Characterisation and Targeting of Stem Cells in Myelodysplastic Syndromes

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Abstract

Understanding which cells within a cancer are responsible for its initiation and propagation is vital if we are to achieve cure. If cancer stem cells are the only population able to sustain a tumour long term, designing therapeutic strategies to target this population will give medical science the best chance of long-term cure. Significant controversy remains over the existence of cancer stem cells, predominantly due to the lack of a sensitive human cancer stem cell assay. This thesis investigates whether two haematological malignancies, myelodysplastic syndromes (MDS) and chronic myelomonocytic leukaemia (CMML) can only be driven by rare and distinct cancer stem cells. We have demonstrated that low and intermediate-1 risk MDS is driven solely by the stem cell (Lin- CD34+ CD38- CD90+ CD45RA-) by developing a novel genetic approach, tracing all somatic mutations and karyotypic abnormalities back to this population.

Prior to this study, very little was known about the clonal architecture of CMML. By performing detailed phenotypic, functional, molecular and genetic analysis of patients with CMML, we were able to demonstrate that the most likely candidate driver cell in these patients was also the stem cell rather than any of the down-stream progenitors.

Currently, effective therapeutic strategies for MDS or CMML are very limited. Allogeneic stem cell transplantation is the only potential cure and not suitable for most patients. Cancer stem cells, including MDS stem cells are known to be highly quiescent and selectively resistant to therapy. Having demonstrated that both MDS and CMML were driven by stem cells, we developed a novel therapeutic targeting strategy. Using the thrombopoietin receptor agonist, Romiplostim, we were able to activate stem cells and enhance their subsequent sensitivity to chemotherapy dramatically. This approach may facilitate improved remission rates and prevent cancer stem cell driven relapse in many diseases.

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Abbreviations

HSC	Haematopoietic stem cell
CFU	Colony forming unit
Lin	Lineage
Sca1	Stem cell antigen 1
LSK	Lineage- Sca1+ cKit+
SLAM	Signalling lymphocyte activation molecule
MPP	Multi-potent progenitor
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
LMPP	Lymphoid primed multi-potent progenitor
MLP	Multi-lymphoid progenitor
GMP	Granulocyte macrophage progenitor
MEP	Megakaryocyte erythroid progenitor
CD	Cluster differentiation
LTC-CFC	Long-term culture colony forming cells
IL-3	Interleukin-3
SCF	Stem cell factor
GM-CSF	Granulocyte macrophage –colony stimulating factor
G-CSF	Granulocyte- colony stimulating factor
TPO	Thrombopoietin
MPL	Myeloproliferative leukaemia virus
NOD/SCID	Non-obese diabetic/Severe combined immune-deficient
NSG	NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ
MDS	Myelodysplastic syndrome
AML	Acute myeloid leukaemia
CMML	Chronic myelomonocytic leukaemia
CML	Chronic myeloid leukaemia

MPD	Myeloproliferative disorder
CSC	Cancer stem cell
BrdU	5-Bromo -2- deoxyuridine
MNC	Mononuclear cells
BM	Bone marrow
PB	Peripheral blood
FACS	Fluorescence activated cell sorting
FMO	Fluorescence minus one
DAPI	4', 6-diamidine-2-phenylidole dihydrochloride
7AAD	7 aminoactinomycin D
FISH	Fluorescence *in situ* hybridisation

1. Introduction

1.1 Normal haematopoiesis

The maintenance and production of blood cells is a carefully orchestrated process. It is imperative that a system exists whereby mature blood cells are replenished regularly and rapidly, as these cells are short-lived but essential for life. It is now over 100 years since it was first proposed that blood cell development may rely on the hierarchical production of blood cells. After examining peripheral blood by light microscopy, a Russian scientist, A Maximow, first suggested that such a wide variety of blood cells was likely to be the result of hierarchical development originating from a stem cell[1]. A heightened interest in understanding haematopoietic development was sparked following the consequences of radiation exposure seen in the Second World War, which resulted in life-threatening bone marrow failure. It later became evident that the inability to produce adequate numbers of blood cells could be rescued by infusing bone marrow from healthy donors[2]. Although this demonstrated that the blood system could be repopulated, it did not enable a distinction to be made regarding whether multiple cell types were required to differentiate into the different lineages, or whether just one type of cell, such as a haematopoietic stem cell, was capable of differentiating into all other cells. Haematopoietic stem cells, by definition, must be able to self-renew whilst also being able to give rise to all the cells of the blood system. Progenitors lie downstream in the hierarchy and gradually lose the ability to self-renew whilst becoming increasingly lineage committed, until they eventually differentiate into specific mature blood cells. Seminal studies of both mouse and human haematopoiesis have provided invaluable insights in to the workings of the blood system.

1.2 Murine Haematopoiesis

Although the existence of a hierarchy between stem and progenitor cells had been hypothesised, it was in 1961 that Till and McCulloch published their pivotal work demonstrating the presence of repopulating cells isolated from mouse bone marrow[3]. Following injection of bulk bone marrow cells into irradiated mice, proliferating colonies (CFU-S), comprised of different lineages, formed in the spleen. This was the first demonstration of transplantability and multipotency of bone marrow cells. However this did not definitively show the presence of a tissue specific haematopoietic stem cell, as long-term repopulation was not demonstrated. Whilst it later became apparent that the CFU-S were derived from downstream progenitors not stem cells[4], this remained an important demonstration of the utility of studying mouse haematopoiesis to understand human biology.

1.2.1 Phenotypic characterisation

These illuminating initial experiments lead on to a more detailed understanding of the structure of mouse haematopoiesis. Fluorescence activated cell sorting (FACS) on the basis of cell surface markers has hugely advanced the ability to purify haematopoietic cell populations (see Chapter 2 Methods). The first study to use flow cytometry to delineate clearly a bone marrow population enriched for stem cells in the mouse, defined the candidate cells as being negative for mature blood cell lineage markers (Lin-), positive for Stem cell antigen 1 (Sca1+) with low level expression of Thy1.1 (Thy1.1 lo)[5]. Using these markers the authors showed that this phenotypically defined population was able to reconstitute lethally irradiated mice, proliferate and also differentiate into myeloid and lymphoid cells. Further purification of repopulating cells was established a few years later with the addition of the stem cell factor receptor (cKit)[6]. However one problem with using

the Lin- Thy1.1 lo Sca1+ cKit+ phenotype to enrich for stem cells was that C57BL/6 mice, the most commonly used mouse strain for *in vivo* transplantation, did not express Thy 1.1. Further studies in C57BL/6 mice established that using Lin- Sca1+ cKit+ (LSK) alone did enrich for stem cells. It has since become increasingly clear that the LSK population is very heterogeneous and comprised of haematopoietic stem and downstream progenitor cells. Further prospective purification of true stem cells has since been achieved using the additional markers, CD34[7] and Flt3[8,9]. The SLAM markers, which define HSCs as being CD150+ (SLAMf1+) CD48- (SLAMf2-) are an alternative set of markers which distinguish stem and progenitor cells[10]. Even more recently, the CD150+ CD48- HSCs have been further sub-fractionated using the additional SLAM markers, CD229 and CD244, into HSC1 (LSK CD150+ CD48- CD229- CD244-) and HSC2 (LSK CD150+ CD48- CD229+ CD244-)[11]. HSC1 cells were found to have longer term self-renewal potential when compared to HSC2 cells.

In addition to using cell surface markers to strictly define HSCs, they have also been used to define possible models of the downstream haematopoietic progenitor hierarchy. The 'classical model' proposes that below the HSC lies a multi-potent progenitor (MPP), which principally differs from the stem cell due to a loss of self-renewal capacity but is still able to give rise to multiple lineages (**Figure 1.1**). The classical model predicted that the first lineage commitment of MPPs was a strict bifurcation between lymphoid and myeloid development with the common lymphoid progenitor (CLP)[12] and common myeloid progenitor (CMP)[13]. However, the revised 'alternative' model established that early lymphoid progenitors retain myelomonocytic capacity but lose megakaryocyte erythroid forming ability (**Figure 1.2**). These are known as lymphoid primed multi-potent progenitors (LMPPs)[14].

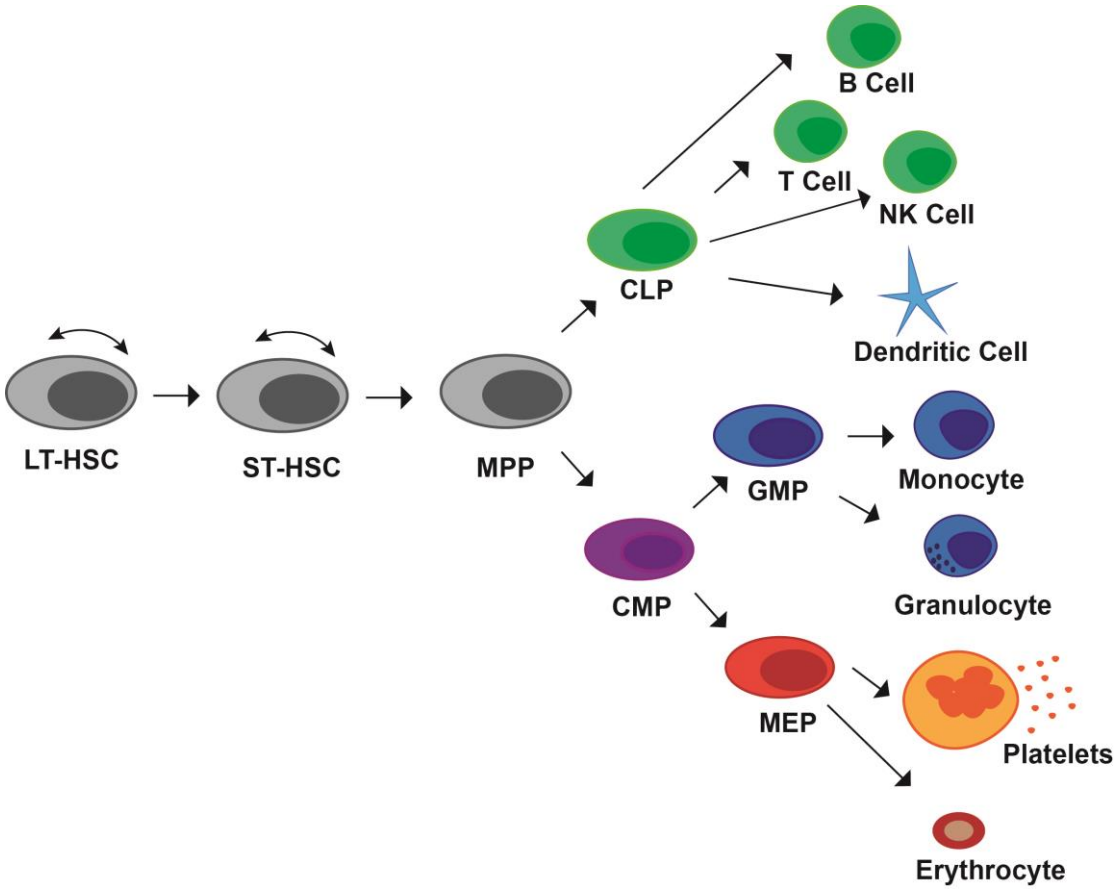


Figure 1.1 Classical model of mouse haematopoietic hierarchy

In this model LT-HSCs (long term haematopoietic stem cells) and ST-HSCs (short term haematopoietic stem cells) are the only populations with prolonged engraftment ability. HSCs give rise to MPPs (multi-potent progenitors) which are multi-potent but have lost significant self-renewal capacity. There is then a strict bifurcation between lymphoid and myeloid commitment with the CLP (common lymphoid progenitor) giving rise to all lymphoid lineages and the CMP (common myeloid progenitor) giving rise to myeloid lineages. Curved arrows indicate self-renewal potential. GMP Granulocyte macrophage progenitor. MEP Megakaryocyte erythroid progenitor. NK Natural Killer.

