Unravelling Haematopoietic Stem Cell Dysfunction in Isolated Del(5q) Myelodysplastic Syndromes

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Laura Stenson

St. Cross College
University of Oxford
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Abstract

Myelodysplastic syndromes (MDS) represent a heterogeneous group of haematological malignancies. A subgroup of MDS patients are characterized by heterozygous deletion of the long arm of chromosome 5, Del(5q), as the only karyotypic abnormality. The commonly deleted region (CDR) on chromosome 5 contains approximately forty-two genes and haploinsufficiency of one or more of these genes is thought to be the basis for Del(5q) MDS pathogenesis.

The 5q deletion originates in the Hematopoietic Stem Cell (HSC) compartment and Del(5q) HSCs have a clonal advantage, outcompeting healthy HSCs in the bone marrow of patients. Although they have a competitive advantage *in situ*, Del(5q) HSCs perform poorly in functional stem cell assays *in vitro* and *in vivo*.

A mouse model of Del(5q) MDS, the *Cd74-Nid67* model, carries a heterozygous deletion of eight genes located within the CDR. *Cd74-Nid67* haploinsufficiency causes macrocytic anaemia and bone marrow dysplasia in mice. However, the impact of *Cd74-Nid67* haploinsufficiency on HSC function has not previously been investigated.

The results presented, herein, demonstrate that haploinsufficiency of *Cd74-Nid67* has a significant impact on HSC self-renewal and repopulation potential. Furthermore, two genes within this region, *Rps14* and *Rbm22*, are identified as likely candidates responsible for *Cd74-Nid67* HSC dysfunction. Finally, we demonstrate that *Cd74-Nid67* HSC dysfunction is driven by a P53-dependent mechanism.

This study provides important insights into the mechanistic basis for disease development and propagation, which may facilitate the development of improved therapeutic avenues for Del(5q) MDS patients.

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Abbreviations

AML	Acute myeloid Leukaemia
BM	Bone Marrow
CAFC	Cobblestone-Area-Forming-Cell
cDNA	Complementary DNA
CFU-E	Colony Forming Unit - Erythroid
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CSC	Cancer Stem Cell
FACS	Fluorescence Activated Cell Sorting
FMO	Fluorescence minus one
GFP	Green Fluorescent Protein
GM	Granulocyte-macrophage
GMP	Granulocyte-macrophage progenitor
HSC	Haematopoietic Stem Cell
Lin	Lineage
LSK	Lineage-Sca-1+c-Kit+
LTC-CFC	Long term culture colony forming cells
MDS	Myelodysplastic Syndrome
MEP	Megakaryocyte-erythroid progenitor
MkP	Megakaryocyte Progenitor
MOI	Multiplicity of Infection
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNAi	RNA interference
Sca-1	Stem cell antigen 1
siRNA	Short interfering RNA
shRNA	Short hairpin RNA
SLAM	Signalling lymphocyte activation molecule

1. Introduction

1.1 The Discovery of Haematopoietic Stem Cells

Billons of blood cells are produced on a daily basis including red blood cells responsible for oxygen transport, platelets which are necessary for blood clotting, and white blood cells, which constitute the innate and adaptive immune system.

Haematopoietic Stem Cells (HSCs) are the source of all blood cells. HSCs are defined by their ability to differentiate into all haematopoietic cell lineages and to self-renew. They continuously replenish blood cells throughout life, supporting the rapid turnover of mature cells. The highly proliferative nature of the haematopoietic system means that the balance between HSC differentiation and self-renewal must be tightly controlled. Seminal studies in mice led to the discovery of HSCs, providing invaluable insights into the structure and function of the haematopoietic system.

During the Second World War many people died from haematopoietic failure due to radiation exposure from atomic bombs. This sparked a surge of interest in haematopoietic research in an effort to identify life-saving treatments. Initial experiments discovered that mice exposed to high doses of radiation could be rescued by bone marrow transplantations (Jacobson, Simmons et al. 1951). It was not until a couple of years later, however, that the blood cells were identified as the important life-saving component (Ford, Hamerton et al. 1956).

In the 1960s, the pioneering work of Till and McCulloch demonstrated the existence of repopulating cells in mouse bone marrow (Till and McCulloch 1961). They discovered that transplanting bulk bone marrow (BM) cells into lethally irradiated mice caused proliferating colonies of multiple lineages to develop in the spleen. The

colonies were described as spleen colony-forming units (CFU-S). The linear correlation between the number of BM cells transplanted and the number of colonies formed led Till and McCulloch to infer that each colony may derive from a single cell (Till and McCulloch 1961). Indeed, further experiments using a mixture of donor BM cells that carried unique chromosomal aberrations established the unicellular origin of each colony. All cells within a colony carried the same unique chromosomal aberration, demonstrating that they had a common precursor (Becker, McCulloch et al. 1963). However, later studies concluded that CFU-S originated from progenitors, not HSCs, as they largely gave rise to myeloid colonies and could not support long-term reconstitution (Schofield 1978). Nevertheless, the studies of Till and McCulloch established key properties of primitive haematopoietic cells and paved the way for the quantitative evaluation of HSC function.

In the 1980s, retroviral-labeling experiments demonstrated that myeloid and lymphoid cells could originate from a single cell (Dick, Magli et al. 1985, Keller, Paige et al. 1985). Moreover, some of these precursors gave rise to long-term haematopoietic reconstitution (Lemischka, Raulet et al. 1986). Since they were capable of self-renewal and producing multiple lineages (multipotent), they fulfilled the definition of HSCs (Weissman and Shizuru 2008).

1.2 Murine Haematopoiesis

1.2.1 Haematopoietic Hierarchy

The haematopoietic system is hierarchically organised with HSCs at the apex and more lineage restricted progenitors downstream. The differentiation into mature blood cells occurs in a stepwise manner. Progenitors gradually lose self-renewal capacity

while becoming increasingly lineage committed until they differentiate into mature blood cells.

The classical model of haematopoiesis proposes that a multi-potent progenitor, MPP, lies directly below the HSC in the hierarchical tree (Figure 1.1). MPPs can produce multiple lineages but differ from HSCs as they have limited self-renewal capacity. The first lineage commitment step, according to the classical model, involves a strict separation of lymphoid and myeloid development with the common lymphoid progenitor (CLP) (Kondo, Weissman et al. 1997) and common myeloid progenitor (CMP) (Akashi, Traver et al. 2000), respectively.

The alternative model of haematopoiesis, however, proposes that the first lineage commitment step involves the separation of GM/lymphoid and GM/MkE development (Figure 1.2). The discovery of lymphoid progenitors that had granulocyte/monocyte (GM) potential but no megakaryocyte/erythroid (MkE) potential, known as lymphoid primed multipotent progenitors (LMPPs), led to the proposal of the alternative model (Adolfsson, Mansson et al. 2005). This was initially controversial (Forsberg, Serwold et al. 2006) but a number of studies have, since, provided support for the alternative model (Lai and Kondo 2006, Yoshida, Ng et al. 2006, Arinobu, Mizuno et al. 2007, Mansson, Hultquist et al. 2007, Bell and Bhandoola 2008, Giebel and Punzel 2008, Boiers, Carrelha et al. 2013)

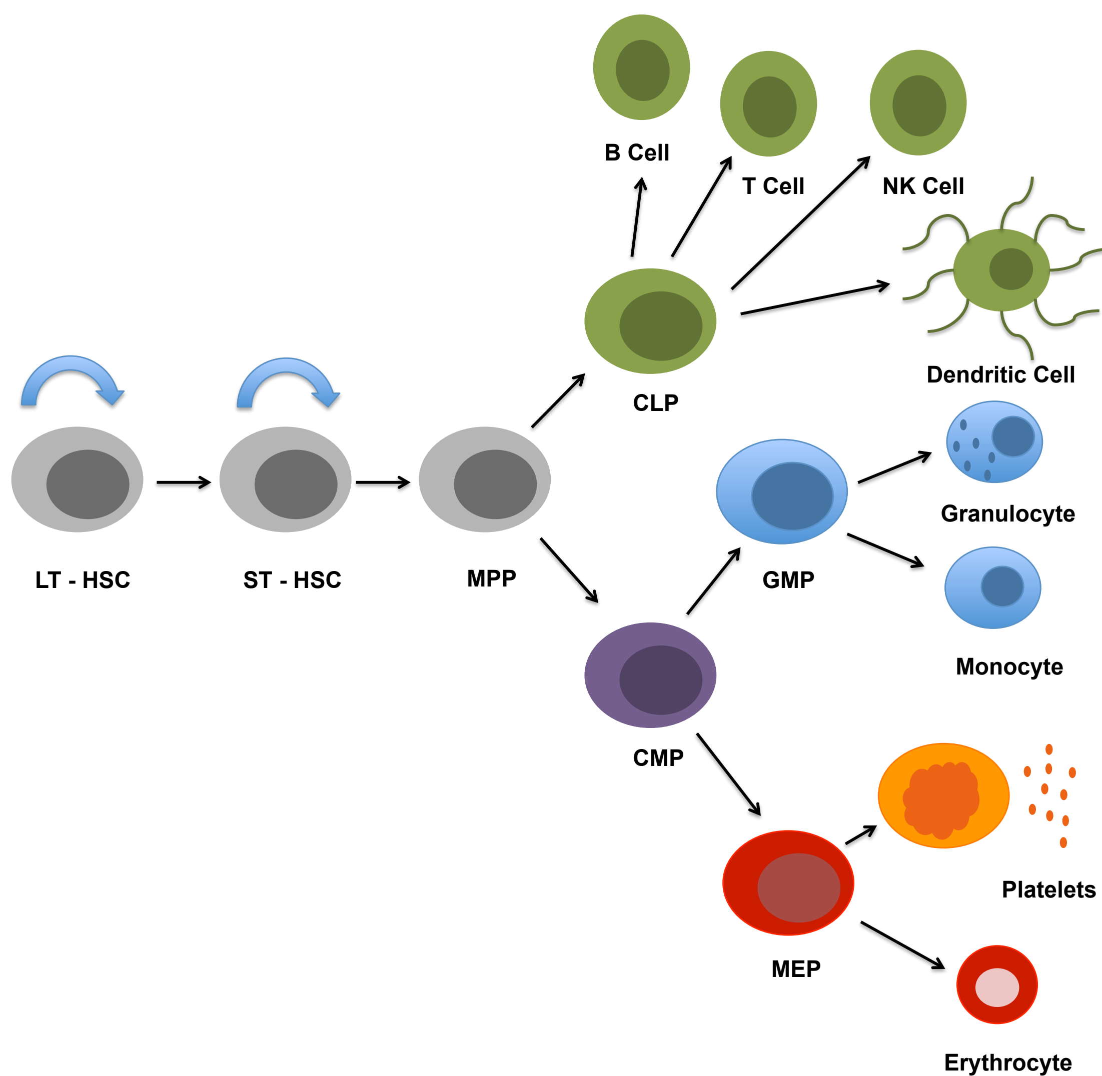


Figure 1.1 Classical model of haematopoietic hierarchy

Long-term Haematopoietic Stem Cells (LT-HSC) and short-term Haematopoietic Stem Cells (ST-HSC) are the only populations with long-term repopulation capacity. MPPs, derived from HSCs, are multipotent but have reduced self-renewal potential. A strict separation between myeloid and lymphoid development then occurs with the common lymphoid progenitor (CLP) producing all mature lymphoid cells and the common myeloid progenitor (CMP) producing all mature myeloid cells. Curved arrows represent self-renewal.

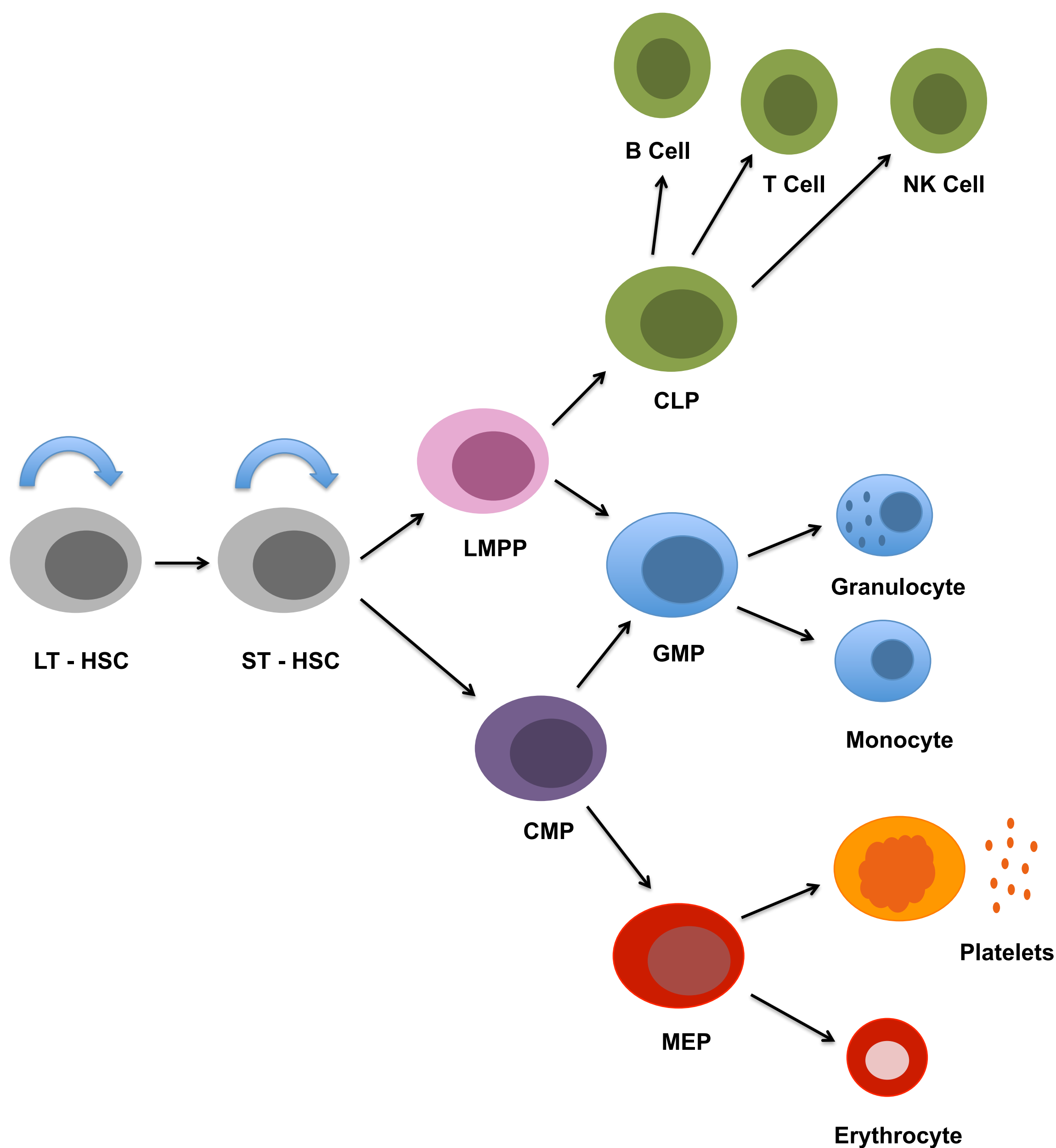


Figure 1.2 Alternative model of haematopoietic hierarchy

Long-term Haematopoietic Stem Cells (LT-HSC) and short-term Haematopoietic Stem Cells (ST-HSC) are the only populations with extensive repopulation potential. The primitive lymphoid primed multipotent progenitor (LMPP) has lymphoid, granulocyte and monocyte potential but lacks megakaryocyte and erythroid potential. The common myeloid progenitor (CMP) can give rise to all myeloid cells, while the common lymphoid progenitor (CLP) can produce all lymphoid cells. Curved arrows represent self-renewal.

1.2.2 Phenotypic Analysis

HSCs are an extremely rare population, representing one in ten-twenty thousand bone marrow cells in mice (Szilvassy, Humphries et al. 1990). It is important, therefore, to identify and purify HSCs in order to study their activity. Cells can be purified based on their expression of cell surface receptors using flow cytometry and fluorescence activated cell sorting (FACS) (Eisenstein 2006).

Initial studies in mice demonstrated that HSCs did not express mature cell lineage markers (lineage negative) but were positive for Stem cell antigen (Sca1+) and c-kit, Lineage-Sca-1+c-kit+ (LSK) (Spangrude, Heimfeld et al. 1988, Ikuta and Weissman 1992, Morrison and Weissman 1994). However, functional studies demonstrated that only one in thirty LSKs could support long-term haematopoietic reconstitution. The LSK markers, therefore, define a heterogeneous population of stem/progenitor cells. In an effort to identify a purer population of HSCs, LSKs were further subdivided using markers such as CD34 (Osawa, Hanada et al. 1996) or the Signaling Lymphocyte Activation Molecule (SLAM) cell surface receptors, CD48 and CD150 (Kiel, Yilmaz et al. 2005). Indeed, the inclusion of the SLAM markers significantly improved HSC purity since one in five had long-term repopulation potential (Osawa, Hanada et al. 1996, Kiel, Yilmaz et al. 2005).

The purification and analysis of stem and progenitor cells by FACS and flow cytometry has been critical to improving our understanding of haematopoiesis. The molecular characterization of phenotypically defined HSCs, in combination with functional assays and genetic experiments, has promoted the identification of key regulators of HSC activity (Mansson, Hultquist et al. 2007, Yoshihara, Arai et al. 2007, Sanjuan-Pla, Macaulay et al. 2013). Furthermore, single cell transplantation assays, which have provided important insights into the heterogeneity of HSCs

(Dykstra, Kent et al. 2007, Kent, Copley et al. 2009, Beerman, Bhattacharya et al. 2010, Challen, Boles et al. 2010, Morita, Ema et al. 2010, Sanjuan-Pla, Macaulay et al. 2013), would not have been possible without prior HSC enrichment using phenotypic markers.

The use of cell surface markers to phenotypically define populations, together with *in vivo* and *in vitro* functional assays, can provide meaningful information about haematopoietic cell activity.

1.2.3 Competitive Transplantation Assays

Long-term transplantation assays are the gold standard method for examining HSC function. They provide information about the capacity of a cell to reconstitute the entire haematopoietic system. Transplantation assays, therefore, provide scope to evaluate the defining features of stem cells, namely multipotency and self-renewal. The repopulation potential of donor HSCs must be evaluated after a minimum of sixteen weeks (Yang, Bryder et al. 2005). Serial transplantations are often performed to examine long-term self-renewal capacity.

The competitive transplantation assay is, perhaps, the most commonly used method for examining HSC potential (Harrison 1980). Bone marrow cells from a donor (experimental) mouse are injected into a lethally irradiated recipient along with a given number of competitor bone marrow cells. The use of congenic mouse strains allows the progeny of donor cells to be distinguished from the progeny of competitor cells. The functionally identical alleles, CD45.1 and CD45.2, allow genetically distinct donor and competitor cells to be differentiated using allele specific antibodies. In this way, the relative contribution of donor cells to haematopoietic lineages can be determined and directly compared to the contribution of competitor cells. Haematopoietic phenotypes can be more readily detected in a competitive transplant

setting. The survival of the recipient does not solely rely on the activity of the donor cells since the competitor cells are sufficient to support haematopoiesis. In this way, the efficiency of donor cell reconstitution can be evaluated and compared to the efficiency of competitor cell reconstitution.

Conversely, in non-competitive transplantation assays, the survival of the recipient relies on the function of the donor cells. In such critical situations, the donor cells may call on their reserve capacity and overcome their inherent deficiencies to promote the survival of the host.

1.2.4 Cell Culture Assays

The activity of cells can also be examined *in vitro* using cell culture assays. This can provide useful information about the lineage potential of distinct populations. The low proliferative potential of progenitors can make it challenging to detect their progeny in a transplantation setting *in vivo*. Cell culture assays are, therefore, especially useful for evaluating the lineage potential of progenitors.

The myeloid, erythroid or megakaryocyte potential of a cell can be determined by adding exogenous growth factors or cytokines to short-term liquid or semi-solid cultures (Coulombel 2004). Lymphoid potential can also be investigated using cell culture assays. B cell potential can be evaluated using a co-culture system with the bone marrow derived stromal cell line, Op9 (Nakano, Kodama et al. 1994). T cell potential, however, can be evaluated using Op9 cells that ectopically express the Notch ligand Delta like 1 (Op9DL1) (Schmitt and Zuniga-Pflucker 2002).

1.3 Human Haematopoiesis

1.3.1 Haematopoietic Hierarchy

Studies using mice have provided valuable insights into the structure and function of the haematopoietic system in man. However, the haematopoietic hierarchy in mouse is much better defined, both phenotypically and functionally. This is largely due to a lack of sensitive assays to examine the function of human haematopoietic cells.

The human haematopoietic hierarchy, as we currently understand it, closely mirrors the hierarchy of the mouse. HSCs, capable of long-term self-renewal and producing all haematopoietic cell lineages, are located at the apex. Downstream multipotent progenitors (MPPs) have reduced self-renewal capacity but can give rise to multiple lineages. The first lineage commitment step occurs downstream of the MPP.

Multi-lymphoid progenitors (MLPs) have the capacity to produce lymphoid cells as well as macrophages, monocytes and dendritic cells (Doulatov, Notta et al. 2010). A similar population was identified in a separate study and described as the equivalent to the mouse LMPP (Goardon, Marchi et al. 2011). Importantly, the human LMPP, unlike the MLP, was found to have granulocyte potential. The short-half life of granulocytes together with the time-point used to assess their production may have prevented their detection in the MLP study.

Lineage restricted myeloid progenitors such as the CMP, GMP and MEP have also been purified. Molecular and functional analysis was used to define the lineage potential of these populations (Manz, Miyamoto et al. 2002). Human lymphoid development, however, is not well understood.

1.3.2 Phenotypic Analysis

The expression of cell surface markers on HSCs and progenitors differ in mouse and

human. CD34, expressed by less than 2% of BM cells, was the first cell surface marker identified that enriched for immature stem/progenitor cells in humans (Civin, Strauss et al. 1984). The phenotypic classification has since been refined, with multi-potent HSCs being defined as CD34+CD38-CD90+ (Baum, Weissman et al. 1992, Bhatia, Bonnet et al. 1998). Importantly, CD34+CD38-CD90+ cells do not represent a pure population of HSCs but define a population enriched with HSCs.

1.3.3 Xenotransplantation

Humanised mouse models are valuable tools for assessing the activity of HSCs or cancer stem cells (CSCs) *in vivo*. Xenotransplantation assays involve transplanting human cells into immunocompromised mice, enabling the establishment of a human graft, without rejection. A number of models have been generated and refined over the past twenty years with the aim of developing a model that supports high levels of long-term, multi-lineage human engraftment (McCune, Namikawa et al. 1988, Mosier, Gulizia et al. 1988, Shultz, Schweitzer et al. 1995). NOG (NOD/Shi-*scid*/IL-2Rγ null) and NSG (NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ) mice, which have truncated or deleted IL-2 receptor common gamma chain, respectively, are currently used models for xenotransplantation studies. NOG and NSG mice lack B, T and NK cells, facilitating long-term, multi-lineage human engraftment (Shultz, Lyons et al. 2005).

There are, however, limitations associated with the use of xenotransplantation assays. Certain growth factors are not cross-reactive between human and mouse, which may inhibit engraftment. Furthermore, xenotransplantation assays involve removing cells from their natural (human) environment and placing them in the unfamiliar environment of a recipient mouse. The activity of HSCs or CSCs upon xenotransplantation may, therefore, not represent their activity in their endogenous environment.

1.3.4 LTC-CFC Assay

The LTC-CFC is the most well established assay for examining human HSC potential *in vitro* (Sutherland, Eaves et al. 1989). Human BM cells that have been co-cultured with a murine stromal cell line are transferred to methylcellulose and myeloid colony growth is evaluated. This is limited not only by the fact that it is an *in vitro* assay but also because multi-lineage potential cannot be assessed. This assay is only capable of supporting myeloid colony growth. Furthermore, it is difficult to examine long-term self-renewal potential since growth is not supported beyond six weeks of culture. LTC-CFC assays are, however, relatively useful when xenotransplantation assays are unsuccessful. Indeed, cells from patients with haematological malignancies such as acute myeloid leukaemia (AML) or myelodysplastic syndromes (MDS) often fail to engraft upon xenotransplantation (Pearce, Taussig et al. 2006, Medyouf, Mossner et al. 2014, Woll, Kjallquist et al. 2014).

1.3.5 CAFC Assay

The cobblestone-area-forming cell (CAFC) is an *in vitro* assay that can be used to measure the activity of stem and progenitor cells. BM cells are co-cultured with a stromal cell line (Bock 1997). The formation of cobblestone areas, defined as colonies of five or more cells that grow underneath the stromal layer, are assessed at sequential time points (such as five weeks and eight weeks). Unlike the LTC-CFC, this assay does not rely on the differentiation potential of HSCs and progenitors (Bock 1997). This is an advantage when investigating the activity of stem/progenitor cells from AML and MDS patients, where differentiation is impaired.

1.4 Cancer Stem Cells

Even though cancer arises from the clonal expansion of one cell that has acquired genetic mutations, there is immense variability in the morphology, function and growth rate of individual cells within a tumour. The cause of intra-tumour heterogeneity, however, is not fully understood (Kreso and Dick 2014).

The stochastic model posits that every cell within a tumour is biologically equivalent and has the potential to drive tumour growth. Heterogeneity occurs as a result of random intrinsic or extrinsic insults acquired by a given cell, which govern whether it can propagate a tumour. According to this model, cancer-initiating cells, capable of driving tumour growth, would not be prospectively identifiable since they would not express consistent, distinguishing markers (Dick 2008).

In contrast, the hierarchical model proposes that cancer is organized hierarchically, similar to the organization of healthy tissues (Kreso and Dick 2014). A rare population of malignant cells, known as cancer stem cells (CSCs) that can be prospectively isolated resides at the apex of the hierarchy. CSCs have extensive self-renewal capacity and can give rise to all other cells of the tumour. CSCs, therefore, are solely responsible for the long-term propagation of a tumour. Furthermore, CSCs are thought to be selectively resistant to many commonly used cancer therapies and their persistence may account for disease relapse (Dick 2008).

The CSC potential of distinct leukaemic or cancer cell populations has primarily been evaluated using xenotransplantation or xenograft assays, respectively. Pioneering studies by John Dick provided the first experimental evidence for the existence of CSCs in acute myeloid leukaemia (Bonnet and Dick 1997). Later studies demonstrated that breast (Al-Hajj, Wicha et al. 2003), brain (Singh, Hawkins et al. 2004), lung (Eramo, Lotti et al. 2008) and pancreatic cancers (Hermann, Huber et al.

2007) were also hierarchically arranged and driven by a rare population of distinct CSCs.

However, the CSC concept has recently been contested by studies that have suggested that cancer-initiating cells in melanoma are not rare or phenotypically distinct (Quintana, Shackleton et al. 2008, Quintana, Shackleton et al. 2010). Furthermore, significant limitations associated with *in vivo* CSC assays have been identified. Many studies have highlighted that *in vivo* CSC assays fail to reliably uncover the tumorigenic potential of cancer cell populations (Pearce, Taussig et al. 2006, Kelly, Dakic et al. 2007, le Viseur, Hotfilder et al. 2008, Taussig, Miraki-Moud et al. 2008, Clevers 2011, Magee, Piskounova et al. 2012). Indeed, a large fraction of AML samples failed to engraft upon xenotransplantation, despite clearly sustaining the malignancy in the patients, themselves (Pearce, Taussig et al. 2006). This suggests that the identity of candidate CSCs must be verified using different experimental approaches.

Interestingly, genetically engineered lineage-tracing technology was recently used to definitively identify and fate-map CSCs in mice (Chen, Li et al. 2012, Driessens, Beck et al. 2012, Schepers, Snippert et al. 2012). Moreover, a recent study demonstrated a hierarchical relationship between stem and progenitor cells in patients with Del(5q) MDS and used DNA mutation tracking to establish the identity of MDS propagating cells *in situ* (Woll, Kjallquist et al. 2014).

1.5 Myelodysplastic Syndromes (MDS)

Myelodysplastic syndromes (MDS) represent a heterogeneous group of haematological malignancies characterized by dysplastic haematopoiesis and cytopenias. MDS is most frequent among elderly patients, with an average age of 75

at presentation. Patients often present with anaemia alone but approximately one third of patients will develop acute myeloid leukaemia (AML). The only curative treatment is allogeneic stem cell transplantation, which is not appropriate for elderly patients with significant comorbidities (Nimer 2008).

The World Health Organisation (WHO) 2008 diagnostic system classifies MDS patients based on morphological and cytogenetic diagnostic criteria (Table 1.1).

Table 1.1 MDS Diagnostic Classification (WHO 2008)

Classification system for patients with Myelodysplastic Syndromes, adapted from (Vardiman, Thiele et al. 2009).

MDS Subtype	Peripheral Blood	Bone Marrow
Refractory cytopaenia with unilineage dysplasia (RCUD)	Anaemia, neutropaenia or thrombocytopaenia	Unilineage dysplasia, <5% blasts
Refractory anaemia with ring sideroblasts (RARS)	Anaemia	Unilineage erythroid dysplasia, >15% erythroid precursors are ring sideroblasts, <5% blasts
Refractory cytopaenia with multilineage dysplasia (RCMD)	Cytopaenias	Multilineage dysplasia +/- ring sideroblasts, <5% blasts
Refractory anaemia with excess blasts type 1 (RAEB I)	Cytopaenias	Unilineage or multilineage dysplasia, 5-9% blasts
Refractory anaemia with excess blasts type 2 (RAEB II)	Cytopaenias	Unilineage or multilineage dysplasia, 10-19% blasts
Isolated Del(5q) Myelodysplastic syndromes	Anaemia. Normal or high platelet count	Isolated 5q31 deletion, hypolobulated megakaryocytes, <5% blasts

1.6 Del(5q) MDS

A subgroup of MDS patients are characterized by heterozygous deletion of the long arm of chromosome 5, Del(5q), as the only karyotypic abnormality. The commonly deleted region (CDR) is located between 5q31 and 5q32 and contains approximately forty-two genes (Table 1.2).

The clinical features of Del(5q) MDS include macrocytic anaemia, normal or elevated platelet counts and hypolobulated megakaryocytes. Del(5q) MDS has a relatively low rate of transformation to AML compared to other subtypes of MDS. Interestingly, the incidence of Del(5q) MDS is higher in females than in males (Nimer 2008).

A rare population of patients with small deletions were used to identify the Del(5q) CDR (Boultwood, Fidler et al. 1994, Boultwood, Fidler et al. 2002). The majority of Del(5q) MDS patients, however, carry larger deletions (Douet-Guilbert, De Braekeleer et al. 2012). The identification of a CDR that spans a large region suggests that this chromosomal section may be susceptible to breakage or, alternatively, that deletion of genes at either end of the CDR are required for disease initiation. Since previous studies have failed to demonstrate that homozygous inactivation occurs for any gene within the CDR, haploinsufficiency of one or more genes is thought to be the basis for Del(5q) MDS pathogenesis (Heinrichs, Kulkarni et al. 2009).

