure 3.25G, second graph) by similar amounts of CD45.2 (mean, MplP: 39.6%, PGK: 64.0%, SF: 56.4%) cells in the LSK fraction compared to the other vectors (Figure 3.25G, third graph). Taken these results together with the vector copy number from Mpl transduced and GFP transduced cells (Figure 3.24B) the data support the hypothesis of a selection advantage for the SIN.MplP.wtMpl vector in the $Mpl^{-/-}$ background.

All mice stayed macroscopically healthy during the whole observation time. However, animals receiving the SF or the PGK promoter driven vectors showed slight dysplasias in the BM with increased mast cell proliferation (Figure 3.24C, D, E), similar to the MDS-like disorder seen in Chapter 3.1. Interestingly these symptoms were absent in the MplP-driven group. To conclude, the MplP-driven construct shows very promising results in long-term reconstitution of lethally irradiated $Mpl^{+/+}$ mice with gene-corrected $Mpl^{-/-}$ cells. PB data did not change during the whole observation time and mice showed no signs of disease after histopathologic analysis. Cells expressing wtMpl via the MplP seem to have a positive selection advantage compared to SF or PGK promoter-driven transgene-expressing constructs most likely due to physiological expression levels and differentiation specificity. This vector construct should mediate a clear therapeutic effect. However, this is still to be shown in BMT experiments with $Mpl^{-/-}$ cells into $Mpl^{-/-}$ recipients.

3.3.7. SIN vector architecture can reduce the risk of malignant clonal dominance

To address the question whether self-inactivating vector architecture has advantages compared to LTR-driven vectors regarding insertional mutagenesis, we compared the diagnosis of all SIN vector Mpl-expressing animals with the LTR animals. Mice receiving SIN vectors (in $Mpl^{+/+}$ and $Mpl^{-/-}$ background) should have a lower susceptibility to insertional mutagenesis resulting in leukemia compared to LTR-driven retroviral vectors (Modlich et al., 2006; Modlich et al., 2008). In total we transplanted 31 (Exp. 9 – 12, Table 3.5) mice with SIN vectors harboring different internal promoters and the wtMpl transgene. None of the animals developed leukemia despite relevant gene marking (Figure 3.21B and Figure 3.24B) compared to the LTR-driven vectors where 3/27 mice developed vector-derived leukemias, as shown in Chapter 3.1. From the *in vivo* studies, we can conclude that the risk of leukemia development seen with the LTR-driven vectors (3/27 mice) is reduced in the SIN vector constructs (0/31). However, cell culture models to determine the replating frequency of the SIN vector constructs should be performed to further analyze the genotoxic risk (Modlich et al., 2006; Zychlinski et al., 2008).

4. Discussion

4.1. Gene therapy for Mpl deficiency is a challenge which can be overcome

The development of a gene therapy for Mpl deficiency is a completely novel strategy to cure CAMT. The only therapy which can be used today is allogeneic BMT. In three previous studies, Mpl was retrovirally overexpressed in the murine BM: Yan et al. found an increase of erythropoiesis with subsequent leukopenia and thrombocytopenia (Yan et al., 1999), similar to our study. However, they did not experimentally address the involvement of primitive hematopoietic cells in disease progression nor perform studies to directly address the dominant negative effect of ectopic Mpl on unmodified bystander cells nor the role of transgene expression levels. Others described the development of a myeloproliferative disease (MPD) when using replication-competent Mpl-expressing retrovirus (Cocault et al., 1996). The third study found no effects on HSC differentiation using the human MPL in a mouse model (Goncalves et al., 1997). We demonstrated that, dependent on vector design, the expression level of Mpl heavily influences the experimental outcome. Even the relatively weak PGK promoter did not prevent severe adverse events of retroviral Mpl expression. In contrast, optimized vectors that express Mpl from its endogenous promoter (MplP) induced no alterations in hematopoiesis, despite substantial vector gene marking in the BM and long-term observation. The potential therapeutic benefit could be shown in $Mpl^{-/-}$ BM *in vitro* and by transplanting animals with Mpl-corrected $Mpl^{-/-}$ BM cells. The risk of side effects from insertional mutagenesis were observed and prevented. Also, a possible use of redesigned MPL protein receptors for therapeutic applications was investigated. To conclude, gene therapy of MPL deficiency might be possible for patients suffering from CAMT despite the small therapeutic window.

4.2. Cytopenias involving the MPL receptor or its ligand THPO

When transplanting mice with cells transduced with the high Mpl-expressing vector constructs, most animals developed a severe pancytopenia involving the whole hematopoiesis. MPL and THPO deregulation is seen in many human diseases. Patients with aplastic anemia and thrombocytopenia have elevated THPO levels similar to that seen in our animal model (Ichikawa et al., 1996; Mukai et al., 1996). Diamond-blackfan anemia presents with increased THPO levels, possibly with an attempt to compensate for

impaired erythropoiesis (Pospisilova et al., 2004). HIV-infected patients have significantly elevated MPL receptor levels on the platelet surface and elevated THPO serum levels which can result in thrombocytopenia (Sundell and Koka, 2006). Thrombocytopenia is also a problem in patients with liver cirrhosis. MPL expression levels on platelets are significantly reduced in cirrhotic patients and seem to correlate with the development of the disease (Ishikawa et al., 2002). Because the liver is the major production site of THPO there may be a connection from cirrhosis and MPL deregulation via THPO, which then correlates with a poor disease prognosis. Following this idea, anemia and thrombocytopenia in liver cirrhotic patients may result from THPO/MPL deregulation.