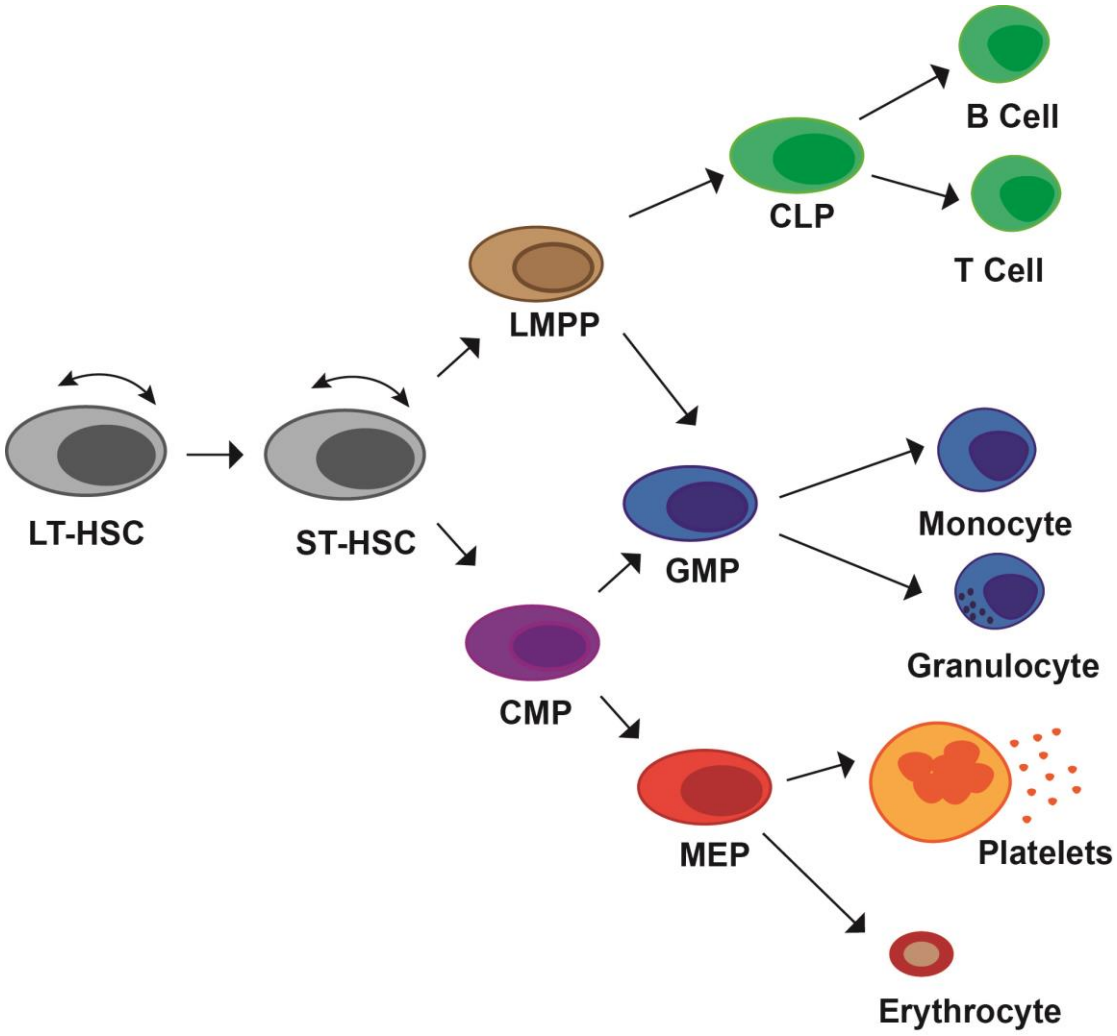


Figure 1.2 Alternative model of mouse haematopoietic hierarchy

In this model there is no strict bifurcation between myeloid and lymphoid commitment after the HSC/MPP stage of development. The primitive LMPP (lymphoid primed multi-potent progenitor) is able to give rise to lymphoid lineages, granulocytes and monocytes but has lost megakaryocytic erythroid potential. The CMP is able to generate all myeloid cells. Curved arrows indicate self-renewal potential.

Although FACS purification of stem and progenitor cells has been extremely important in aiding our understanding of the haematopoietic hierarchy, all such markers require substantial functional validation. Only in combination with *in vivo* transplantation and clonal *in vitro* assays, have these developments in phenotypic purification advanced the field.

1.2.2 *In vivo* transplantation assays

As the key features of an HSC are self-renewal and the ability to produce all the cells which constitute the haematopoietic system, the gold standard for determining functional stem cell activity of mouse HSCs is the long-term repopulating assay. Perhaps the most frequently used assay for measuring stem cell potential, is the competitive transplantation assay[15]. In this assay bone marrow cells from a donor mouse are injected into a lethally irradiated recipient mouse along with a known number of competitor mouse bone marrow cells. Genetic markers are used to distinguish progeny for the two different sources of HSCs. The most commonly used genetic markers are the CD45.1/CD45.2 system. CD45 has two functionally identical alleles in mice, which enables identification of genetically distinct donor and competitor cells by allele specific antibodies. The repopulating ability of the donor cells is used as a surrogate for stem cell activity, both short and long term. Long-term repopulation is considered to be engraftment of donor cells for a minimum of 16 weeks[9]. Peripheral blood from the recipient mouse can be analysed using flow cytometry to establish the percentage contribution of the donor cells to the recipient's multi-lineage haematopoiesis. In order to establish self-renewal, serial transplantations are often performed. However the success of this approach is dependent on the donor stem cells having adequate homing and engrafting ability.

1.2.3 *In vitro* colony forming cell assays

Colony forming cell (CFC) assays determine short-term progenitor cell activity after plating cells in a semi-solid media, such as methylcellulose supplemented with cytokines. These assays provide information regarding a given cell population's ability to produce a variety of short-term myeloid colonies (erythroid, megakaryocytic, granulocytic and myelomonocytic) but are not ideal for assessing lymphoid potential, where alternative co-culture systems are better. They do not estimate stem cell potential.

Long-term *in vitro* colony forming cell assays have also been developed, which are particularly useful when haematopoietic defects in a test population result in poor homing or engraftment in a murine transplantation system. The first of these assays was the cobblestone area forming cell assay. This was followed by the development of the long-term culture-initiating cell (LTC-IC) assay also known as the long-term culture-colony forming cell (LTC-CFC) assay[16]. In this assay, haematopoietic cells are co-cultured with irradiated murine stromal cells for 5-6 weeks prior to transferring to methylcellulose, and scored 14 days later for the presence of myeloid and lymphoid colonies. During the 5-6 weeks co-culture, only cells with significant self-renewal will sustain ability to form colonies when transferred to a CFC assay. Clearly this is limited by the fact that it is an *in vitro* measure of self-renewal. The final readout is also usually performed after only 5-6 weeks of cell culture, suggesting enrichment of more primitive cells than are identified in standard CFC assays but not necessarily true stem cells.

1.3 Human Haematopoiesis

Mouse studies form a key part in understanding human haematopoiesis. However, understanding normal human haematopoiesis is also vital, in order to gain fundamental insights into the aberrations which ensue at malignant transformation. There are clear similarities between mouse and human haematopoietic roadmaps but the stem and progenitor hierarchy is much more poorly defined in the latter, primarily due to a historical lack of sensitive functional assays.

1.3.1 Phenotypic characterisation

Mouse and human stem and progenitor cell surface markers have significant differences. The first cell surface marker demonstrated to enrich for a human immature progenitor population was CD34[17]. This was found to be present on only 1-2% of normal bone marrow cells, which morphologically appeared to be primitive cells. In this initial study, over 95% of the CFC-GM (colony forming cell-granulocyte macrophage) and BFU-E (burst forming unit-erythroid) activity resided in the CD34+ population[17]. However no long-term repopulating assays were performed. As the field has progressed, the phenotype has become increasingly refined with the markers CD34+CD38-CD90+ enriching for multi-potent human stem cells[18,19]. However it is also worth noting that stem cell potential has been demonstrated in the CD34- compartment[20,21]. These studies have suggested that such cells may in fact be more primitive than CD34+ cells.

1.3.2 *In vitro* assays

The most well established *in vitro* assay for determining human stem cell potential is the LTC-CFC[22]. As is the case with the murine version, it involves the co-culture of human bone marrow cells on murine stromal cells for 5-6 week followed by transfer to methylcellulose and the counting of myeloid progenitor colonies. This is limited not only by the fact that it is an *in vitro* assay but also because it is only able to determine myeloid potential and not multi-lineage differentiation. It does not routinely enable estimation of self-renewal capacity much beyond 6 weeks of culture, although extended protocols are available. However it is particularly valuable in settings where xenotransplantation is not successful.