The 5q deletion in isolated Del(5q) MDS is an early event that originates in the HSC compartment and drives disease development (Nilsson, Astrand-Grundstrom et al. 2000, Woll, Kjallquist et al. 2014). Del(5q) CD34+CD38- HSCs have a clonal advantage, outcompeting "healthy" HSCs in the bone marrow of patients (Nilsson, Astrand-Grundstrom et al. 2000). At diagnosis, the majority of purified CD34+CD38- HSCs (94%) in Del(5q) MDS patients carry the 5q deletion. Furthermore, the bulk of purified HSCs (99%) from MDS patients with complex karyotypes (5q deletion and

other cytogenetic abnormalities) harbour the 5q deletion (Nilsson, Astrand-Grundstrom et al. 2000). Although Del(5q) HSCs have a competitive advantage *in situ*, they perform extremely poorly in stem cell assays *in vitro* (LTC-CFC) and *in vivo* (xenotransplantation) (Nilsson, Astrand-Grundstrom et al. 2000, Tehranchi, Woll et al. 2010).

There is no curative treatment for Del(5q) MDS. However, patients respond remarkably well to treatment with an immunomodulatory drug, Lenalidomide. This agent directly inhibits the Del(5q) malignant clone. Lenalidomide triggers cell death by inducing ubiquitination and degradation of *CSKN1A1*, a gene located within the CDR in Del(5q) MDS (Kronke, Fink et al. 2015). Since Del(5q) cells only have one copy of *CSKN1A1*, they are selectively depleted over healthy cells. However, half of Del(5q) MDS patients relapse within three years of Lenalidomide treatment (List, Kurtin et al. 2005, List, Dewald et al. 2006). An important study examined the impact of Lenalidomide treatment on the disease course of Del(5q) MDS patients (Tehranchi, Woll et al. 2010). The total number of Del(5q) cells in the BM of patients was efficiently reduced following treatment with Lenalidomide. However, resistant Del(5q) cells persisted in the rare CD34+CD38-CD90+ HSC compartment, even during clinical remission. Over time, most patients stopped responding to treatment and experienced clinical and cytogenetic relapse. The few Del(5q) HSCs that survived therapy were, therefore, sufficient to re-initiate disease.

Table 1.2 Genetic Annotation of the CDR in Del(5q) MDS

List of protein-coding genes within the CDR in Del(5q) MDS, (Boultwood, Fidler et al. 2002) and Ensembl version 83.

Gene Name	Gene Description
SH3TC2	SH3 domain and tetratricopeptide repeats 2
ABLIM3	Actin binding LIM protein family member 3
AFAP1L1	Actin filament associated protein 1-Like 1
GRPEL2	GrpE-Like 2, Mitochondrial
PCYOX1L	Prenylcysteine Oxidase 1 Like
IL17B	Interleukin 17 beta
CSKN1A1	Casein kinase 1, alpha 1
ARHGEF37	Rho Guanine Nucleotide Exchange Factor 37
PPARGC1B	Peroxisome Proliferator-Activated Receptor Gamma, Coactivator 1
PDE6A	Phosphodiesterase 6A
SLC26A	Solute carrier Family 26 (Anion exchanger) member 2
TIGD6	Tigger Transposable Element Derived 6
HMGXB3	HMG-box containing 3
CSF1R	Macrophage colony stimulating factor-1 receptor
PDGFRB	Platelet-derived growth factor receptor beta
CDX1	Caudal type Homeobox 1
SLC6A7	Solute Carrier Family 6, member 7
CAMK2A	Calcium/calmodulin dependent protein kinase II alpha-B subunit
ARSI	Arylsulfatase Family, member 1
TCOF1	Treacher Collins-Franceschetti syndrome 1
CD74	CD74 antigen
RPS14	40S ribosomal protein S14
NDST1	Heparan sufhate N-deacetylase/N-sulphotransferase 1
SYNPO	Synaptopodin, actin-associated protein
MYOZ3	Myozenin 3
RBM22	RNA Binding Motif Protein 22
DCTN4	Dynactin, p62 subunit
SMIM3 (NID67)	Small Integral Membrane Protein 3
IRGM	Immunity Related GTPase Family, M
ZNF300	Zinc finger protein 300
GPX3	Glutathione peroxidase 3
TNIP1	TNFAIP3 interacting protein 1
ANXA6	Annexin A6
CCDC69	Coiled-coil domain containing 69
GM2A	Ganglioside G-M2 activator protein
SLC36A3	Solute Carrier Family 36, member 3
SLC36A2	Solute Carrier Family 36, member 2
SLC36A1	Solute Carrier Family 36, member 1
MEGF1 (FAT2)	Human homologue of the Drosophila fat tumour suppressor gene
SPARC	Secreted protein acidic cysteine-rich
ATOX1	Antioxidant 1 copper chaperone
G3BP	Ras-GTPase –activating protein SH3-domain-binding protein

1.6.1 The Mouse as a Model of Del(5q) MDS

CSCs in Del(5q) MDS reside in the HSC compartment. Del(5q) HSCs are, therefore, both required and sufficient to propagate disease (Woll, Kjallquist et al. 2014). Human HSCs are not well characterised phenotypically and the assays used to evaluate their function are limited by their lack of sensitivity.

Mouse HSCs are much better defined, both phenotypically and functionally, than their human counterparts. Studies using mouse HSCs have provided important biological insights that have helped provide a better understanding of human HSCs and their role in disease pathogenesis. Furthermore, the use of mouse models provides the opportunity to examine the impact of genetic changes on the function of HSCs *in vivo*, which is not possible in human. Since significant phases of disease development have occurred before patients are clinically diagnosed with MDS (Nimer 2008), the crucial initiating events responsible for disease pathogenesis are often masked by secondary events, complicating the interpretation of results. The study of a mouse model provides scope to examine the initiating events/mechanisms responsible for disease propagation, which may promote the development of improved therapeutics that target the cause of disease.

1.7 *Cd74-Nid67* Mouse Model

The CDR in Del(5q) MDS corresponds to two regions of synteny on mouse chromosomes 11 and 18, with complete conservation of gene order and orientation. Barlow and colleagues generated four mouse models that harboured heterozygous deletions of large chromosomal regions corresponding to distinct parts of the CDR in Del(5q) MDS patients (Barlow, Drynan et al. 2010). The analysis of such models provides scope to identify the regions responsible for Del(5q) MDS pathogenesis. The

regions targeted were *Sparc-Gpx3*, *Sparc-Nmu2r*, *Arsi-Csf1r* and *Cd74-Nid67*. The impact of deficiency of each region on mature blood cell production was examined. Deficiency of *Sparc-Gpx3*, *Sparc-Nmu2r* or *Arsi-Csf1r* did not affect red blood cell (RBC) count or haemoglobin concentration. The impact of deficiency of such loci on stem/progenitor populations in the BM, however, was not examined.

Haploinsufficiency of *Cd74-Nid67* was found to replicate important features of Del(5q) MDS such as macrocytic anaemia and bone marrow dysplasia (Barlow, Drynan et al. 2010). Moreover, the differentiation capacity of myeloid, erythroid and megakaryocyte progenitors was significantly impaired in *Cd74-Nid67* mice. Interestingly, deficiency of *Cd74-Nid67* led to increased expression of P53 and elevated levels of apoptosis in BM cells. This suggests that *Cd74-Nid67* haploinsufficiency may account for some of the defining clinical features of Del(5q) MDS. However, the impact of *Cd74-Nid67* haploinsufficiency on HSC function has yet to be explored (Barlow, Drynan et al. 2010).

1.7.1 Candidate Genes within *Cd74-Nid67*

The *Cd74-Nid67* region contains eight known genes (Figure 1.3). The function of each gene within this region was investigated with the aim of identifying potential candidates that may contribute to Del(5q) MDS.

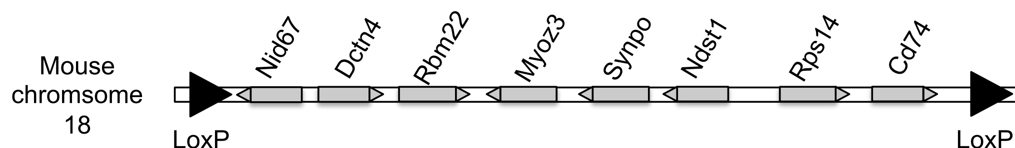


Figure 1.3 Targeted region in *Cd74-Nid67* mice

Schematic diagram of genes located within *Cd74-Nid67,* flanked by loxP sites, on mouse chromosome 18. Arrows indicate the direction of transcription.

1.7.1.1 Rps14

RPS14 encodes a protein that forms part of the 40S ribosomal subunit. Studies in yeast demonstrated that rpS14 is essential for the processing of 18S pre-rRNA (Ferreira-Cerca, Poll et al. 2005). Indeed, reduced expression of *RPS14* in a human erythroid cell line disrupted 18S pre-rRNA processing and inhibited the formation of the 40S subunit (Ebert, Pretz et al. 2008).

Interestingly, an RNA interference screen in CD34+ human progenitor cells revealed that *RPS14* plays an important role in erythroid development *in vitro* (Ebert, Pretz et al. 2008). *RPS14* haploinsufficiency caused a significant decrease in erythroid cell production relative to megakaryocyte or myeloid cell production (Ebert, Pretz et al. 2008). Furthermore, impaired ribosome subunit assembly in human erythroid cells activated the P53 checkpoint, leading to the accumulation of *CDKN1A* and subsequent cell cycle arrest (Dutt, Narla et al. 2011). These studies suggest that *RPS14* deficiency accounts for the erythroid differentiation deficit in Del(5q) MDS.

Importantly, HSCs also seem to be susceptible to disruptions in ribosome biogenesis. A recent study demonstrated that a mutation in the *Rpl24* ribosome subunit, *Rpl24*

Bst/+, impaired the repopulation potential of mouse HSCs (Signer, Magee et al. 2014). This study also highlighted that the rate of protein synthesis must be tightly controlled in HSCs in order to maintain optimal activity.

Furthermore, recurrent mutations in distinct ribosomal genes have been identified in congenital disorders such as Diamond Blackfan Anaemia, Schwachman-Diamond syndrome (SDS), X-linked dyskeratosis congenita and Treacher Collins syndrome; collectively termed ribosomopathies. This suggests that defects in ribosome biogenesis are sufficient to drive disease (Narla and Ebert 2010). Interestingly, both erythroid differentiation and HSC function were impaired as a result of *Rps19* deficiency in a mouse model of Diamond Blackfan Anaemia (Jaako, Flygare et al. 2011). *Rps14* deficiency may, therefore, not only account for impaired erythroid differentiation but may also contribute to HSC dysfunction in Del(5q) MDS.

1.7.1.2 Rbm22

RBM22 encodes an RNA binding protein that is required for the first catalytic step of pre-mRNA splicing. Depletion of RBM22 in HeLa nuclear extracts inhibited pre-mRNA splicing, resulting in a significant decrease of spliced products (Rasche, Dybkov et al. 2012). RBM22 is thought to be a key link between the catalytic RNA network and factors that regulate spliceosome fidelity. Since merely altering the concentration of general splicing factors affects the kinetics of spliceosome assembly and splice site recognition (Park, Parisky et al. 2004), it is likely that haploinsufficiency of *RBM22* would affect pre-mRNA splicing.

Aberrations in pre-mRNA splicing have previously been associated with cancer (Venables 2004) and, more recently, MDS (Yoshida, Sanada et al. 2011). A large number of MDS patients were found to have recurrent point mutations in genes that

encode core components of the pre-mRNA splicing machinery, such as *U2AF35*, *SRSF2* and *SF3B1* (Yoshida, Sanada et al. 2011). This discovery may represent a major breakthrough in understanding the underlying biology of MDS.

It has been proposed that such mutations may lead to the generation of aberrantly spliced mRNA species. Indeed, splicing was disrupted in myeloid progenitors from mice expressing mutant *U2af35* (S34F) and, interestingly, splicing-induced changes were enriched in ribosomal genes and genes involved in RNA processing (Shirai, Ley et al. 2015). The splicing pattern of genes was also altered in progenitors from mice expressing mutant *Srsf2* (P95H). In fact, recurrent mis-splicing of a key haematopoietic mediator, *Ezh2,* led to impaired cellular differentiation (Kim, Ilagan et al. 2015). Importantly, the repopulation potential of HSCs from *U2af35* or *Srsf2* mutant mice was also significantly altered (Kim, Ilagan et al. 2015, Shirai, Ley et al. 2015). This raises the possibility that deficiency of *Rbm22* may, in a similar manner, alter splicing and disrupt HSC function.

1.7.1.3 Cd74

Cd74 encodes a polypeptide known as the invariant chain, Ii, which is involved in the formation and trafficking of the MHC class II protein (Borghese and Clanchy 2011). *Cd74* also plays a role in positive and negative selection in the thymus. Loss of *Cd74* in mice resulted in altered numbers of CD4+ T cells and an attenuated response to peptide and protein antigens (Wong and Rudensky 1996). Further studies using knockout mice demonstrated that *Cd74* is also involved in B cell maturation in the spleen (Matza, Lantner et al. 2002).

1.7.1.4 Ndst1

Ndst1 encodes an enzyme (N-deacetylase/N-sulfotransferase) involved in the synthesis of heparan sulfate, which plays a role in the inflammatory response. Homozygous loss of *Ndst1* in mice can result in death during gestation. Interestingly, heparan sulfate levels were increased in mast cells from embryos with heterozygous or homozygous loss of *Ndst1*. Furthermore, mast cells from *Ndst1*+/- and *Ndst1*-/- embryos contained increased amounts of mast cell mediators. In the absence of *Ndst1*, the more efficient isoform, *Ndst2*, bound to enzyme complexes, leading to increased heparan sulfation. This, in turn, enhanced the capacity of the polysaccharide to bind to histamine and proteases (Dagalv, Holmborn et al. 2011).

1.7.1.5 Dctn4

Dctn4 encodes the P62 subunit of the dynactin complex, which binds to dynein and promotes the transport of vesicles and organelles along microtubules. Dynactin is also involved in mitosis (Schroer 2004). Knockdown and overexpression studies in a fibroblast cell line revealed that *Dctn4* facilitates the binding of dynactin to the nuclear envelope prior to mitosis (Yeh, Quintyne et al. 2012). The impact of heterozygous or homozygous loss of *Dctn4* on cellular function, however, has yet to be examined.

1.7.1.6 Nid67

Limited studies have been performed to elucidate the function of *Nid67*. It is expressed by a neural cell line in response to FGF or NGF treatment and may be involved in neuronal differentiation (Vician, Silver et al. 2001). The impact of

heterozygous or homozygous loss of *Nid67 in vitro* or *in vivo* has not previously been examined.

1.7.1.7 Synpo

Synpo encodes Synaptopodin, an actin-binding protein that regulates cell shape and motility. *Synpo* knockout mice were used to investigate its function in neuronal development. Neuronal dendritic spine growth was impaired in young (two-three week old) *Synpo* null mice (Zhang, Poschel et al. 2013). *Synpo*, therefore, is thought to mediate the shape and growth of dendritic spines in postnatal brain development. The impact of heterozygous loss of *Synpo* was not investigated.

1.7.1.8 Myoz3

Myoz3 encodes Myozenin-3, an intracellular protein that modulates calcineurin signaling. Members of the myozenin family bind to calcineurin and help to tether it to the sarcomere of skeletal muscle (Frey and Olson 2002). The function of *Myoz3* has not previously been investigated *in vivo*.

1.7.2 Candidate Genes Outside *Cd74-Nid67*

Some studies have demonstrated that haploinsufficiency of genes outside *Cd74-Nid67* also affect haematopoietic development. Indeed, deficiency of individual genes such as *Cskn1a1* (Schneider, Adema et al. 2014), *Egr1* (Joslin, Fernald et al. 2007) or *Apc* (Lane, Sykes et al. 2010, Wang, Fernald et al. 2010) can disrupt haematopoietic differentiation or HSC function. However, the full clinical spectrum of Del(5q) MDS has not been recapitulated by deficiency of any individual gene. Furthermore, the majority of genes that have been investigated are located more proximally on 5q, outside the Del(5q) MDS CDR (Joslin, Fernald et al. 2007, Min, Pietramaggiori et al.

2008, Lane, Sykes et al. 2010, Wang, Fernald et al. 2010). While deficiency of genes outside the CDR may contribute to the clinical phenotype in Del(5q) MDS patients with larger deletions, it cannot be the prominent driver of disease. The proximal deletion of 5q is more common in patients with therapy related MDS (t-MDS) or AML.

1.7.2.1 Cskn1a1

CSKN1A1, encoding casein kinase 1a (CK1a), is located within the CDR (5q32) defined in isolated Del(5q) MDS. CK1a controls the activity of beta-catenin and acts as a tumour suppressor in melanoma (Sinnberg, Menzel et al. 2010).

A recent study examined the impact of *Cskn1a1* haploinsufficiency on adult haematopoiesis by transplanting *Cskn1a1* fl/+ *MxCre* tg/+ "not yet deleted" BM cells into lethally irradiated recipients and inducing deletion four weeks post transplantation (Schneider, Adema et al. 2014). Haploinsufficiency of *Cskn1a1* did not affect BM cellularity or haemoglobin levels. However, an increase in WBC counts due to T cell lymphocytosis was evident as a result of *Cskn1a1* haploinsufficiency. Furthermore, HSC frequency was increased while the number of quiescent HSCs was slightly decreased in mice transplanted with *Cskn1a1* haploinsufficient cells.

Competitive repopulation assays revealed that *Cskn1a1* haploinsufficient stem/progenitor cells (but also mature myeloid and T cells) outcompeted *wild-type* counterparts in both primary and secondary recipients. Haploinsufficiency of *Cskn1a1*, therefore, did not impair haematopoietic differentiation. In fact, mature *Cskn1a1* haploinsufficient cells had a growth advantage. The growth advantage in Del(5q) MDS patients, however, is exclusive to the HSC compartment. The results of this study suggest that haploinsufficiency of *CSKN1A1* may account for the clonal

advantage of Del(5q) HSCs but cannot explain the full disease spectrum.

1.7.2.2 miRNA-145 and miRNA-146a

MicroRNAs (miRNAs) are short noncoding RNAs that suppress the expression of specific messenger RNA targets. The expression of miR-145 and miR-146a, located on chromosome 5q, were significantly reduced in CD34+ cells from Del(5q) MDS patients (Starczynowski, Kuchenbauer et al. 2010). The targets of miR-145 and miR-146a were identified as Toll–interleukin-1 receptor domain–containing adaptor protein (TIRAP) and tumor necrosis factor receptor–associated factor-6 (TRAF6), respectively. TIRAP lies upstream of TRAF6 in innate immune signaling.

Reduced activity of miR-145 and miR-146a together caused thrombocytosis, mild neutropenia and megakaryocytic dysplasia in mice. Furthermore, enforced expression of TRAF6 caused a similar phenotype. Interestingly, a fraction of mice transplanted with TRAF6-expressing marrow developed either bone marrow failure or acute myeloid leukemia. The results of this study suggest that aberrant activation of innate immune signals in the BM can recapitulate some of the clinical features seen in Del(5q) MDS patients. However, it is important to note that the expression of miRNA-145 was reduced by approximately 90% in transduced BM cells, differing from the ~50% reduction in BM cells from Del(5q) MDS patients. Furthermore, while miRNA-145 is located within the Del(5q) MDS CDR, miRNA-146a is located outside the CDR.

1.7.2.3 Tcof1

TCOF1, encoding Treacle, is located within the CDR defined in isolated Del(5q) MDS. Treacle is a component of one of the pre-ribosomal ribonucleoprotein complexes and is required for the transcription of ribosomal DNA (1996, Valdez,

Henning et al. 2004). Mutations in Treacle have been shown to cause Treacher Collins Syndrome (TCS), a rare congenital disorder of craniofacial development (1996). Importantly, haploinsufficiency of *Tcof1* in mice disrupted ribosome biogenesis and inhibited the production of neural crest precursors (Jones, Lynn et al. 2008). Interestingly, inhibition of P53 prevented the development of craniofacial abnormalities.

The impact of *Tcof1* haploinsufficiency on haematopoietic development has not previously been investigated. However, its involvement in ribosome biogenesis and its impact on cellular function at haploinsufficiency, suggests that *Tcof1* is a candidate gene that may contribute to the pathogenesis of Del(5q) MDS.

1.7.2.4 Egr1

EGR1 is located on chromosome 5q, outside of the CDR defined in isolated Del(5q) MDS. *EGR1* encodes a zinc finger transcription factor that regulates the transcription of several tumor suppressor genes such as *TP53*, *CDKN1A/p21*, *TGFB*, and *PTEN*.

Mice with heterozygous or homozygous deletion of *Egr1* were treated with a potent DNA alkylating agent, N-ethyl-nitrosourea (ENU), to induce secondary cooperating mutations (Joslin, Fernald et al. 2007). *Egr1*+/− and *Egr1*−/− mice treated with ENU developed T-cell lymphoma or a myeloproliferative disorder (MPD) at an increased rate with a shorter latency period than *wild-type* littermates. This suggests that deficiency of *Egr1* may enhance malignant transformation. However, the findings of this study are most relevant to therapy-related MDS/AML (t-MDS/AML) rather than isolated Del(5q) MDS since patients that develop t-MDS/AML have been treated with alkylating agents. Furthermore, *EGR1* lies within the CDR on chromosome 5q defined in t-MDS/AML.

Another study demonstrated that complete loss of *Egr1* in mice stimulated HSC division (Min, Pietramaggiori et al. 2008). HSCs were also readily mobilized to the peripheral blood in *Egr1* null mice. This suggests that *Egr1* may regulate HSC quiescence and migration. However, the impact of *Egr1* haploinsufficiency on HSC activity, the most relevant setting for Del(5q) MDS, was not examined.

1.7.2.5 Apc

APC is located at chromosome band 5q23, outside the Del(5q) MDS CDR. APC, similar to CK1a, is a negative regulator of beta catenin, which is involved in stem cell self-renewal.

Haploinsufficiency of *Apc* in mice caused the development of anaemia with macrocytosis and monocytosis (Wang, Fernald et al. 2010). Furthermore, there was an expansion of HSCs in the BM of *Apc* mutant mice. However, competitive transplantations revealed that *Apc* haploinsufficient HSCs had decreased long-term repopulation capacity.

Another study investigated if introducing a point mutation into the *Apc* gene, *Apc* min, had an impact on HSC function (Lane, Sykes et al. 2010). BM cells from *Apc* min mice had increased repopulation potential in primary transplants but impaired repopulation potential in secondary transplants (Lane, Sykes et al. 2010). These studies suggest that deficiency of *Apc* leads to HSC exhaustion upon transplantation, which may be attributed to their exit from quiescence.

1.8 Aims

In this study, we used a mouse model of Del(5q) MDS, the *Cd74-Nid67* model, to explore the initiating events responsible for disease development.

We investigated whether haploinsufficiency of *Cd74-Nid67* conferred aberrant self-renewal and disease propagating ability to HSCs. The use of the *Cd74-Nid67* mouse model provided scope to investigate whether haploinsufficiency of eight genes (out of over forty located within the CDR in Del(5q) MDS) could recapitulate the HSC dysfunction seen in patients.

If, indeed, *Cd74-Nid67* HSCs acted as cancer stem cells, the mechanisms responsible for disease initiation and propagation could be explored. The identification of the gene(s) responsible for HSC dysfunction would provide important insights into the mechanistic basis of disease development. Further mechanistic insight could be provided by examining if P53, previously implicated in Del(5q) MDS pathogenesis, was involved in driving *Cd74-Nid67* HSC dysfunction.

In summary, the aims of this study were:

1. To investigate the impact of *Cd74-Nid67* haploinsufficiency on HSC function
2. To identify the genes within *Cd74-Nid67* that are responsible for HSC dysfunction
3. To examine if a P53-dependant mechanism drives *Cd74-Nid67* HSC dysfunction

2. Materials and Methods

2.1 Mouse Studies

C57BL/6 (CD45.2) and B6SJL (CD45.1) mice were bred at the Biomedical Sciences Centre, John Radcliffe Hospital, Oxford.

Cd74-Nid67 mice (Barlow, Drynan et al. 2010) were kindly provided by Andrew McKenzie (University of Cambridge, UK) and were crossed to *VavCre* transgenic mice to induce deletion in all haematopoietic cells (Georgiades, Ogilvy et al. 2002). *Cd74* knockout mice (Bikoff, Huang et al. 1993), were kindly provided by Elizabeth Robertson (University of Oxford, UK).

Trp53 mutant mice (Marino, Vooijs et al. 2000) were crossed to *Cd74-Nid67* and *VavCre* mice.

All experiments were done with the approval of the UK Home Office, Project License 30/3103. All mice used were between 8-12 weeks of age, unless otherwise stated.

2.2 Genotyping

DNA was isolated from earclips using the E.Z.N.A Tissue DNA Kit (Omega bio-tek), according to manufacturers instructions. PCRs were performed using the primers outlined in Table 2.1 to determine the genotype of mice.

Table 2.1 Genotyping Analysis of Transgenic Mice

	Primer Sequences	**Annealing Temp.**	**Polymerase**
Cd74-Nid67 floxed allele	Forward Primer: CACGTGACCCAGGAGACTAC Reverse Primer: GCTATTGGGAAGGCTCTGACA	60°C	Taq DNA Polymerase (Thermofisher Scientific)
VavCre transgene	Forward Primer: AGATGCCAGGACATCAGGAACCTG Reverse Primer: ATCAGCCACACCAGACACAGAGATC	62°C	Phusion High-Fidelity DNA Polymerase (New England Biolabs)
Cd74 KO	Forward Primer: GGCTAGGTCCCAGTGTAGGC Reverse Primer: CGCTGACAGCCGGAACACGG	60°C	Kapa2G Robust DNA Polymerase (Kapabiosystems)
Trp53 floxed allele	Forward Primer: AAGGGGTATGAGGGACAAGG Reverse Primer: GAAGACAGAAAAGGGGAGGG	60°C	Kapa2G Robust DNA Polymerase (Kapabiosystems)

2.3 Peripheral Blood Count Analysis

Blood samples were collected from tail veins in EDTA coated tubes and cellularity was measured using the haematology analyser, Sysmex KX-21N.

2.4 Flow Cytometry

2.4.1 Phenotypic HSC and Progenitor analysis

Femora and tibiae from experimental mice were crushed using a pestle and mortar and resuspended in PBS supplemented with 5% Fetal Calf Serum (staining medium). Cells were counted using the haematology analyser, Sysmex KX-21N. Bone marrow cells were centrifuged, resuspended in Fc-block, stained with monoclonal antibodies (2X) and incubated for fifteen minutes on ice. Cells were then washed and resuspended in staining medium. Flow cytometric analysis was performed using the BD LSRII Flow Cytometer. See Tables 2.2, 2.3 and 2.4 for details on the instrument settings and antibodies used for staining and identification of cell populations. All experiments included fluorescent-minus-one (FMO) and single-stained controls (Maecker and Trotter, 2006).

Table 2.2 FACS Aria IIu and LSRII configurations

Instrument	Laser Wavelength	Laser Power	PMTS and filter configuration
BD FACS Aria IIlu	Violet 407nm	100mW	QD605 610/20; Pacific Blue: 450/50; Pacific Orange 585/42;
	Blue 488 nm	100mW	FITC: 525/50; SSC: 488/10
	Green 532 nm	150 mW	PE-Cy7: 780/60; PE-Cy5: 685/35; PETexasRed: 610/20; PE: 575/26
	Red 638nm	40mW	Alexa Fluor 700: 730/45; APC:670/14
BD LSRII	Violet 407nm	50mW	Pacific Blue: 450/50; Pacific Orange: 585/42;
	Blue 488 nm	100 mW	FITC: 525/50; SSC: 488/10
	Green 532 nm	150 mW	PE-Cy7: 780/60; PE-Cy5: 685/35; PETexas Red: 610/20; PE: 575/25
	Red 640 nm	40 mW	Alexa Fluor 700: 730/45; APC:670/14

2.4.2 Cell Cycle analysis

Bone marrow cells were resuspended in Fc block and stained with cell surface antibodies (Table 2.3). The stained cells were incubated in a fixation and permeabilisation solution (BD) on ice for 30 minutes. The cells were washed and stained with anti-Ki67 antibody (BD) and left shaking overnight at 4°C in the dark. The following day samples were stained with DAPI and analysed on the LSRII.

Table 2.3 FACS antibody staining panels for HSC, cell cycle and myeloid progenitor analysis

Antigen	Conjugate	Company	Clone	Staining Panel
Sca-1	FITC	BD	E13-161.7	HSC, Cell Cycle
c-kit	APCeF780	eBioscience	2B8	HSC, Cell Cycle
CD150	PECy7	Biolegend	TC15-12F12.2	HSC, Cell Cycle
CD48	APC	Biolegend	HM48-1	HSC, Cell Cycle
CD34	Biotin	eBioscience	RAM34	HSC
Streptavidin	PETxRed	BD	n/a	HSC
CD8	PECy5	Biolegend	53-6.7	HSC, Cell Cycle, Myeloid Progenitor
CD5	PECy5	Biolegend	53-7.3	HSC, Cell Cycle, Myeloid Progenitor

B220	PECy5	Biolegend	RA3-6B2	HSC, Cell Cycle, Myeloid Progenitor
Gr-1	PECy5	Biolegend	RB6-8C5	HSC, Cell Cycle, Myeloid Progenitor
Ter119	PECy5	Biolegend	TER-119	HSC, Cell Cycle
DAPI				HSC (viability) Cell Cycle (DNA stain)
Ki67	PE	BD	B56	Cell Cycle
CD16/32	PE	eBioscience	93	Myeloid Progenitor
CD150	APC	Biolegend	TC15-12F12.2	Myeloid Progenitor
CD105	Biotin	Biolegend	MJ7/18	Myeloid Progenitor
CD41	PECy7	eBioscience	MWReg30	Myeloid Progenitor
Ter119	PerCP Cy5.5	eBioscience	TER-119	Myeloid Progenitor
Mac-1	APC	Biolegend	M1/70	Mature Myeloid Cells
Gr-1	FITC	eBioscience	RB6-8C5	Mature Myeloid Cells

2.5 Competitive Transplantation Experiments

Recipient C57BL/6 CD45.1 mice were lethally irradiated (9 Gy) and injected with five million *wild-type* competitor (CD45.1) cells and the volume equivalent of five million donor (CD45.2) cells (Figure 2.1). Peripheral blood and bone marrow reconstitution was analysed after sixteen weeks.