Recently, the influence of the MPL receptor expressed on endothelial cells was investigated. However, the amount of receptor did not contribute to the regulation of circulating THPO levels (Geddis et al., 2006). The model of soluble cytokine receptors (Heaney and Golde, 1996) leading to a dominant negative effect, like circulating antibodies, may also be true for MPL. Naturally occurring truncated forms of the MPL receptor are known, possibly caused by alternative splicing (Millot et al., 2002). Truncated isoforms of Mpl with essential C-terminal peptides target the full-length receptor for degradation and might be a new mechanism for receptor regulation (Coers et al., 2004; Sabath et al., 2002). It is also known that cytoplasmic Mpl receptors can transfer signals like the membrane-bound ones (Otto et al., 2001). Increased numbers of these soluble forms can also be the reason for thrombocytopenia.

Patients treated with recombinant human THPO (rhTHPO) developed THPO-specific antibodies that also acted against endogenous THPO. Their hematopoietic system was severely affected and they eventually developed pancytopenia (Basser et al., 2002; Li et al., 2001). The dominant negative effect conducted by the anti-THPO antibodies is similar to that found in our experiments and results in similar phenotypes. This side effect observed in patients confirms that deregulation of THPO signaling has profound effects on humans. Of note, genetic deficiency of MPL also has a much more striking effect on humans than on mice, since all CAMT patients with a complete absence of MPL develop aplastic anemia in early childhood (Germeshausen et al., 2006), in contrast to the more subtle HSC defect observed in Mpl-deficient mice (Alexander et al., 1996c; Kimura et al.,

1998; Petit-Cocault et al., 2007). We thus postulate that disturbing the regulation of MPL expression in patients may also lead to profound effects involving the regulation and regeneration of the HSC pool. In agreement with that, in preleukemic and leukemic situations, the unregulated expansion of cells that express MPL or other receptors that may sequestrate a crucial niche factor, may contribute to the selective displacement of normal hematopoiesis and further aggravate the disease.

4.3. CMPD and MDS are clonal events in humans and often show deregulation of MPL or THPO

It is important to point out that in our mice developing CMPD and MDS-like disorders no selection for a single transformed clone occurred. Therefore – although the symptoms mimic the respective human pathologies – they do not completely resemble the human pathogenesis. However, recent calculations for clonal dominance in mice suggesting the transformation of one HSC estimate 2.5 ± 0.5 years latency for clonality in contrast to an expected lifespan of a maximum of two years (Catlin et al., 2005). In our murine model, ectopic Mpl expression alone is sufficient to induce those pathologies, thus abrogating the need for clonal selection in the pathogenesis of CMPD and MDS.

CMPD is characterized by excessive hematopoiesis which results from one or more hematopoietic growth factors (Kaushansky, 2006a). In our experiment, CMPD was caused by the overexpression of the Mpl receptor on non-target cells. In human CMPD, Mk and erythroid cells exhibit increased Mpl expression levels (Bock et al., 2004) and abnormal megakaryopoiesis is frequently found in the BM (Duensing et al., 1999). Some patients with CMPD develop acute transformation of blasts with megakaryocytic features similar to our model (Hashimoto et al., 2000). In familial essential thrombocythemia, platelets are strongly increased. This is determined in the upregulation by the hematopoietic growth factor THPO which is caused by a mutation in the promoter region of the THPO gene (Kondo et al., 1998).

The role of THPO signaling in human MDS is largely unknown. Because one typical feature of MDS is abnormal megakaryopoietic development and thrombocytopenia, some studies have focused on the expression of MPL and THPO in MDS patients (Adams et al., 1997; Hofmann et al., 1999). In MDS, deregulation of THPO levels (up- or down

regulation depending on the involved population) were found (Emmons et al., 1996; Ichikawa et al., 1996; van den Oudenrijn et al., 2002) while expression levels of MPL were unchanged or lowered (Tamura et al., 1998). In addition, the intracellular signal transduction of THPO signals appeared to be altered. STAT3 and STAT5 activation was enhanced in CD34 cells in the late phase of MDS progression (Kalina et al., 2000). Dysmegakaryopoiesis in MDS patients could not be treated with addition of THPO (Hofmann et al., 2000). Inadequate THPO response in MDS patients possibly results from overexpression of MPL on the preleukemic clone leading to a dominant negative effect (Zwierzina et al., 1998).

The Mpl receptor is expressed on murine mast cells and deletion of Thpo increases mast cell proliferation (Martelli et al., 2008; Migliaccio et al., 2007). THPO alone also supports mast cell growth from human hematopoietic progenitor cells (Kirshenbaum et al., 2005) which could explain the excessive mast cell proliferation in our model. Osteosclerosis, similar to our case, was also found in mice overexpressing Thpo which could be reduced with proteasome inhibitors (Villeval et al., 1997; Wagner-Ballon et al., 2007). Nevertheless, it may be worthwhile investigating consumption of crucial niche factors, other than THPO, by aberrant cell clones as a confounding factor of human MDS. Competition for microenvironmental support can lead to the suppression of normal hematopoiesis (Catlin et al., 2005). Recently, a potential new ligand for the MPL receptor was found. The human homologue of a fungal nuclear migration protein (hNUDC) was shown to bind to the extracellular domain of MPL and activate proliferation of Mk and platelets (Pan et al., 2005; Wei et al., 2006). Consequently, factors which were not brought into sight of MPL interference so far could also be responsible for CMPD and MDS in patients.

4.4. Hematopoietic population crisis induced by Mpl overexpression resembles gradation effect in population ecology

In previous work, the study of hematopoietic cytokine receptor expression has mostly focused on cell-intrinsic effects regulating cell survival, differentiation and transformation. We chose ectopic expression of Mpl by gamma-retroviral vectors in a competitive BMT model to address both cell-intrinsic and population effects of ectopic

receptor expression. We demonstrate that Mpl-overexpressing hematopoietic cells acquire an initial growth advantage with massively elevated multi-lineage hematopoiesis resembling CMPD with proliferation of blasts in extramedullary sites. From this stage of hyperproliferative hematopoiesis, two different outcomes occurred. A minority of animals (~10%) developed erythroid leukemia, which was dependent on secondary events such as the retroviral insertion sites in known proto-oncogenes encoding transcription factors. In the majority of mice, however, the CMPD resulted in a fatal population crisis, leading to lethal pancytopenia with crucial aspects of MDS. As unmodified hematopoietic cells were also involved in the population crisis, and a dominant-negative Mpl receptor expressed in a chimeric situation induced the same phenotype, we hypothesize that consumption of a crucial niche factor, e.g. Thpo, was the major cause of the population crisis.