1.3.3 Xenotransplantation

The development of humanised mouse models over 20 years ago has provided a valuable tool for assessing stem cell potential. Although the exact models and the success rates have varied, the principle rests on the ability to transplant human cells into immunocompromised mice, enabling the establishment of a human graft, without rejection. Three slightly different approaches were used initially in the 1980s. One group injected peripheral blood leucocytes into severe combined immune deficient (SCID) mice, which resulted in lymphoid reconstitution as evidenced by the presence of circulating donor B and T cells[23]. McCune et *al* surgically engrafted human fetal liver into SCID mice but again no significant myelopoiesis was evident[24]. The third group used a different approach, sub-lethally irradiating SCID mice prior to intravenous injection of bone marrow cells in combination with the human cytokines IL-3, GM-CSF and SCF[25]. Using this model, both lymphoid and myeloid engraftment were evident for the first time. To generate improved reconstitution ability, the SCID mouse was crossed with the Non-Obese Diabetic (NOD) mouse which resulted in higher levels of engraftment[26]. However the NOD/SCID mice developed thymic lymphomas which limited

the length of time experiments could continue. NK cells also persisted in this model, inhibiting human engraftment[26]. This latter limitation has been overcome by the generation of the NOG (NOD/Shi-scid/IL-2Rγnull) and NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice, which have a truncated or deleted IL-2 receptor common gamma chain, respectively. This results in a complete absence of mouse B, T and NK cells, enabling a 5-fold improvement in human engraftment[27]. Importantly, due to aberrant cytokine signalling, these mice did not develop lymphomas and thus long-term experiments are possible. In order to improve human engraftment capacity further, attempts have also been made to engineer knock-in mice which express human cytokines such as thrombopoietin (TPO), IL-3 and GM-CSF[28].

1.3.4 Autologous Stem Cell Transplantation

Patients with a variety of malignancies have provided important functional evidence for the ability of certain cell surface markers to potentially enrich for stem cells. Successful autologous transplantation of CD34+ CD90+ peripheral blood mobilised stem cells in breast cancer patients resulted in long-term multi-lineage engraftment[29]. Similar studies have since been performed in patients with multiple myeloma and non-Hodgkin's lymphoma[30,31]. However this does not definitively demonstrate stem cell ability as transplanted cells are not genetically marked and cannot be distinguished from endogenous reconstitution.

Together, both mouse and human studies support the concept that the majority of human stem cells lie within the CD34+ CD38- CD90+ compartment.

1.3.5 Haematopoietic Hierarchy

The structure of normal human haematopoiesis has not been as clearly elucidated as mouse haematopoiesis. However substantial evidence does now support a similar hierarchy of stem and progenitor cells.

Majeti *et al* characterised human stem and multi-potent progenitor cells, using the phenotypic markers CD34, CD38, CD90 and CD45RA[32]. *In vitro* and *in vivo* functional assays were used to determine a hierarchy which concluded that CD34+CD38-CD90+CD45RA- HSCs, resided at the apex (**Figure 1.3**). These cells were capable of self-renewal and multi-potent differentiation. The same set of phenotypic markers could also be used to define downstream multi-potent progenitors (MPPs, CD34+CD38-CD90-CD45RA-) which were capable of far more limited self-renewal but still retained the capacity to generate lymphoid and myeloid cells. Importantly this population did not give rise to HSCs, demonstrating that MPPs are downstream of HSCs in the hematopoietic hierarchy. CD34+CD38-CD90-CD45RA+ cells were not capable of self-renewal but could generate limited numbers of myeloid colonies *in vitro*. Further interrogation of this population, later named the multi-lymphoid progenitor (MLP), revealed that single cells were able to generate lymphoid colonies but myelomonocytic cells were present too[33]. Subsequently a similar population was identified by a different group and termed equivalent to the mouse LMPP[34].

Downstream lineage restricted myeloid progenitors have also been prospectively isolated from the CD34+CD38+ population, by sub-fractionation based on IL3-R (CD123) and CD45RA cell surface expression. Colony forming assays and gene expression analysis have been used to discern lineage restricted populations: CMP (CD34+CD38+CD123+CD45RA-) GMP (CD34+CD38+CD123+CD45RA+) and MEP (CD34+CD38+CD123-CD45RA-)[35]. These

populations have been found to have very limited LTC-CFC activity and are not able to differentiate *in vitro* in to lymphoid cells.

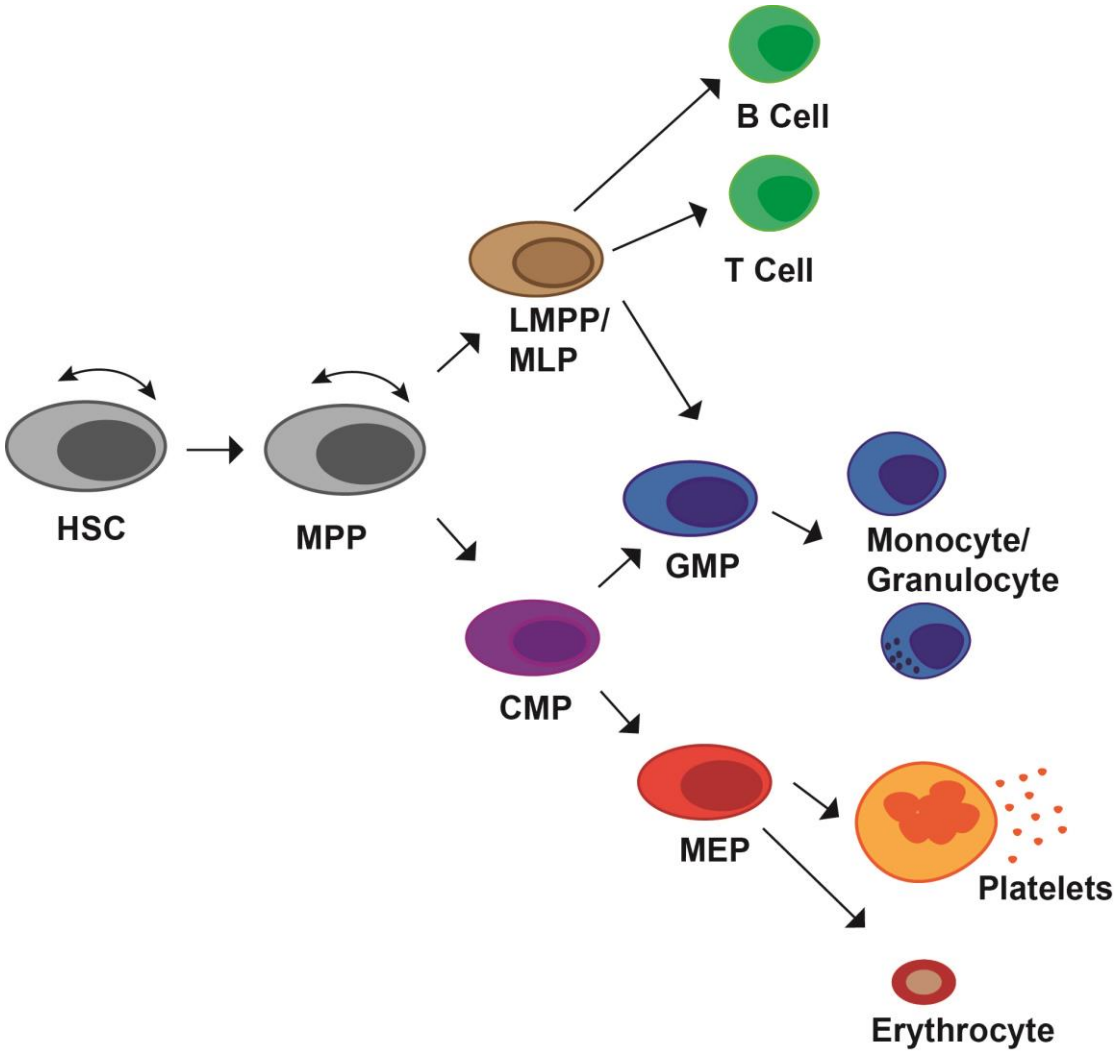


Figure 1.3 Model of human haematopoietic hierarchy

Human haematopoiesis is thought to develop in a manner similar to the mouse alternative model. However lineage priming and commitment steps are far less well established. Heterogeneity with regards to long- and short-term repopulating ability of HSCs is unknown. Human lymphoid development is also far less well understood in comparison to the mouse. MLP multi-lymphoid progenitor. Curved lines indicate self-renewal.

1.4 Malignant Haematopoiesis

It has been clearly demonstrated that normal human haematopoiesis is hierarchically arranged. However there is still significant controversy regarding whether malignant haematopoiesis remains hierarchically arranged, or whether this process is disrupted. Understanding whether all cells, or only rare and distinct cells, are capable of replenishing a cancer is vital not only for understanding the mechanisms of disease but also to enable the design of effective targeted therapies.

1.4.1 Are cancers sustained in a stochastic or hierarchical manner?

Two principle models of cancer propagation have been put forward for many years[36-39]. The stochastic model proposes that all cells within a tumour are biologically equivalent and have equal potential to propagate the tumour. Random intrinsic and extrinsic insults on a particular cell will determine whether it acquires the ability to drive the malignancy, resulting in heterogeneous tumours. Importantly in such a model it would not be possible to prospectively isolate the driver cells as they have no consistent distinguishing markers[36]. Alternatively, the hierarchical model proposes that only a distinct sub-population of cells are able to self-renew and drive the malignancy. In order to fulfil the definition of a 'cancer stem cell', these cells must have unique self-renewal potential, and the ability to give rise to all non-stem cell types in the malignant tissue[36] (**Figure 1.4**). Cells which are able to propagate cancers when transplanted into immune compromised mice are characteristically referred to as 'cancer propagating cells' rather than cancer stem cells. It is important to note that different cancers and even different patients with similar cancers may follow different models. Seminal studies in leukaemia and solid organ malignancies over the past 15 years have suggested that AML, MDS, breast, gastrointestinal and certain brain cancers are

hierarchically arranged and driven by rare distinct cancer stem cells[40-46]. However detailed studies in melanoma have not found a population of distinct cancer stem cells but instead demonstrated that 1 in 4 unselected single melanoma cells are able to reconstitute the tumour in mice[47,48]. This suggests that in the case of melanoma, cells which can drive the tumour are not rare or phenotypically distinct. Further studies also determined that reconstituting melanoma cells were not hierarchically arranged. This is in sharp contrast to the preceding studies on other malignancies and suggests that patterns of development are likely to vary between tumour types.