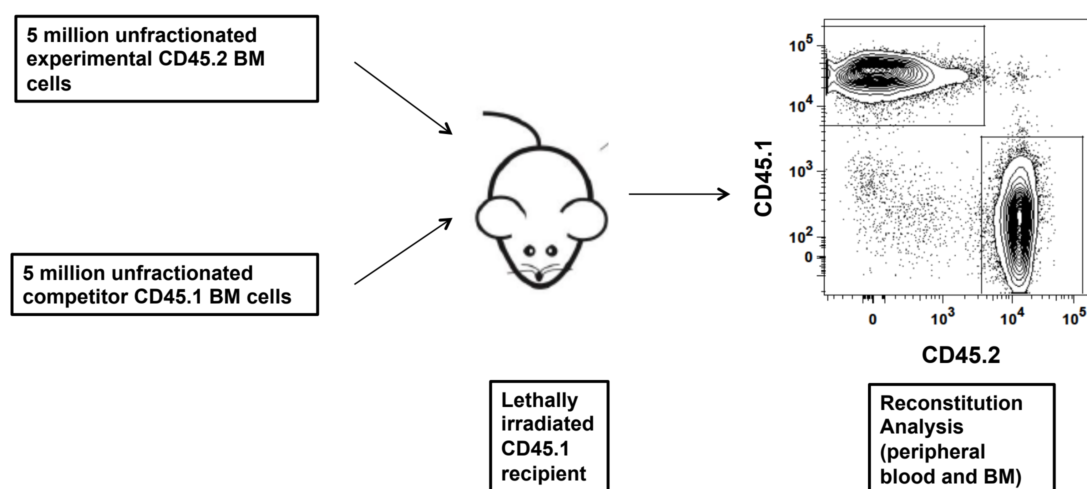


Figure 2.1 Experimental Design of Competitive Transplantation Assays

Schematic diagram of competitive transplantation experiments. 5 million donor (CD45.2) cells were harvested from experimental mice, mixed with 5 million competitor (CD45.1) cells and injected into lethally irradiated recipient (CD45.1) mice. The reconstitution of donor CD45.2 cells in blood and BM was assessed by flow cytometry.

2.5.1 Peripheral Blood Reconstitution Analysis

Blood samples were collected from tail veins in EDTA coated tubes. WBCs were separated from erythroid cells using 2% Dextran. Ammonium Chloride was used to lyse remaining erythroid cells. WBCs were stained using the antibodies listed in Table 2.4 and analysed using the LSR II flow cytometer (BD). Viable cells were identified

by 7AAD exclusion. All experiments included fluorescent-minus-one (FMO) and single-stained controls.

Table 2.4 FACS antibody panel for reconstitution analysis of HSCs, myeloid progenitors and peripheral blood

Antigen	Conjugate	Company	Clone	Staining Panel
CD45.2	AF700	Biolegend	104	HSC, myeloid progenitor, blood reconstitution
CD45.1	FITC	Biolegend	A20	HSC, myeloid progenitor, blood reconstitution
CD11b (Mac1)	APC	Biolegend	M1/70	blood reconstitution
Gr1	PO	Invitrogen	RB6-8C5	blood reconstitution
CD19	PECy7	BD	1D3	blood reconstitution
CD4/CD8	PE	Biolegend	H129.19/53-6.7	blood reconstitution
NK1.1	PB	Biolegend	PK136	blood reconstitution
7AAD (Viability)				

2.6 *In vitro* Assays to Examine Myeloid and Megakaryocyte Potential

Seventy-five HSCs were sorted into 1.5 ml of X-Vivo 15 (SLS Life Science) supplemented with 10% FCS (HyClone), 10^{-4} M 2-mercaptoethanol (Sigma), 1% penicillin/streptomycin (PAA Laboratories) and the following cytokines: 50 ng/ml murine stem cell factor (mSCF; PeproTech), 50 ng/ml human FLT3 ligand (hFL; Immunex), 50 ng/ml human thrombopoietin (hTPO; PeproTech), 20 ng/ml murine interleukin 3 (mIL-3; PeproTech), 10 ng/ml human erythropoietin (hEpo). Twenty microliters were transferred to each well of a 60-well Terasaki plate (VWR), seeding on average one cell per well. Density of cell growth in each well was evaluated after 7 days of culture.

2.7 shRNA Lentivirus Experiments

shRNA lentiviral supernatants were prepared by the Vector Unit, Lund University in collaboration with Jonas Larsson. Titres and target sequences are outlined in Table 2.5 below.

Purified LSK cells from *wild-type* mice (C57/BL6) were pre-stimulated in serum free medium (StemSpan SFEM, Stem Cell Technologies) supplemented with penicillin/streptomycin (GIBCO), mSCF (100ng/ml), human thrombopoietin (mTPO; 100ng/ml), mIL-3 (20ng/ml) and hFlt3 (100ng/ml) in 96 well plates for 3 hours at 37°C (0.5 million cells per ml). shRNA lentiviruses (at a final dilution of 1/25) were added to cells and incubated for 48 hours at 37°C. 20,000 LSK cells were harvested, washed with PBS + 1% FCS and mixed with 200,000 unfractionated competitor cells and injected into a lethally irradiated CD45.1 recipient. HSC reconstitution was analysed ~12 weeks post transplant using the antibodies listed in Table 2.7.

Transduction efficiency, as measured by frequency of GFP+ cells, was determined 5 days after transduction. Cell viability was measured by DAPI exclusion. Transduced, GFP+ cells were sorted on the BD FACS Aria Illu for gene expression analysis.

Table 2.5 Titres and target sequences of *Rps14* shRNA lentiviruses

shRNA ID	Target Sequence	Titre (TU/ml)	MOI
TRCN0000104360 (shRNA 60)	CCGATCTTTCTGGCAAGGAAA	7.7x10^7	6
TRCN0000104361 (shRNA 61)	GCATCCTTCAATGACACCTTT	4.5x10^8	36

2.8 cDNA Lentivirus Experiments

cDNA lentiviral preparations were prepared by the Vector Unit, Lund University in collaboration with Jonas Larsson. Titres and codon optimised cDNA sequences are outlined in Table 2.6.

Purified LSK cells from *Cd74-Nid67* mice (C57/BL6) were pre-stimulated in serum free medium (StemSpan SFEM, Stem Cell Technologies) supplemented with penicillin/streptomycin (GIBCO), mSCF (100ng/ml), human thrombopoietin (mTPO; 100ng/ml), mIL-3 (20ng/ml) and hFlt3 (100ng/ml) in 96 well plates for 3 hours at 37°C (0.5 million cells per ml). cDNA lentiviruses (at a final dilution of 1/25) were added to cells and incubated for 48 hours at 37°C. 5,000 LSK cells were harvested, washed with PBS + 1% FCS, mixed with 200,000 unfractionated competitor cells and injected into a lethally irradiated CD45.1 recipient. Myeloid reconstitution in peripheral blood was analysed ~4 weeks post transplant using the antibodies listed in Table 2.7.

Transduction efficiency, as measured by frequency of GFP+ cells, was determined 5 days after transduction. Cell viability was measured by DAPI exclusion. Transduced, GFP+ cells were sorted on the BD FACS Aria Illu for gene expression analysis.

Table 2.6 Titres and sequences of cDNA lentiviruses

Gene	Codon Optimised Sequence	Titre (TU/ml)
Rps14	ATGGCACCCAGGAAAGGAAAAGAGAAGAAGGAAGAGCAGG TCATCAGCCTCGGTCCCCAGGTCGCAGAAGGAGAAAACGTG TTCGGGGTGTGCCACATCTTCGCCAGCTTTAACGACACTTTC GTGCATGTCACCGATCTGTCTGGGAAAGAGACAATTTGTCGA GTGACTGGCGGAATGAAGGTCAAAGCTGACAGGGATGAAAG CTCCCCATACGCAGCTATGCTGGCAGCACAGGACGTGGCAC AGCGGTGCAAGGAGCTCGGAATCACCGCACTGCACATCAAG CTCCGGGCCACAGGGGGTAATCGAACCAAGACACCAGGACC TGGAGCACAGTCTGCTCTGAGGGCTCTCGCAAGAAGTGGGA TGAAGATCGGTAGAATTGAAGATGTGACACCCATTCCATCC GACTCAACAAGACGAAAAGGGGGGCGACGAGGTAGAAGGC TCTGA	4.5x10^8 MOI 36
Rbm22	ACCGGTATGGCTACCTCCCTGGGCTCCAATACCTACAATCGG CAGAACTGGGAAGATGCTGACTTTCCTATCCTCTGTCAGACC TGTCTCGGGGAAAACCCCTACATCCGGATGACAAAGGAAAA ATATGGAAAAGAGTGCAAGATTTGTGCACGCCCCTTCACCGT GTTCAGGTGGTGCCCAGGGGTCCGGATGCGCTTCAAGAAAA CAGAAGTGTGCCAGACTTGTTCTAAGCTGAAAAATGTCTGCC AGACCTGTCTGCTCGACCTGGAGTACGGGCTCCCAATCCAGG TGCGCGATGCTGGTCTGAGCTTCAAGGACGATATGCCCAAG AGCGACGTGAACAAGGAGTACTATACTCAGAACATGGAAAG AGAGATTAGCAATTCCGACGGAACCCGGCCTGTGGGAATGC TGGGAAAGGCTACCTCTACAAGTGATATGCTGCTCAAGCTCG CAAGGACCACACCATACTATAAACGAAACAGGCCCCACATC TGCAGCTTCTGGGTGAAAGGCGAATGCAAGCGGGGAGAGGA ATGTCCATATCGCCATGAGAAGCCCACAGACCCTGACGATC CACTGGCCGACCAGAACATCAAGGATAGATACTACGGAATT AATGACCCCGTGGCAGATAAACTGCTCAAGAGGGCCTCCAC CATGCCTAGACTGGACCCCCCTGAGGATAAGACAATCACTA CCCTGTACGTCGGCGGACTCGGGGACACTATTACCGAAACA GATCTGCGCAATCACTTCTATCAGTTTGGCGAGATCCGAACT ATTACCGTGGTCCAGAGGCAGCAGTGTGCTTTCATCCAGTTT GCAACCCGACAGGCCGCTGAAGTGGCAGCCGAGAAATCCTT TAACAAGCTGATTGTGAATGGCAGGAGACTCAACGTCAAGT GGGGCAGAAGTCAGGCTGCACGGGGAAAGGAAAAAGAGAA GGACGGGACAACTGATTCAGGTATCAAGCTGGAGCCAGTGC CAGGACTGCCTGGTGCCCTCCCACCACCTCCAGCCGCTGAGG AAGAGGCATCCGCCAACTACTTCAATCTGCCACCTTCTGGAC CACCCGCCGTGGTCAACATCGCTCTCCCTCCACCACCTGGTA TTGCACCACCACCTCCACCAGGATTCGGTCCACACATGTTTC ATCCTATGGGCCCTCCACCCCCTTTTATGAGGGCTCCTGGAC CAATCCATTACCCTTCACAGGACCCTCAGAGAATGGGGGCTC ACGCTGGCAAACATAGCTCACCTTAACCGCGG	5.3x10^8 MOI 42

Table 2.7 FACS antibody panel for reconstitution analysis of HSCs and mature myeloid cells in lentiviral experiments

Antigen	Conjugate	Company	Clone	Staining Panel
CD45.1	FITC	Biolegend	A20	HSC and peripheral blood reconstitution
CD45.2	AF700	Biolegend	104	HSC and peripheral blood reconstitution
Sca-1	PB	Biolegend	D7	HSC reconstitution
c-kit	APCeF780	eBioscience	2B8	HSC reconstitution
CD150	PECy7	Biolegend	TC15-12F12.2	HSC reconstitution
CD48	APC	Biolegend	HM48-1	HSC reconstitution
CD8	PECy5	Biolegend	53-6.7	HSC reconstitution
CD5	PECy5	Biolegend	53-7.3	HSC reconstitution
B220	PECy5	Biolegend	RA3-6B2	HSC reconstitution
Gr-1	PECy5	Biolegend	RB6-8C5	HSC reconstitution
Ter119	PECy5	Biolegend	TER-119	HSC reconstitution

Antigen	Conjugate	Company	Clone	Staining Panel
Mac-1	APC	Biolegend	M1/70	Peripheral blood (myeloid) reconstitution
Gr-1	PO	eBioscience	RB6-8C5	Peripheral blood (myeloid) reconstitution

2.9 Gene expression analysis

Cells were sorted into tubes containing 5ul Cells Direct 2x reaction mix (Invitrogen), 0.1ul SUPERase 12-In RNAse inhibitor (Ambion), 1.2ul TE buffer (Sigma), 1.2ul RT Taq mix (Invitrogen) and 2.5ul 0.2x gene specific Taqman assay mix. This was in a total volume of 10ul. Reverse transcription and amplification of target transcripts was performed on a thermal cycler (Tetra II Biorad) using the following protocol: RT reaction time at 50°C for 15 minutes, inactivation of reverse transcriptase at 95°C for 2 minutes, target gene amplification in 22 cycles at 95°C for 15 seconds followed by 60°C for 4 minutes. Samples were then loaded on to a primed Biomark 48.48 dynamic array. The following quantitative PCR reaction was performed: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

TaqMan primers used for the analysis are shown in Table 2.8 below. Data was analysed using the ΔCt method and results were normalized to *Gapdh* expression. Two replicates (50-100 cells/replicate) were included for each cell population per mouse.

Table 2.8 Taqman gene expression assays used for quantification of relative gene expression

Gene	Name	Probe ID
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (p21)	Mm04205640_g1
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1
Rbm22	RNA binding motif protein 22	Mm01266642_m1
Rps14	Ribosomal protein S14	Mm00849906_g1

2.10 RNA Sequencing, Sample preparation and Analysis

Samples for RNA sequencing were prepared using the SMARTer Ultra Low RNA kit for Illumina Sequencing (Clontech) according to manufacturers instructions as previously described (Ramskold, Luo et al. 2012). Briefly, 150 murine HSCs were sorted directly into lysis buffer supplemented with RNase Inhibitor (Clontech). cDNA library was prepared according to manufacturer instructions with 15 cycles of amplification. Amplified cDNA libraries validated with a distinct peak spanning 400 bp to 9000 bp in size as measured by High Sensitivity DNA kit (Agilent) on Agilent 2100 Bioanalyzer were further processed for tagmentation and Nextera indexing (Illumina). The size of the amplified fragments was confirmed using the Agilent DNA1000 Kit (Agilent). 8 samples with different indexes were pooled per lane and sequenced on HiSeq 2000 (Illumina) generating single–end, 50 bp reads. Nikolaos Barkas, University of Oxford, performed bioinformatic analysis. QC analysis was performed and the individual sample reads were mapped to the UCSC mouse (GRCm38) genome. Gene transcript expression levels were quantified using the RPKM (Reads Per Kilobase of transcript per Million mapped reads) metric.

2.11 Patient Studies

RNA sequencing data from Del(5q) MDS patients and healthy controls was included in this study (Figure 3.8 and Figure 4.1). Dr. Petter Woll, University of Oxford performed all experiments with human samples, as previously described (Woll, Kjallquist et al. 2014).

Bone marrow and peripheral blood specimens from Del(5q) MDS patients and normal age-matched volunteers, were collected from Karolinska Institute, Sweden; Lund University Hospital, Sweden; Oslo University Hospital, Norway, Aarhus University Hospital, Denmark and Hopital Saint Louis Paris, France. All patients and volunteers provided written informed consent and the study was approved by the relevant local ethics committees.

2.12 Data Analysis

FlowJo (Version 8) analysis software (TreeStar) was used to analyse all flow cytometry data. Graphpad prism was used for all statistical analysis. Unless otherwise stated in the main text, statistical 2-way comparisons were made using a student's t-test and multiple comparisons using the One Way Anova.

3. The Impact of *Cd74-Nid67* Haploinsufficiency on HSC Function

3.1 Introduction

Deletion of 5q is an initiating event in Del(5q) MDS that is both required and sufficient to in propagate disease (Woll, Kjallquist et al. 2014). The commonly deleted region (CDR) spans approximately 40 genes, located between 5q31 and 5q32 (Boultwood, Fidler et al. 1994) and is distinct from the more proximal 5q deletion in patients with therapy-related MDS (t-MDS) and AML (Lai, Godley et al. 2001). Previous studies have failed to demonstrate that homozygous inactivation occurs for any gene within the CDR, suggesting that haploinsufficiency of one or more genes within this region is responsible for Del(5q) MDS pathogenesis (Heinrichs, Kulkarni et al. 2009).

The 5q deletion originates in the CD34+ CD38- CD90+ multipotent haematopoietic stem cell (HSC) compartment in patients (Tehranchi, Woll et al. 2010, Woll, Kjallquist et al. 2014). Del(5q) HSCs have a clonal advantage, outcompeting "healthy" HSCs in the bone marrow of patients (Nilsson, Astrand-Grundstrom et al. 2000). Although Del(5q) HSCs have a clonal advantage *in situ*, they perform extremely poorly in functional stem cell assays *in vitro* (in LTC-IC assays) and *in vivo* (upon transplantation into immune deficient mice) (Nilsson, Astrand-Grundstrom et al. 2000, Tehranchi, Woll et al. 2010).

In addition to impairing HSC function, the 5q deletion restricts erythroid and megakaryocyte differentiation (Nimer 2008). Macrocytic anaemia and hypolobulated

megakaryocytes are, therefore, prominent features of Del(5q) MDS. The molecular basis underlying the differentiation deficit in Del(5q) MDS has previously been investigated, identifying *Rps14* (Ebert, Pretz et al. 2008) and miR-145 and miR-146a (Starczynowski, Kuchenbauer et al. 2010) as important mediators of erythroid and megakaryocyte differentiation, respectively. The identification of key molecular drivers of HSC dysfunction, however, requires further exploration.

Deficiency of *Apc,* a gene located on 5q, in mice, altered HSC self-renewal and repopulating ability (Wang, Fernald et al. 2010). However, *Apc* lies outside the CDR defined in isolated Del(5q) MDS and is, therefore, unlikely to drive disease pathogenesis. Deficiency of *Apc* may contribute to the clinical phenotype in t-MDS and AML patients with more proximal 5q deletions or, indeed, in Del(5q) MDS patients with larger deletions.

A recent study demonstrated that haploinsufficiency of *Cskn1a1*, a gene located within the commonly deleted region in Del(5q) MDS, conferred a modest competitive advantage to LSK stem/progenitor cells upon serial transplantation (Schneider, Adema et al. 2014). However, unlike Del(5q) MDS, the growth advantage was not exclusive to HSCs but was also seen in mature myeloid and T cells. Furthermore, *Cskn1a1* haploinsufficiency did not affect haemoglobin levels but caused an increase in white cell counts with lymphocytosis (Schneider, Adema et al. 2014). This suggests that *Cskn1a1* deficiency may contribute to aberrant HSC proliferation in Del(5q) MDS but does not account for the full disease spectrum.

The identification of the gene(s) responsible for HSC dysfunction in Del(5q) MDS will be key to understanding the mechanistic basis for disease development and propagation. The *Cd74-Nid67* mouse model of Del(5q) MDS allows conditional deletion of eight genes (including *Rps14*) corresponding to part of the CDR in

patients (Barlow, Drynan et al. 2010). This provides scope to identify the gene(s) responsible for HSC dysfunction.

Cd74-Nid67 deficiency targeted to haematopoietic cells using the *Lmo2Cre* line has previously been shown to cause macrocytic anaemia and bone marrow dysplasia (Barlow, Drynan et al. 2010). This suggests that deficiency of this region is important in Del(5q) MDS pathogenesis. The impact of *Cd74-Nid67* haploinsufficiency on HSCs, however, has yet to be explored.

In this study, we crossed *Cd74-Nid67* fl/+ mice to the *VavCre* mouse line (Georgiades, Ogilvy et al. 2002) to target the deletion to all haematopoietic cells. The principal aim was to investigate the impact of *Cd74-Nid67* haploinsufficiency on HSC self-renewal and repopulation potential. This may provide important insights into mechanisms of disease initiation and promote the development of improved therapeutic avenues for patients.

3.2 Results

3.2.1 Haploinsufficiency of *Cd74-Nid67* Alters RBC Number and Size

To confirm that haploinsufficiency of *Cd74-Nid67* has an effect on RBC parameters, blood samples from *Cd74-Nid67* mice (*Cd74-Nid67* fl/+ *VavCre* tg/+) and littermate controls (*Cd74-Nid67* +/+ *VavCre* tg/+) were analysed. There was no change in white blood cell (WBC) count (Figure 3.1 A), haemoglobin concentration (Figure 3.1 C) or platelet count (Figure 3.1 F) in *Cd74-Nid67* mice. However, the number of RBCs was significantly reduced in *Cd74-Nid67* mice compared to controls (Figure 3.1 B). Furthermore, the mean corpuscular volume (MCV) of RBCs (Figure 3.1 D) and the standard deviation of RBC distribution width (Figure 3.1 E) were significantly increased in *Cd74-Nid67* mice.

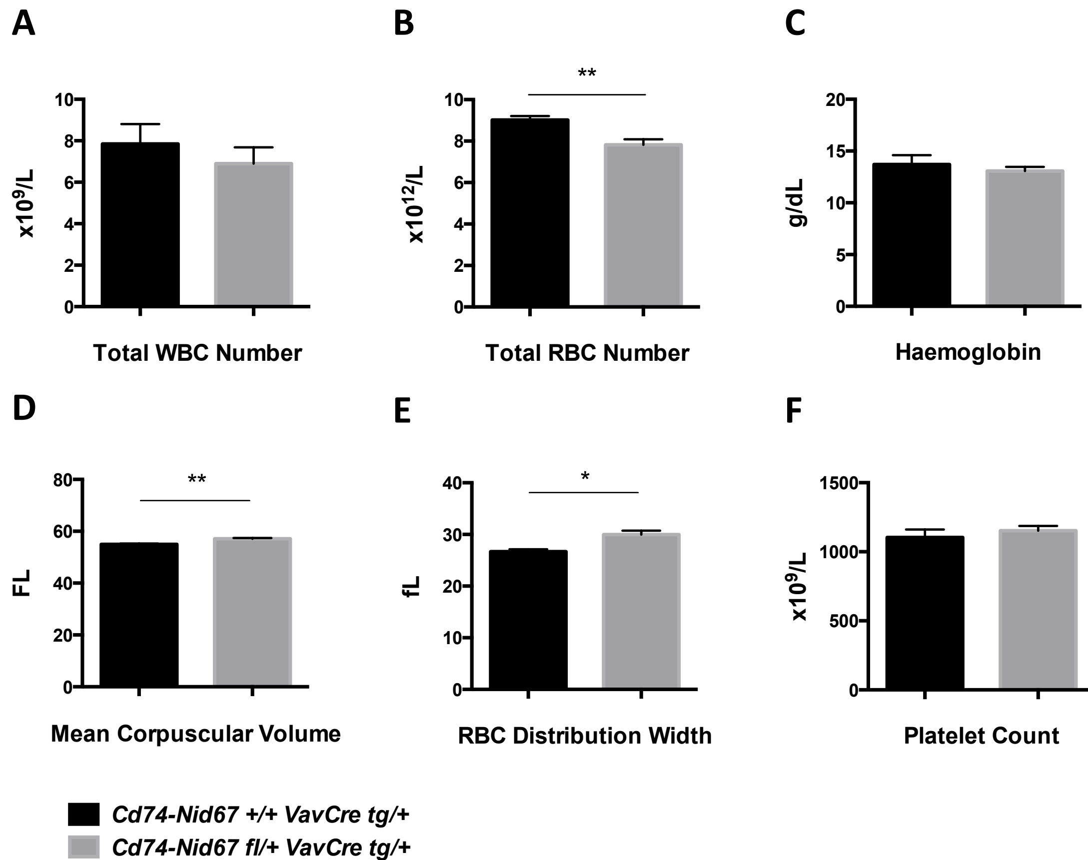


Figure 3.1 Peripheral blood measurements of *Cd74-Nid67* mice

Blood from adult (8 week old) mice with the indicated genotypes was analysed (n=6). **A.** White Blood Cell (WBC) number. **B**. Red Blood Cell (RBC) number. **C**. Haemoglobin concentration. **D**. Mean corpuscular volume. **E.** Red Blood Cell (RBC) distribution width. **F**. Platelet Count.

Data is expressed as mean +/- SEM. * $P<0.05$, ** $P<0.01$.

3.2.2 *Cd74-Nid67* Haploinsufficiency Causes an Increase in HSC Number

To examine the impact of *Cd74-Nid67* deficiency at the stem cell level, flow cytometric analysis was performed to determine the number of HSCs in the BM of *Cd74-Nid67* mice.

The gating strategy used for analysis is shown (Figure 3.2 A and B). Deficiency of *Cd74-Nid67* caused a significant increase in the number of LSK (Lineage-, Sca-1+, c-kit+) stem/progenitor cells (Figure 3.2 C). The number of Short-Term (ST-HSC) and Long-Term HSCs (LT-HSC), defined as Lineage-, Sca-1+, c-kit+, CD48-, CD150+ and CD34+ or CD34-, respectively, were also significantly increased in *Cd74-Nid67* mice (Figure 3.2 D).

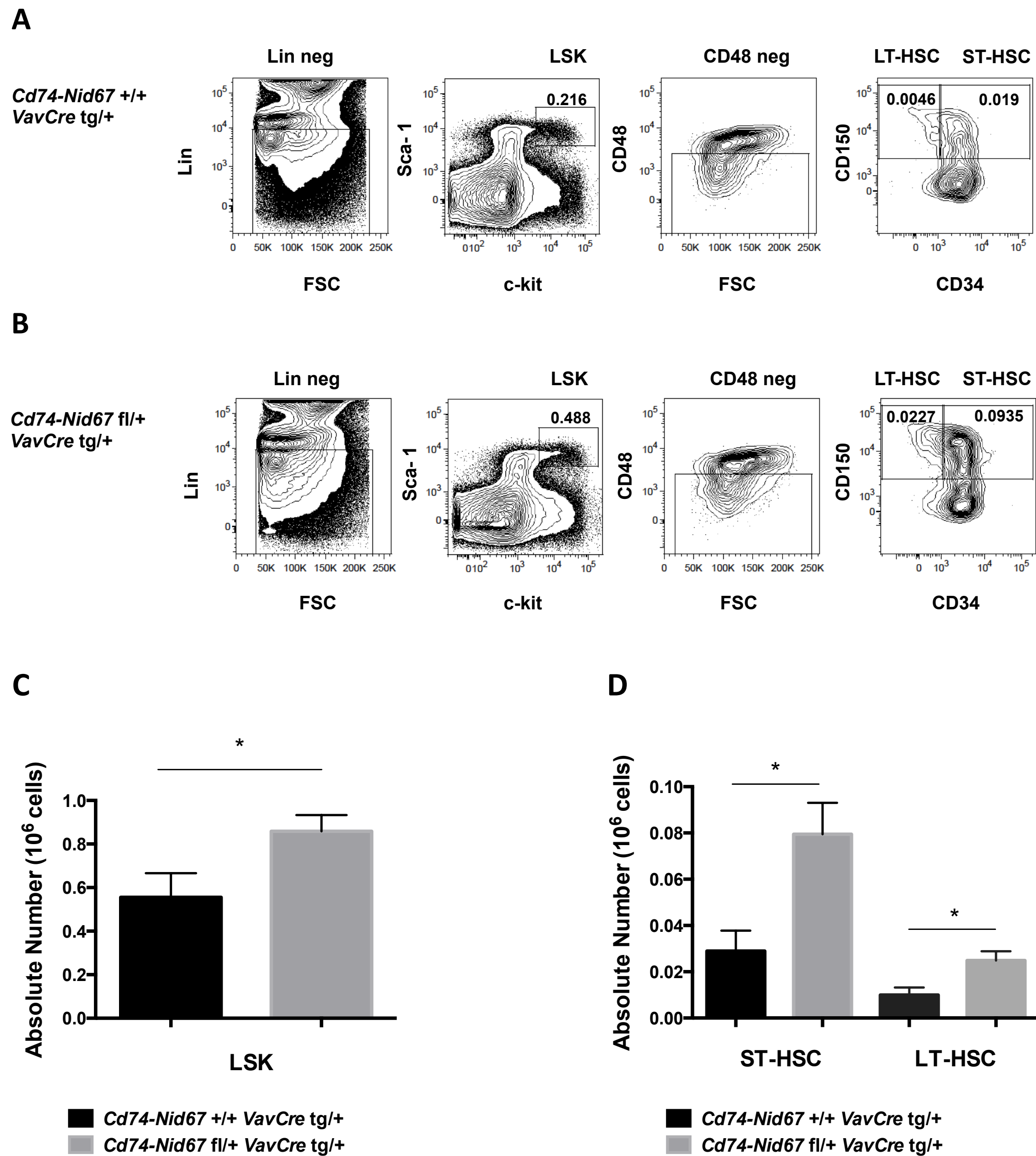


Figure 3.2 Impact of *Cd74-Nid67* haploinsufficiency on stem/progenitor cells

Bone Marrow (BM) from adult mice (8-12 weeks old) with the indicated genotypes was analysed (n=8). **A.** and **B.** Representative FACS profiles of stem and progenitor populations from a control mouse and a *Cd74-Nid67* mouse, respectively. Numbers within quadrants indicate the frequency of the population in total BM. **C.** Absolute number of stem/progenitor (LSK) cells. **D.** Absolute number of Short Term HSCs (ST-HSC) and Long Term HSCs (LT-HSC). Data is expressed as mean +/- SEM. P<0.05, ** P<0.01, *** P<0.001

3.2.3 *Cd74-Nid67* Haploinsufficiency Disrupts HSC Quiescence

To determine if *Cd74-Nid67* haploinsufficiency affects stem cell quiescence, the cell cycle status of HSCs was examined (Figure 3.3 A and B). HSCs were defined as Lineage-, Sca-1+, c-kit+, CD48-, CD150+. The cell surface marker, CD34, which distinguishes long-term HSCs from short-term HSCs, was not included since its expression is directly linked to the activation status of HSCs (Ogawa, Tajima et al. 2001).

Haploinsufficiency of *Cd74-Nid67* resulted in a significant reduction of HSCs in G0 phase of the cell cycle and a corresponding increase in the number of HSCs in G1. However, there was no change in the frequency of HSCs in S/G2/M (Figure 3.3 C).