Recent reports define the function of THPO signaling in the maintenance of HSC quiescence (Qian et al., 2007; Yoshihara et al., 2007). These studies suggest that the depletion of THPO results in higher HSC cycling with potential exhaustion. Analysis of the stem and progenitor cells in mice during the different stages of disease progression in our study showed an augmentation of CMP, GMP and MEP shortly after transplantation and reduction of LSK as well as CMP, GMP and MEP cells during pancytopenia development, whereas mice expressing the dominant negative receptor lacked the early increase of hematopoiesis. Even prior to the onset of pancytopenia, we could show in CFU assays of wtMpl-transplanted mice that BM cells of these animals showed a significantly reduced colony formation capacity compared to GFP-expressing cells. All these phenotypes involved co-existing unmodified cells, arguing against a predominant role of cell-intrinsic effects such as exhaustion in the development of pancytopenia and MDS.

The successive rise and fall of hematopoiesis induced by overexpression of Mpl on a subset of cells perfectly matches the phenomenon of gradation in population ecology, with a phase of rapid population expansion addressed as prograd ation, until the peak of the population (culmination) occurs with a subsequent decline addressed as retrogradation (Schwerdtfeger, 1932; Stoecker and Dietrich, 1986). The term gradation, originally used in the context of population ecology, can describe the progression seen in our model from

CMPD (progradation) to pancytopenia and MDS (retrogradation) after ectopic expression of Mpl on a subset of BM cells (Figure 4.1). To our knowledge, this striking transition from cell-intrinsic to population effects is unprecedented in studies addressing the role of cytokine receptors. We would therefore suggest to include population dynamics as an important readout in future studies addressing the role of cytokines and their receptors in tightly balanced regenerative systems such as hematopoiesis.

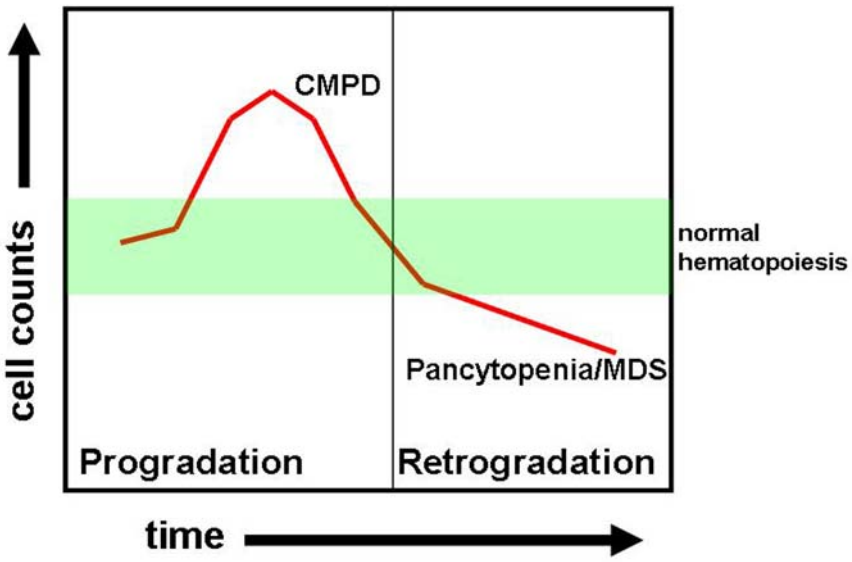

Figure 4.1: Schematical description of the population effect (adapted from Stoecker and Dietrich, 1986). Cell counts of hematopoiesis rise during progradation phase which resembles CMPD in mice some weeks after transplantation with wtMpl transduced BM cells. When a crucial niche factor (e.g. Thpo) is exhausted, HSC reduce proliferation and differentiation. Cell counts of PB start to decrease. When sequestering effects are too high cell counts of all hematopoietic lineages are reduced below normal hematopoiesis level. Mice then turn into retrogradation phase which we could describe as pancytopenia with MDS. The rise and fall of hematopoiesis resembles the gradation effect used in ecology for many years to describe the progradation and retrogradation phases of a whole animal species (especially insects) to describe their dependence on nutrition and other ecologic factors resulting in high population rates with subsequent decrease after niche factors are exhausted.

4.5. The adaptive mechanism of THPO and MPL influences hematopoietic stem cells

In our experimental system involving hematopoietic cells of regular C57Bl6/J mice, the expression of Mpl under the control of a retroviral LTR resulted in an unrestricted expression in all major hematopoietic lineages. In contrast, endogenous MPL starts to be