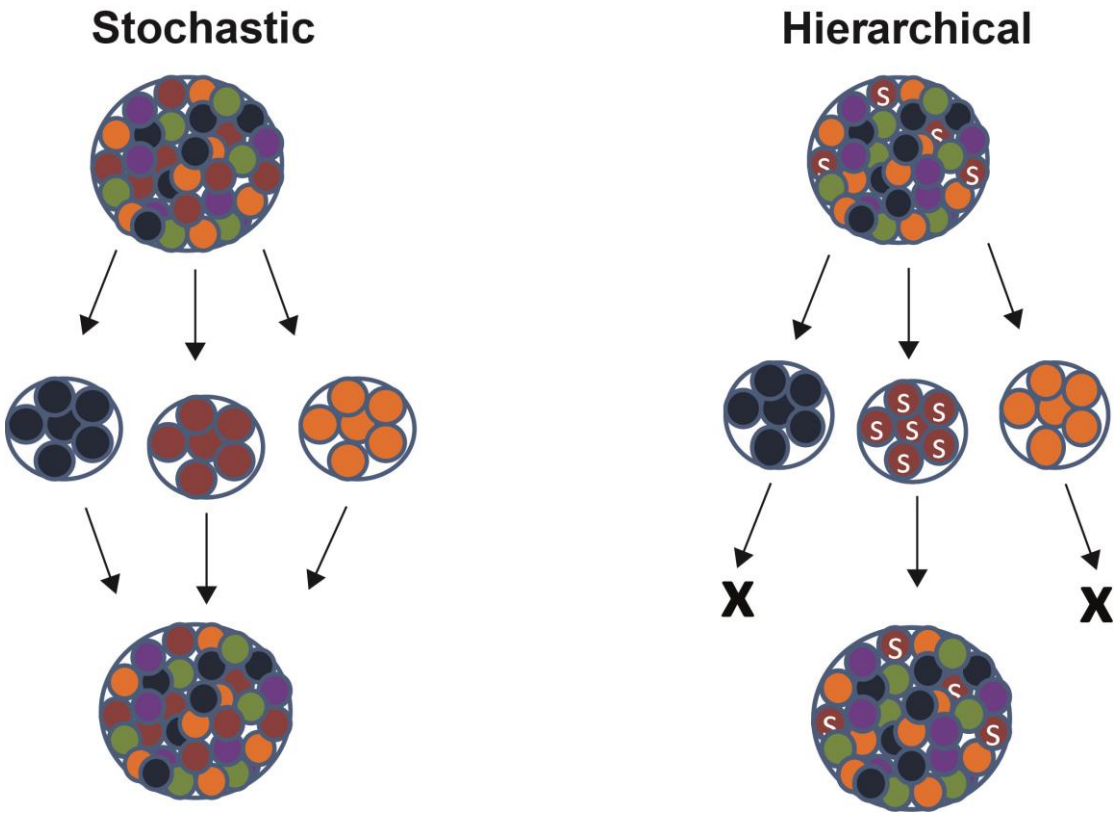


Figure 1.4 Stochastic and hierarchical models of cancer development

The stochastic model (left panel) proposes that a wide variety of cell types which comprise the cancer, are able to self-renew and propagate the cancer with equipotent ability. In contrast, the hierarchical model (right panel) is defined by rare and distinct cancer stem cells (S) which reside at the top of the hierarchy. These are the only cells with self-renewal

capacity and therefore the ability to replenish the tumour. Coloured circles represent different populations of cells. Red cells labelled 'S' represent cancer stem cells. X represents the inability of the blue and orange cells to propagate the tumour (right panel).

1.4.2 Cell of Origin

It is important to note that the term cancer stem cell (CSC) does not inherently imply that a CSC must have originated from a normal tissue-specific stem cell. It does not delineate the cell of origin. Cancer stem cells can originate from normal stem cells or alternatively from committed progenitors which acquire aberrant self-renewal ability. There is a need to clarify the relationship between malignant and normal stem and progenitor cells. Normal haematopoietic stem cells are CD34+CD38-CD90+CD45RA-[32]. The pioneering work of John Dick and colleagues initially demonstrated that when bone marrow cells from AML patients were sorted using CD34 and CD38, only the CD34+CD38- bone marrow cells were able to reconstitute NOD/SCID mice, supporting the idea that they might arise from normal HSCs[40,41]. However this was later challenged, as it became apparent that anti-CD38 antibodies could have a significant impact on preventing the engraftment of CD38+ cells[49]. Further studies have since definitively demonstrated that there is significant leukaemia initiating capacity within the CD34+CD38+ population, which serves to highlight the difficulties in relying on particular phenotypic markers or functional assays. Goardon *et al* demonstrated that in a sub-group of patients with CD34+ AML, there resided a GMP-like leukaemia initiating cell which was CD34+CD38+CD45RA+. Along with substantial molecular and functional data, this supported the idea that this down-stream progenitor population was likely to be required to sustain the leukaemia[34]. Interestingly a GMP-like leukaemia initiating cell had previously also been proposed in blast crisis CML[50].

A recent study demonstrated the existence of pre-leukaemic stem cells in AML[51]. Pre-leukemic stem cells were defined as HSCs containing some but not all the mutations found in the bulk AML cells. These pre-leukemic stem cells, like normal stem cells, were able to self-renew and differentiate in to all myeloid and lymphoid cells. The authors propose that additional genetic changes then occur within the expanding population of pre leukaemic stem cells resulting in transformation to a true leukaemic stem cell. This was a particularly important discovery, emphasising that early stages of AML are likely to originate within the HSC compartment.

1.4.3 Do all malignancies follow the cancer stem cell model?

Prospective isolation of cancer stem cells has proven to be much harder in solid organ malignancies, due to the enormous intra- and inter-tumour heterogeneity. Studies in breast cancer demonstrated that CD44+CD24low cells were the only cell population able to reconstitute NOD/SCID mice serially and also give rise to non-tumourigenic progeny[42]. However numerous attempts to demonstrate that melanoma is hierarchically organised have failed. Although CD133 was thought to enrich for cancer stem cells, it became apparent that both CD133+ and CD133- melanoma cells were able to reconstitute immunocompromised mice and give rise to both CD133+ and CD133- cells, failing to demonstrate a hierarchy. When NSG mice were used instead of NOD/SCID mice, 1 in 4 unselected single melanoma cells engrafted, demonstrating that melanoma reconstituting ability was not limited to a rare population of cells at all and did not appear to be hierarchically arranged[47]. This suggested that modifications to xenotransplantation may reveal tumourigenic capacity in a larger number of cells than previously established. This leads on to a number of caveats in the cancer stem cell model which have largely not been addressed due to the lack of alternatives to xenotransplantation.

1.4.4 Are cancer stem cells always rare?

Although the seminal work by John Dick and colleagues reported that only a rare population of cells were able to propagate AML, importantly the definition of a cancer stem cell does not *require* that all proposed cancer stem cells are rare. In addition to studies performed using melanoma cells, work by Andreas Strasser has demonstrated that a large proportion (>10%) of primary pre B and B lymphoma cells derived from transgenic mice are able to propagate disease in non-irradiated histocompatible recipients[52]. Low frequencies of cancer propagating cells in xenotransplantation may reflect the inability of certain human cancer cells to engraft in mice. A tumour may still conform to the cancer stem cell model even if putative cancer stem cells are present at a high frequency[37].

1.4.5 Limitations of xenotransplantation

One of the major limitations of human stem cell studies has been the reliance on xenotransplantation. As this assay is dependent on taking a cell out of its natural environment, it may not equate to cancer stem cell activity in a patient. The transplantation procedure itself also disrupts steady state haematopoiesis. There is no direct evidence to suggest that a candidate human cancer stem cell in the mouse would behave hierarchically in the same manner in the patient. Definitive evidence of distinct cell types acting as cancer stem cells, within a tumour *in situ* in mouse models of cancer, was only recently established through genetic lineage tracing experiments[53-55]. In addition, although particular growth factors and adhesion molecules may be species cross-reactive, many are not, which may also limit engraftment. Life span and size difference between man and mouse may also impact on the ability to use the mouse model as an accurate model of human tumourigenesis.

Demonstrating the existence of human cancer stem cells has proven even more difficult due to the lack of sensitivity of xenotransplantation. The fact that only around 50% of samples

from an aggressive malignancy such as AML engraft at all despite clearly sustaining the tumour in the patient, demonstrates that this is not a robust model[56]. It is essential that assays are developed which can investigate the cancer stem cell ability of candidate cells within the patient. The ideal proof of a human cancer stem cell would be to demonstrate its presence in patients thus removing the limitations of existing *in vitro* and *in vivo* mouse data. If the cancer stem cell theory is correct then eradication of this driving population is the only way to prevent relapse and cure disease.

1.5 Cancer stem cell resistance and therapeutic targeting

Over the years, the mainstay of chemotherapy has been designed to kill rapidly proliferating bulk cancer cells, treating all cancer cells as a homogeneous target. Over the past few years, drug development has been moved towards the molecular and genetic drivers of disease but again assumes all cells will respond in a similar manner. However if cancer cells are not all biologically equivalent, perhaps we need to think more carefully about eliminating the cells which are driving the disease rather than the bulk tumour. Normal stem cells have a number of features which may render them more resistant to drug targeting compared to more mature cells. Features such as high expression of drug efflux transporters, anti-apoptotic proteins and resistance to DNA damage, have been proposed as putative mechanisms of cancer stem cell resistance[57].

1.5.1 Cancer stem cell quiescence

Cancer stem cell quiescence has been proposed as a major cause of drug resistance, as the majority of cytotoxic drugs target proliferating cells[58]. It is well established that the majority of normal HSCs are quiescent, with the vast majority residing in the G_0 phase of cell cycle,

unlike progenitors which are more actively cycling[59]. This feature enables normal stem cells to survive life long and provide a reservoir of potent but well protected HSCs for rapid reconstitution of peripheral blood cells when needed. The fact that certain malignancies such as breast cancer can relapse many years after the initial remission, suggests that dormancy is likely to be a significant clinical problem with malignant stem cells and not just their normal counterparts[60].

Resistance of normal HSCs to chemotherapy has been demonstrated by examining the impact of the anti-proliferative chemotherapy Fluorouracil (5-FU) on mouse HSCs. In an initial experiment a single dose of 5-FU was delivered and resulted in elimination of bone marrow progenitor cells but the dormant HSC pool remained intact. In response to the loss of progenitors, a positive feedback loop ensued, triggering the remaining HSCs to cycle actively in order to replenish the bone marrow. When a second dose of 5FU was then administered, the residual cycling HSCs were eliminated[61]. These seminal experiments raised the exciting potential for the use of drugs which are able to cycle stem cells, as agents to activate dormant leukemic stem cells thus rendering them more susceptible to subsequent chemotherapy[62,63]. This may enable previously resistant leukaemic stem cells to be eliminated by combination treatment with growth factors followed by chemotherapy (**Figure 1.5**).