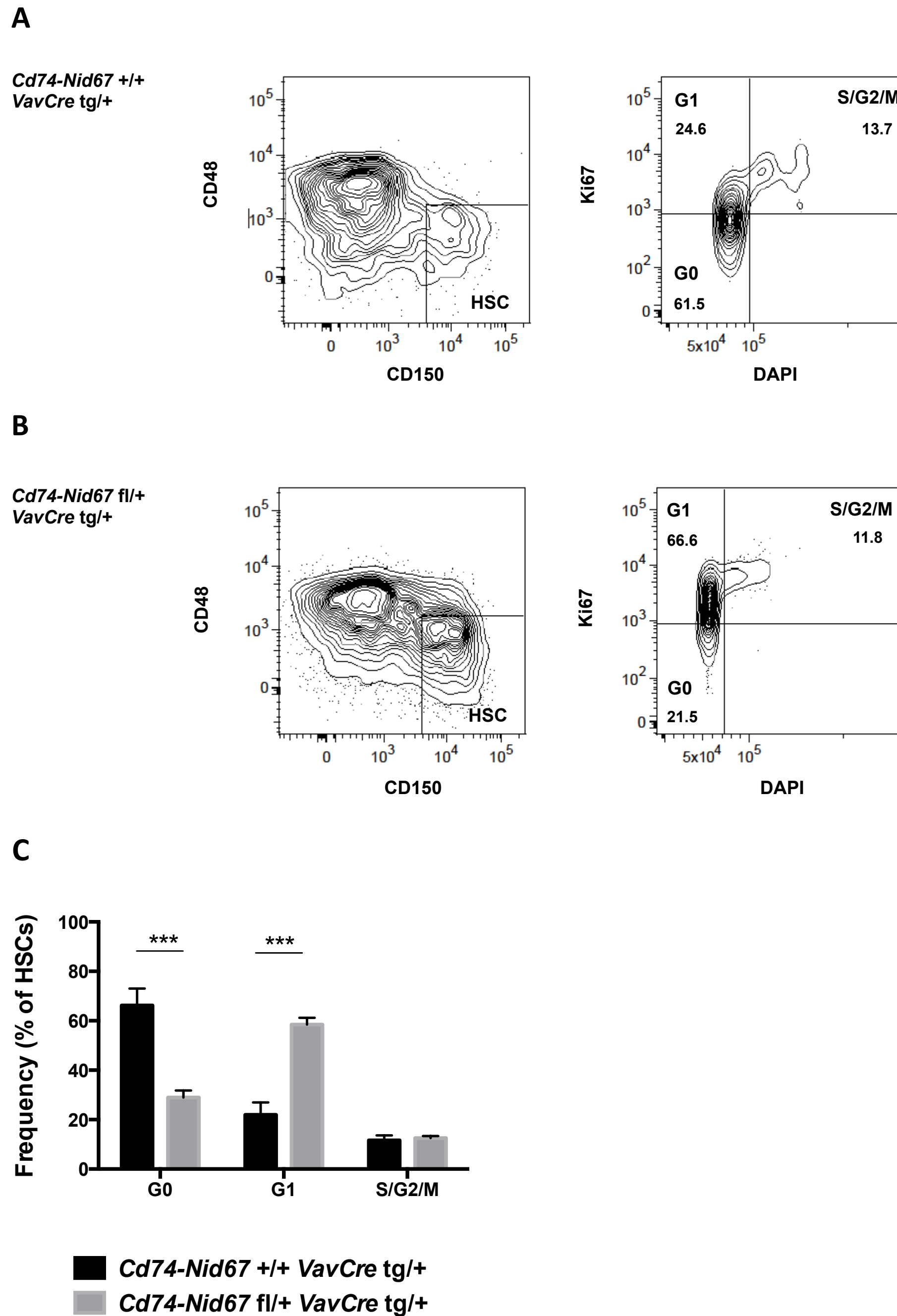


Figure 3.3 Cell cycle status of *Cd74-Nid67* HSCs

Cell cycle status of HSCs in adult mice with the indicated genotypes was analysed using Ki67 and DAPI staining (n=6). **A.** and **B.** Representative FACS profiles from a control mouse and a *Cd74-Nid67* mouse, respectively. Numbers within quadrants indicate the frequency of HSCs in each phase of the cell cycle. **C.** Frequency of HSCs in G0 (Ki67-, DAPI-), G1 (Ki67+, DAPI-) and S/G2/M (Ki67+, DAPI+). Data is expressed as mean +/- SEM. * P<0.05, ** P<0.01, *** P<0.001

3.2.4 *Cd74-Nid67* Hapolinsufficiency Specifically Affects Erythroid Progenitors

To investigate the effect of *Cd74-Nid67* deficiency on myeloid, erythroid and megakaryocytic progenitors, flow cytometric analysis was performed and LSK cells were separated based on the expression of CD150, CD41, FcgRII, CD105 and Ter119, as previously described (Pronk, Rossi et al. 2007). The gating strategy used for analysis is shown (Figure 3.4 A and B).

Cd74-Nid67 haploinsufficiency did not alter the number of early or mature pre-granulocyte monocyte progenitors (PreGM and GMP, respectively). There was also no change in pre-megakaryocyte erythroid progenitors (Pre-MegE) or megakaryocyte progenitors (MkP) in *Cd74-Nid67* mice (Figure 3.4 C). Early erythroid progenitors (Pre CFU-E) were also unaffected. However, *Cd74-Nid67* mice exhibited a notable decrease in colony forming unit-erythroid (CFU-E) progenitors and more mature pro-erythroid (Pro-Ery) progenitors.

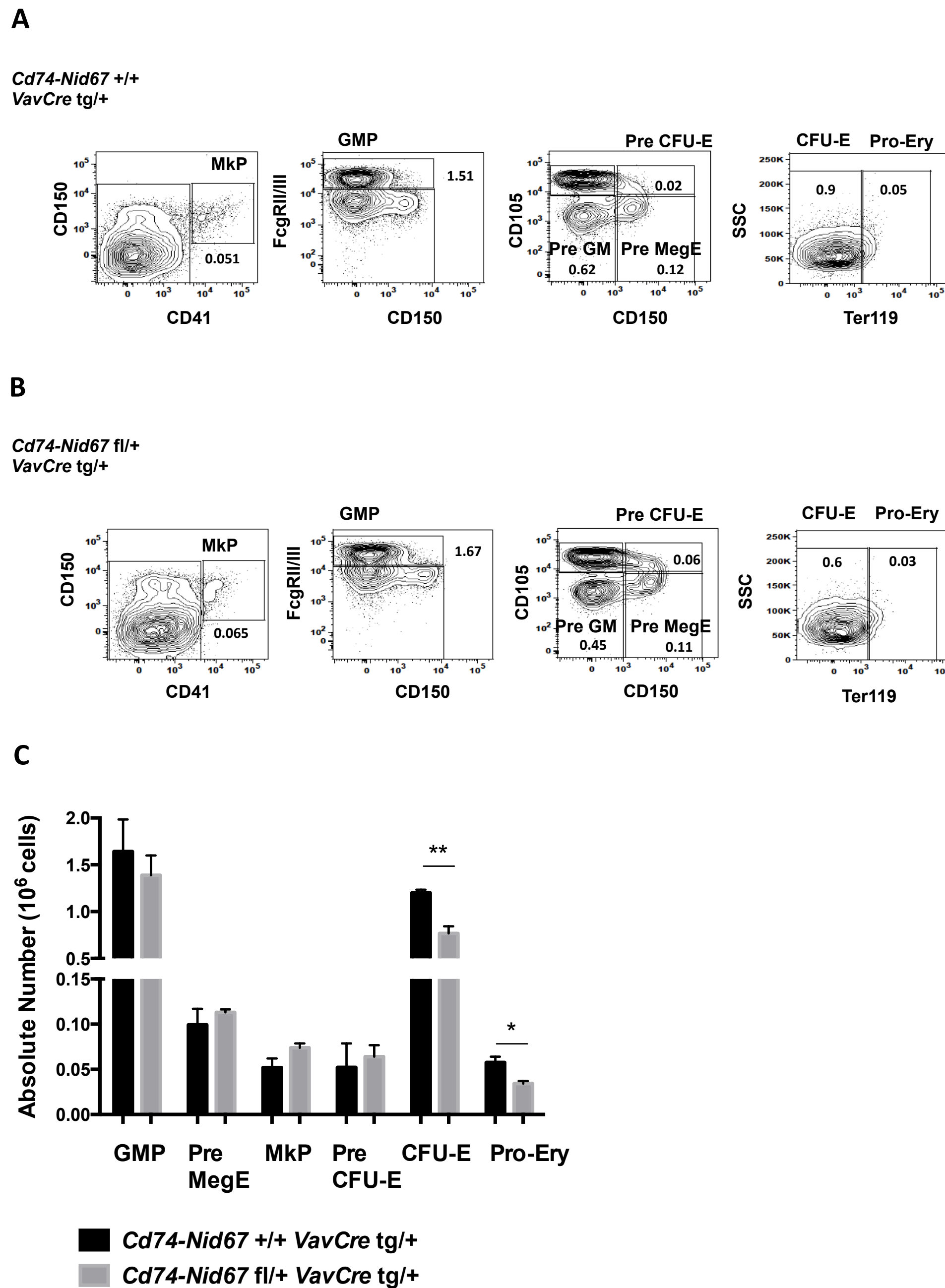


Figure 3.4 Impact of *Cd74-Nid67* haploinsufficiency on myeloid and erythroid progenitors

Bone Marrow (BM) from adult mice (8 weeks old) with the indicated genotypes was analysed (n=6). **A.** and **B.** Representative FACS profiles of myeloid and erythroid progenitors from a control mouse and a *Cd74-Nid67* mouse, respectively. Numbers within quadrants indicate the frequency of the population in total BM. **C.** Absolute number of myeloid and erythroid progenitors. Data is expressed as mean +/- SEM. * P<0.05, ** P<0.01, *** P<0.001

3.2.5 *Cd74-Nid67* Haploinsufficiency Impairs the Production of Mature Myeloid Cells

The impact of *Cd74-Nid67* haploinsufficiency on BM cellularity and mature myeloid cell production was investigated. The BM cellularity was significantly reduced in *Cd74-Nid67* mice compared to controls (Figure 3.5 A). A significant reduction in mature myeloid cells (Mac1+, Gr1+) in the BM of *Cd74-Nid67* mice was also evident (Figure 3.5 B).

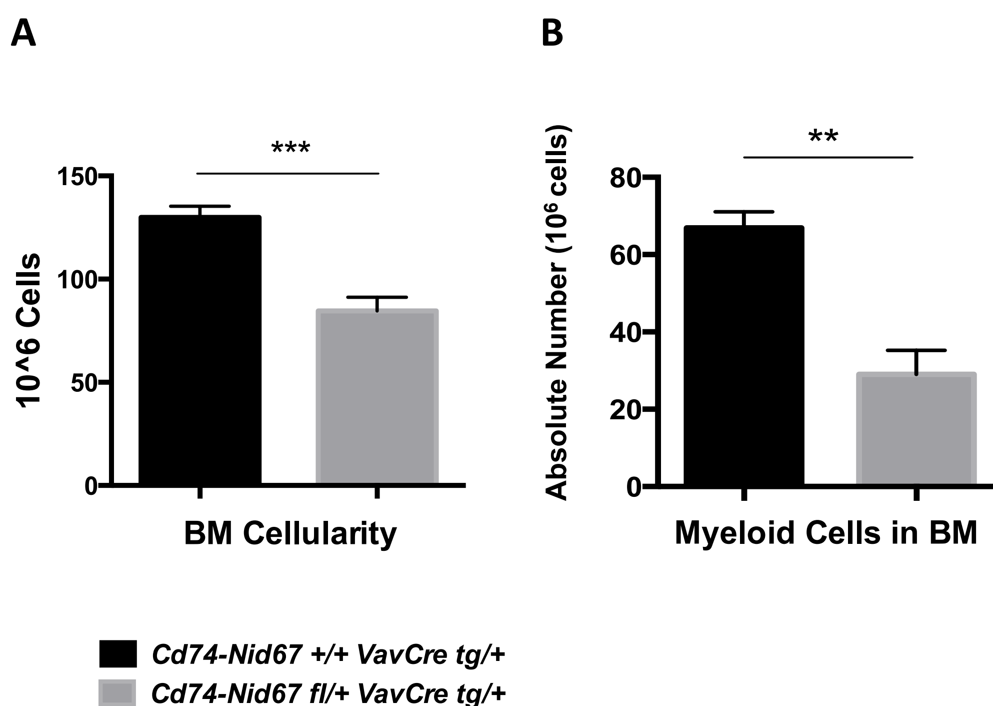


Figure 3.5 Impact of *Cd74-Nid67* haploinsufficiency on BM cellularity and mature myeloid cell production

Bone Marrow (BM) from adult mice (8 weeks old) with the indicated genotypes was analysed (n=8). **A.** Number of bone marrow mononuclear cells (BM cellularity) of 2 femora and 2 tibiae per mouse. **B.** Absolute number of mature myeloid cells in BM.
Data is expressed as mean +/- SEM. * P<0.05, ** P<0.01, *** P<0.00

3.2.6 *Cd74-Nid67* Haploinsufficiency Alters Myeloid and Megakaryocyte Potential *in vitro*

To investigate the effect of *Cd74-Nid67* haploinsufficiency on myeloid and megakaryocyte potential, single HSCs (LSK, CD48-, CD150+) from *Cd74-Nid67* mice and controls were placed in liquid culture with cytokines promoting myeloid and megakaryocyte differentiation. *Cd74-Nid67* HSCs produced fewer colonies that were smaller in size than control HSCs (Figure 3.6A and B).

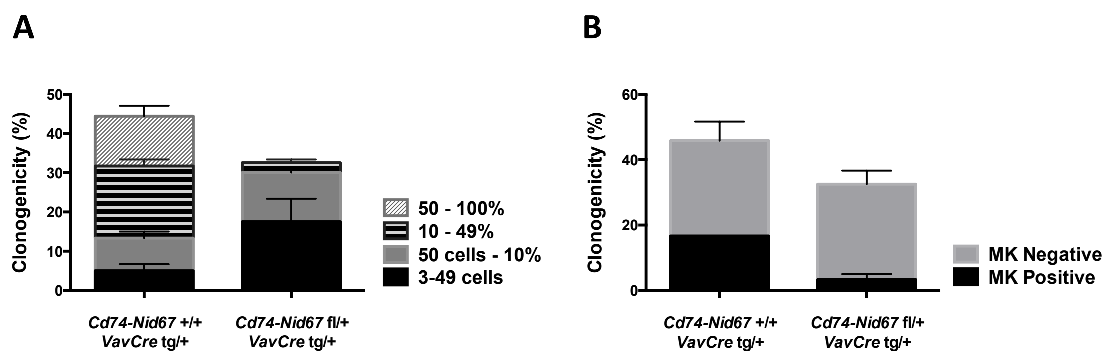


Figure 3.6 Myeloid and megakaryocyte potential of *Cd74-Nid67* HSCs

GM and MK potential of LSK, CD48-, CD150+ HSCs at the single cell level from mice (8-12 weeks old) with the indicated genotypes. **A.** Colony growth (number of cells or percentage of well covered by cells) and **B.** MK potential were scored after eight days of culture. Data is expressed as mean +/- SEM.

3.2.7 *Cd74-Nid67* Haploinsufficiency Impairs HSC Function and Differentiation Capacity *in vivo*

To assess the impact of *Cd74-Nid67* deficiency on HSC function, long-term competitive transplantations were performed and multi-lineage reconstitution in peripheral blood, and stem/progenitor reconstitution in bone marrow were analysed. Deficiency of *Cd74-Nid67* dramatically impaired the reconstitution of myeloid, T and B cell lineages in blood (Figure 3.7 A). In fact, less than 1% of cells were derived from *Cd74-Nid67* HSCs. Similarly, the contribution of *Cd74-Nid67* HSCs to total BM cells and myeloid progenitors was severely reduced (Figure 3.7 B and C). *Cd74-Nid67* LSK and HSC reconstitution was also significantly reduced compared to controls (Figure 3.7 D), although not to the same extent as other populations. In fact, a sizeable number of *Cd74-Nid67* LSKs and HSCs (LSK, CD48-, CD150+) managed to engraft long-term.

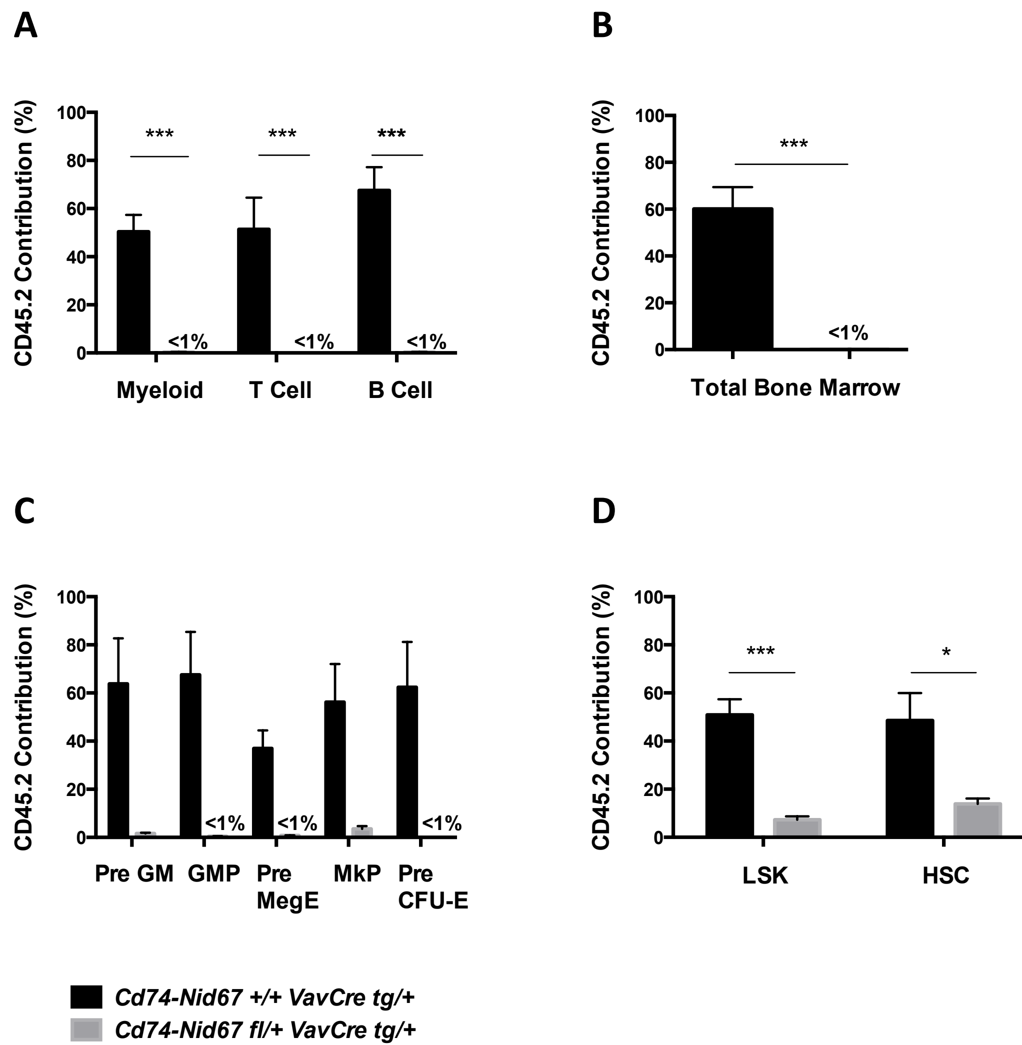


Figure 3.7 Impact of *Cd74-Nid67* haploinsufficiency on HSC function

Reconstitution of cells from *Cd74-Nid67* +/+ *VavCre* tg/+ and *Cd74-Nid67* fl/+ *VavCre* tg/+ in peripheral blood and bone marrow one-year post competitive transplantation (n=6). **A.** Contribution of donor cells to myeloid, B and T cells in peripheral blood **B.** Contribution of donor cells to total BM cells. **C.** Reconstitution of donor myeloid and erythroid progenitors in bone marrow. **D.** Reconstitution of donor LSK and HSCs in bone marrow.
Data is expressed as mean +/- SEM. * P<0.05, ** P<0.01, *** P<0.001

3.2.8 HSCs from *Cd74-Nid67* Mice and Del(5q) MDS Patients Exhibit Common Molecular Changes

Cd74-Nid67 HSC dysfunction was further explored by comparing *Cd74-Nid67* HSCs (Figure 3.8 A) and Del(5q) HSCs (Figure 3.8 B) at the molecular level. Purified *Cd74-Nid67* HSCs (LSK, CD48-, CD150+) and Del(5q) patient HSCs (CD34+, CD38-, CD90+) were subjected to global RNA sequencing and gene expression profiling. The expression of *Cdkn1a*, involved in cell cycle regulation and a downstream mediator of P53, is significantly increased in both *Cd74-Nid67* HSCs and Del(5q) HSCs. The expression of *Plac8* and *Pim2*, involved in cell growth and apoptosis, are also significantly elevated in both *Cd74-Nid67* and Del(5q) HSCs. Furthermore, an important regulator of HSC function, *Hlf,* is significantly decreased.

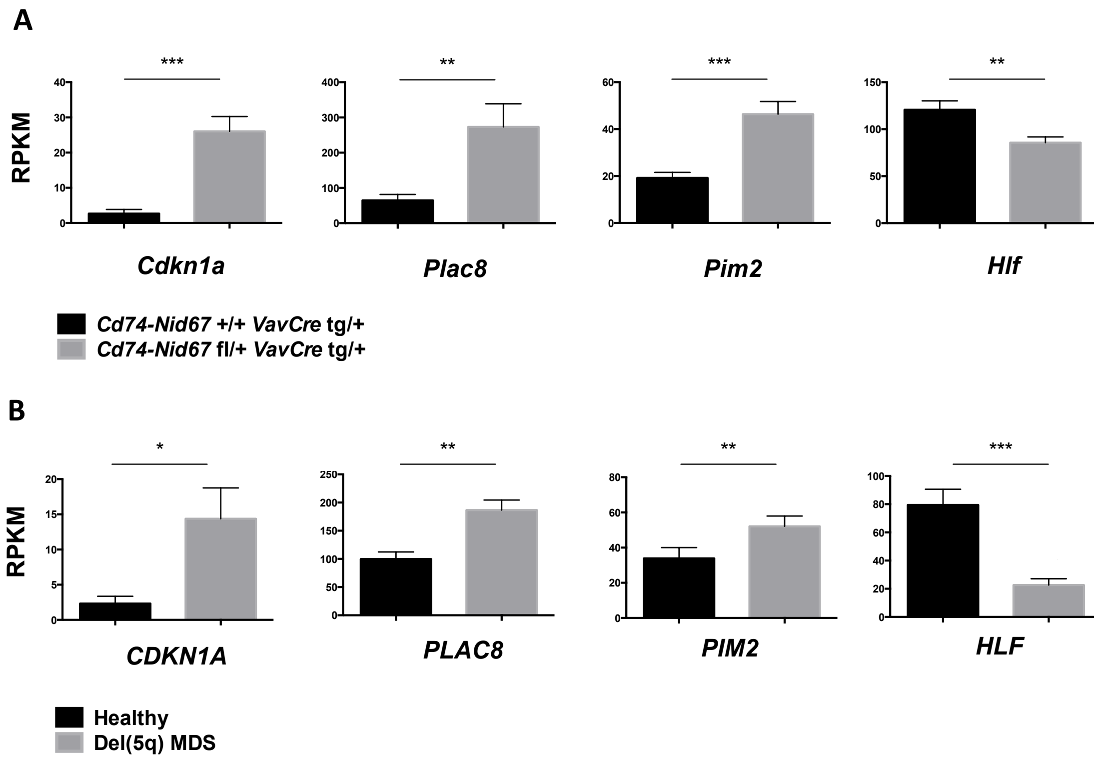


Figure 3.8 Molecular Changes in murine *Cd74-Nid67* HSCs and human Del(5q) HSCs

A. Mean reads per kilobase per million (RPKM) of differentially expressed genes in purified HSCs from 8-12 week old *Cd74-Nid67 +/+ VavCre* tg/+ controls and *Cd74-Nid67 fl/+ VavCre tg/+* mice (n=9). **B**. Mean reads per kilobase per million (RPKM) of selected genes in purified HSCs from healthy controls and isolated Del(5q) MDS patients (n=6).
* P<0.05, ** P<0.01, ***P<0.001

3.2.9 Haploinsufficiency of *Cd74-Nid67* in Aged Mice Causes Mild Anaemia

Since Del(5q) MDS is a disease of the elderly, the effect of *Cd74-Nid67* haploinsufficiency on mature blood cells was examined in aged mice. Peripheral blood samples from *Cd74-Nid67* mice and littermate controls were analysed. There were no significant changes in white blood cell (WBC) count (Figure 3.9 A), mean corpuscular volume (Figure 3.9 D), RBC distribution width (Figure 3.9 E) or platelet count (Figure 3.9 F) in *Cd74-Nid67* mice compared to controls. However, the RBC number (Figure 3.9 B) and haemoglobin concentration (Figure 3.9 C) were significantly reduced in aged *Cd74-Nid67* mice.

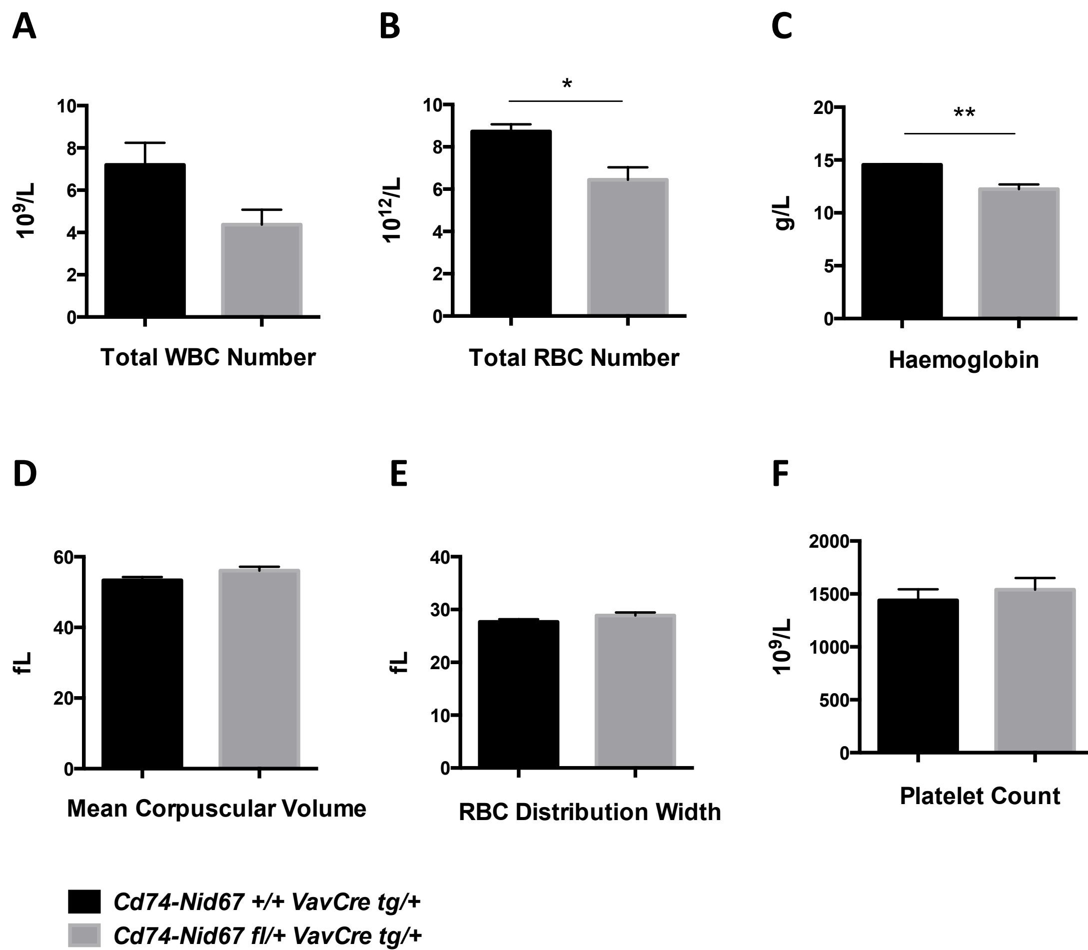


Figure 3.9 Peripheral blood measurements of aged *Cd74-Nid67* mice

Blood from aged (~18 months old) mice with the indicated genotypes was analysed (n=4). **A.** White Blood Cell (WBC) number. **B**. Red Blood Cell (RBC) number. **C**. Haemoglobin concentration. **D**. Mean corpuscular volume. **E.** Red Blood Cell (RBC) distribution width. **F**. Platelet Count.

3.2.10 Myeloid, Erythroid and Megakaryocyte Progenitors are Altered in Aged *Cd74-Nid67* Mice

BM samples from aged *Cd74-Nid67* mice and littermate controls were analysed to examine if the phenotype was progressive. Similar to young adult *Cd74-Nid67* mice, BM cellularity was reduced (Figure 3.10 A) and LT-HSCs (LSK, CD48-, CD150+, CD34-) were increased (Figure 3.10 B) in aged *Cd74-Nid67* mice.

Haploinsufficiency of *Cd74-Nid67* in aged mice resulted in a significant reduction of HSCs in G0 phase of the cell cycle and a corresponding increase in the number of HSCs in G1 and S/G2/M (Figure 3.10 C).

Cd74-Nid67 haploinsufficiency in aged mice did not alter the number of early granulocyte monocyte, megakaryocyte or erythroid progenitors (PreGM, Pre-MegE and Pre CFU-E, respectively), as in young *Cd74-Nid67* mice. However, aged *Cd74-Nid67* mice exhibited a significant decrease in more mature granulocyte monocyte progenitors (GMP), megakaryocyte progenitors (MkP) and erythroid progenitors (CFU-E and Pro-Ery) (Figure 3.10 D). In contrast, young adult *Cd74-Nid67* mice exhibited a reduction exclusively in mature erythroid progenitors (CFU-E and Pro-Ery).

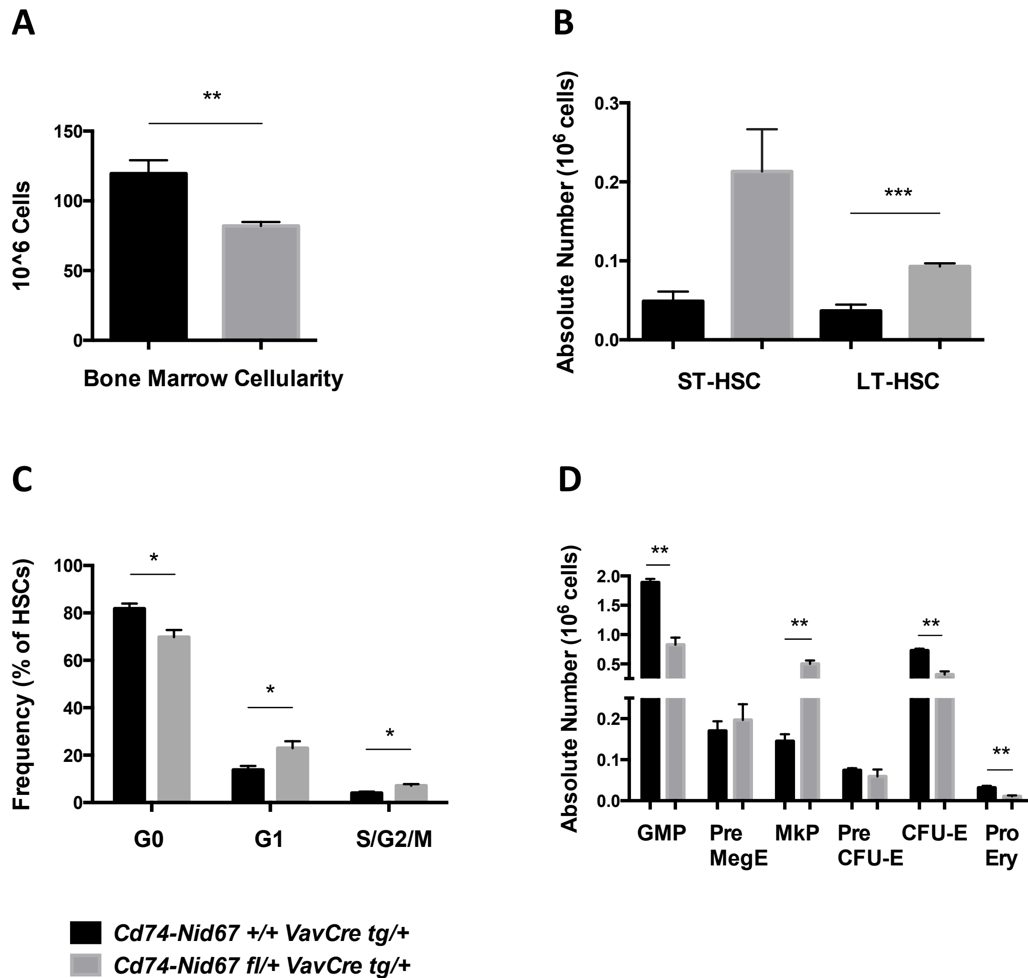


Figure 3.10 Impact of *Cd74-Nid67* haploinsufficiency on HSCs and progenitors in aged mice

Bone Marrow samples from aged mice (~18 months old) with the indicated genotypes were analysed (n=4). **A.** Number of bone marrow mononuclear cells (BM cellularity) of 2 femora and 2 tibiae per mouse. **B.** Absolute number of Short-Term HSCs (ST-HSC) and Long-Term HSCs (LT-HSC). **C**. Frequency of HSCs in G0 (Ki67-, DAPI-), G1 (Ki67+, DAPI-) and S/G2/M (Ki67+, DAPI+). **D.** Absolute number of myeloid and erythroid progenitors.