expressed in HSC and is then restricted to the megakaryocytic lineage and platelets which regulate THPO levels in the periphery. HSC reside in their special niche in the BM (Yin and Li, 2006) and chemokine-mediated interaction, in addition to THPO/MPL, of hematopoietic progenitors with the BM niche is required for thrombopoiesis (Avecilla et al., 2004). THPO/MPL signaling increases HSC proliferation and self-renewal (Seita et al., 2007; Tong et al., 2007; Yagi et al., 1999). $Mpl^{-/-}$ mice have reduced numbers of HSC because of defective stem cell amplification and self-renewal (Petit-Cocault et al., 2007). Mpl might support early expansion of differentiating stem cells but is not regulating the HSC numbers (Abkowitz and Chen, 2007). THPO induces HoxA9 nuclear transport in immature hematopoietic cells and stimulates HoxB4 expression (Kirito et al., 2003; Kirito et al., 2004) which could be a potential mechanisms by which THPO supports HSC. The discovery that a diverse range of transcription factors are downstream effectors of THPO helps to explain the molecular mechanisms by which the cytokine affects hematopoiesis (Kirito and Kaushansky, 2006). As THPO is constitutively produced in the liver and in the kidneys, intact megakaryopoiesis and high platelet levels are likely to reduce the availability of THPO in the BM niche (Kaushansky, 2006b). This creates a feedback in the regulation of HSC survival and expansion, which is dependent on MPL expression. Strikingly, if Thpo/Mpl signaling is disabled by targeted genetic knock-out in mice, the phenotype is much less severe than the one obtained in our model by unregulated overexpression of either wtMpl or dnMpl. This suggests the existence of adaptive mechanisms of HSC survival as demonstrated by studies in which Mpl knock-out cells engraft and repopulate equally efficiently as wild-type BM cells when transplanted in a Thpo-deficient background (Abkowitz and Chen, 2007). However, such adaptive mechanisms appear to be unavailable or insufficient if Thpo/Mpl homeostasis is perturbed in adult wild type animals as in our studies.

4.6. Activating MPL mutants can induce proliferative disorders in patients: MPD and leukemia

Inadequate THPO levels may serve as an *in vivo* myeloid leukemic growth factor in a significant number of AML patients (Corazza et al., 2006). In AML cells, MPL was shown to be overexpressed on leukemic blasts and expression levels correlated with poor

prognosis from a potential dominant negative effect (Albitar et al., 1999; Schroder et al., 2000; Wetzler et al., 1997). Recently, activating mutations in the human MPL gene have been identified in a subset of MPD patients that were negative for JAKV617F mutations (Pardanani et al., 2006; Pikman et al., 2006). Intracellular crucial regions for point mutations in the MPL receptor are known which can give rise to constitutive activation (Staerk et al., 2006). MPD are grouped in several disorders, some of which emanate from receptor mutations like MPLW515LK (Tefferi and Gilliland, 2007). Constitutive MPL activation leads to JAK-STAT activation and supports the concept of constitutive JAK-STAT signaling in human MPD. MPL may even play a role as part of an autocrine pathway in MPD (Kaushansky, 1998). In addition, THPO overexpression can lead to supraphysiological signaling resulting in similar disorders in mice (Villeval et al., 1997) as also seen in our model.

To address the importance of one other major pathway in MPL-induced leukemia seen with the MPLW515LK mutation, we analyzed the Raf-Ras-MAPK-ERK1/2 pathway. Mutated NRAS can function as an initiating oncogene by inducing myeloid malignancies in mice (Parikh et al., 2006). Oncogenic HRAS promotes abnormal proliferation of CFU-E progenitors and early erythroblasts and supports their erythropoietin (Epo)-independent growth (Zhang et al., 2003) which we also observed *in vitro* with our caMpl mutant. Furthermore, oncogenic KRAS in hematopoietic cells initiates a rapidly fatal myeloproliferative disorder (Braun et al., 2004). These pivotal features of increased RAS signaling lead us to the hypothesis that RAS is the major pathway for leukemia development in constitutively active Mpl. Following this assumption, a truncated Mpl receptor that could not activate the Ras signaling pathway was not leukemogenic in our experiments, which confirmed our hypothesis.

Other pathways, like PI3K signaling, are also crucial to many aspects of cell growth, survival and apoptosis, and their constitutive activation has been implicated in both the pathogenesis and the progression of a wide variety of neoplasias (Martelli et al., 2006). Furthermore, acquired activating mutations in JAK-STAT signaling other than JAKV617F are involved in the pathophysiology of hematological malignancy (Khwaja, 2006). It is thus of great interest to perform detailed analysis on these pathways downstream of Mpl. Small molecule inhibitors (Pardanani et al., 2007) and peptide

agonists of THPO (Cwirla et al., 1997; Erickson-Miller et al., 2005) are tested for possible clinical application in MPD resulting from activating mutations in MPL. These products can become very potent therapeutics in future as therapeutics for MPD where MPL is involved.

In human gene therapy the risk for the patients to develop an MPD-like disorder from a constitutive active mutation is very low. In our mouse model we transplanted BM cells where the majority of these cells was transduced with the caMpl mutant resulting in a fast outgrowth of leukemia. Despite the low possibility to gain a point mutation in one of the critical regions of the MPL receptor, additionally only one cell would carry this mutation and had to undergo selection to become a transformed clone.

4.7. The proto-oncogene Mpl is not leukemogenic *per se*

In our study, only three out of 27 mice developed acute leukemia with an unusual erythroid phenotype. 0/31 mice transplanted with wild-type Mpl receptor in the SIN vector backbone harboring high expressing promoters (SF) or low expressing promoters (PGK, MplP) developed leukemia. SIN vectors and vectors with low expressing promoters have a greatly reduced risk to activate neighboring proto-oncogenes by insertional mutagenesis (Ryu et al., 2008; Zychlinski et al., 2008). This indicates that enhanced Mpl signaling determines the leukemic phenotype but is not the exclusive factor for leukemia induction. In addition, the selection of the specific leukemogenic clones strongly supports the finding that insertional activation of proto-oncogenes by retroviral vector integration was a necessary cofactor. The selection of clones with retroviral vector insertions in the ETS transcription factors Fli-1 or Sfpi-1 (Pu.1) is reminiscent of the pathogenesis of erythroleukemia induced by the friend murine leukemia virus or the friend virus complex, the latter involving a replication-defective virus expressing a glycoprotein, gp55, that stimulates the Mpl-related receptor for erythropoietin (EpoR) (Paul et al., 1991; Schuetze et al., 1993; Starck et al., 1999). The transcription factor Klf3, which plays a pivotal role in erythroid cell differentiation (Funnell et al., 2007), is shown to be deregulated after retroviral insertion *in vitro* (Kirberg et al., 2005).