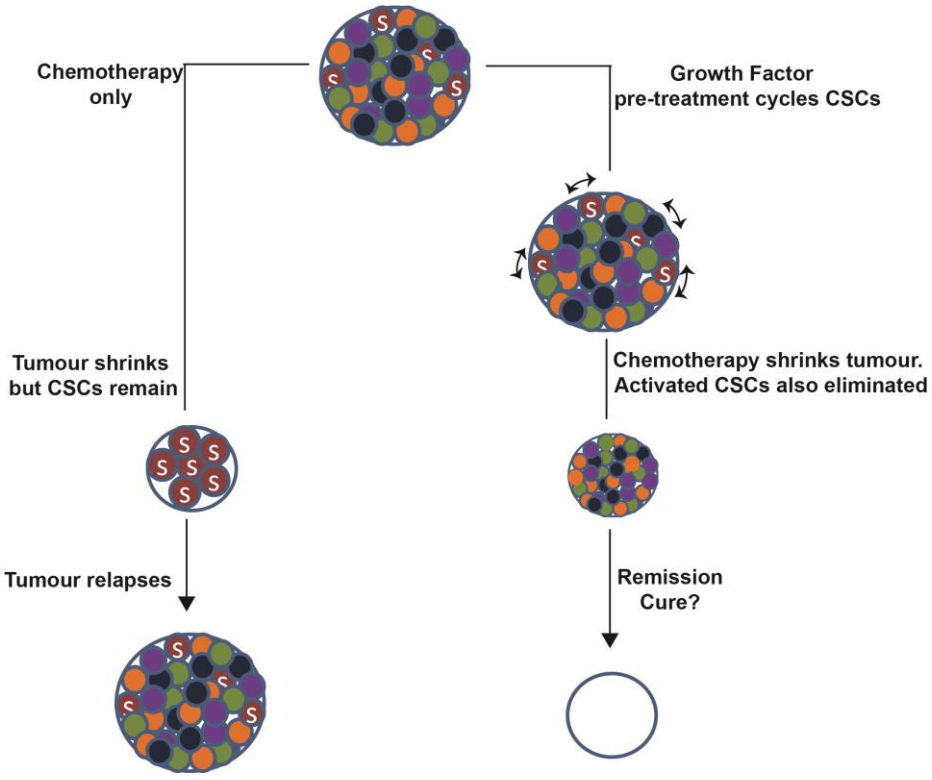


Figure 1.5 Targeting of malignant stem cells with growth factors

In this model, standard chemotherapy cannot target quiescent stem cells (left). Following chemotherapy alone, the bulk tumour shrinks but the cancer stem cells (S) remain intact and repopulate the tumour. If stem cell specific growth factors are given prior to chemotherapy, enhanced elimination of the now activated stem cells occurs, resulting in the potential for long-term cure (right). Coloured circles represent bulk tumour cells and red cells labelled 'S' are cancer stem cells.

1.5.2 Granulocyte colony stimulating factor

Granulocyte Colony Stimulating Factor (G-CSF) is a haematopoietic cytokine important for the differentiation and proliferation of myeloid progenitors. *G-csf*$^{-/-}$ and G-csf receptor knockout (*Csf3-r*$^{-/-}$) mice demonstrate profound deficiencies in neutrophil number, mobilisation and function[64,65]. Although a reduction in myeloid progenitors has been demonstrated in these mice, numbers or function of purified HSCs were not examined. G-CSF receptor (*Csf3-r*) mRNA expression on mouse HSCs is relatively low when compared to known stem cell affiliated genes, suggesting high levels of expression are not required for adequate stem cell function[66]. Overall, these findings do not support a critical role for G-CSF in stem cell maintenance and function.

There has been considerable interest in using G-CSF to cycle dormant stem cells thus rendering them more susceptible to chemotherapy. There is clear evidence that G-CSF can mobilise stem cells from the bone marrow to the peripheral blood by disrupting interactions between the bone marrow niche and stem cells[67]. However a key question is whether G-CSF is able to cycle, rather than just mobilise, purified stem cells. Studies performed before the discovery of cell surface markers which purified stem cells rather than progenitors suggested that populations enriched for stem and progenitor cells (Thy1.1 lo Lin- Sca1+ cKit+ MAC1- CD4-) did cycle more actively post G-CSF administration[68]. However these studies had a number of limitations. Only phenotypic analysis was performed and as these studies predated purification of stem cells using the SLAM or CD34 markers, they were looking at heterogeneous populations of stem and progenitor cells. In addition, they only investigated the cycling effect of a combination of Cyclophosphamide and G-CSF and did not compare with single agent G-CSF. In a second study performed by the same group, mice were initially treated with Cyclophosphamide and G-CSF. A second dose of Cyclophosphamide was then administered, in order to see if the stem and progenitor cells were now more susceptible to

chemotherapy. Phenotypic analysis suggested the Thy1.1 lo Lin- Sca1+ cKit+ MAC1- CD4- cells were almost all eliminated after this second hit but no functional stem cell assays were performed to determine if stem cells were truly eliminated[69].

An alternative technique for determining whether cells are quiescent is treatment of mice with the thymidine analogue BrdU[59]. DNA is labelled with BrdU when a cell divides and then followed by a 'chase' period where no further BrdU is administered. Almost all stem and progenitor cells will be labelled within 12-14 days of continuous BrdU exposure, as BrdU is thought to stimulate cell division and therefore BrdU incorporation. However, during the chase period, cycling cells which continue to divide will lose BrdU staining intensity until it is not detectable by flow cytometry. Only long-term non-dividing quiescent cells will remain BrdU+ and are termed label-retaining cells (LRCs). Wilson *et al* demonstrated that post *in vivo* G-CSF administration, the frequency of BrdU+ LRCs (CD34-CD150+CD48-) was lower than in control mice. This is perhaps the most compelling evidence that G-CSF may be able to put phenotypic HSCs in to cell cycle[59].

There is limited data looking at the bone marrow cell cycle status of normal human stem cells post G-CSF. One important study demonstrated enhanced cycling of CD34+ CD38- AML cells engrafted into mice after administration of G-CSF [70]. Further studies in these mice suggested an enhanced kill in response to Cytarabine, after activation by G-CSF. However clinical studies using this 'priming' approach have not been as successful[71,72]. This may be due to the fact that G-CSF may only cycle progenitors rather than true stem cells in patients. There are a number of additional limitations to these clinical studies, including variations in patient selection, G-CSF dose, treatment schedules and chemotherapy combinations which may also explain the lack of efficacy.

1.5.3 Interferon alpha

Interferons are cytokines which have significant immune-modulatory capabilities. Interferon (IFN) alpha has been used to treat viral infections, multiple sclerosis and a variety of malignancies. Studies have also demonstrated that *in vivo* administration of interferon alpha cycles mouse HSCs (LSK+CD150+CD34-) and progenitors (LSK+150+)[62]. In the same study, similar effects were seen following the injection of PolyI-PolyC which mimics IFN alpha. Subsequent treatment of the mice with 5-FU resulted in severe anaemia and death within 2 weeks. This was presumed to be due to a loss of HSCs, providing evidence for INF alpha activation enhancing HSC elimination.

In the 1990s, IFN alpha was routinely used to treat chronic myeloid leukaemia (CML). It was superseded by Imatinib which was found to have far greater targeted efficacy and fewer side effects[73]. The majority of patients, even those with undetectable minimal residual disease (MRD), relapsed when taken off Imatinib[74]. It is widely thought that this is due to selective treatment resistance of dormant CML stem cells[75,76]. Studies have also demonstrated that combined IFN alpha and Imatinib treatment may result in improved remission rates[77]. This may be due to the stem cell cycling effect of INF alpha enabling the elimination of previously dormant stem cells by Imatinib. However it is important to note that in these studies IFN alpha was often difficult for patients to tolerate, indicating the need for an alternative cycling agent.

1.5.4 Thrombopoietin

Thrombopoietin (TPO) is perhaps the most attractive candidate growth factor to specifically target stem cells. Activation of TPO signalling through its receptor MPL is essential for megakaryocyte and platelet proliferation. Importantly it is also a key regulator of normal stem cell maintenance in the mouse and human[78]. *Tpo*$^{-/-}$ and *Mpl*$^{-/-}$ mice demonstrate profound deficiencies in stem cell number, function and alterations in cell cycle properties[79,80]. This combined effect on platelet and stem cell maintenance is clinically evident in patients with congenital amegakaryocytic thrombocytopenia (CAMT) who have compound heterozygous or homozygous lack of function mutations of the *MPL* gene[81]. Patients are born with a profound isolated thrombocytopenia which progresses rapidly to multi-lineage bone marrow failure within the first few years of life, cured only by allogeneic stem cell transplantation. Interestingly a very recent case study reported the first CAMT patient with compound heterozygous mutations of the *MPL* gene, who developed pancytopenia within the first month of life, emphasising the importance of this gene in neonatal stem cell maintenance[82]. TPO is also an established mobiliser of CD34+ bone marrow cells in normal volunteers and cancer patients[83].

There is some controversy in the field regarding whether TPO can induce stem cells to cycle. On the one hand, one study demonstrated that TPO was able to act synergistically with other cytokines to induce proliferation of mouse HSCs[84]. A more recent study used *in vivo* HSC divisional tracking with CFSE (5(6)-carboxyfluorescein diacetate N-succinimidyl ester) to determine enhanced cell division of LSK CD150+ cells post injection of a TPO receptor agonist (Romiplostim)[85]. However, alternative studies have suggested that TPO may have the opposite effect, inducing stem cell quiescence. This is supported by the fact that *Tpo*$^{-/-}$ mice not only have a reduction in the number of stem cells but the remaining stem cells (LSKFlt3-) were cycling more actively, as determined by both increased BrdU incorporation and a

reduction in the number of stem cells with a side population phenotype[79]. These findings were somewhat supported by an additional study where administration of the TPO/MPL signalling pathway inhibitor, AMM2, also resulted in an expansion of cycling stem cells. However in the same study, administration of TPO only resulted in a transient expansion of quiescent stem cells (2 days after injection) but analysis 6 days after administration revealed no significant difference when compared to non-injected controls[86]. A more recent study primarily looking at the effect of thrombopoietin on DNA damage responses, found no impact of thrombopoietin on the cell cycle status of stem cells, although a much lower dose of TPO was used in this study[87].

1.6 Myelodysplastic Syndromes

1.6.1 Clinical Features and Classification

Myelodysplastic syndromes (MDS) are a heterogeneous group of malignant blood disorders characterised by inadequate, dysplastic haematopoiesis and resultant cytopenias[88,89]. The majority of patients are elderly with a median age of 75 at presentation. Patients often present with anaemia alone but around 30% of patients will progress to acute myeloid leukaemia. The only curative treatment is allogeneic stem cell transplantation which is not suitable for most patients given their advanced age and often significant comorbidities. Very few alternative effective treatments are available, resulting in the majority of patients being managed supportively with blood products and antibiotics. The median survival for patients with advanced MDS who transform to AML is less than 7 months, with a cure rate <10% in over 65 year olds[90].

Over the past 25 years, two diagnostic classification systems have been established. Initially the French American British (FAB) classification was used but this has been superseded by the World Health Organisation (WHO) 2008 system, which uses morphological and cytogenetic diagnostic criteria (**Table 1.1**). A number of prognostic classification systems have also evolved, the most widely used being the International Prognostic Scoring System (IPSS)[91] (**Table 1.2 and 1.3**) However this has now been refined to further stratify untreated patients with MDS (IPSS-Revised) and continues to be updated[92].