Data is expressed as mean +/- SEM. * P<0.05, ** P<0.01, *** P<0.001

3.3 Discussion

Cancer stem cells in Del(5q) MDS lie within the HSC compartment. Del(5q) HSCs are, therefore, both necessary and sufficient to propagate disease (Nilsson, Astrand-Grundstrom et al. 2000, Tehranchi, Woll et al. 2010, Woll, Kjallquist et al. 2014). Previous studies have identified individual genes and regions on 5q that contribute to the erythroid and megakaryocyte phenotype in Del(5q) MDS (Ebert, Pretz et al. 2008, Barlow, Drynan et al. 2010, Starczynowski, Kuchenbauer et al. 2010). The molecular drivers of HSC dysfunction and their involvement in disease pathogenesis, however, required further investigation.

In this study, we used the *Cd74-Nid67* mouse model to examine the impact of deficiency of this region on HSC function. Understanding the molecular mechanisms that alter HSC activity in Del(5q) MDS will provide important insights into the initial events responsible for disease development.

Haploinsufficiency of *Cd74-Nid67* (specifically targeted to haematopoietic cells using the *VavCre* mouse line) resulted in significant changes in RBC number and size, indicative of a mild macrocytic anaemia. This is consistent with previous findings (Barlow, Drynan et al. 2010). However, Barlow and colleagues also identified a small yet significant reduction in haemoglobin concentration in *Cd74-Nid67* mice, which was not reproduced in this study. Barlow's study examined the peripheral blood of much larger numbers of mice of each genotype (n=28-52) (Barlow, Drynan et al. 2010). Since the difference between *Cd74-Nid67* mice and controls is subtle, larger numbers of mice may be required in this study for differences to reach statistical significance.

Haploinsufficiency of *Cd74-Nid67* caused a significant expansion of both short-term and long-term HSCs, similar to the HSC expansion seen in Del(5q) MDS patients

(Nilsson, Astrand-Grundstrom et al. 2000, Woll, Kjallquist et al. 2014). The cell cycle status of *Cd74-Nid67* HSCs also closely mirrors that of Del(5q) HSCs (Tehranchi, Woll et al. 2010), namely reduced HSC quiescence. Similar to Del(5q) HSCs, there was a decreased number of *Cd74-Nid67* HSCs in G0, an increased number of HSCs in G1 but no corresponding increase in S/G2/M. This suggests that there may be a block in progression through the cell cycle. It is tempting to speculate that the increased expression of *Cdkn1a,* a cell cycle regulator that acts at the G1 checkpoint (Gartel and Radhakrishnan 2005)*,* prevents entry of *Cd74-Nid67* and Del(5q) HSCs into S phase. Alternatively, the failure to detect a change in the frequency of *Cd74-Nid67* HSCs in S/G2/M may reflect a technical limitation. Cell cycling is a dynamic process but phenotypic cell cycle analysis captures cells at one particular time point only. It fails to capture cells that have divided and returned to quiescence (G0 phase). BrdU incorporation experiments may be pursued to overcome this limitation. BrdU is a thymidine analogue that incorporates into newly synthesised DNA and cells that have incorporated BrdU will retain staining even if they return to quiescence (Wilson, Laurenti et al. 2008). BrdU incorporation experiments will, therefore, capture cells that have entered active S phase at any time point.

The reduction in BM cellularity in *Cd74-Nid67* mice is consistent with previous findings (Barlow, Drynan et al. 2010). In this study, a significant decrease in mature myeloid cells (defined as Mac1+, Gr1+) was seen in the BM of *Cd74-Nid67* mice, likely accounting for the reduced BM cellularity.

This study identified that, in steady state conditions, the number of erythroid progenitors was reduced in young *Cd74-Nid67* mice compared to controls. Myeloid and megakaryocyte progenitors were, however, unaffected. The reduction of erythroid progenitors (CFU-E and Pro-Ery) in *Cd74-Nid67* mice suggests a block in

differentiation in this lineage. Interestingly, in conditions of stress (transplantation or aging), a differentiation deficit was seen in myeloid, megakaryocyte and erythroid lineages. The specific disruption to erythroid progenitors, even in homeostatic conditions, suggests that this lineage is most sensitive to *Cd74-Nid67* deficiency. This is consistent with the enhanced sensitivity of the erythroid lineage to *Rps14* deficiency, relative to myeloid and megakaryocyte lineages in human (Ebert, Pretz et al. 2008, Dutt, Narla et al. 2011).

The myeloid and megakaryocyte potential is, however, somewhat impaired in young *Cd74-Nid67* mice. This is evidenced by the impaired growth of these lineages *in vitro*. The reduction in the number of myeloid and megakaryocyte colonies produced by *Cd74-Nid67* HSCs, together with the significant decrease in colony size, mimics the output of Del(5q) HSCs in similar assays (Nilsson, Astrand-Grundstrom et al. 2000).

Haploinsufficiency of *Cd74-Nid67* severely compromised HSC function since *Cd74-Nid67* HSCs reconstituted extremely poorly upon competitive transplantation. Although significantly reduced compared to controls, a significant population of *Cd74-Nid67* HSCs and LSKs reconstituted the BM long term but failed to contribute to mature cells in the BM or blood. The failure of *Cd74-Nid67* HSCs to produce mature cells of any lineage reveals a block in differentiation that is extremely relevant to Del(5q) MDS pathogenesis (Nilsson, Astrand-Grundstrom et al. 2000). Furthermore, the deficit in repopulating activity upon transplantation resembles the poor engraftment of Del(5q) patient HSCs when transplanted into immune deficient mice (Nilsson, Astrand-Grundstrom et al. 2000, Pang, Pluvinage et al. 2013).

It is interesting that transplantation experiments with *Cd74-Nid67* BM cells revealed a multi-lineage differentiation block as most studies have focused specifically on the erythroid differentiation deficit in Del(5q) MDS (Ebert, Pretz et al. 2008, Dutt, Narla

et al. 2011). Nilsson and colleagues, however, suggested that Del(5q) HSCs were also deficient in their ability to differentiate towards lymphoid cells (Nilsson, Astrand-Grundstrom et al. 2000). They demonstrated that the 5q deletion was present in almost all HSCs and a reasonable frequency of B cell progenitors but could not be detected in mature lymphocytes from Del(5q) MDS patients,. This highlights that mature lymphocytes were not part of the Del(5q) clone and suggests that Del(5q) is incompatible with lymphoid differentiation (Nilsson, Astrand-Grundstrom et al. 2000). The lack of lymphopenia in Del(5q) MDS patients implies that there is an effective compensatory mechanism for the production of lymphocytes. Long lived lymphoid progenitors generated before the 5q deletion may be the source of "normal" lymphocytes in Del(5q) MDS patients.

The full extent to which HSC function was impaired by *Cd74-Nid67* haploinsufficiency was only observed in a transplantation setting. Reconstituting the haematopoietic system of a lethally irradiated mouse places HSCs under immense replicative stress with a high demand for rapid protein synthesis. A recent study demonstrated that aged HSCs survived replication in homeostatic conditions but their dysfunction became evident when confronted with a strong replication challenge such as transplantation (Flach, Bakker et al. 2014). Similarly, *Cd74-Nid67* HSCs managed to support the production of all lineages in steady-state conditions. However, when confronted with replicative stress (competitive transplantation), the severe functional impairment of *Cd74-Nid67* HSCs became apparent.

Preliminary molecular analysis of *Cd74-Nid67* HSCs and Del(5q) HSCs revealed common changes in the expression of genes associated with cell growth and cell cycle regulation. Further analysis, using an unbiased approach, will be performed to generate a detailed list of dysregulated genes. The identification of genes that are

dysregulated in both *Cd74-Nid67* and Del(5q) HSCs may represent key therapeutic targets and may provide important insights into the molecular basis underlying HSC dysfunction.

Since Del(5q) MDS is a disease of the elderly, it was important to examine the impact of *Cd74-Nid67* deficiency in aged mice. The disruption of myeloid and megakaryocyte progenitors, in steady state conditions, exclusively in aged *Cd74-Nid67* mice, suggests that the differentiation deficit becomes worse with age. The impact of *Cd74-Nid67* haploinsufficiency on HSCs in aged mice, however, was similar to that seen in adult *Cd74-Nid67* mice. It will be interesting to pursue transplantation experiments to examine the effect of *Cd74-Nid67* haploinsufficiency on HSC function in aged mice.

This study used the *VavCre* transgenic mouse line to induce deletion of the *Cd74-Nid67* region in all haematopoietic cells at the earliest stages of embryonic development. The aged *Cd74-Nid67* mice examined in this study, therefore, harboured the deletion from an early embryonic stage. Since the 5q deletion in Del(5q) MDS is a somatic mutation that arises in elderly patients, it will be important to examine the impact of inducing the deletion in aged mice, a more clinically relevant setting. The use of an inducible *Cre* line such as *Mx1Cre* (Kuhn, Schwenk et al. 1995) would provide the opportunity to induce *Cd74-Nid67* deletion in aged mice upon treatment with PolyIC.

In this study, we have demonstrated for the first time that haploinsufficiency of a distinct region, *Cd74-Nid67,* corresponding to part of the CDR in Del(5q) MDS causes severe HSC dysfunction, similar to that seen in patients. Further studies will aim to address the mechanisms underlying HSC dysfunction in *Cd74-Nid67* mice.

4. Identification of the Genes Responsible for *Cd74-Nid67* HSC Dysfunction

4.1 Introduction

We have found that deficiency of *Cd74-Nid67* severely impairs HSC function in mice. This suggests that deficiency of one or a combination of genes located within this region may contribute to HSC dysfunction in Del(5q) MDS patients. Identifying the gene(s) within *Cd74-Nid67* required for normal HSC activity may provide important insights into the mechanisms responsible for the development of Del(5q) MDS.

There are eight genes (*Cd74*, *Rps14*, *Ndst1*, *Synpo*, *Myoz3*, *Rbm22*, *Dctn4*, *Nid67*) located within the *Cd74-Nid67* region. The impact of deficiency of any of these genes on HSC activity has not previously been explored.

The erythroid differentiation deficit in Del(5q) MDS has been attributed to haploinsufficiency of the ribosomal protein, *Rps14* (Ebert, Pretz et al. 2008). Furthermore, HSCs deficient in other ribosomal proteins, *Rps19* (Jaako, Flygare et al. 2011) and *Notchless* (Le Bouteiller, Souilhol et al. 2013), engraft poorly upon competitive transplantation, a phenotype similar to *Cd74-Nid67* HSCs. A recent study demonstrated that the rate of protein synthesis was significantly reduced in haematopoietic cells from mice that carry a hypomorphic mutation in the *Rpl24* ribosome protein (*Rpl24 Bst/*+) (Signer, Magee et al. 2014). Interestingly, HSCs were particularly sensitive to changes in protein synthesis and *Rpl24 Bst/*+ HSC function was severely impaired in the face of replicative stress (Signer, Magee et al. 2014).

Haploinsufficiency of *Rps14* may, therefore, affect the rate of protein synthesis and account for the *Cd74-Nid67* HSC reconstitution defect.

Rbm22 encodes an RNA binding protein that is required for pre-mRNA splicing (Rasche, Dybkov et al. 2012). Mutations in spliceosomal components have recently been identified in a large number of MDS patients (Papaemmanuil, Cazzola et al. 2011, Yoshida, Sanada et al. 2011). This suggests that dysregulated splicing may be a key driver of disease. Since merely altering the concentration of splicing factors is proposed to affect the kinetics of spliceosome assembly and splice site recognition (Park, Parisky et al. 2004), haploinsufficiency of *Rbm22* may result in significant disruptions to pre-mRNA splicing.

Cd74 encodes the invariant chain, a transmembrane polypeptide, involved in the formation and transport of MHC class II protein (Cresswell 1994). *Cd74* has not previously been linked to HSC or MDS biology. However, previous studies have demonstrated that it plays a role in lymphoid maturation (Wong and Rudensky 1996, Matza, Lantner et al. 2002). Given its involvement in haematopoietic development, *Cd74* deficiency may contribute to the phenotype seen in *Cd74-Nid67* mice.

The remaining five genes within *Cd74-Nid67* have not previously been linked to cellular pathways associated with MDS pathogenesis. *Ndst1* encodes an essential enzyme (N-deacetylase/N-sulfotransferase) that participates in the biosynthesis of heparan sulfate (Ringvall and Kjellen 2010). *Synpo* encodes Synaptopodin, an actin-binding protein and *Myoz3* encodes Myozenin-3, an intracellular binding protein expressed in skeletal muscle. *Dctn4* encodes part of the dynactin complex, which is essential for mitosis and intracellular trafficking of organelles (Schroer 2004). The function of *Nid67*, encoding a small membrane protein expressed by a rat neuronal cell line (Vician, Silver et al. 2001), has not been studied in detail.

The identification of the gene(s) responsible for HSC dysfunction in Del(5q) MDS will be key to understanding the mechanistic basis for disease development and propagation. The gold standard method to investigate the function of a gene *in vivo* involves using a knockout model to determine the impact of its loss. Knockout mouse models of *Cd74* (Bikoff, Huang et al. 1993) and *Ndst1* (Grobe, Inatani et al. 2005), have previously been described. However, the impact of their loss on HSCs has not been explored.

In this study, we used a knockout mouse model, an shRNA knockdown strategy and a cDNA "rescue" approach to examine the contribution of candidate genes within *Cd74-Nid67* to HSC dysfunction. Short hairpin lentiviruses targeting *Rps14* (a likely candidate gene) were generated and their knockdown efficiency and impact on HSC function was examined. Since off-target effects are common with shRNAs (Jackson and Linsley 2004), complementary "rescue" experiments were performed to determine if restoring expression of *Rps14* or *Rbm22* could reverse *Cd74-Nid67* HSC dysfunction.

The identification of the genes involved in driving HSC dysfunction in Del(5q) MDS will provide important insights into the mechanisms responsible for disease initiation.

4.2 Results

4.2.1 Expression of Genes within *Cd74-Nid67* in HSCs from Mice and Man

To explore which genes within *Cd74-Nid67* may contribute to HSC dysfunction in Del(5q) MDS, their expression in mouse and human HSCs was examined. Purified human HSCs (Lineage-, CD34+, CD38-, CD90+, CD45RA-) and mouse HSCs (LSK, CD48-, CD150+) were subjected to global RNA sequencing and gene expression profiling.

All genes that lie within *Cd74-Nid67,* except *Myoz3* and *Synpo,* are expressed in HSCs from *wild-type* mice (Figure 4.1 A.) Similarly, all genes within *CD74-NID67,* except *SYNPO,* are expressed in human HSCs (Figure 4.1 B).

The expression of *Nid67*, *Dctn4*, *Rbm22*, *Ndst1*, *Rps14* and *Cd74* in HSCs from *Cd74-Nid67* mice was, as predicted, reduced by ~50% compared to HSCs from controls (Figure 4.1 C). Similarly, their expression in Del(5q) patient HSCs was approximately 50% their level of expression in healthy HSCs (Figure 4.1 D).

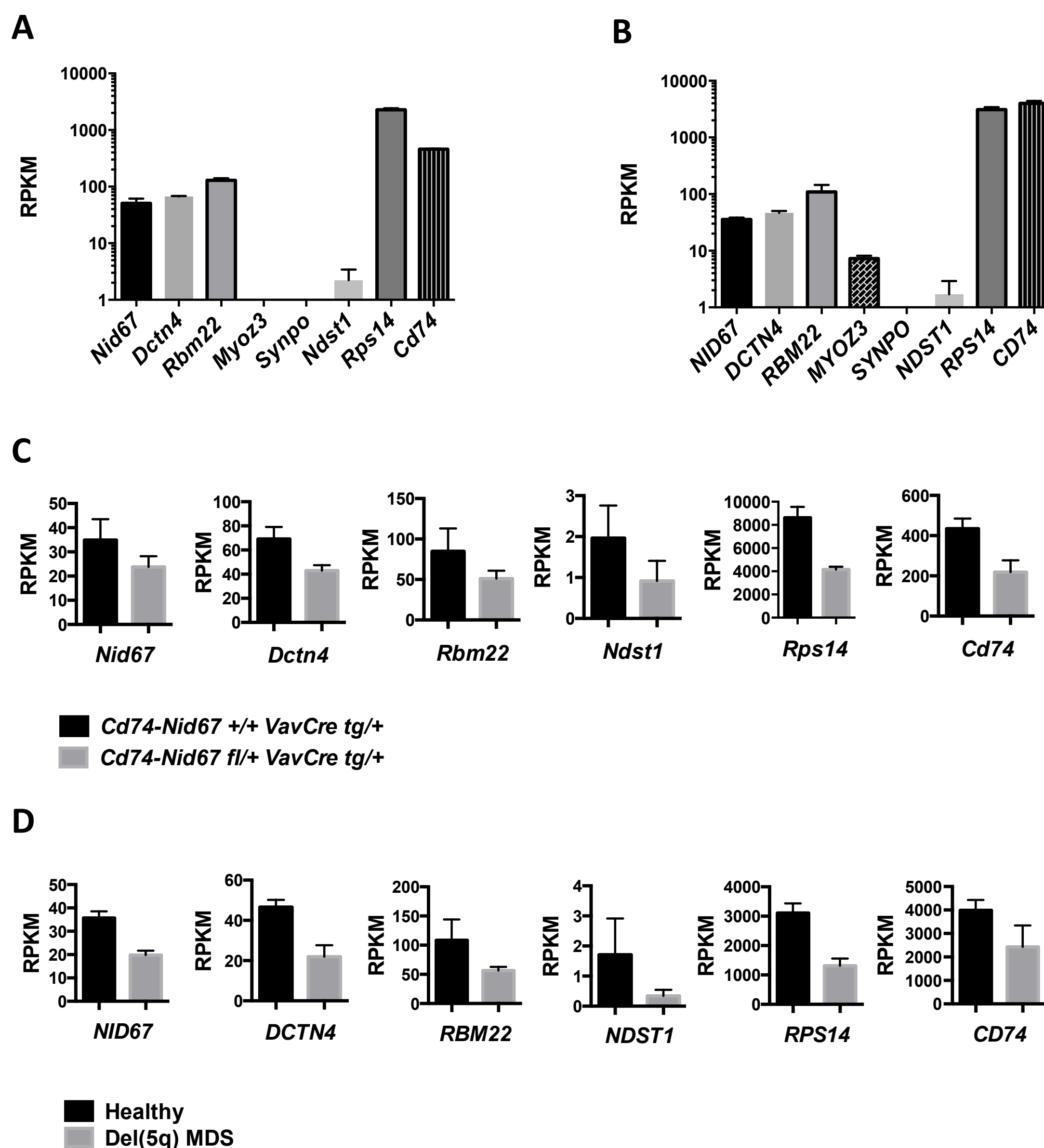


Figure 4.1 Expression of genes within *Cd74-Nid67* in HSCs from mouse and man

A. Reads per kilobase per million (RPKM) of genes within *Cd74-Nid67* in purified (LSK, CD48neg, CD150+) HSCs from 8-12 week old *wild-type* mice (n=9). **B**. Reads per kilobase per million (RPKM) of genes within *CD74-NID67* in purified HSCs from healthy controls (n=5). **C.** Reads per kilobase per million (RPKM) of genes within *Cd74-Nid67* in purified HSCs from 8-12 week old *Cd74-Nid67 +/+ VavCre* tg/+ controls and *Cd74-Nid67 fl/+ VavCre tg/*+ mice (n=9). **D.** Reads per kilobase per million (RPKM) of genes within *CD74-NID67* in purified HSCs from healthy controls and Del(5q) MDS patients (n=5).
Data is expressed as mean +/- SEM.

4.2.2 Complete or Partial Loss of *Cd74* Does Not Alter HSC Number or Function

To determine whether *Cd74* deficiency alone is responsible for *Cd74-Nid67* HSC dysfunction, the impact of heterozygous and homozygous loss of *Cd74* on HSCs was examined using a knockout mouse model (Bikoff, Huang et al. 1993).

There was no change in BM cellularity in *Cd74* +/- or *Cd74* -/- mice compared to controls (Figure 4.2 A). The number of Long Term HSCs (LT-HSC) and Short Term HSCs (ST-HSC), defined as LSK, CD48-, CD150+ and CD34- or CD34+, respectively, were not changed in *Cd74* +/- or *Cd74* -/- mice (Figure 4.2 B and C). Furthermore, reconstitution of *Cd74* +/- and *Cd74* -/- mature cells in peripheral blood is similar to the reconstitution of control cells (Figure 4.2 D).

LSK (Figure 4.2 E) and HSC (Figure 4.2 F) reconstitution in BM was also not affected by heterozygous or homozygous loss of *Cd74*.

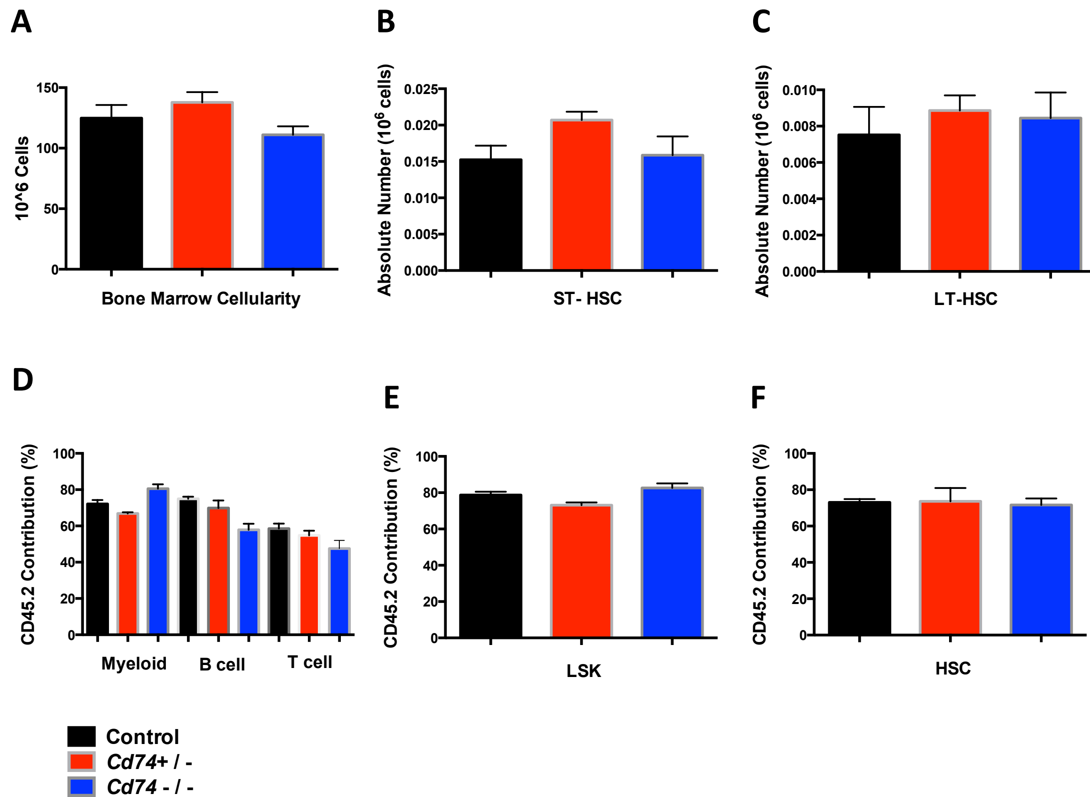


Figure 4.2 Impact of *Cd74* loss on HSC number and function

A. Bone Marrow (BM) cellularity (2 femora and 2 tibiae) of 8-12 week old control (*wild-type*), *Cd74* +/- and *Cd74* -/- mice (n=4). **B.** Number of short-term HSCs (ST-HSC) and long-term HSCs (LT-HSC) in BM of 8-12 week old control (*wild-type*), *Cd74* +/- and *Cd74* -/- mice (n=4). **C.** Reconstitution of mature cells in peripheral blood from mice of indicated genotypes sixteen weeks post competitive transplant (n=4). **D**. Reconstitution of control (*wild-type*), *Cd74* +/- and *Cd74* -/- LSKs and HSCs (LSK, CD48-, CD150+) sixteen weeks post competitive transplant (n=4). Data is expressed as mean +/- SEM.

4.2.3 shRNAs Targeting *Rps14* Reduce Its Expression by ~50% in LSK Stem/Progenitor Cells

To identify which gene targeted in *Cd74-Nid67* mice causes HSC dysfunction, we employed an shRNA knockdown strategy to reduce the expression of *Rps14*, a likely candidate gene, in *wild-type* LSKs. Hairpin lentiviruses with the pLKO.1 HIV-based vector backbone (Figure 4.3 A) were used to reduce the expression of *Rps14* in *wild-type* stem/progenitor (LSK) cells. The transduction efficiencies, as measured by the frequency of GFP+ cells, of control lentiviruses (Luciferase) or shRNA hairpins targeting *Rps14* (shRNA 60 and 61) were high at the tested dose (Figure 4.3 B). The expression of *Rps14* was reduced by approximately 50% in transduced (GFP+) LSK cells expressing shRNAs 60 and 61 *in vitro* (Figure 4.3 C). Interestingly, the expression of *Cdkn1a*, a downstream mediator of P53, was significantly increased in LSK cells expressing shRNAs 60 and 61 (Figure 4.3 D), similar to its upregulation in *Cd74-Nid67* HSCs.

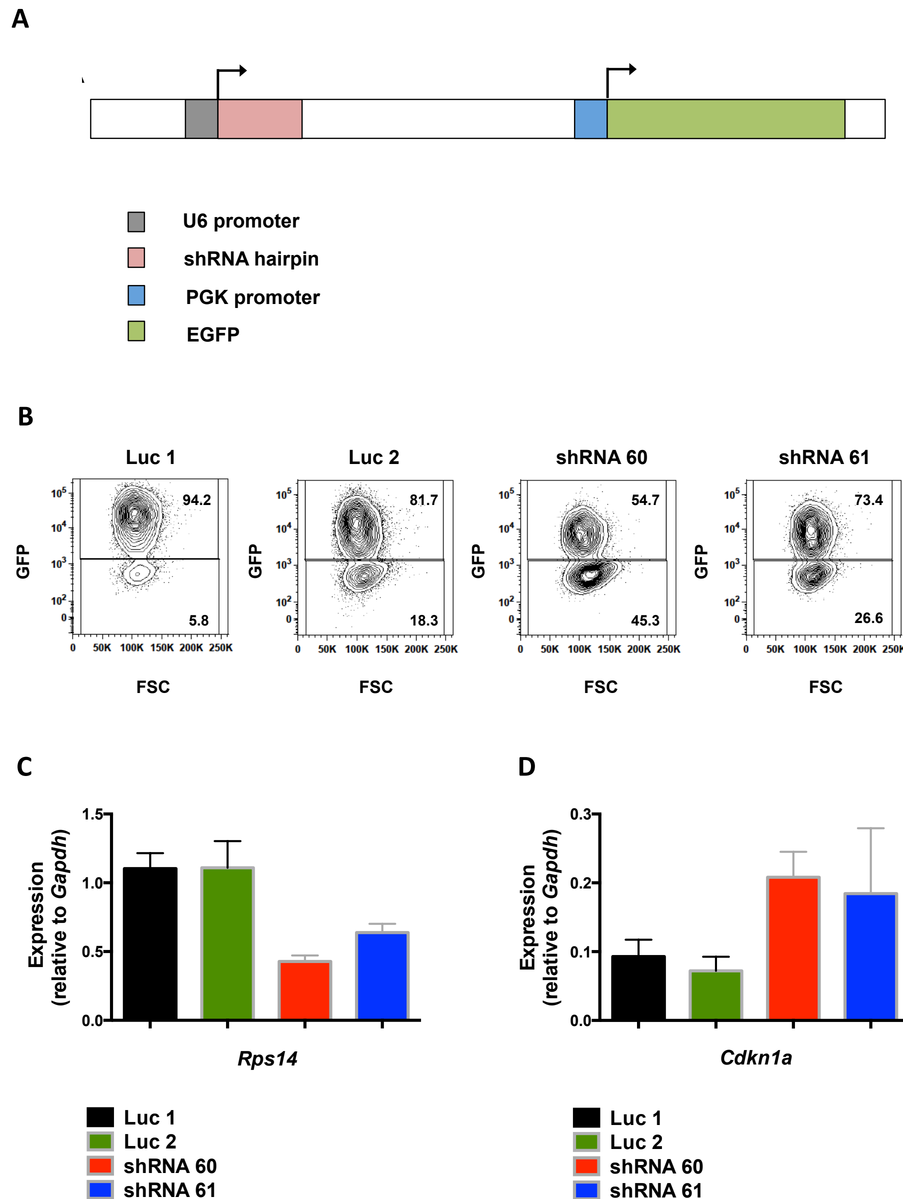


Figure 4.3 Transduction and knockdown efficiency of *Rps14* shRNAs in LSK cells *in vitro*

A. Schematic diagram of pLKO.1 lentiviral construct. U6 promoter drives expression of shRNA hairpin and PGK promoter drives expression of EGFP protein. **B**. Representative FACS plots of GFP levels in *wild-type* LSK cells transduced with Luciferase control (Luc1, Luc2) or *Rps14* shRNAs (shRNA 60, shRNA 61) *in vitro*. **C.** mRNA expression of *Rps14* (normalised to *Gapdh*) in GFP+ LSKs treated with control lentiviruses (Luc1, Luc2) or shRNAs targeting *Rps14* (shRNA 60, shRNA 61) (n=3). **D.** mRNA expression of *Cdkn1a* (normalised to *Gapdh*) in GFP+ LSKs treated with control lentiviruses or shRNAs targeting *Rps14* (n=3). Data is expressed as mean +/- SEM.