4.8. Conclusion

In conclusion, we showed that ectopic and unregulated expression of Mpl leads to severe deregulation of hematopoiesis. By expression of a dominant negative Mpl receptor that can bind but not transfer Thpo signals, we could show that Mpl expression inhibits Thpo signaling by a dominant negative effect. An initial, CMPD-like increase of peripheral blood cell counts was restricted to the full-length receptor and therefore induced by Mpl signaling. However, ectopic Mpl expression was not leukemogenic *per se*. Our results highlight the importance for regulated expression of Mpl, and as such probably for many molecules involved in hematopoietin receptor signaling. This has strong relevance for the development of gene therapies for hematopoietic disorders that are based on deficiencies in hematopoietin receptor signaling molecules.

In addition, we showed that the expression of a constitutively active Mpl receptor mutant is highly leukemogenic. Mice developed erythroid- and myeloid leukemias with massive hepatosplenomegaly and strong infiltration in hematopoietic organs. Some human MPD are closely connected to MPL mutants that makes research for THPO/MPL inhibiting targets an up-to-date topic. Downstream MPL signaling inhibitors may be potential therapeutics for Mpl-induced diseases. We showed that truncated receptor mutants lacking defined domains that activate signaling pathways of Mpl, like Ras signaling, are less mutagenic. Mice did not develop leukemia despite constitutive activity.

Thirdly, the determination of the underlying mechanisms that cause pancytopenia and leukemia lead the way to new vector design. Using the MplP in SIN constructs were shown to mediate physiological expression levels and lack the pathogenic side effects. We further showed that $Mpl^{-/-}$ BM cells transduced with these new vectors could successfully repopulate mice after BMT and participate in hematopoiesis. This is a great step towards a therapeutic gene therapy for Mpl deficiency.

4.9. Outlook

We performed a profound molecular and histological analysis of the Mpl-transplanted animals and found no side effects and no dysplasias in mice transplanted with SIN.MplP.wtMpl-transduced BM cells. However, further analysis of secondary transplantations of gene-corrected cells should be performed. It has to be shown that there

are no upcoming side effects like leukemia in secondary recipients resulting from further stress on the transduced cells. Another important result which the secondary transplantation will show is if long-term HSC were transduced and functionally corrected.

Furthermore, the correction of $Mpl^{-/-}$ BM by BMT into $Mpl^{-/-}$ recipients should be shown. Gene-corrected $Mpl^{-/-}$ mice should present normal platelets and HSC numbers. $Mpl^{-/-}$ mice have reduced platelet counts and reduced HSC numbers ten-fold. The rescue of HSC number with the gene therapy of $Mpl^{-/-}$ mice is the proof-of-principle for gene therapy of CAMT with gamma-retroviral or lentiretroviral vectors. This would also be the first evidence of the correction of HSC by function caused by a mutated cytokine receptor.

HSC have low cycling rates and reside relatively quiescently in their niche. MLV vectors - used in this study - are only able to integrate into dividing cells and gain access to the host genome when the nuclear membrane breaks down during mitosis. Lentiviral vectors have the advantage of also transducing quiescent cells. By using lentiviral vectors it may be possible to target more HSC, which is very important for CAMT gene therapy because of the already reduced HSC numbers in patients. An alternative to deal with this problem would be an early collection of HSC from patients. The number of HSC is reduced at birth and further decreases with disease development, finally leading to an aplastic anemia. The continuing reducing numbers of HSC make it difficult for a gene therapy approach to target enough HSC in patients already in final stages using retro- or lentiviral vectors. With HSC collection shortly after birth, the important HSC for a lifetime curative therapy would be very useful. Also new cytokine cocktails are described which maintain the HSC ability *ex vivo* and stimulate the expansion of HSC (Huynh et al., 2008; Zhang et al., 2006). A serum-free culture containing SCF, TPO, FGF-1, angiopoietin-like 5, and IGFBP2 supports an approximately 20-fold net expansion of repopulating human cord blood HSC, a number potentially applicable to several clinical processes including HSC transplantation for CAMT (Zhang et al., 2008).

The differentiation-specific expression of the Mpl promoter fragment could be improved. 2 kb is a large size for a promoter compared to the smaller SF and PGK promoters (391 bp and 512 bp). Identification of a minimally sized promoter is critical for the design of viral vectors in gene therapy. Deletion mutants of the MplP fragment may

give insights into the important regions for the promoter activity. It may be possible to reduce the size of the MplP and increase the differentiation-specificity even further. Geller et al. recently published an algorithm to predict promoter important sites to reduce the size of promoters. They showed that shorter promoter fragments in Müller cells predicted by their program showed increased mean fluorescence intensity of transduced cells by more positive cells (Geller et al., 2008). Another possibility to mediate site-specific activity is the use of small molecular RNA target sequences (Brown et al., 2007). These microRNA target sequences can be recognized by host cell-specific miRNAs which then target the viral mRNA for degradation and thereby restrict the expression to only a particular cell lineage. This approach could be an alternative if the MplP mediates too low expression or if it still is too nonspecific. A stronger ubiquitous promoter like the PGK promoter may be used with the RNA target sequence with restricted expression only to express receptors in HSC and Mk.

As described in the literature, CAMT results from inactivating mutations in the *c-mpl* gene. These mutations can lead to a block of receptor dimerization or if the mutations are in the THPO binding region they can inhibit ligand binding. For patients suffering from the latter mutations, an alternative therapy could be considered. Novel small molecules which mimetic activities of Thpo can be used. These mimetics specifically activate the Mpl receptor and induce increased platelet numbers in mice (Inagaki et al., 2004). They may also bind to the mutated MPL receptor and activate dimerization even if THPO is not able to bind to these sites. Thereby, mimitics can have therapeutic benefits compared to direct THPO application. For these specific groups of CAMT patients, mimetics might have a therapeutic effect which makes a difficult and risky gene therapy approach unnecessary.