Subtype	Peripheral Blood	Bone marrow
Refractory cytopenia with unilineage dysplasia (RCUD)	**Anaemia, neutropaenia or thrombocytopenia**	**Unilineage dysplasia, <5% blasts**
Refractory anaemia with ring sideroblasts (RARS)	**Anaemia**	**Unilineage erythroid dysplasia, >15% erythroid precursors are ring sideroblasts, <5% blasts**
Refractory cytopenia with multilineage dysplasia (RCMD)	**Cytopenias**	**Multilineage dysplasia +/- ring sideroblasts, <5% blasts**
Refractory anaemia with excess blasts type 1 (RAEB I)	**Cytopenias**	**Unilineage or multilineage dysplasia, 5-9% blasts**
Refractory anaemia with excess blasts type 2 (RAEB II)	**Cytopenias**	**Unilineage or multilineage dysplasia, 10-19% blasts**
Myelodysplastic syndrome associated with isolated del (5q)	**Anaemia. Normal or high platelet count**	**5q31 deletion, hypolobulated megakaryocytes, <5% blasts**

Table 1.1 MDS diagnostic classification (WHO 2008)

Classification system for patients with Myelodysplastic Syndromes. (Adapted from Vardiman JW *et al* Blood 2009[93]).

Prognostic variable	Score Value				
	0	0.5	1	1.5	2
BM Blasts Karyotype Cytopenias	<5 Good 0/1	5-10 Intermediate 2/3	- Poor	11-20	21-30

Table 1.2 International prognostic scoring system (IPSS)

Prognostic scoring system for untreated patients with primary MDS. Score for risk groups: Low, 0; Intermediate-1, 0.5-1.0; Intermediate-2, 1.5-2.0; High, >2.5. Karyotype: Good, –Y, del (5q), del (20q); Poor, Complex ≥3 cytogenetic abnormalities, all chromosome 7 anomalies; Intermediate, other abnormalities. (Adapted from Greenberg P *et al* Blood 1997[91]).

IPSS score	Low	Int-1	Int-2	High
Median survival (year)	5.7	3.5	1.2	0.4
25% AML evolution (year)	9.4	3.3	1.1	0.2

Table 1.3 AML evolution of MDS patients within IPSS subgroups.

(Adapted from Greenberg P *et al* Blood 1997[91]).

1.6.2 Cellular Biology

The biology of myelodysplastic syndromes is still not well understood. The most well studied subgroup is isolated del (5q) MDS. It is characteristically more common in women, presents with refractory anaemia, a normal or raised platelet count, dysplasia and an isolated del (5q) on cytogenetic analysis. The prognosis of patients is relatively good when compared to other MDS subgroups. However, one study did find a higher than expected transformation rate to leukaemia of 10% within the first 5 years after diagnosis[94].

Previous studies suggest that in patients with isolated del (5q) MDS, disease is very likely to be driven by clonally involved MDS stem cells[43]. Isolated del (5q) MDS has been shown to originate in the phenotypic and functional CD34+ CD38- CD90+ haematopoietic stem cell. Strong supporting evidence for the stem cell origin of this disease includes the fact that over 94% of the phenotypic and functional stem cells contain the del (5q) and seem to outcompete normal stem cells[43]. In addition, both the lymphoid and myeloid compartments are clonally involved, supporting a multi-potent origin of disease. To date the molecular signature of such phenotypically defined del (5q) stem cells is very similar to that of normal haematopoietic stem cells and very distinct from haematopoietic progenitors[95]. However these studies had significant limitations. Due to the availability of techniques at the time, these studies relied predominantly on *in vitro* assays as a surrogate for stem cell activity. Studies focussed entirely on the rare MDS sub-type, del (5q) MDS, due to the availability of a clonal marker in a relatively homogenous patient population. It is important to study patients in alternative low and high risk subgroups of MDS before we can definitively say that all MDS is hierarchically arranged and driven by an MDS stem cell.

1.6.3 The Genetics Landscape of MDS

Until recently, very little was known about the genetic abnormalities underlying MDS. However, recent landmark studies have used next generation sequencing to show that recurrent DNA mutations are present in over 70% of MDS patients, including those with normal cytogenetics[96-99]. Mutations in splice factors (SF3B1, SRSF2, U2AF2, and ZRSR2), epigenetic regulators (ASXL1, TET2, EZH2) transcription factors (RUNX1, ETV6 and NRAS), signalling molecules (JAK2) and apoptosis regulators (TP53) have been described. The first major study utilised next generation sequencing and mass spectrometry based genotyping to identify somatic point mutations in 439 patients with MDS[96]. Mutations in 18 genes were detected. When mutation status was correlated with clinical characteristics, it became clear that mutations in RUNX1, TP53 and NRAS were strongly associated with profound thrombocytopenia and increased bone marrow blasts, demonstrating that mutation status may be useful for stratifying patients. It was also clear in this study that patients who had a large number of mutations (>5) had a poorer prognosis compared to patients with fewer mutations. The mutations identified in this study were not specific for MDS as they were also identifiable in patients with other myeloid neoplasms. However, subsequent exome and whole genome sequencing approaches revealed novel splice factor mutations which appeared to be far more specific for myelodysplastic disorders[97,98]. Yoshida and colleagues reported exome sequencing of 29 MDS patients and revealed novel splice factor mutations[97]. Of particular interest was the fact that specific mutations were associated with certain clinically distinct disorders when validated in a larger group of patients. Mutations in SF3B1 were present in 75% of patients with MDS and ring sideroblasts and mutations in SRSF2 were present in 28.4% of patients with CMML. At the same time an alternative study also demonstrated a similarly high prevalence of the same SF3B1 mutation in patients with MDS and ring sideroblasts (65% 53 of 82 patients)[98].

More recently Pappaemenauil and colleagues also performed targeted sequencing of MDS patients in order to look further at the relationship between mutations and clinical phenotype[99]. One clear and clinically important conclusion from this work was that the more mutations a patient had, the worse the overall outcome. RNA splice factor mutations appeared to be very early events and were mutually exclusive and resulted in a different pattern of cooperating mutations. However mutation profiling did not seem to add predictive value to current standard clinical variables when predicting leukaemia free survival.

These seminal studies and many more have been an important step in understanding the molecular basis of the MDS and have also provided clonal markers in patients with a normal karyotype.

The identification of such driver mutations has potentially provided unique genetic tools to track clonal diversity, for the first time enabling 'fate mapping' of genetic changes within MDS patients. This would provide an approach to determine definitively the cell of origin in MDS patients without cytogenetic aberrations. It could also be extended to other sub-groups of myeloid malignancies such as the Myelodysplastic/Myeloproliferative overlap syndromes, which are biologically poorly characterised.

1.6.4 MDS Stem Cell Quiescence and Therapy Resistance

Lenalidomide is a synthetic derivative of Thalidomide but the precise mechanism of action and molecular targets of Lenalidomide are unknown[100]. Lenalidomide is particularly effective in del(5q) MDS, resulting in a rapid resolution of anaemia in many patients[101,102]. Responses are characteristically not long lived, with most patients relapsing within 2-3 years. Tehranchi *et al* analysed the stem and progenitor cell compartment in patients with del (5q) MDS, treated with Lenalidomide over time[103]. Prior to Lenalidomide, the vast majority of MDS stem (CD34+ CD38- CD90+) and progenitor cells (CD34+ CD38+) contained the del (5q) as had been shown in previous studies[43]. However when the compartments were re-examined once the patients were in a complete clinical and cytogenetic remission, the del (5q) had largely been eliminated from the progenitor compartment but still remained in the stem cells. This demonstrated that the stem cells were selectively resistant to treatment. Further clinical significance of the stem cells was elicited by bone marrow examination at disease progression, when a considerable expansion of del (5q) stem cells was noted, with greater than 90% of the stem cells now being clonally involved[103].

The causes of such stem cell resistance may be multifactorial. High level expression of ATP binding cassette (ABC) multidrug-resistance transporters was found on stem cells relative to progenitors, which may mean that Lenalidomide concentrations were sub-therapeutic in the stem cells. An alternative mechanism of resistance may be stem cell quiescence. Cell cycle analysis of del (5q) stem cells prior to Lenalidomide demonstrated that they were highly quiescent, as were normal HSCs, with the majority of cells residing in G_0. In sharp contrast, the progenitors were actively cycling[103]. This implicated dormancy as being a highly possible mechanism of stem cell resistance to Lenalidomide.

The majority of previous studies have focussed on the ability of growth factors to activate and cycle quiescent stem cells in AML and CML. We propose that MDS may be an ideal disease model in which to investigate whether activation of quiescent MDS stem cells will enable more effective chemotherapeutic targeting.

1.7 Project Aims

The first aim of this thesis was to characterise fully the bone marrow stem and progenitor compartments of patients with low and intermediate-1 (low/int-1) risk MDS, at the phenotypic, functional and molecular level. This detailed characterisation would then enable determination of the hierarchical relationship between proposed stem and progenitor populations, beyond del (5q) MDS. If a functional hierarchy could be established, tracking somatic DNA mutations found in bulk bone marrow cells, back to candidate stem cells would provide unique fate mapping opportunities to establish the identity of MDS propagating cells *in situ*.

Almost all studies on MDS stem cell biology have focussed on low/intermediate-1 risk MDS patients. My second aim was to fully characterise the stem and progenitor cell compartments in patients with the more advanced and aggressive myeloid disorder, chronic myelomonocytic leukaemia (CMML). CMML became particularly interesting during my studies as a number of articles were published demonstrating the mutational complexity of CMML[97,104,105], providing unique genetic markers to track through cell populations. CMML is essentially an incurable disease. Identifying the CMML cancer stem cell may provide improved opportunities for more effective targeted therapies.

Cancer stem cell resistance is an area of intense scientific and clinical scrutiny at present. It has been clearly shown that normal and MDS stem cells are highly quiescent and selectively resistant to therapy[103]. This may be a key mechanism by which such stem cells evade drug targeting. Thrombopoietin is known to be a key mediator of stem cell quiescence[79]. As such my third aim was to see if clinically available TPO receptor agonists could enhance cycling of previously dormant and thus unreachable stem cells. Such priming may then enable more effective subsequent chemotherapeutic elimination of these characteristically resistant cells.

2. Materials and Methods

2.1 Patient studies

2.1.1 Collection of patient material

Bone marrow and peripheral blood specimens from patients with MDS, CMML, 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.