4.2.4 Reconstitution of HSCs transduced with *Rps14* shRNAs is Significantly Impaired

To examine the impact of *Rps14* deficiency on HSC function, purified LSK cells were treated with control Luciferase lentiviruses or shRNA hairpins targeting *Rps14* and transplanted into lethally irradiated mice (Figure 4.4 A). There was no difference in total CD45.2 HSC (Lineage-, Sca-1+, c-kit+, CD48-, CD150+) reconstitution between the control (Luciferase) and *Rps14* (shRNA 60 and 61) groups (Figure 4.4 C). However, the engraftment of GFP+ HSCs transduced with shRNAs 60 and 61 was significantly reduced compared to HSCs transduced with Luciferase (Figure 4.4 B and D). Moreover, the small population of purified GFP+ LSK cells transduced with shRNAs 60 and 61 that engrafted *in vivo* did not exhibit any change in *Rps14* expression (Figure 4.4 E). This suggests that there was a selection against the engraftment of *Rps14* deficient cells.

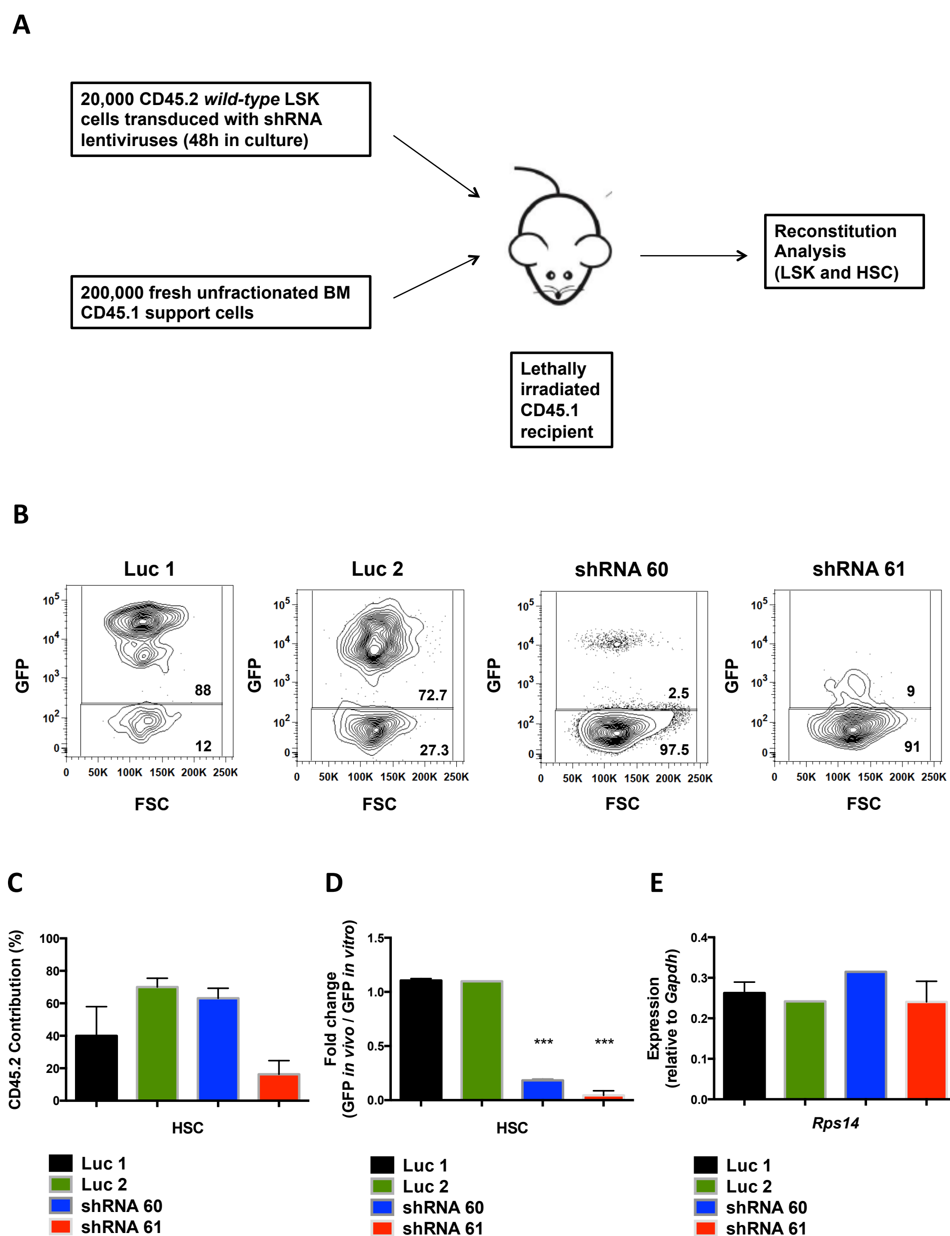


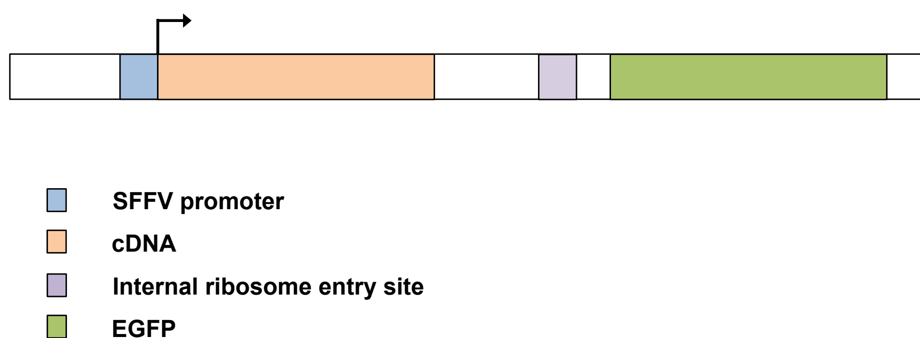
Figure 4.4 Impact of shRNA knockdown of *Rps14* on HSC function

A. Schematic diagram of experimental design of shRNA transplantation experiments. **B.** Representative FACS plots of GFP levels of engrafted HSCs transduced with Luciferase control (Luc1, Luc2) or *Rps14* shRNAs (shRNA 60, shRNA 61) *in vivo*. **C.** Total CD45.2 reconstitution of *wild-type* HSCs treated with Luciferase or shRNAs targeting *Rps14,* 12 weeks post transplant (n=3). **D.** Frequency of GFP+ donor CD45.2 HSCs 12 weeks post transplant (normalised to GFP frequency *in vitro*) (n=3). **E.** mRNA expression of *Rps14* (normalised to *Gapdh*) in engrafted GFP+ LSKs treated with control lentiviruses (Luc1, Luc2) or shRNAs targeting *Rps14* (shRNA 60, shRNA 61) (n=4). Data is expressed as mean +/- SEM.

4.2.5 Efficient Transduction of *Cd74-Nid67* LSKs with cDNA Lentiviruses

Since off-target effects are common with shRNAs, a complementary "rescue" approach was devised. Purified *Cd74-Nid67* LSK cells were transduced with Control (empty vector), *Rps14* or *Rbm22* cDNA pRRL-SFFV-IRES-EGFP lentiviral constructs to determine if restoring expression of these genes could rescue *Cd74-Nid67* HSC dysfunction (Figure 4.5 A). The transduction efficiencies, as measured by frequency of GFP+ cells, were high for Control, *Rbm22* and *Rps14* cDNA lentiviruses at the dose tested (Figure 4.5 B).

A



B

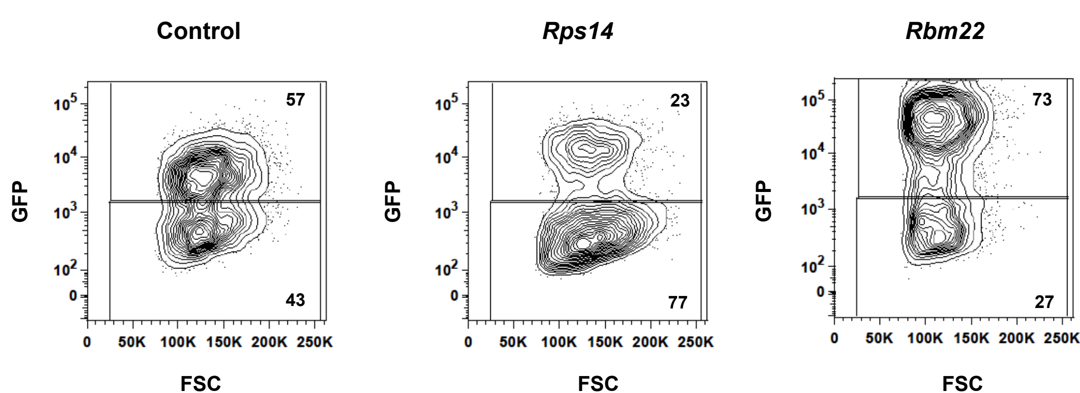


Figure 4.5 Transduction efficiencies of cDNA lentiviruses in LSKs *in vitro*

A. Schematic diagram of cDNA lentiviral construct. SFFV promoter drives expression of codon optimized mouse cDNA and an internal ribosome entry site allows translation of the EGFP protein. **B**. Representative FACS plots of GFP levels in *Cd74-Nid67* LSK cells transduced with Control, *Rps14* or *Rbm22* cDNA lentiviruses *in vitro*.

4.2.6 Lentiviral Transduction with *Rbm22* cDNA Improves *Cd74-Nid67* Myeloid Reconstitution

To determine if introducing *Rps14* or *Rbm22* could improve *Cd74-Nid67* HSC function, purified LSKs from *Cd74-Nid67* mice were treated with Control, *Rps14* or *Rbm22* cDNA lentiviruses and transplanted into lethally irradiated recipients (Figure 4.6). The reconstitution of myeloid cells expressing control, *Rps14* or *Rbm22* cDNAs was examined by flow cytometric analysis (Figure 4.7 A, B and C). Preliminary experiments revealed that the overall myeloid reconstitution in peripheral blood was increased in the *Rps14* and *Rbm22* groups compared to the Control group (Figure 4.7 D). Furthermore, almost all donor myeloid cells in the *Rbm22* group were GFP positive, suggesting a strong selection for cells expressing *Rbm22* cDNA (Figure 4.7 E).

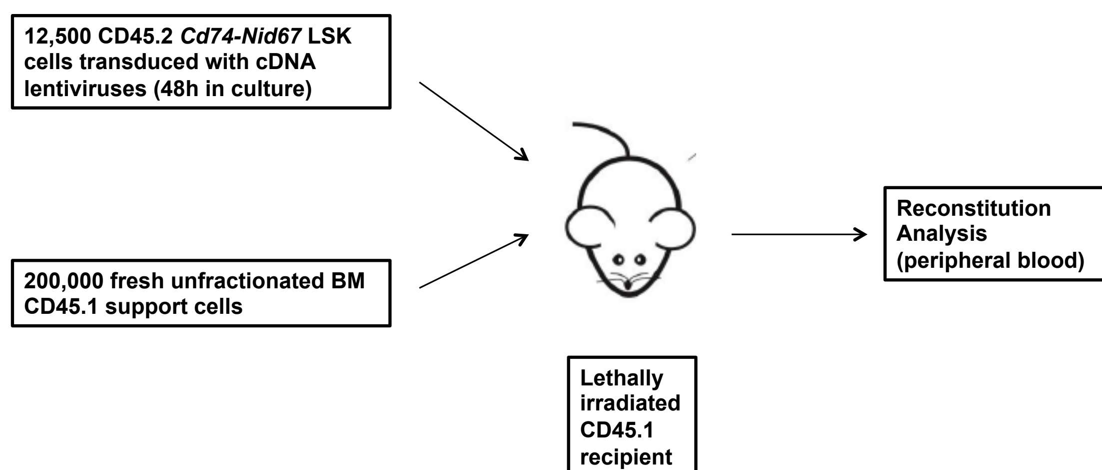
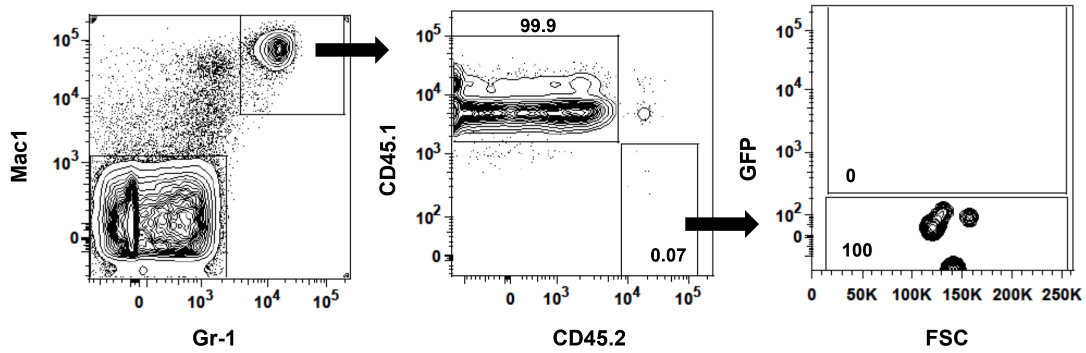


Figure 4.6 Experimental design for “rescue” experiments

Schematic diagram of “rescue” cDNA lentiviral transplantation experiments. Donor *Cd74-Nid67* (CD45.2) LSKs were treated with Control, *Rps14* or *Rbm22* mouse codon optimised cDNA lentiviruses for 48 hours and transplanted along with unfractionated bone marrow support (CD45.1) cells into lethally irradiated recipients (CD45.1).

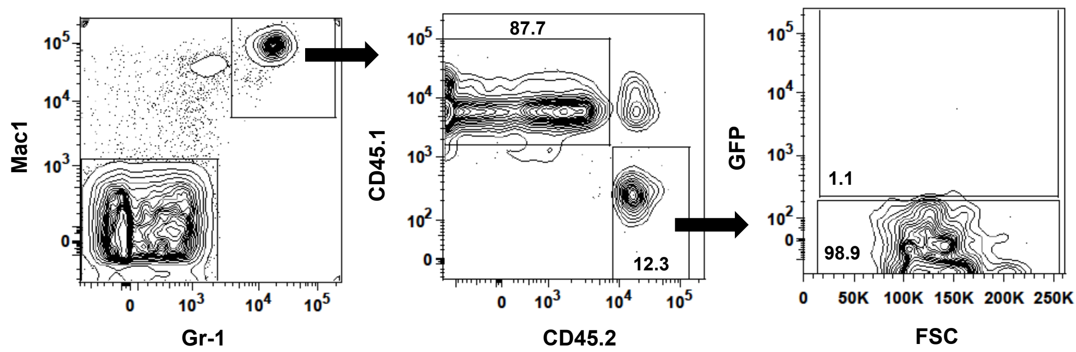
A

Control



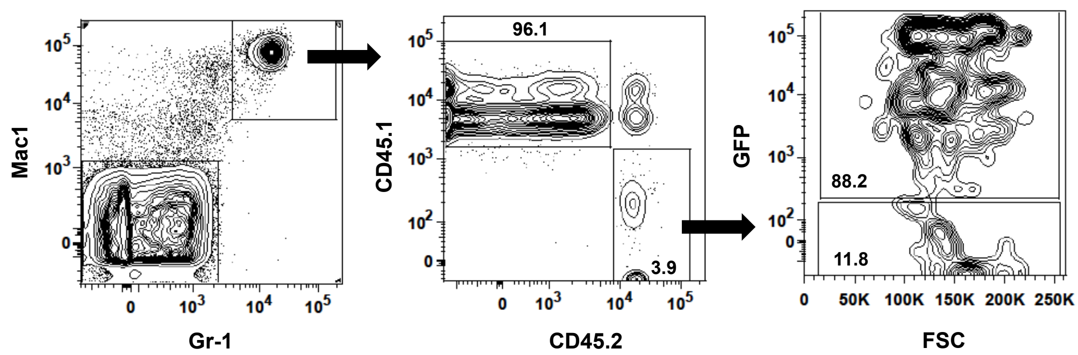
B

Rps14



C

Rbm22



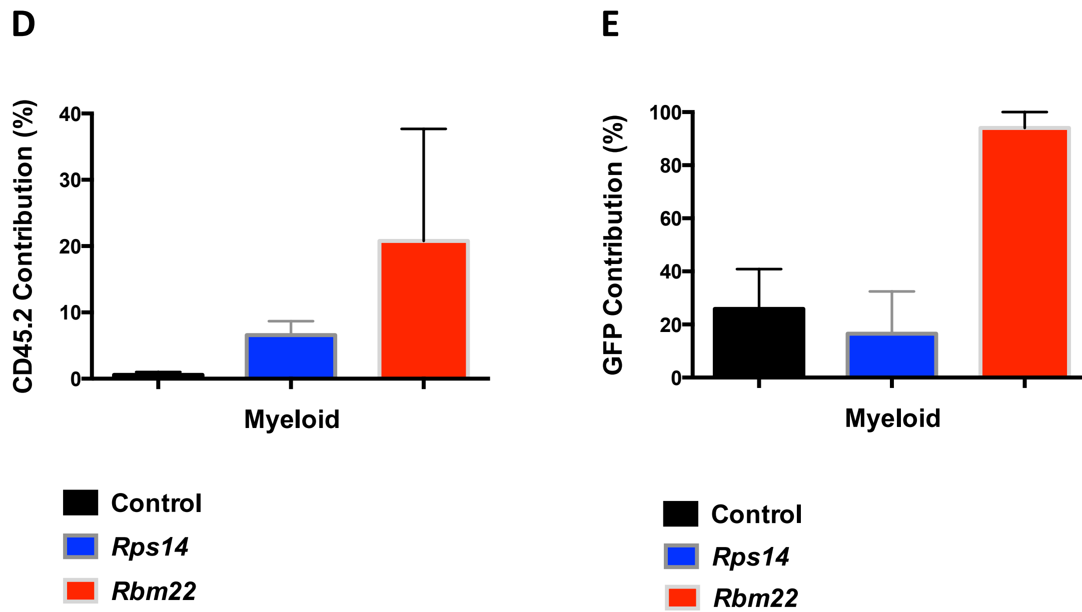


Figure 4.7 Myeloid reconstitution of *Cd74-Nid67* cells transduced with *Rps14* or *Rbm22* cDNA lentiviruses

Representative FACS plots of reconstituted myeloid (Mac1+, Gr1+) *Cd74-Nid67* cells (CD45.2) expressing Control, *Rps14* or *Rbm22* cDNA (**A**, **B** and **C**, respectively) in blood. **D.** Donor reconstitution (CD45.2) of *Cd74-Nid67* myeloid cells treated with Control, *Rps14* or *Rbm22* cDNA lentiviruses in peripheral blood 4 weeks post transplant (n=3). **E.** GFP contribution of donor *Cd74-Nid67* myeloid cells treated with Control, *Rps14* or *Rbm22* cDNA lentiviruses in peripheral blood 4 weeks post transplant (n=3). Data is expressed as mean +/- SEM.

4.2.7 Transduction of *wild-type* Cells with *Rps14* or *Rbm22* Lentiviruses Does Not Affect Their Reconstitution

As an important control experiment, *Rps14* or *Rbm22* cDNA were introduced into *wild-type* cells to examine if their overexpression affects reconstitution capacity. Purified LSK cells from *wild-type* mice were treated with Control, *Rps14* or *Rbm22* cDNA lentiviruses and transplanted into lethally irradiated recipients. The reconstitution of myeloid cells in peripheral blood was similar in all groups (Figure 4.8 A). Moreover, the frequency of engrafted myeloid cells that were GFP positive was similar to the frequency of transduced (GFP positive) LSKs *in vitro* (Figure 4.8 B).

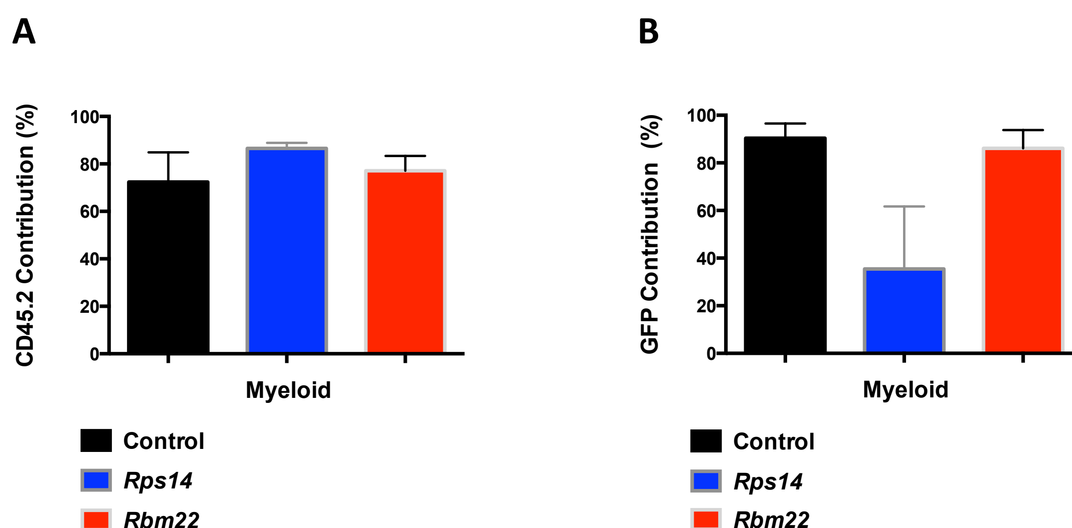


Figure 4.8 Impact of *Rps14* and *Rbm22* cDNA expression on the reconstitution of *wild-type* myeloid Cells

A. Donor reconstitution (CD45.2) of *wild-type* myeloid cells treated with Control, *Rps14* or *Rbm22* cDNA lentiviruses in peripheral blood 4 weeks post transplant (n=3). **B.** GFP contribution of donor *wild-type* myeloid cells treated with Control, *Rps14* or *Rbm22* cDNA lentiviruses in peripheral blood 4 weeks post transplant (n=3). Data is expressed as mean +/- SEM.

4.3 Discussion

Pinpointing the genes within *Cd74-Nid67* that, at haploinsufficiency, disrupt HSC function would provide a better understanding of the primary events leading to Del(5q) MDS development and may promote the establishment of targeted therapeutic approaches for patients.

We found that only six of the eight genes within *Cd74-Nid67* are expressed in *wild-type* mouse HSCs. Moreover, the expression pattern was similar in HSCs from mice and humans. *Myoz3* and *Synpo* were not expressed in HSCs from *wild-type* mice. Similarly, *SYNPO* was not expressed in human HSCs while *MYOZ3* was expressed at very low levels. The expression of genes within *Cd74-Nid67* in HSCs was determined by RNA sequencing analysis and confirmed by qPCR analysis. Our results suggest that *Synpo* and *Myoz3* are not required for normal HSC function in mice and cannot account for the altered function of *Cd74-Nid67* HSCs.

Gene expression profiling in this study revealed that the expression of genes within *Cd74-Nid67* were reduced by approximately 50% in HSCs from *Cd74-Nid67* mice and Del(5q) MDS patients compared to HSCs from *wild-type* mice and healthy controls, respectively. This supports previous studies demonstrating that haploinsufficiency, not homozygous inactivation, of one or more genes on 5q underlies Del(5q) MDS pathogenesis (Heinrichs, Kulkarni et al. 2009).

In this study, we used an available *Cd74* knockout mouse model (Bikoff, Huang et al. 1993) to examine the impact of *Cd74* loss on haematopoiesis. Neither heterozygous nor homozygous loss of *Cd74* had an effect on HSC number or repopulating ability, demonstrating that haploinsufficiency of *Cd74*, alone, is not responsible for *Cd74-Nid67* HSC dysfunction. However, these findings do not exclude the possibility that

haploinsufficiency of *Cd74*, in combination with other genes, may account for *Cd74-Nid67* HSC dysfunction.

Since there were no knockout mice available for one of the most likely candidate genes*, Rps14,* an shRNA strategy was employed to investigate the impact of its deficiency on HSC activity. We found that *Cdkn1a* was significantly upregulated in stem/progenitor cells expressing *Rps14* shRNAs, similar to its upregulation in HSCs from *Cd74-Nid67* mice and Del(5q) MDS patients. Moreover, the repopulation potential of GFP+ HSCs treated with *Rps14* shRNAs was severely compromised upon transplantation. This suggests that haploinsufficiency of *Rps14* may be sufficient to disrupt HSC function. However, there are caveats associated with this finding. The total (CD45.2) engraftment of HSCs did not correlate with the engraftment of GFP+ HSCs. There was no difference in total (CD45.2) HSC reconstitution between the control and *Rps14* shRNA groups. Based on the dramatic reduction in the engraftment of GFP+ HSCs in the *Rps14* group, a corresponding decrease in CD45.2 HSC reconstitution would have been expected. The discrepancy between total (CD45.2) reconstitution and GFP+ reconstitution may be attributed to the variability in viral supernatant toxicity. Indeed, cells treated with different viral preparations of the same Luciferase construct (Luc1 and Luc2) differed in their reconstitution (CD45.2) capacity.

Importantly, the experimental design used in this study (transplanting non-transduced cells along with transduced cells) allowed us to identify this issue. The non-transduced CD45.2 cells served as an internal control. The toxicity issue, therefore, must be resolved in order to conclusively determine the impact of *Rps14* haploinsufficiency on HSC function. It will, then, also be important to use shRNA hairpins to knockdown the expression of *Rbm22* and examine the impact on HSC repopulation potential.

The use of an inducible shRNA system provides scope to overcome the issue of viral supernatant toxicity or, at least, control for it. shRNA expression could be induced upon treatment with Doxycycline, for example, in transduced cells after transplantation. The impact of gene knockdown on reconstitution could, therefore, be examined relative to the initial reconstitution.

Since non-specific, off-target effects are often a problem with shRNAs (Jackson and Linsley 2004), complementary "rescue" experiments were performed. Purified *Cd74-Nid67* HSCs transduced with *Rps14* and *Rbm22* cDNA lentiviruses were transplanted into lethally irradiated recipients to determine if restoring expression of either gene could improve HSC function. Preliminary peripheral blood analysis revealed that the reconstitution of myeloid cells was increased in the *Rbm22* and *Rps14* groups. The improvement in reconstitution following introduction of *Rps14* and *Rbm22* into *Cd74-Nid67* cells indicates that deficiency of these genes may account for *Cd74-Nid67* HSC dysfunction. The improvement in reconstitution was particularly striking in the *Rbm22* group, as almost all reconstituted cells were GFP positive, suggesting a clear selection for cells expressing *Rbm22* cDNA.

It is surprising that many of the reconstituted cells in the *Rps14* group were not GFP positive. This may simply reflect the reduced transduction efficiency of the *Rps14* cDNA lentivirus compared to the Luciferase and *Rbm22* cDNA lentiviruses. Alternatively, *Rps14* cDNA lentiviral expression may not have been sufficient to restore endogenous *Rps14* levels in *Cd74-Nid67* cells. The lack of a clear selection for the engraftment of GFP+ cells in the *Rps14* group may suggest that *Cd74-Nid67* HSC dysfunction was not, in fact, rescued, which could be due to inadequate expression of *Rps14*. It will be crucial to examine the expression of *Rps14* in engrafted HSCs transduced with the cDNA lentivirus to determine if expression was fully restored to

endogenous levels. Since *Rps14* is expressed at extremely high levels in mouse HSCs, fully restoring endogenous levels of expression will likely be challenging. We used a strong SFFV viral promoter to drive expression of cDNA sequences that were codon-optimised in an effort to achieve the highest expression levels possible.

Since there are only a small number of replicates per group (n=3), the "rescue" experiments must be repeated. Furthermore, the analysis was performed relatively soon after transplantation (four weeks). Since lymphoid cells are long-lived, we focused on myeloid reconstitution as, at this early time point, it better reflects HSC reconstitution. It will be important to follow the reconstitution in peripheral blood over time and, ultimately, to examine the reconstitution of HSCs in the BM.

Although *Cd74-Nid67* reconstitution was improved in the *Rps14* and *Rbm22* groups, it is clear that the reconstitution capacity was not fully restored to *wild-type* levels. This was not entirely unexpected. The recombination of *Cd74-Nid67* occurred early in the embryo while cells from adult mice were transduced with *Rps14* and *Rbm22* cDNAs. The *Cd74-Nid67* cells were, therefore, deficient in these genes from the earliest stages of development and may have been too dysfunctional at the stage of transduction to be completely rescued. A strategy that involves transducing *Cd74-Nid67 Mx1Cre* "not yet deleted" cells with cDNA lentiviruses and inducing deletion in cells already expressing *Rps14* or *Rbm22* cDNAs after transplantation must, therefore, be considered.

Alternatively, the partial "rescue" of the *Cd74-Nid67* repopulation deficit may reflect variability in the levels of cDNA expression by individual cells. The "rescued" cells that manage to engraft may be the only cells that express *Rps14* or *Rbm22* at sufficient levels and their function may be completely restored. It will be interesting to analyse the "rescued", reconstituted *Cd74-Nid67* HSCs both molecularly and functionally to

determine if their gene expression signature and engraftment capacity upon secondary transplantation has been fully restored to healthy levels.

The results from this study suggest that deficiency of *Rps14* and *Rbm22* may account for *Cd74-Nid67* HSC dysfunction, and therefore, may be centrally involved in the development of Del(5q) MDS. Future studies will use *Rps14* and *Rbm22* conditional knockout mouse models, the gold standard method for examining gene function, to confirm the findings presented in this chapter. This will provide important insights into the mechanisms of disease initiation in Del(5q) MDS.

5. The Impact of P53 Loss on *Cd74-Nid67* HSC Function

5.1 Introduction

Mutations in P53 are strongly associated with Del(5q) MDS, occurring in almost one fifth of patients, and are linked with an increased risk of leukaemic transformation (Jadersten, Saft et al. 2011). Furthermore, a P53-dependent mechanism has been implicated in driving the erythroid differentiation block in Del(5q) MDS (Ebert, Pretz et al. 2008, Barlow, Drynan et al. 2010, Dutt, Narla et al. 2011).

Impaired ribosome biogenesis has been linked to Del(5q) MDS, as well as other diseases such as Diamond Blackfan Anaemia (DBA) and Treacher Collins Syndrome (TCS) (Narla and Ebert 2010). Interestingly, suppressing P53 activity in genetically engineered animal models for each of these diseases completely reverses the clinical phenotypes. Deficiency of *Rps19,* a gene commonly mutated in DBA patients, caused a block in erythropoiesis in zebrafish that was alleviated by suppressing P53 activity (Danilova, Sakamoto et al. 2008). Similarly, loss of P53 completely reversed the leukocytopenia and macrocytic anaemia in *Rps19* deficient mice (Jaako, Flygare et al. 2011). Removal of P53 also rescued footpad hyperpigmentation and macrocytic anaemia in mice with germline mutations in *Rps19* and *Rps20* (McGowan, Li et al. 2008). Similarly, inhibition of P53 by both genetic and chemical means corrected the craniofacial abnormalities caused by deficiency of *Tcof1* in a mouse model of TCS (Jones, Lynn et al. 2008). Furthermore, insufficiency of *Rps14*, responsible for the erythroid differentiation deficit in Del(5q) MDS (Ebert, Pretz et al. 2008), caused P53

accumulation, cell cycle arrest and apoptosis in human erythroid progenitors *in vitro*. Treatment with the P53 inhibitor, pifithrin- α, completely rescued the erythroid phenotype (Dutt, Narla et al. 2011).