Designing an artificial receptor construct as a therapeutic molecule is another approach for gene therapy of CAMT. F36VMpl is such a molecule and induces a sustained expansion of mouse BM cells *ex vivo* and erythroid cells *in vivo* and also induces a response sufficient to accelerate recovery from irradiation-induced anemia (Weinreich et al., 2006). The mutant F36VMpl lacks the ability to bind THPO but can be activated with CID (chemical inducers of dimerization) which should reduce side effects, but also makes it necessary for the patient to take a drug throughout his/her life time

without knowing the consequences (Richard et al., 2004). Also, our nrMpl and cnnMpl constructs, mediating proliferation of transduced cells but lacking leukemogenic potential as the caMpl receptor, could be used to i.e. expand HSC in a transcriptionally regulated promoter vector system or when expressed transiently only.

Finally, a novel strategy for a curative therapy is homologous recombination in induced pluripotent cells (iPS). The therapeutic benefit was recently demonstrated in a murine model of sickle cell disease (Hanna et al., 2007). It was shown that reprogramming of fibroblasts to a pluripotent state could be induced *in vitro* through ectopic expression of four transcription factors (Oct4, Sox2, c-Myc and Klf4). These data demonstrate that pluripotent HSC can be directly generated from fibroblast cultures by the addition of only a few defined factors (Takahashi and Yamanaka, 2006; Wernig et al., 2007). The use of iPS cells as a curative approach for many disorders is thinkable, also for CAMT.

All these above-mentioned strategies are novel potential therapeutic options for curing MPL deficiency resulting in CAMT. However, more work on safety studies and correct dosage finding is needed. This work took the strategy of classical gene therapy and showed the advantages as well as the disadvantages of this technique. It could well be that in the end the combination of two or more strategies will prove to be successful.

5. Literature

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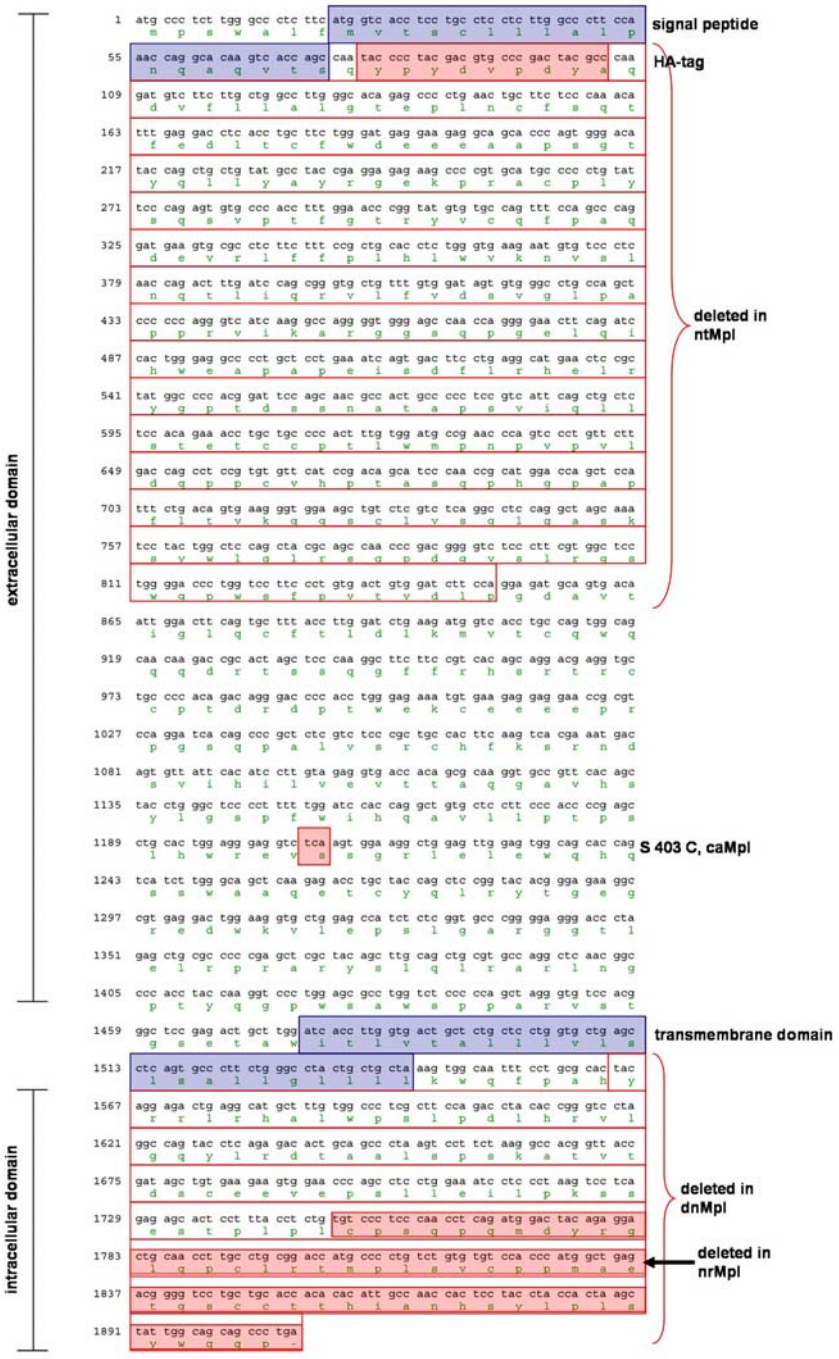
6. Appendix