2.1.2 Processing of patient material

Bone marrow and peripheral blood samples were obtained fresh and mononuclear cells isolated using Lymphoprep (Axis Shield) separation, before being viably frozen in 10% dimethylsulfoxide (DMSO; Sigma Aldrich) and 90% fetal calf serum (FCS; Thermo Fisher). CD34 enrichment was performed in some cases prior to freezing using human CD34 micro beads (MACS Miltenyi Biotec). Cells were thawed at 37^{o}C in a water bath and resuspended in 70% Iscoves Modified Dulbecco Medium (IMDM; Invitrogen), 20% FCS and 10% DNAse (Merck). Cell pellets were then resuspended in phosphate buffered saline (PBS; Dulbeccos) and FCS for analysis.

2.1.3 Flow Cytometry Analysis and Cell Sorting

2.1.3.1 Stem and progenitor cell analysis

Cryopreserved bone marrow mononuclear cells (MNCs) or CD34 enriched cells were thawed and prepared for flow cytometry analysis as described above.

Cells were incubated with human Fc Block (Miltenyi Biotec) and stained with antibodies to human antigens[106]. Stem cells (Lineage-CD34+CD38-CD90+CD45RA-), CMPs (Lineage-CD34+CD38+CD45RA-CD123+) GMPs (Lineage-CD34+CD38+CD45RA+CD123+), MEPs (Lineage-CD34+CD38+CD45RA-CD123-), Mature T cells (CD11b-CD14-CD19-CD34-CD3+CD4+/CD8 +)) and Pro B cells (CD34+CD19+) were purified. Fluorescence Activated Cell Sorting (FACS) was performed on live MNCs or CD34-enriched cells as identified by DAPI exclusion after staining with the monoclonal antibodies. All experiments included fluorescence-minus-one (FMO) and single-stained controls. Cells were analysed on an LSR II flow cytometer (Becton Dickinson) and sorted on a FACS ARIA IIu cell sorter (Becton Dickinson). See **Table 2.1** and **Table 2.2** below for details on the antibodies and instrument settings used for staining and identification of cell populations.

2.1.3.2 Cell cycle analysis

The cell cycle status of CMML patients was analysed after fixing and permeabilising the cells with 3.2% paraformaldehyde and 90% ice cold methanol (Sigma). Cells were then stained with surface markers CD19 (exclusion of quiescent B cells), CD34, CD38 and CD90. Intracellular markers Ki67 and 4',6-diamidine-2-phenylidole dihydrochloride (DAPI) were used as a marker of cell proliferation and a DNA stain, respectively. Samples were analysed on an LSRII (BD). The following markers were used to determine cell cycle status: G_0 (Ki67−DAPI−), G_1 (Ki67+DAPI−), and SG_2M (Ki67+DAPI+)[103].

Antigen	Conjugate	Company	Clone	Staining panel
CD34	APC	BD	8G12	Stem cell, Lymphoid and cell cycle
CD38	PE Texas Red	Invitrogen	HIT2	Stem cell and cell cycle
CD90	PE	BioL	5E10	Stem cell and cell cycle
CD45RA	FITC	Invitrogen	MEM56	Stem cell
CD123	PECy7	BioL	6H6	Stem cell
CD2	PECy5	BioL	RPA-2.10	Lineage cocktail Stem cell
CD3	PECy5	BioL	HIT3a	Lineage cocktail Stem cell
CD3	FITC	BioL	SK7	Lymphoid
CD4	PECy5	BioL	RPA-T4	Lineage cocktail Stem cell
CD4	PECy7	BioL	RPA-T4	Lymphoid
CD7	PECy5	BioL	MEM86	Lineage cocktail Stem cell
CD8	PECy5	BioL	RPA-T8	Lineage cocktail Stem cell
CD8	APCCy7	BioL	RPA-T8	Lymphoid
CD10	PECy5	BioL	SJ5-1B4	Lineage cocktail Stem cell
CD11b	PECy5	BioL	ICRF44	Lineage cocktail Stem cell and Lymphoid
CD14	PECy5	Beckman-Coulter	TUK4	Lineage cocktail Stem cell and Lymphoid
CD19	PECy5	BioL	HIB19	Lineage cocktail Stem cell, Lymphoid and cell cycle
CD20	PECy5	BioL	2H7	Lineage cocktail Stem cell
CD56	PECy5	BD	B159	Lineage cocktail Stem cell
CD235	PECy5	BioL	HIR2	Lineage cocktail Stem cell
Ki67	FITC	BD	B56	Cell cycle
DAPI		Invitrogen		Viability and cell cycle

Table 2.1 FACS Antibody staining for human stem, progenitor and cell cycle analysis

Instrument	Laser Wavelength	Laser Power	PMTS and filter configuration
BD FACS Aria IIu	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

Table 2.2 FACS Aria IIu and LSRII configurations

2.1.4 Tissue culture procedures

2.1.4.1 Myeloid progenitor assays

FACS purified GMPs and MEPs were plated in complete human methocult medium (Stem cell technologies H4434) in 35mm gridded petri dishes. Cells were incubated at 37^{o}C, 5% CO_2 for 14 days[103]. Colonies were then counted, picked and immediately frozen on dry ice before being stored at -80^{o}C. 150-300 cells were sorted per population per patient and plated in 2 replicates

2.1.4.2 In vitro stem cell assays

Stromal feeder layers of M210B4 and SL/SL murine fibroblasts were plated at least 18 hours prior to co-culturing purified populations[22,103]. 200-1000 cells per purified stem or progenitor population per patient, were plated in 1-3 replicates, depending on the number of cells available. Weekly half-medium changes of Myelocult H5100 (Stem Cell Technologies), supplemented with 10^{-6}M Hydrocortisone were performed for 6 weeks. At 6 weeks, each well was then examined by light microscopy before transferring all the cells from one well to one methylcellulose dish (H4100). The methylcellulose (H4100) was supplemented with 20% FCS, 2 mM L-Glutamine (PAA), 10^{-4}M 2-mercaptoethanol (Sigma), 100 U/mL penicillin/streptomycin (PAA), recombinant human (rh) stem cell factor (SCF; Amgen), rhFLT3-ligand (FL; Immunex), rhIL-3 (Peprotech), rhG-CSF (Amgen), rhGM-CSF (Immunex) (all at 10 ng/mL), and rhEPO (5 U/mL, Boehringer Mannheim). Cells were then incubated at 37^{o}C 5% CO_2 for 14 days. Colonies were then counted, picked and immediately frozen on dry ice before being stored at -80^{o}C for future DNA mutation screening. Importantly all colonies derived from one methycellulose dish may be derived from the same stem cell and cannot be viewed as independent. Therefore, only colonies derived from separate methycellulose dishes can be defined as having arisen from independent stem cells.

2.1.4.3 Fluorescence in situ hybridisation

For interphase FISH, cells were centrifuged onto slides and hybridized with a specific probe against the 5q31 region or the centromeric region of chromosome 8. Images were obtained with the use of fluorescence microscopy. For chromosome 5, the Vysis D5S23 D5S721/LSI EGR1 dual color probe (Abbot Molecular) was used, hybridising to 5p15.2 and 5q31 respectively. In the nuclei of normal cells, the probe hybridising to 5q31 region appears as 2 orange signals, whereas in cells with a 5q deletion, there is one orange signal. Both normal and 5q deleted cells express 2 green signals. For chromosome 8, the Vysis CEP 8 (D8Z2) was used, hybridizing to 8p11.1-q11.1 (Abbot Molecular). In the nuclei of normal cells, the probe hybridizing to the 8p11.1-q11.1 region appears as 2 green signals, whereas in a cell with trisomy 8, there are 3 green signals. Whenever possible at least 100 nuclei were analysed per population.

2.1.4.4 Single cell tracking

In order to determine whether Romiplostim could activate and enhance the rate of division of human HSCs, single cell tracking was performed[103]. Single human HSCs (CD34+ CD38- CD90+ CD45RA-) were FACS purified from a normal bone marrow sample. Cells were sorted in to a serum free medium, Stem Span (Stem Cell Technologies), supplemented with either 100ng/ml stem cell factor (SCF) (Amgen), 100ng/ml SCF + 100ng/ml rhTPO (Genentech) or 100ng/ml SCF + 100ng/ml Romiplostim (Amgen). 750 cells were sorted in to 1.5ml of media and 20ul of cell suspension was then plated into individual Terasaki wells. At 24 hours wells were scored under an inverted light microscope and only wells which contained single cells were tracked. Cell division was then tracked in these wells at 48, 72 and 96 hours post sorting and scored as viable single cells or viable divided cells. All cells scored at baseline were still viable at 96 hours.

2.1.4.5 In vitro Romiplostim + Cytarabine

To determine if Romiplostim could enhance the ability of Cytarabine (Hospira) to eliminate human HSCs in vitro, 600 normal HSCs were sorted into 300ul Stem span + 100ng/ml SCF and a further 600 HSCs into 300ul of Stem span + 100ng/ml SCF + 100ng/ml Romiplostim. 10uL of cell suspension was transferred into single terasaki wells (20 cells per well). A baseline cell count of all wells was performed at 24 hours, demonstrating that each well contained 10-20 cells. A dose titration of Cytarabine was performed at 48 hours. 10ul of 2X Cytarabine was added to give a final concentration of 16nM, 32nM, 64nM, and 128nM. 3-5 replicates of each Cytarabine concentration were plated. Viable cells were counted at 72 hours and 96 hours post sort.

2.1.5 Gene Expression

Fluidigm analysis was performed by Dr Petter Woll[103]. Gene expression was analysed with the use of a Biomark 48.48 dynamic array (Fluidigm) for multiplex quantitative real time PCR of purified populations. 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^{o} for 15 minutes, inactivation of reverse transcriptase at 95^{o} for 2 minutes, target gene amplification in 22 cycles at 95^{o} for 15 seconds followed by 60^{o} for 4 minutes. Samples were then loaded on to a primed Biomark 48.48 dynamic array. The following quantitative PCR reaction was performed: 95^{o} for 10 minutes followed by 40 cycles of 95^{o} for 15 seconds and 60^{o} for 60 seconds.

Data were analysed using the ΔCt method and results were normalized to *GAPDH* expression. Two replicates (50-100 cells/replicate) were included for each cell population for each patient. A list of TaqMan assays is provided (**Table 2.3**).