Studies demonstrating the link between ribosomal protein deficiency and P53 activation in the haematopoietic system have focused almost exclusively on the erythroid lineage (Dutt, Narla et al. 2011, Jaako, Flygare et al. 2011). The P53 response to ribosomal protein deficiency in HSCs, however, has yet to be explored.

Cd74-Nid67 mice have an increased number of P53 positive BM cells and an elevated level of apoptosis (Barlow, Drynan et al. 2010). Inhibition of P53 reversed the macrocytic anaemia and BM dysplasia in *Cd74-Nid67* mice. Moreover, myeloid progenitor frequency and myeloid and erythroid colony forming ability (CFU-GM and CFU-E, respectively) were restored in the absence of P53 (Barlow, Drynan et al. 2010). This indicates that a P53 dependant mechanism underlies the haematopoietic differentiation deficit in *Cd74-Nid67* mice. The increased expression of *Cdkn1a* (or P21), a downstream mediator of P53 activity, in purified HSCs from *Cd74-Nid67* mice and Del(5q) MDS patients suggests that P53 accumulation may also be responsible for aberrant HSC function.

In this study, we aimed to examine whether loss of P53 could restore self-renewal and repopulation potential to *Cd74-Nid67* HSCs. Conditional *Trp53* null mice (Marino, Vooijs et al. 2000) were crossed with *Cd74-Nid67* mice and HSC number and function were examined. Delineating whether P53 is involved in driving Del(5q) HSC dysfunction may provide important insights into the mechanisms responsible for disease initiation.

5.2 Results

5.2.1 Loss of P53 Restores Red Blood Cell Counts in *Cd74-Nid67* Mice

Peripheral blood samples from *Cd74-Nid67* mice with heterozygous or homozygous loss of *Trp53 (Trp53* fl/+ *Cd74-Nid67* fl/+ *VavCre* tg/+ or *Trp53* fl/fl *Cd74-Nid67* fl/+ *VavCre* tg/+, respectively) were analysed and compared to *Cd74-Nid67* mice with *wild-type* P53 (*Trp53* +/+ *Cd74-Nid67* fl/+ *VavCre* tg/+) and *VavCre* only (*Trp53* +/+ *Cd74-Nid67* +/+ *VavCre* tg/+) controls.

Heterozygous loss of *Trp53* in *Cd74-Nid67* mice did not have any effect on any of the parameters measured.

Homozygous loss of *Trp53* restored red blood cell (RBC) number (Figure 5.1 B) and mean corpuscular volume (MCV) (Figure 5.1 D) to normal levels in *Cd74-Nid67* mice. However, there was no effect on white blood cell (WBC) number (Figure 5.1 A), haemoglobin concentration (Figure 5.1 C) or RBC distribution width (Figure 5.1 E).

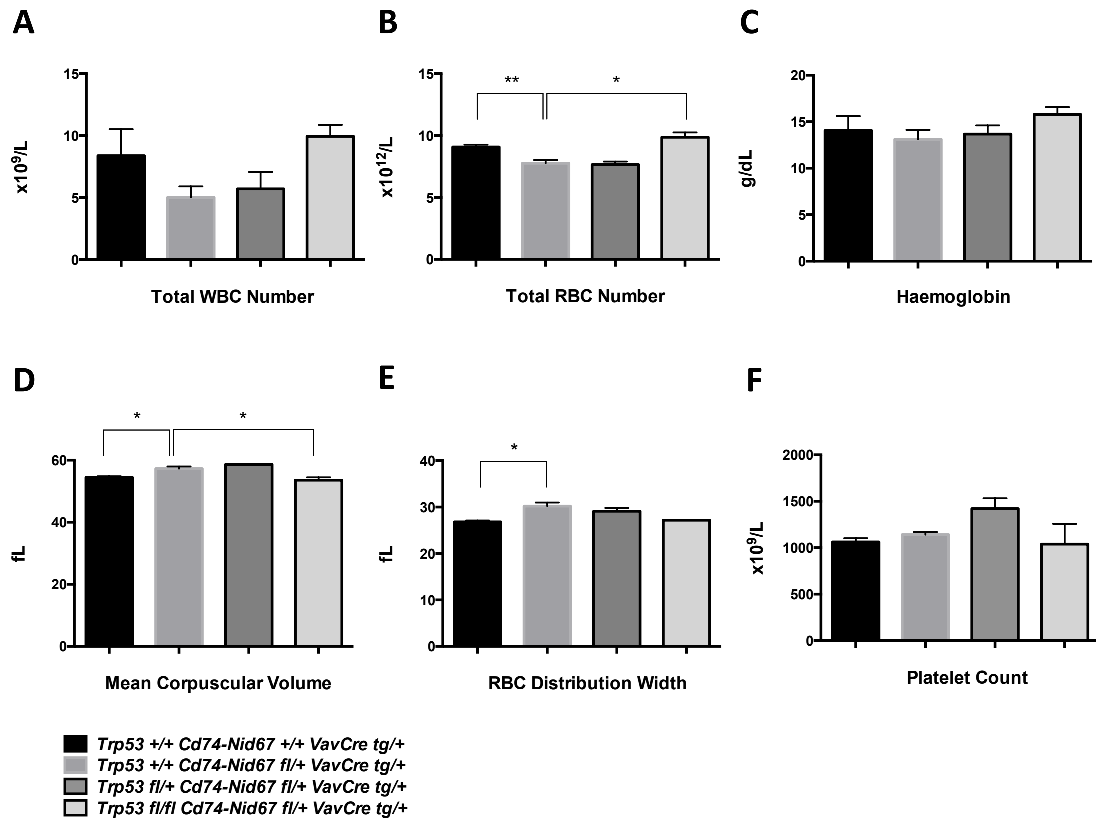


Figure 5.1 Peripheral blood measurements of *Cd74-Nid67* mice in the presence or absence of P53

Blood from adult (8 week old) mice with the indicated genotypes was analysed (n=5). **A.** White Blood Cell (WBC) number. **B**. Red Blood Cell (RBC) number. **C**. Haemoglobin concentration. **D**. Mean corpuscular volume. **E.** Red Blood Cell (RBC) distribution width. **F**. Platelet Count.

Data is expressed as mean +/- SEM. * $P<0.05$, ** $P<0.01$.

5.2.2 Loss of P53 Restores BM Cellularity and HSC numbers in *Cd74-Nid67* mice

To further explore the impact of P53 deficiency in *Cd74-Nid67* mice, BM cellularity and HSC number were examined. The gating strategy used for flow cytometric analysis of HSCs is shown (Figure 5.2 A-D).

A significant increase in bone marrow cellularity was evident in *Cd74-Nid67* mice with homozygous loss of *Trp53* compared to *Cd74-Nid67* mice with *wild-type Trp53* (Figure 5.3 A). However, no change in BM cellularity was seen in *Cd74-Nid67* mice with heterozygous loss of *Trp53*.

Homozygous loss of *Trp53* restored the number of short-term and long-term HSCs (ST-HSC and LT-HSC), defined as LSK, CD48-, CD150+ and CD34- or CD34+, to normal levels in *Cd74-Nid67* mice (Figure 5.3 B and C). Interestingly, heterozygous loss of *Trp53* resulted in an intermediate rescue of ST-HSC and LT-HSC number in *Cd74-Nid67* mice.

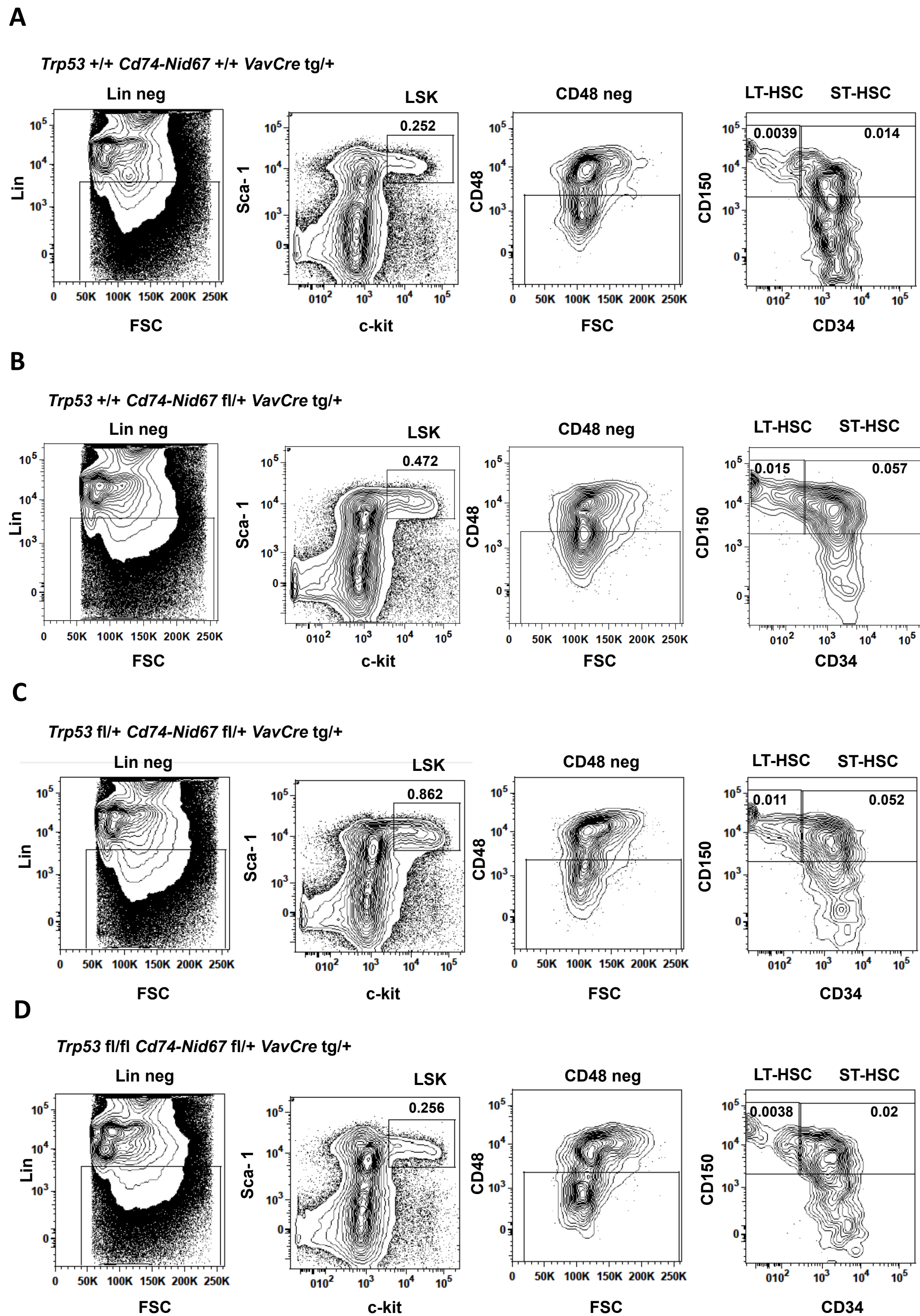


Figure 5.2 Gating strategy for HSC analysis

Representative FACS profiles of stem and progenitor populations from mice with the indicated genotypes (A-D). Numbers within quadrants indicate the frequency of the population in total BM.

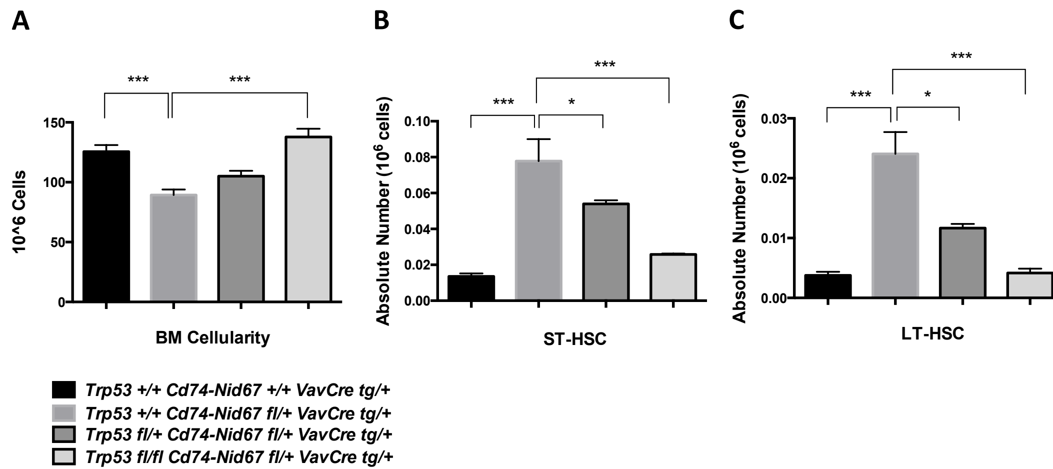


Figure 5.3 Impact of P53 loss on long-term and short-term HSC Numbers in *Cd74-Nid67* Mice

Bone Marrow (BM) from mice with the indicated genotypes was analysed. **A.** Number of bone marrow mononuclear cells (BM cellularity) of 2 femora and 2 tibiae per mouse (n=5). **B.** and **C.** Absolute number of Long-Term HSCs (LT-HSC) and Short-Term HSCs (ST-HSC), respectively (n=3).

Data is expressed as mean +/- SEM. * P<0.05, ** P<0.01, *** P<0.001

5.2.3 Loss of P53 Restores *Cd74-Nid67* Mature Lineage Reconstitution

To assess the impact of P53 deficiency on *Cd74-Nid67* HSC function, long-term competitive transplantations were performed and multi-lineage reconstitution was analysed in the peripheral blood of recipients. Complete loss of P53 fully restored the reconstitution of *Cd74-Nid67* myeloid cells (Figure 5.4 A), B cells (Figure 5.4 B) and T cells (Figure 5.4 C) in peripheral blood. Partial loss of P53, however, failed to restore reconstitution of *Cd74-Nid67* mature cells in blood.

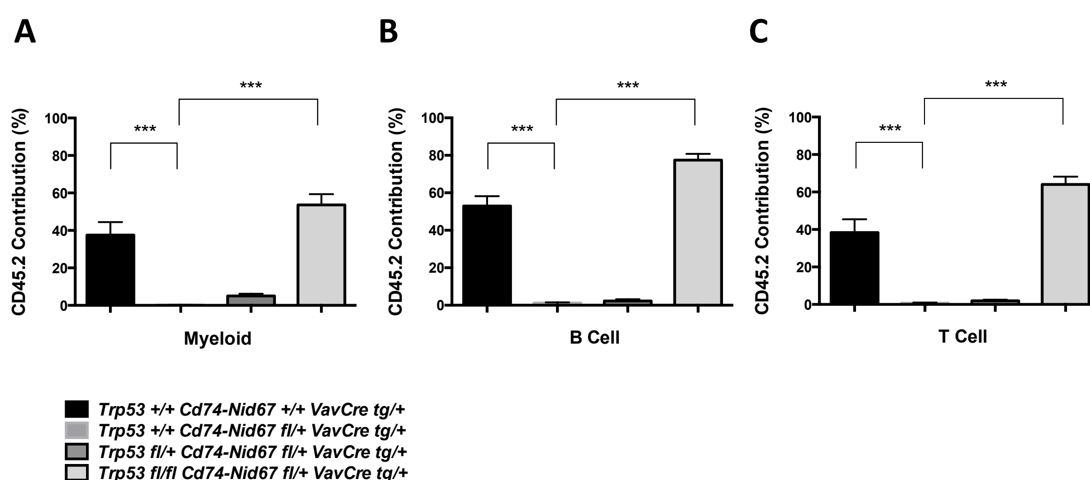


Figure 5.4 Impact of P53 Loss on Reconstitution of *Cd74-Nid67* Mature Lineages

Reconstitution of mature cells from mice with the indicated genotypes was analysed (n=3). Reconstitution of **A.** myeloid, **B.** B cells and **C.** T cells in peripheral blood sixteen weeks post competitive transplantation.
Data is expressed as mean +/- SEM. * P<0.05, ** P<0.01, *** P<0.001

5.2.4 Loss of P53 Rescues the Reconstitution of *Cd74-Nid67* Myeloid and Erythroid Progenitors

To assess the impact of P53 deficiency on *Cd74-Nid67* myeloid and erythroid progenitor reconstitution, their contribution to the BM of recipients was examined sixteen weeks after transplantation.

The reconstitution of *Cd74-Nid67* PreGM (Figure 5.5 A), GMP (Figure 5.5 B), Pre MegE (Figure 5.5 C), MkP (Figure 5.5 D), Pre CFU-E (Figure 5.5 E) and CFU-E (Figure 5.5 F) cells with or without P53 was examined.

Loss of P53 completely rescued the reconstitution of *Cd74-Nid67* myeloid and erythroid progenitors. Heterozygous loss of *Trp53* improved the reconstitution of myeloid and megakaryocyte progenitors (PreGM, GMP, Pre MegE and MkP) but had no effect on reconstitution of erythroid progenitors (Pre CFU-E and CFU-E), suggesting a partial rescue in this context.

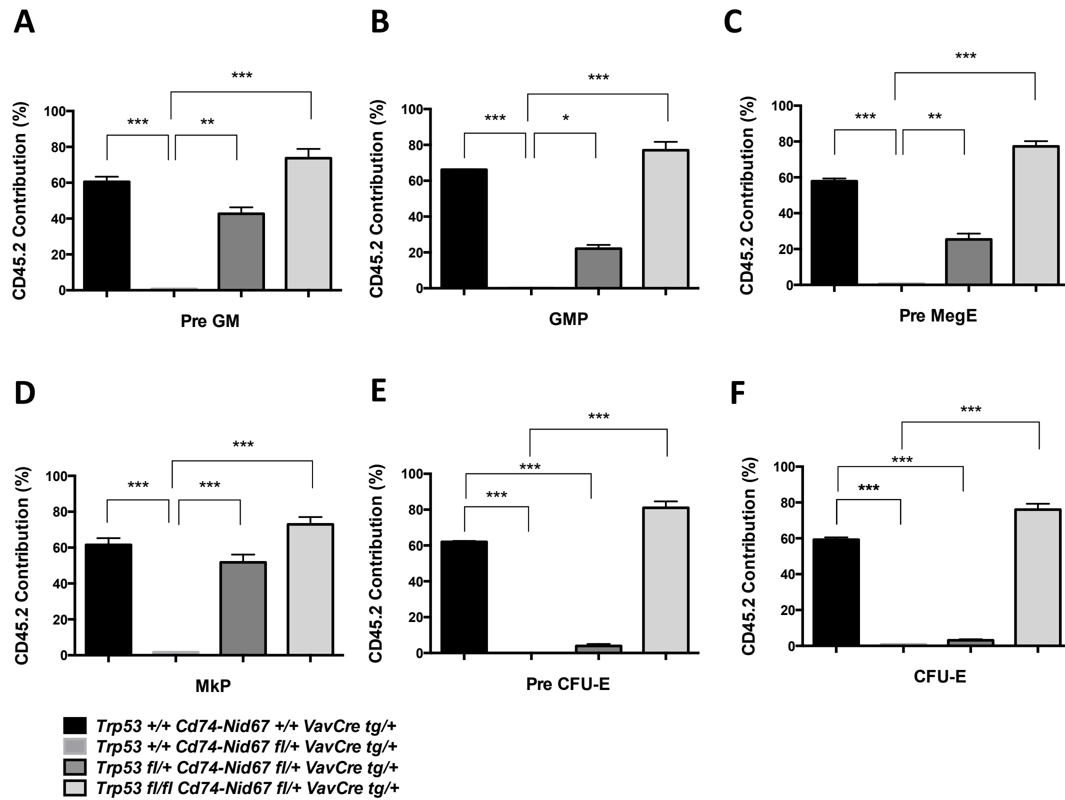


Figure 5.5 Impact of P53 Suppression on reconstitution of *Cd74-Nid67* myeloid and erythroid progenitors

Donor (CD45.2) reconstitution of **A.** Pre GM, **B.** GMP, **C.** Pre MegE**, D.** MkP**, E.** Pre CFU-E and **F.** CFU-E populations in bone marrow of recipients (CD45.1) sixteen weeks post competitive transplant (n=3).

Data is expressed as mean +/- SEM. * P<0.05, ** P<0.01, *** P<0.001

5.2.5 Loss of P53 Rescues *Cd74-Nid67* Haematopoietic Stem Cell Engraftment

To determine the impact of P53 deficiency on *Cd74-Nid67* stem/progenitor repopulating ability, flow cytometry analysis was performed to examine the engraftment of donor HSCs (CD45.2) sixteen weeks after transplantation. Both homozygous and heterozygous loss of *Trp53* completely reversed the engraftment deficit of *Cd74-Nid67* LSK stem/progenitor cells (Figure 5.6 A) and LSK, CD48-, CD150+ HSCs (Figure 5.6 B).

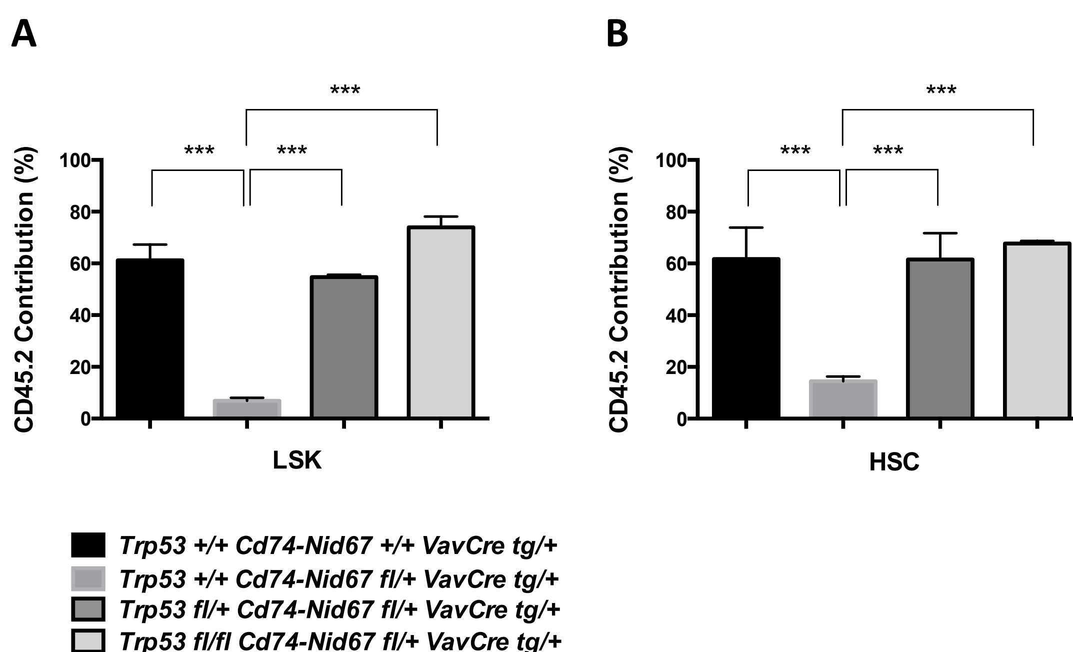


Figure 5.6 ***Cd74-Nid67*** **HSC Engraftment in the presence or absence of P53**

Donor contribution (CD45.2) of **A.** stem/progenitor (LSK) cells and **B.** HSCs in bone marrow of recipients (CD45.1) sixteen weeks post competitive transplant (n=3).
Data is expressed as mean +/- SEM. * P<0.05, ** P<0.01, *** P<0.001

5.3 Discussion

Mutations in TP53 are strongly associated with 5q deletions and are found in almost 20% of low-risk Del(5q) MDS patients (Saft, Karimi et al. 2014). Such mutations are associated with strong expression of P53 and render them at higher risk to leukaemic transformation (Saft, Karimi et al. 2014). Moreover, P53 is activated in response to *Rps14* deficiency in erythroid cells, leading to a block in differentiation (Dutt, Narla et al. 2011). Taken together, these findings suggest that P53 may be an important contributor to the pathogenesis of Del(5q) MDS and the progression to AML. However, P53's contribution to HSC dysfunction in Del(5q) MDS remained to be explored. In this study, we demonstrated, for the first time, that P53 is required to confer aberrant self-renewal and repopulation potential to *Cd74-Nid67* HSCs.

We found that loss of P53 reversed the *Cd74-Nid67*-mediated changes in RBC size and number. This is in agreement with previous findings (Barlow, Drynan et al. 2010). Furthermore, we demonstrated that loss of P53 reversed the expansion of HSCs in *Cd74-Nid67* mice. This highlights that P53, not only mediates the erythroid differentiation deficit but also mediates HSC dysfunction in *Cd74-Nid67* mice.

Importantly, the repopulation and differentiation capacity of *Cd74-Nid67* stem and progenitor cells was fully restored in the absence of P53. This suggests that *Cd74-Nid67* HSC dysfunction may be dependent on the activity of P53, a finding that has important implications for understanding the biology of Del(5q) MDS. In support of this, the expression of P53 was significantly increased in BM cells from *Cd74-Nid67* mice (Barlow, Drynan et al. 2010). Moreover, the increased expression of *Cdkn1a*, a downstream mediator of P53, in HSCs from *Cd74-Nid67* mice and Del(5q) MDS patients is compatible with P53 activation.

Interestingly, heterozygous loss of *Trp53* completely rescued reconstitution of HSCs,

partially rescued reconstitution of myeloid progenitors but failed to rescue reconstitution of erythroid progenitors. Homozygous loss of *Trp53,* however, rescued reconstitution of progenitors and mature cells of all lineages. This highlights the impact of P53 gene dosage in different cell populations. The highly proliferative nature of erythroid progenitors together with the large demand for haemoglobin production places a burden on their translational machinery. This may sensitise them to ribosomal haploinsufficiency and subsequent P53 activation. The involvement of the P53 pathway in erythroid maturation may also enhance the sensitivity of this lineage to further P53 activation (Peller, Frenkel et al. 2003). Importantly, a recent study demonstrated that human erythroid cells (defined as CD71+) had a particularly low threshold for P53 activation compared to megakaryocytes (defined as CD41+) or myeloid cells (defined as CD13+, CD33+, CD45+) in response to *RPS14* knockdown (Dutt, Narla et al. 2011). The enhanced sensitivity of the erythroid lineage is consistent with the clinical presentation of Del(5q) MDS.

Since eight genes lie within *Cd74-Nid67*, we cannot conclude from this study that *Rps14* deficiency is solely responsible for accumulation of P53 in *Cd74-Nid67* HSCs.

Although the activation of P53 in response to ribosomal stress has been well documented in multiple cell types including erythroid progenitors (Fumagalli, Di Cara et al. 2009, Dutt, Narla et al. 2011), it has not been demonstrated in HSCs. It is, however, tempting to speculate that *Rps14* insufficiency is also responsible for driving P53 accumulation in HSCs. This is supported by our finding that shRNA knockdown of *Rps14* in stem/progenitor cells (LSK) resulted in increased expression of *Cdkn1a*, a downstream mediator of P53 activity.

The mechanistic basis for P53 activation in response to ribosomal stress was examined in human haematopoietic progenitor cells *in vitro* (Fumagalli, Di Cara et al.

2009, Dutt, Narla et al. 2011). The primary negative regulator of P53, MDM2, serves as a link between ribosome biogenesis and the P53 pathway. Mutation or deficiency of ribosomal proteins can disrupt ribosome assembly. This can lead to an accumulation of free ribosomal proteins that can bind and block MDM2. This, in turn, leads to P53 activation. Further studies using shRNA knockdown strategies or *Rps14* knockout mice will be critical to determine if *Rps14* insufficiency and the ribosomal stress-MDM2 axis are, indeed, responsible for P53 activation in HSCs.

Deficiency of other genes within *Cd74-Nid67* may also play a role in p53 activation. This, however, remains to be investigated. Aberrant splicing, linked to DNA damage, can drive stem/progenitor dysfunction and myelodysplasia in mice (Colla, Ong et al. 2015). *Rbm22* deficiency could, therefore, induce DNA damage and activate P53, impairing HSC function. Indeed, disrupting the pre-mRNA splicing machinery in a human melanoma cell line was shown to cause P53 accumulation and cell cycle arrest (Allende-Vega, Dayal et al. 2013).

A recent study demonstrated that exit from quiescence and replicative stress is associated with DNA damage and impaired function of HSCs upon transplantation (Walter, Lier et al. 2015). *Cd74-Nid67* HSCs may, therefore, harbour excessive DNA damage, given their reduced quiescence and impaired function. In the face of replicative stress (transplantation), P53 may trigger cell cycle arrest and apoptosis in *Cd74-Nid67* HSCs, hindering their engraftment. Removal of P53 may prevent normal cell death responses, allowing damaged HSCs to survive and exposing them to the risk of acquiring new genetic or karyotypic changes. This, in turn, could drive leukaemic transformation. This is compatible with studies that have demonstrated an increased incidence of karyotypic changes in Del(5q) MDS patients with TP53 mutations (Sebaa, Ades et al. 2012, Kulasekararaj, Smith et al. 2013). Indeed,

aberrant self-renewal activity was identified in progenitor cells from a Del(5q) MDS patient that had acquired a TP53 mutation. Moreover, this patient later developed AML (Woll, Kjallquist et al. 2014). It will be important to examine if DNA damage has accumulated in *Cd74-Nid67* HSCs and to determine whether loss of P53 results in genomic instability and, ultimately, malignant transformation.

Although P53 suppression appears to rescue the function of *Cd74-Nid67* HSCs, elimination of P53 activity is unlikely to be a promising therapeutic approach for Del(5q) MDS patients. The important tumour suppressor role of P53 together with its involvement in a wide range of cellular processes suggests that serious consequences may result from altering its expression.

Eliminating P53 activity is known to cause genomic instability (Meek 2009, Negrini, Gorgoulis et al. 2010) and mice that lack or express a mutant form of P53 are extremely prone to tumour development, especially lymphomas (Donehower, Harvey et al. 1992, Jacks, Remington et al. 1994, Lang, Iwakuma et al. 2004). Moreover, TP53 mutations are particularly common in MDS patients with Del(5q) and are associated with an increased risk of progression to acute myeloid leukaemia (AML) (Jadersten, Saft et al. 2011). These findings emphasise the consequences of disrupting p53 activity and highlight the requirement for tight regulation of this pathway.

The results of this study provide important insights into the critical role that P53 plays in *Cd74-Nid67* HSC dysfunction. Further studies will delineate the molecular events that trigger P53 activation, which will be key to understanding the aetiology of Del(5q) MDS.