6.1. Mpl sequence



6.2. Insertion sites of caMpl leukemias (Table 3.4)

#	gene name	CIS (IDDb)	CIS (RTCGD)	gene ID	distance	orientation	name and function
1	1500011H22Rik			68948	131531	R	RIKEN cDNA 1500011H22 gene
2	1500019G21Rik			66245	130979	R	RIKEN cDNA 1500019G21 gene
3	2210408F21Rik	2		73652	54893	F	RIKEN cDNA 2210408F21 gene
4	5730449L18Rik			66637	80930	R	hypothetical protein RIKEN cDNA 5730449L18 gene
5	6030429G01Rik			436022	-21542	R	RIKEN cDNA 6030429G01 gene
6	AI597468			103266	101392	R	expressed sequence AI597468
7	Anapc13			69010	-86482	R	anaphase promoting complex subunit 13
8	Anxa6			11749	60387	F	annexin A6
9	Apobec3			80287	2914	F	apolipoprotein B editing complex 3
10	Arpc2		1	76709	-7954	R	actin related protein 2/3 complex, subunit 2
11	Atpbd1c		1	68080	-131682	F	ATP binding domain 1 family, member C
12	BC013712		1	230787	437	R	hypothetical protein LOC230787 isoform 1, ICB-1
13	BC022224			192970	-63574	R	cDNA sequence BC022224
14	Brsk1			381979	-136944	F	BR serine/threonine kinase 1
15	Btbd14b			66830	27540	F	BTB (POZ) domain containing 14B
16	Car4			12351	-65283	F	carbonic anhydrase 4
17	Cbx7			52609	17618	R	chromobox homolog 7
18	Ccdc63			330188	-102762	R	coiled-coil domain containing 63
19	Ccdc69			52570	105104	F	coiled-coil domain containing 69
20	Cep63			28135	82843	F	Cep63 protein
21	Cry1			12952	-19112	F	cryptochrome 1 (photolyase-like)
22	Dnaic2			432611	-112413	F	dynein, axonemal, intermediate chain 2
23	Dusp1		1	19252	11327	R	dual specificity phosphatase 1
24	Eltd1			170757	-12525	F	EGF, latrophilin seven transmembrane domain containing 1
25	Eps8l1			67425	89242	F	EPS8-like 1
26	Evi1	14	25	14013	-99574	R	ecotropic viral integration site 1
27	Fhl4			14202	-101758	F	four and a half LIM domains 4
28	Ggnbp2			217039	-21822	R	ZN403_MOUSE Isoform 3 of Q5SV77 - Mus musculus (Mouse)
29	Gm2a			14667	-125090	R	GM2 ganglioside activator protein
30	Golga5			27277	25625	F	Golgi autoantigen, golgin subfamily a, 5

#	gene name	CIS (IDDb)	CIS (RTCGD)	gene ID	distance	orientation	name and function
31	Gpx3			14778	70077	R	glutathione peroxidase 3
32	Hint1		1	15254	106589	R	histidine triad nucleotide binding protein 1
33	Hivep3		4	16656	144029	R	human immunodeficiency virus type I enhancer binding protein 3
34	Hoxa7	2		15404	67	R	homeo box A7
35	Hvcn1			74096	31087	F	hydrogen voltage-gated channel 1
36	Ier2		4	15936	2553	F	immediate early response 2
37	IL1r1	1		16177	45225	R	Interleukine 1 receptor type I, CD121a, b
38	Il8ra			227288	-34080	F	interleukin 8 receptor, alpha
39	Itgam		1	16409	-514	F	Integrin alpha M, CD11b, myeloid marker, similar to centaurin, gamma 2
40	Kif22		1	110033	13298	F	kinesin family member 22
41	Ky		1	16716	37143	R	kyphoscoliosis peptidase
42	LOC667832			667832	145784	F	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1
43	Lyl1		3	17095	-41162	R	lymphoblastomic leukemia
44	Lyrm7			75530	-112111	F	LYR motif containing 7
45	Maz			17188	-1854	F	MYC-associated zinc finger protein (purine-binding transcription factor)
46	Mrm1			217038	-73045	R	mitochondrial rRNA methyltransferase 1 homolog (S. cerevisiae)
47	Mterfd3			74238	-76165	F	MTERF domain containing 3
48	Myl2			17906	139845	F	myosin, light polypeptide 2, regulatory, cardiac, slow
49	Ndst1			15531	34686	F	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1
50	Nfix		6	18032	140045	F	nuclear factor I/X
51	Pigw			70325	-12275	R	phosphatidylinositol glycan anchor biosynthesis, class W
52	Plau			18792	-7549	F	plasminogen activator, urokinase
53	Ppp1cc			19047	82545	F	protein phosphatase 1, catalytic subunit, gamma isoform
54	Ppp1r12c		2	232807	-52304	R	protein phosphatase 1, regulatory (inhibitor) subunit 12C
55	Pptc7			320717	-43574	F	PTC7 protein phosphatase homolog (S. cerevisiae)
56	Prkd2			101540	-888	R	protein kinase D2
57	Prr16			71373	-104128	F	proline rich 16
58	Ptprh			545902	50057	R	protein tyrosine phosphatase, receptor type, H
59	Rad9b			231724	113371	R	RAD9 homolog B (S. cerevisiae)
60	Rarg		3	19411	14049	F	retinoic acid receptor, gamma

#	gene name	CIS (IDDb)	CIS (RTCGD)	gene ID	distance	orientation	name and function
61	Rdh13			108841	-108375	R	retinol dehydrogenase 13 (all-trans and 9-cis)
62	Rpl38		1	67671	-44123	F	ribosomal protein L38
63	Saps1		2	243819	104966	R	SAPS domain family, member 1
64	Sertad1			55942	3292	F	SERTA domain containing 1
65	Smndc1		1	76479	-14996	F	survival motor neuron domain containing 1
66	Specc1			432572	101743	R	spectrin domain with coiled-coils 1
67	Spon1		1	233744	14981	R	spondin 1, (f-spondin) extracellular matrix protein
68	Synpo			104027	-26672	F	synaptopodin
69	Syt5			53420	-7417	R	synaptotagmin V
70	Tmem86b			68255	76498	R	transmembrane protein 86B
71	Tnip1			57783	-10115	F	TNFAIP3 interacting protein 1
72	Tnni3			21954	-31541	R	troponin I, cardiac
73	Tnnt1			21955	-38009	R	troponin T1, skeletal, slow
74	Trmt1			212528	-28983	R	TRM1 tRNA methyltransferase 1 homolog (S. cerevisiae)
75	Ttyh2			117160	-60469	F	tweety homolog 2 (Drosophila)
76	Vps29			56433	-113589	F	vacuolar protein sorting 29 (S. pombe)
77	Znhit3			448850	23796	R	zinc finger, HIT type 3

Table 3.4: Insertion sites. List of insertion sites of SF91.caMpl, SF91.RRE.caMpl and SF91.ntMpl transplanted animals (n=26). All animals developed leukemia. Insertion sites were detected by LM-PCR. Closest genes in a frame of plus-minus 150 kbp from insertion site are listed. CIS common integration site, IDDb Insertional dominance database, RTCGD Retroviral tagged cancer gene database, distance negative number = 5' from transcription start site, distance positive number = 3' from transcription start site, R reverse, F forward.

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6.4. Beteiligungen /Contributions

Histopathology:	Guntram Büsche Chapters: 2.3. Mouse experiments and analysis 3.1.2. Mpl expression confers a selective growth advantage to murine bone marrow cells 3.1.3. Leukemias developing in wtMpl expressing mice require insertional mutagenesis 3.1.6. Pancytopenic mice develop an MDS-like bone marrow failure syndrome 3.3.3. Differentiation-specific and low Mpl expression reduce the risk of side effects in mice after BMT 3.3.6. Mpl gene-corrected $Mpl^{-/-}$ bone marrow cells contribute to the LSK fraction after BMT
SKY analysis:	Cornelia Rudolph, Tanja Hinrichsen Chapters: 2.2. Cell culture techniques and analysis 3.2.3 caMpl-induced leukemia selects for clones with retroviral vector insertions in proto-oncogenes
Lineage Mpl expression level:	Ute Modlich, Sabine Knöß Chapter: 3.3.6. Mpl gene-corrected $Mpl^{-/-}$ bone marrow cells contribute to the LSK fraction after BMT
Statistical analysis:	Martijn Brugman Chapters: 2.3. Mouse experiments and analysis 3.1.2. Mpl expression confers a selective growth advantage to murine bone marrow cells 3.1.5. Pancytopenia after transplantation is induced by a dominant negative effect

6.5. Lebenslauf / Curriculum Vitae

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Hannover, 16.06.2008

Daniel Wicke

6.6. Publications

Peer Reviewed Articles

Daniel C. Wicke, Johann Meyer, Guntram Buesche, Hans Kreipe, Zhixiong Li, Karl H. Welte, Matthias Ballmaier, Christopher Baum, Ute Modlich. *Hematopoietic population crisis induced by ectopic expression of the thrombopoietin receptor Mpl.* Submitted.

Ute Modlich, Axel Schambach, Martijn H. Brugman, **Daniel C. Wicke**, Sabine Knoess, Zhixiong Li, Cornelia Rudolph, Brigitte Schlegelberger and Christopher Baum. *Leukemia induction after a single retroviral vector insertion in Prdm16 or Evi1.* Leukemia, 2008.

Meeting Abstracts

Daniel C. Wicke, Johann Meyer, Guntram Buesche, Hans Kreipe, Zhixiong Li, Axel Schambach, Matthias Ballmaier, Karl H. Welte, Christopher Baum and Ute Modlich. *Transcriptonally regulated vectors reduce the risk of serious adverse events in gene therapy for Mpl deficiency*. 11th Annual Meeting of the American Society of Gene Therapy, Boston, USA, 29th May 2008.

Daniel Wicke, Ute Modlich, Johann Meyer, Matthias Ballmaier, Cornelia Rudolph, Brigitte Schlegelberger, Karl H. Welte and Christopher Baum. *Gene therapy of MPL deficiency: a narrow window between pancytopenia and leukemia.* 36th Annual Meeting of the International Society of Hematology, Hamburg, Germany, 28th September 2007.

Daniel Wicke, Ute Modlich, Johann Meyer, Matthias Ballmaier, Cornelia Rudolph, Brigitte Schlegelberger, Karl H. Welte and Christopher Baum. *Gene therapy of MPL deficiency: a paradigm for gene addition therapy of growth factor receptors.* 14th Annual Meeting of the German Society of Gene Therapy, Heidelberg, Germany, 19th July 2007.

Daniel Wicke, Ute Modlich, Johann Meyer, Matthias Ballmaier, Cornelia Rudolph, Brigitte Schlegelberger, Karl H. Welte and Christopher Baum. *Gene therapy of MPL deficiency: a paradigm for gene addition therapy of growth factor receptors.* 10th Annual Meeting of the American Society of Gene Therapy, Seattle, USA, 31th May 2007.

Ute Modlich, Axel Schambach, **Daniel Wicke**, Sabine Knoess and Christopher Baum. *Insertional mutagenesis by retroviral self-inacitvating vectors containing the viral SFFV promoter.* 10th Annual Meeting of the American Society of Gene Therapy, Seattle, USA, 31th May 2007.

Daniel Wicke, Ute Modlich, Johann Meyer and Christopher Baum. *Gene therapy of Mpl deficiency: a paradigm for the therapeutic expression of growth factor receptors.* 3rd Leukerbad Meeting of the European CONSERT, Leukerbad, Switzerland, 03rd February 2007.

Daniel Wicke, Ute Modlich, Johann Meyer and Christopher Baum. *Gene therapy of CAMT: a difficult balance to cure MPL deficiency.* 13th Annual Meeting of the German Society of Gene Therapy, Duesseldorf, Germany, 13th July 2006.

Daniel Wicke, Ute Modlich, Johann Meyer and Christopher Baum. *Gene therapy of Mpl deficiency: a difficult balance.* Transatlantic Gene Therapy Retreat, Baltimore, USA, 30th May 2006.