6. Discussion

6.1 Technological Considerations

The development and enhancement of scientific tools and technologies in recent decades has greatly improved the depth and breadth with which scientific questions can be addressed. However, the use of innovative scientific tools often presents major challenges. It is important to consider these challenges so that the appropriate controls can be identified and the implications of the results understood.

6.1.1 Transgenic *Cre* Lines

In this study, the *VavCre* mouse line (Georgiades, Ogilvy et al. 2002) was used to delete *Cd74-Nid67* in all haematopoietic cells. Since *Cd74-Nid67* contains eight known genes and spans a large region (~32 kilobases), a constitutive *Cre* was selected to achieve optimal deletion efficiency. The expression of genes within *Cd74-Nid67* was reduced by 50% in purified HSCs from targeted mice, as would be expected from heterozygous deletion. We also confirmed that *Cd74-Nid67* deletion had occurred in the BM of targeted mice using a PCR that detected the recombination event, although this approach was not quantitative. The complete failure (<1%) of *Cd74-Nid67* mature cells to engraft upon competitive transplantation further supports that deletion was extremely efficient.

The drawback of the *VavCre* line, however, is that recombination occurs early in the embryo. Since Del(5q) is a somatic mutation that occurs in elderly patients (Nimer 2008), the induction of *Cd74-Nid67* haploinsufficiency in aged mice using an inducible *Cre* line, such as *Mx1Cre* (Kuhn, Schwenk et al. 1995), is more clinically

relevant. *Mx1Cre* is under the control of an interferon-responsive promoter so that treating targeted mice with PolyIC will induce its expression. However, *Mx1* is expressed in tissues outside the haematopoietic system and *Mx1Cre*-mediated recombination has been shown to occur in the liver, heart, kidney and duodenum (Kuhn, Schwenk et al. 1995). In order to examine the cell intrinsic effect of *Cd74-Nid67* haploinsufficiency, "not yet deleted" *Cd74-Nid67 Mx1Cre* BM cells must be transplanted into lethally irradiated recipients and deletion induced post transplantation.

Preliminary experiments were performed with the *Mx1Cre* line but the deletion of *Cd74-Nid67* seemed inefficient based on recombination PCR results and the phenotype of the mice. Further experiments with *Cd74-Nid67 Mx1Cre* mice were, therefore, not pursued. The large distance (~32 kilobases) between the loxP sites flanking *Cd74-Nid67* may explain the inefficient recombination with an inducible *Cre* line, such as *Mx1Cre*. It will be important to try to improve the efficiency of *Cd74-Nid67* deletion with the *Mx1Cre* line to model the disease in a setting that is most relevant to Del(5q) MDS patients. Careful titration of PolyIC may allow the deletion efficiency to be improved without increasing the risk of cytotoxicity.

It will also be important to develop a quantitative method to assess deletion efficiency. Digital PCR technology allows the absolute quantification of DNA molecules. This method could, therefore, be used to quantitatively evaluate *Cd74-Nid67* deletion efficiency. This would provide important information about whether increasing the dose of PolyIC improves deletion efficiency. However, even if the deletion was not extremely efficient, the use of digital PCR provides scope to track the number of deleted cells over time. This could provide meaningful insights into the impact of the deletion on cellular function.

There are a number of technical challenges associated with the use of transgenic *Cre* lines. It has previously been suggested that, in some cases, the expression of *Cre* alone can cause a phenotype (Schmidt-Supprian and Rajewsky 2007, Li, Choi et al. 2014). This is due the presence of cryptic loxP sites located within the mouse genome. These studies recommend using *Cre* only mice as controls in an effort to identify and overcome this issue. In this study, we used the appropriate controls, *VavCre* only littermates, to ensure that the phenotype was not a result of *Cre* off-target effects.

6.1.2 Phenotypic and Functional Definition of HSCs

Despite significant advances in understanding the biology of HSCs, it is still not possible to isolate a pure population of HSCs using phenotypic markers. However, phenotypic markers can highly enrich for HSC activity in mice. Indeed, one in five SLAM-defined HSCs (LSK, CD48-, CD150+) has long-term repopulation potential (Kiel, Yilmaz et al. 2005).

In this study, the use of phenotypic HSC markers (in combination with functional assays) revealed that *Cd74-Nid67* HSCs were the only population capable of engraftment upon transplantation. This finding provided important information about the impact of *Cd74-Nid67* haploinsufficiency on haematopoiesis. The detection of *Cd74-Nid67* HSC engraftment would have been impossible without the use of phenotypic markers due to their rarity within the BM. Indeed, *Cd74-Nid67* reconstitution was not detected when unfractionated BM cells were analysed.

The classification of cells as functional HSCs relies on their ability to support long-term, multi-lineage reconstitution upon transplantation. However, there are limitations associated with this definition of HSCs. Transplantation assays involve removing cells from their natural environment and subjecting them to replicative stress upon

injection into lethally irradiated recipients. This assay, therefore, does not provide scope to evaluate the function of HSCs in homeostatic conditions. Furthermore, transplantation assays are limited by their dependence on the homing and engraftment capacity of HSCs. Nevertheless, transplantation assays are the best available method to evaluate HSC activity and have provided important insights into HSC regulation and heterogeneity (Dykstra, Kent et al. 2007, Beerman, Bhattacharya et al. 2010, Challen, Boles et al. 2010, Morita, Ema et al. 2010, Sanjuan-Pla, Macaulay et al. 2013).

Indeed, the use of transplantation assays in this study helped to improve our understanding of HSC dysfunction in *Cd74-Nid67* mice. The results highlight that *Cd74-Nid67* HSCs act differently in different environments. *Cd74-Nid67* HSCs, although increased in number and less quiescent, manage to produce downstream progenitors and mature cells of all lineages in their endogenous environment in steady-state conditions. However, upon transplantation, *Cd74-Nid67* HSCs engraft poorly and fail to produce any mature cells. The competitive transplantation assay, therefore, revealed important information about the repopulation capacity of *Cd74-Nid67* HSCs during stress haematopoiesis.

6.1.3 shRNA and cDNA Lentiviruses

In this study, shRNA experiments were performed to identify genes within *Cd74-Nid67* that are important for HSC function. However, there are limitations associated with the use of shRNAs as experimental tools. A number of studies have demonstrated that RNA interference (RNAi) in mammalian cells can have off-target silencing effects and these off-target effects can cause measurable phenotypes (Lin, Ruan et al. 2005, Fedorov, Anderson et al. 2006). Furthermore, certain sequence motifs and dsRNA lengths can activate the interferon response causing non-specific effects and

complicating the interpretation of results (Bridge, Pebernard et al. 2003, Semizarov, Frost et al. 2003).

An interesting study demonstrated that expressing shRNAs at high levels inhibited miRNA activity, causing tissue damage and frequent lethality in mice. The overexpression of shRNAs saturated the cellular miRNA/short hairpin RNA pathways, disrupting the endogenous processing of miRNAs (Grimm, Streetz et al. 2006). Indeed, other studies have also demonstrated that RNAi off-target effects are concentration dependent (Jackson, Bartz et al. 2003, Semizarov, Frost et al. 2003).

In an effort to identify and overcome shRNA off-target effects, the gene of interest is often re-introduced into cells using cDNA lentiviruses to try to rescue the shRNA-driven phenotype. The cDNA of the gene of interest contains synonymous substitutions so that it is not targeted by the shRNA for knockdown. If shRNA-mediated effects can be rescued following the introduction of the gene of interest, this provides convincing evidence that the phenotype was not caused by off-target effects.

In this study, we introduced individual genes into *Cd74-Nid67* LSKs using cDNA lentiviral constructs to determine if the repopulation deficit could be rescued. The introduction of both *Rps14* and *Rbm22* seemed to significantly improve the total reconstitution (CD45.2) of *Cd74-Nid67* cells, supporting their involvement in *Cd74-Nid67* HSC dysfunction. However, the results were not conclusive since there did not seem to be a selection for the engraftment of transduced (GFP+) cells in the *Rps14* group. This may simply reflect the poor transduction efficiency of the *Rps14* cDNA lentivirus. On the other hand, the expression of *Rps14* may not have been sufficient to restore endogenous levels in *Cd74-Nid67* cells. The lack of a clear selection for the engraftment of GFP+ cells in the *Rps14* group may suggest that *Cd74-Nid67* HSC dysfunction was not, in fact, rescued. This may be due to insufficient expression of

Rps14. Further studies will examine *Rps14* expression in GFP+ reconstituted cells with the aim of addressing this issue.

The reliability of results may also be affected by the cytotoxicity of the viral supernatant, which can contain cellular debris from the packaging process. In this study, there may have been variability in the cytotoxicity of different shRNA viral preparations. There was a significant difference in the total reconstitution (CD45.2) of cells treated with different viral preparations of an identical Luciferase construct. This difference was not visible, however, if the reconstitution was solely measured by the engraftment of GFP+ cells. Transduced cells were not purified based on GFP expression before transplantation, as they are in many other studies. The non-transduced cells (transplanted along with GFP+ cells) served as an internal control and allowed us to identify that there may have been an issue with toxicity.

The use of shRNA lentiviruses presents a number of technical challenges. However, the inclusion of appropriate controls can help to overcome these issues. Indeed, previous studies have successfully used shRNA technology to identify genes that play important roles in haematopoietic development (Ebert, Pretz et al. 2008, Jaako, Flygare et al. 2011). The use of shRNA and cDNA lentiviral constructs in this study provided interesting preliminary data supporting the involvement of *Rps14* and *Rbm22* in *Cd74-Nid67* HSC dysfunction.

6.2 *Cd74-Nid67* Mouse Model

We have found that the *Cd74-Nid67* mouse model recapitulates key features of Del(5q) MDS. It was previously shown that *Cd74-Nid67* haploinsufficiency causes macrocytic anaemia and BM dysplasia (Barlow, Drynan et al. 2010). We have, for the first time, demonstrated that it also significantly impairs HSC function. Furthermore,

preliminary data from this study suggests that deficiency of *Rps14* and *Rbm22* are responsible for the aberrant activity of *Cd74-Nid67* HSCs. Finally, we discovered that a P53-dependent mechanism drives *Cd74-Nid67* HSC dysfunction. The use of the *Cd74-Nid67* model of disease has, therefore, provided novel insights into the aetiology of Del(5q) MDS.

6.2.1 Understanding *Cd74-Nid67* HSC Dysfunction

The complex Del(5q) HSC phenotype seems to be recapitulated in *Cd74-Nid67* mice. The significant increase in phenotypically defined HSCs in *Cd74-Nid67* mice is similar to the expansion of HSCs in Del(5q) MDS patients. This suggests that deficiency of *Cd74-Nid67* disrupts HSC self-renewal. Although Del(5q) HSCs have a growth advantage in the BM of patients, they perform extremely poorly in LTC-CFC assays and upon xenotransplantation (Nilsson, Astrand-Grundstrom et al. 2000, Woll, Kjallquist et al. 2014). The *Cd74-Nid67* HSC repopulation deficit mimics the dysfunctional activity of Del(5q) patient HSCs. The cause of Del(5q) HSC dysfunction, however, is not well understood.

6.2.1.1 Replicative Stress

The HSC phenotype seen in *Cd74-Nid67* mice (8-12 weeks old) is similar to that described in aged (22+ months old) *wild-type* mice (Flach, Bakker et al. 2014). Indeed, the numerical expansion but functional decline of HSCs is common to both. Furthermore, the expression of *Cdkn1a* is increased in aged HSCs, as in *Cd74-Nid67* HSCs. Interestingly, aged HSCs, in steady state conditions, have heightened levels of replication stress. They survive replication unless they are confronted with a strong replication challenge, such as transplantation (Flach, Bakker et al. 2014). A similar mechanism may explain the reduced capacity of *Cd74-Nid67* HSCs, and indeed

Del(5q) HSCs, to engraft upon transplantation. Moreover, the increased levels of replication stress in aged HSCs led to the transcriptional silencing of ribosomal genes and a decrease in ribosome biogenesis (Flach, Bakker et al. 2014). This further suggests that deficiency of the ribosomal protein, *Rps14*, and subsequent disruption to ribosome biogenesis may account for *Cd74-Nid67* HSC dysfunction.

It will be important to examine if the impaired reconstitution capacity of *Cd74-Nid67* HSCs reflects their failure to overcome severe replicative challenges. *Cd74-Nid67* HSCs will be examined for markers of replication stress, previously identified in aged HSCs (Flach, Bakker et al. 2014), such as cell cycle defects and chromosome gaps. The function of *Cd74-Nid67* HSCs will also be examined after treatment with a HSC mobilising agent, Fluorouracil (5-FU). This would place HSCs under severe replicative stress. If, indeed, excessive levels of replicative stress account for the functional decline of *Cd74-Nid67* HSCs upon transplantation, similar disruptions to haematopoiesis would be expected in this setting.

6.2.1.2 MDS Niche

The impaired repopulation potential of HSCs from both *Cd74-Nid67* mice and Del(5q) MDS patients may suggest that a specific environment or niche is required to support their growth. A recent study demonstrated that transplanting MDS HSCs along with MDS mesenchymal stromal cells significantly improved the levels of engraftment (Medyouf, Mossner et al. 2014). However, transplanting MDS cells along with stromal cells from healthy donors did not have any effect. This suggests that MDS HSCs may reprogram their environment, creating a niche that facilitates their abnormal development. Indeed, stromal cells from healthy donors were found to produce excessive quantities of growth factors when co-cultured with HSCs from MDS patients (Medyouf, Mossner et al. 2014).

It would be interesting to examine if *Cd74-Nid67* HSCs influence their environment to promote their survival. If this were the case, the engraftment of a minor population of *Cd74-Nid67* HSCs, initially, would be expected to increase over time, after reprogramming the niche. Future studies will investigate the reconstitution kinetics of *Cd74-Nid67* HSCs following transplantation.

6.2.1.3 Homing to the BM

The impaired engraftment of *Cd74-Nid67* and Del(5q) MDS HSCs upon transplantation may reflect their inability to home to the BM. Further experiments will be performed to address this possibility. *Cd74-Nid67* stem/progenitor cells will be labelled with a fluorescent dye and the number of fluorescently labelled cells in the BM will be evaluated shortly after transplantation, as previously described (Yusuf and Scadden 2009). However, homing is a complex process. HSCs must not only home to the BM, but also find the appropriate niche within the BM (Lo Celso and Scadden 2011) and there is currently no good assay to evaluate this.

6.2.1.4 Differentiation Block

Although *Cd74-Nid67* HSCs do not outcompete healthy competitor cells upon transplantation, they have a growth advantage relative to downstream *Cd74-Nid67* progenitors and mature cells. Almost no *Cd74-Nid67* progenitors or mature cells manage to engraft while a significant population of *Cd74-Nid67* HSCs reconstitute the BM long-term. The failure of *Cd74-Nid67* HSCs to produce any mature cells reveals a multi-lineage differentiation block. This suggests that the balance between HSC self-renewal and differentiation is skewed. The differentiation of multiple (myeloid, B and T cell) lineages also seems to be impaired in Del(5q) MDS patients (Nilsson, Astrand-Grundstrom et al. 2000). The neutropenia often seen in Del(5q)

MDS patients highlights an impairment in myeloid differentiation (Nimer 2008). Furthermore, the failure to detect the 5q deletion in mature B cells from Del(5q) MDS patients, despite its presence in almost all HSCs and a reasonable frequency of Pro-B cells suggests that Del(5q) is incompatible with lymphoid differentiation (Nilsson, Astrand-Grundstrom et al. 2000).

The findings from this study suggest that deficiency of *Cd74-Nid67* is sufficient to disrupt multi-lineage differentiation in conditions of stress (competitive transplantation).

6.3 Molecular Determinants of *Cd74-Nid67* HSC Dysfunction

We have found that haploinsufficiency of eight genes (out of over forty within the CDR) has a dramatic impact on HSC function. This suggests that deficiency of one or more genes within this region may contribute to the pathogenesis of Del(5q) MDS. Our study then aimed to pinpoint the gene responsible for the observed HSC phenotype. Two genes within *Cd74-Nid67* (*Myoz3* and *Synpo*) were eliminated as potential candidates since they were not expressed in purified HSCs from *wild-type* mice. The impact of deficiency of three (*Cd74*, *Rps14* and *Rbm22*) of the remaining six genes was examined. These genes were selected from the literature based on their propensity to cause a phenotype at haploinsufficiency, their involvement in cellular processes related to MDS or their role in haematopoietic development. Indeed, our preliminary analyses suggested that *Rps14* and *Rbm22* are important mediators of HSC function and supported their involvement in Del(5q) MDS pathogenesis.

6.3.1 *Rps14*

The results from both the shRNA and rescue experiments in this study suggest that *Rps14* deficiency may be responsible for the *Cd74-Nid67* HSC repopulation defect.

shRNA experiments revealed that reduced expression of *Rps14* in stem/progenitor cells caused a significant increase in the expression of *Cdkn1a*, a downstream mediator of P53. This is similar to the upregulation of *Cdkn1a* in *Cd74-Nid67* HSCs. Since P53 activity is controlled at the translational level, the expression of *Cdkn1a* at the transcriptional level serves as a marker of P53 activity. It seems, therefore, that *Rps14* deficiency leads to P53 activation. This is not surprising since shRNA knockdown of *RPS14* in human CD34+ cells *in vitro* resulted in a dramatic increase in P53 levels (Dutt, Narla et al. 2011). Furthermore, deficiency of another ribosomal protein, *Rps6*, in primary mouse liver cells and a human cell line led to the induction of P53 (Volarevic, Stewart et al. 2000, Fumagalli, Di Cara et al. 2009). Indeed, ribosome biogenesis is thought to be controlled by a P53 dependent surveillance mechanism (Narla and Ebert 2010).

In this study, loss of P53 was sufficient to completely correct the *Cd74-Nid67* HSC repopulation deficit, identifying it as a driver of HSC dysfunction. Interestingly, a number of studies have demonstrated that phenotypes induced by deficiency of ribosomal proteins can be rescued by the removal of P53 (Danilova, Sakamoto et al. 2008, Jones, Lynn et al. 2008, McGowan, Li et al. 2008, Dutt, Narla et al. 2011, Jaako, Flygare et al. 2011). Taken together, these data further implicate that deficiency of a ribosomal protein such as *Rps14*, which can activate P53, may account for the HSC phenotype in *Cd74-Nid67* mice.

The loss of *Rps6* in liver cells disrupted ribosome biogenesis, activating a ribosomal stress-MDM2 pathway. This, in turn, led to P53 activation and cell cycle arrest (Volarevic, Stewart et al. 2000, Fumagalli, Di Cara et al. 2009). A similar mechanism may mediate *Cd74-Nid67* HSC dysfunction. The accumulation of P53 in response to *Rps14* deficiency may lead to cell cycle arrest. The increased number of *Cd74-Nid67*

HSCs in G1 without a corresponding increase in S/G2/M indicates suggests that there may be a block in their progression through the cell cycle. Furthermore, the increased expression of *Cdkn1a* in *Cd74-Nid67* HSCs is compatible with a G1 phase cell cycle arrest. The removal of P53 may allow *Cd74-Nid67* HSCs to progress through the cell cycle when challenged to replicate, thereby "rescuing" the repopulation deficit.

The functional impairment of *Cd74-Nid67* HSCs upon transplantation is compatible with a phenotype caused by the deficiency of a ribosomal protein. Cells deficient in ribosomal proteins and, therefore, translational capacity are likely to perform poorly upon confrontation with a major replication challenge. In support of this, cell proliferation and regeneration of liver mass was completely abrogated in *Rps6* null mice following partial hepatectomy (Volarevic, Stewart et al. 2000). Moreover, *Rps6* haploinsufficiency led to induction of P53 and cell cycle arrest in stimulated but not resting T cells (Sulic, Panic et al. 2005). This suggests that the impact of ribosomal deficiency on cellular function may not be evident in steady-state conditions but becomes apparent when rapid proliferation is required.

The preliminary shRNA and rescue data presented in this study, together with findings from previous studies, strongly implicate *Rps14* as the gene responsible for *Cd74-Nid67* HSC dysfunction. Future studies will assess the impact of *Rps14* haploinsufficiency on HSC function using a conditional *Rps14* knockout mouse.

If, indeed, studies with knockout mice confirm that *Rps14* haploinsufficiency causes HSC dysfunction, further mechanistic studies will be performed. The impact of *Rps14* deficiency on ribosome biogenesis in primary haematopoietic cells will be explored. Indeed, a defect in the processing of pre-rRNA was identified upon shRNA knockdown of RPS14 in an erythroblast cell line (Ebert, Pretz et al. 2008). The rate of protein synthesis in HSCs from *Rps14* haploinsufficient mice will also be examined.

It has previously been shown that HSCs are particularly sensitive to changes in protein synthesis relative to other haematopoietic cells (Signer, Magee et al. 2014). Interestingly, the rate of protein synthesis was significantly reduced in HSCs from mice that have a hypomorphic mutation in the *Rpl24* ribosome protein (*Rpl24 Bst/*+). Moreover, the reduced protein synthesis affected the repopulation potential of *Rpl24 Bst/*+HSCs upon transplantation (Signer, Magee et al. 2014). Perhaps haploinsufficiency of *Rps14* disrupts protein synthesis and HSC function in a similar manner to the *Rpl24* mutation. It will also be critical to determine if P53 is activated in response to *Rps14* deficiency in HSCs and, if so, to examine the mechanism of activation.

6.3.2 *Rbm22*

The results from this study indicate that *Rbm22* haploinsufficiency may contribute to aberrant HSC activity in *Cd74-Nid67* mice. *Rbm22* was identified as a likely candidate based on its involvement in pre-mRNA splicing, a pathway of relevance to MDS pathogenesis (Yoshida, Sanada et al. 2011). Indeed, mutations in splicing genes such as *U2AF35*, *SRSF2* and *SF3B1* are classified as driver mutations due to their occurrence early in disease and their high prevalence in MDS patients (Papaemmanuil, Cazzola et al. 2011).

Interestingly, although common in patients with other subtypes of MDS, mutations in genes involved in pre-mRNA splicing are rare in Del(5q) MDS patients (Papaemmanuil, Gerstung et al. 2013). This suggests that a common mechanism, dysregulated pre-mRNA splicing, may lead to a similar outcome in patients with Del(5q) MDS and other subtypes of MDS. *Rbm22* deficiency alone may be sufficient to cause significant alterations in splicing. Mutations in other splicing genes would, therefore, not be required to disrupt this pathway.

In support of this, the impaired reconstitution capacity of *Cd74-Nid67* HSCs, although more dramatic, is similar to the impaired repopulation potential of cells from mice expressing mutant *U2af35* (Shirai, Ley et al. 2015). Interestingly, splicing defects were enriched in genes involved in RNA and ribosomal processing in stem/progenitor cells from *U2af35* mutant mice, further emphasising the importance of these pathways in MDS pathogenesis (Shirai, Ley et al. 2015). Moreover, studies from our lab revealed that pre-mRNA splicing was disrupted in HSCs from Del(5q) MDS patients and that genes involved in translation and RNA processing were particularly affected (Petter Woll, personal communication).

Since P53 is a critical mediator of *Cd74-Nid67* HSC dysfunction, it is interesting to note that disruptions to pre-mRNA splicing have also been linked to P53 activation. A recent study demonstrated that the splicing factor, SRSF1, can bind to an RPL5-MDM2 complex and stabilise P53 (Fregoso, Das et al. 2013). This study suggested that the ribosomal stress-MDM2-P53 signalling pathway might also serve to detect aberrations in pre-mRNA splicing. Deficiency of *Rbm22* may, therefore, disrupt pre-mRNA splicing and lead to the accumulation of P53, causing HSC dysfunction. Future studies will investigate if *Rbm22* haploinsufficiency leads to P53 activation.

6.3.3 Combination of Genes

The HSC phenotype observed in Del(5q) MDS patients and *Cd74-Nid67* mice could be the result of haploinsufficiency of multiple genes within the CDR, rather than the effect of haploinsufficiency of a single gene. Indeed, deficiency of three genes was required to confer a phenotype that allowed growth at high temperatures in yeast (Steinmetz, Sinha et al. 2002). Deficiency of any one gene, in isolation, was neither necessary nor sufficient to have an effect. However, previous studies have demonstrated that deficiency of individual genes on 5q can cause a phenotype (Ebert,

Pretz et al. 2008, Schneider, Adema et al. 2014). Furthermore, the shRNA and "rescue" experiments described in this study suggest that *Rps14* or *Rbm22*, in isolation, can have an effect on HSC activity. It would, however, be interesting to examine the impact of the combined deficiency of *Rps14* and *Rbm22* on HSC function. Their involvement in related cellular processes, namely pre-mRNA splicing and translation, raises the possibility that their effect on HSC function may be additive.

The relative contribution of a combination of genes could be investigated by transducing *Cd74-Nid67* HSCs with a pool of combined lentiviral cDNA constructs for all genes within *Cd74-Nid67*. The "rescued" *Cd74-Nid67* HSCs would engraft upon transplantation. The contribution of each gene to the "rescued" HSC pool could be determined by performing next-generation sequencing targeted towards the lentiviral backbone and the rescue constructs. A similar approach was previously used to identify a combination of six genes that could impart HSC repopulation potential to mature blood cells (Riddell, Gazit et al. 2014). However, the use of multiple lentiviral constructs presents significant technical challenges related to cytotoxicity and appropriate expression levels. These challenges must be overcome in order to use this approach successfully. Polycistronic lentiviral constructs have previously been used to reduce the cytotoxicity associated with multiple lentiviral supernatants and to ensure that multiple cDNAs are expressed within the same cell. (Riddell, Gazit et al. 2014).

6.3.4 Non-Protein Coding Elements

This study evaluated the protein coding genes located within *Cd74-Nid67* as potential candidates responsible for the HSC phenotype. However, deficiency of other entities such as miRNAs, lncRNAs or promoters and enhancers for genes outside *Cd74-Nid67*

could account for HSC dysfunction. Indeed, deficiency of miRNAs located on 5q has previously been shown to impair megakaryocyte development (Starczynowski, Kuchenbauer et al. 2010). There are, however, no miRNAs located within the targeted region in *Cd74-Nid67* mice, although miRNA- 5107 lies just downstream of *Cd74*.

It would be difficult to assess the impact of disrupting promoters or enhancers for genes outside *Cd74-Nid67* on HSC function. Preliminary analysis suggests that *Rps14* and *Rbm22* are the major contributors to the HSC phenotype seen in *Cd74-Nid67* mice. Future studies using conditional knockout mice for individual genes will aim to definitively examine their contribution to the dysfunctional activity of HSCs in *Cd74-Nid67* mice.

6.3.5 Other Genes within Del(5q) CDR

The results of this study demonstrate that the *Cd74-Nid67* region is important for normal HSC function in mice. However, this does not rule out the possibility that other genes that lie within the Del(5q) CDR (outside *Cd74-Nid67*) are involved in mediating HSC function. Barlow and colleagues generated four mouse models (*Sparc-Gpx3*, *Sparc-Nmu2r*, *Arsi-Csf1r* and *Cd74-Nid67*), each with a heterozygous deletion of a region corresponding to part of the CDR in Del(5q) MDS patients (Barlow, Drynan et al. 2010). The RBC counts and haemoglobin concentrations were only altered in *Cd74-Nid67* mice. The other targeted mice were, therefore, not examined in further detail.

Although deficiency of *Sparc-Gpx3*, *Sparc-Nmu2r* or *Arsi-Csf1r* did not cause a peripheral blood phenotype, their deficiency may have an effect on stem/progenitor cells in the BM. Future studies will assess the impact of haploinsufficiency of these regions on HSC function.

It is important to note that the targeted regions in the four mouse models (combined) span approximately twenty four out of ~forty genes described within the Del(5q) CDR (Barlow, Drynan et al. 2010). Indeed, genes that are not targeted in these mice may also contribute to the HSC dysfunction seen in Del(5q) MDS patients.

6.4 Future Studies

6.4.1 Molecular Comparison of HSCs from *Cd74-Nid67* Mice and Del(5q) MDS Patients

In this study, the molecular basis of HSC dysfunction was examined by subjecting *Cd74-Nid67* murine HSCs and Del(5q) MDS patient HSCs to global RNA sequencing and gene expression profiling. Preliminary gene expression analysis identified genes involved in cell cycle regulation, HSC function and cell growth that were dysregulated in both *Cd74-Nid67* HSCs and Del(5q) HSCs. Further in-depth analysis will be performed to identify additional dysregulated targets and pathways, which may provide valuable insights into the mechanisms underlying HSC dysfunction.

Future studies will also examine if splicing patterns are altered in HSCs from *Cd74-Nid67* mice and Del(5q) MDS patients. This is particularly relevant since deficiency of *Rbm22* may account for aberrant HSC function in *Cd74-Nid67* mice and Del(5q) MDS patients. RNA sequencing technology was recently used to identify splicing alterations in haematopoietic progenitors from *U2af35* (Shirai, Ley et al. 2015) and *Srsf2* (Kim, Ilagan et al. 2015) mutant mice. The RNA sequencing data from the mutant mice was integrated with data sets from MDS patients with the corresponding mutations to pinpoint common changes in splicing. Interestingly, *Ezh2*, a haematopoietic regulator, was mis-spliced in *Srsf2* mutant stem/progenitor cells,

which provided a mechanistic insight into the haematopoietic impairment (Kim, Ilagan et al. 2015).

6.4.2 Identification of Genes that Contribute to HSC Dysfunction in Isolated Del(5q) MDS Patients

Preliminary results from this study suggest that deficiency of *Rps14* and *Rbm22* account for the HSC dysfunction seen in *Cd74-Nid67* mice. If further experiments support their involvement, it will be important to directly relate these findings to Del(5q) MDS patients.

Future studies will investigate the impact of restoring the expression of *RPS14* and *RBM22* on the HSC phenotype in patients with isolated Del(5q) MDS. Whole exome sequencing of patient samples will be performed to identify Del(5q) MDS patients that lack additional driver mutations. The expression of *RPS14* and *RBM22* will be restored in Del(5q) stem/progenitor cells using cDNA lentiviruses. The activity of transduced Del(5q) HSCs will be examined using *in vivo* (xenotransplantation) and *in vitro* (LTC-CFC) assays to determine whether restoring the expression of *RPS14* and *RBM22* can correct Del(5q) HSC dysfunction.

6.5 Conclusions

In this study we used a mouse model of Del(5q) MDS, the *Cd74-Nid67* model, to investigate the initiating events that lead to disease development. We have demonstrated that haploinsufficiency of eight genes, *Cd74-Nid67*, out of over forty located within the Del(5q) CDR has a significant impact on HSC quiescence, self-renewal and repopulation potential. Furthermore, we identified two genes within this region, *Rps14* and *Rbm22*, as likely candidates that may contribute to *Cd74-Nid67* HSC dysfunction. Finally, we demonstrated that a P53-dependent mechanism

mediates *Cd74-Nid67* HSC dysfunction. Future studies will aim to delineate the mechanistic basis for *Cd74-Nid67* HSC dysfunction, which will provide important insights into the aetiology of Del(5q) MDS and may promote the development of improved therapeutic avenues for patients.

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