Gene	Name	Probe ID
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	Hs00184500_m1
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	Hs00175938_m1
CSF1R	colony stimulating factor 1 receptor	Hs00911250_m1
CSF3R	colony stimulating factor 3 receptor (granulocyte)	Hs01114427_m1
EPOR	erythropoietin receptor	Hs00181092_m1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
GATA1	GATA binding protein 1 (globin transcription factor 1)	Hs01085823_m1
HLF	hepatic leukemia factor	Hs00171406_m1
HOXA9	homeobox A9	Hs00365956_m1
KLF1	Kruppel-like factor 1 (erythroid)	Hs00610592_m1
MPL	myeloproliferative leukemia virus oncogene	
MPO	Myeloperoxidase	Hs00165162_m1
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	Hs00232074_m1
TAL1	T-cell acute lymphocytic leukemia 1	Hs01097987_m1

Table 2.3 List of Taqman gene expression assays used for quantification of relative gene expression

2.1.6 NSG Transplantation

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice 10-14 weeks of age were sub-lethally irradiated by exposure to two doses of 1.25 Gy (Cs source) four hours apart. Cells were delivered intra-femorally within 24 hours of the last radiation dose. Purified stem and progenitor cells were injected in to each mouse. The cell populations were injected according to their ratios within the patient's BM. Human engraftment and lineage distribution was monitored by flow cytometry by BM aspiration where human engraftment was calculated based on the frequency of human CD45 from the sum of human and mouse CD45 within the total BM. Secondary transplantations were performed using 5-10 million total bone marrow cells from engrafted primary mice. All mice were bred and maintained at the Oxford Biomedical Services and all experiments were done with the approval of the UK Home Office, Project License 30/2570.

2.1.6.1 Bone marrow aspiration and analysis

In order to monitor engraftment, bone marrow aspirations were performed every 5 weeks for human reconstitution and lineage analysis. Mice were anaesthetised with inhalational isoflurane. Bone marrow (5ul) was aspirated from the femur using a 30 gauge 0.3ml syringe and transferred to an eppendorf containing PBS+2%FCS. The aspirates were then centrifuged and erythroid cell lysis was performed using ammonium chloride. Cells were then stained with human and mouse antibodies (**Table 2.4**) and analysed on an LSRII flow cytometer (BD).

Antigen	Conjugate	Company	Clone
CD15	FITC	BD	MMA
CD33	FITC	BD	P67.6
CD66b	FITC	BD	G10F5
DAPI (Viability)			
Mouse CD45	Pacific Orange	Life Technologies	30-F11
CD19	PE	BD	SJ25C1
CD34	APC	BD	8G12
Human CD45	AF700	Biolegend	HI30

Table 2.4 FACS Antibody staining for human engraftment and lineage distribution in engrafted NSG mice.

2.1.7 DNA Sequencing

2.1.7.1 Cell preparation and freezing

For bulk bone marrow cell mutation screening, 500,000 - 1,000,000 mononuclear cells were centrifuged. The supernatant was aspirated and the cell pellet frozen at -80^{o}C. For purified populations, two replicates of 100 cells were sorted per patient and immediately pelleted and frozen on dry ice for storage at -80^{o}C. For LTC-CFC mutation analysis, colonies were carefully picked from methylcellulose plates and transferred to 200ul of sterile PBS. Cells were then centrifuged and following aspiration of the supernatant, frozen at -80^{o}C. DNA was later isolated (**Figure 2.1**).

2.1.7.2 DNA isolation

The subsequent aspects of the DNA sequencing protocol, until variant detection, were performed at the Karolinska Institute, Stockholm, Sweden by Dr Petter Woll. I then performed analysis.

Genomic DNA was either isolated from unfractionated BM cells, or was whole genome amplified (WGA) from purified cells and single LTC-CFC colonies using GenomiPhi V2 DNA Amplification Kit (GE Healthcare). The isolated DNA was then enzymatically digested and validated using 2200 TapeStation High Sensitivity D1K assay (Agilent).

2.1.7.3 Haloplex DNA sequencing

The genomic DNA restriction fragments were hybridized to HaloPlex probes targeting regions of interest. Coding regions of genes, known to be mutated in myeloid neoplasms and other cancers, were selected for targeted screening (**Table 2.5**). The circular fragments generated by the HaloPlex probes were captured on magnetic beads and amplified by PCR. The enriched targets were validated by TapeStation High Sensitivity D1K assay and quantified using Qubit dsDNA HS kit (Invitrogen). After adjusting the samples to 2nM, the samples were pooled and sequenced on HiSeq 2000 (Illumina) generating paired-end, 100 bp reads.

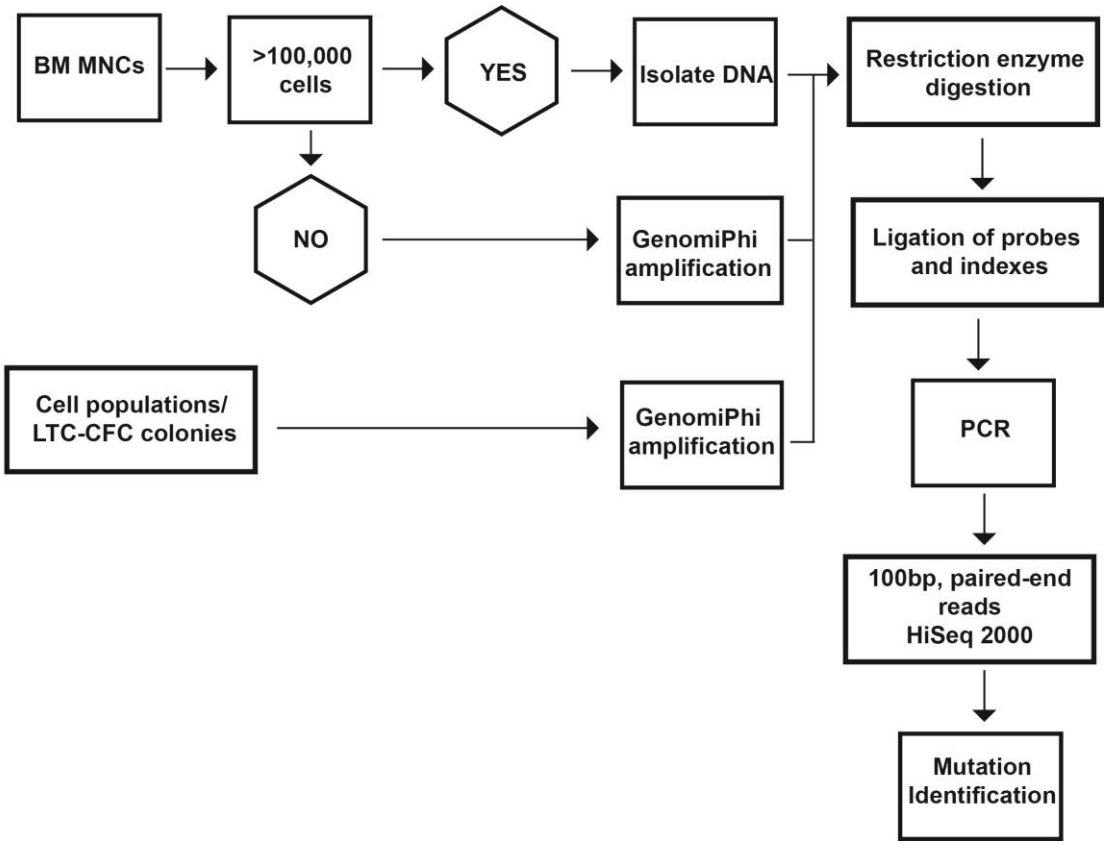


Figure 2.1 Overview of sample processing for Haloplex screening

DNA was isolated from bone marrow mononuclear cells, purified populations and LTC-CFC colonies. Following restriction enzyme digestion, oligonucleotide probes for selected genes were ligated. PCR amplification was then performed prior to sequencing on an Illumina HiSeq 2000.

2.1.7.4 Variant detection

Mapping to a human genome (hg19) and variant detection were performed. Variant allele frequencies were summarised and compared using VarScan after conversion to MPileup format using SAMtools3, 4[107,108]. Somatic mutations were identified by comparing bulk MDS cells and FACS purified (CD11b-CD14-CD19-CD34-CD3+CD4+/CD8+) T cell controls. Identified

mutations were subsequently traced back to purified MDS stem/progenitor cells and individually picked LTC-CFCs.

Non-synonymous genetic changes in coding regions of selected genes with less than 5% of mutant reads detected in T cell controls and at least 5 times higher mutant read frequency in the bulk BM sample were considered as somatic changes. In cases where T cell controls were not available, reported recurrent mutations in the coding regions of known genes implicated in leukaemia, MDS/MPD or cancer in general were selected for analysis in purified populations and individually picked LTC-CFCs.

2.1.7.5 LTC-CFC mutation detection

As individually picked LTC-CFCs are clonally derived, these were only scored as positive or negative. However, as there is potential for contamination with cells from other colonies when picking clones, we considered LTC-CFC clones with mutation read frequencies below 5% (<10% of cells involved) as negative and read frequencies above 25% (>50% of cells involved) as positive (when modelling heterozygous mutations). If mutation read frequencies fell in between these thresholds, the clones were regarded as inconclusive. In most cases, based on data generated from individual and clonally derived LTC-CFCs, mutations were found to be heterozygous. However, in the case of a *JAK2* V617F mutation in Patient 3 (Chapter 3) and a *CBL* I383M mutation in Patient 8 (Chapter 4) these mutations appeared to be carried in a homozygous state.

2.1.7.6 5q Deletion

For detection of chromosome 5q deletion, frequent SNPs within the common deleted region (CDR) based on information from dbSNP build 135 were used. Informative SNPs located within the 5q common-deleted-region and detected as heterozygous within T cell controls

were used to track loss of heterozygosity in purified cell populations and LTC-CFC clones. For SNPs where del(5q) resulted in loss of the reference allele, the percentage of the variant reads was used. However, when SNPs resulted in loss of the variant allele, the reported frequency was adjusted by subtraction from 100%, and the average for all the SNPs was used to calculate the percentage of reads supporting loss of heterozygosity.

Gene ID	**Gene name**
ASXL1	additional sex combs like 1 (Drosophila)
ATRX	alpha thalassemia/mental retardation syndrome X-linked
BCOR	BCL6 co-repressor
CBL	Cbl proto-oncogene, E3 ubiquitin protein ligase
CDKN2A-P16INK4A/P14ARF	cyclin-dependent kinase inhibitor 2A
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha
CSF1R	colony stimulating factor 1 receptor
CSF3R	colony stimulating factor 3 receptor (granulocyte)
DDX39B	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39B
DDX46	DEAD (Asp-Glu-Ala-Asp) box polypeptide 46
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha
ELANE	elastase, neutrophil expressed
EPOR	erythropoietin receptor
ETV6	ets variant 6
EZH2	enhancer of zeste homolog 2 (Drosophila)
FLT3	fms-related tyrosine kinase 3
GATA1	GATA binding protein 1 (globin transcription factor 1)
GATA2	GATA binding protein 2
GNAS	GNAS complex locus

IDH1	isocitrate dehydrogenase 1 (NADP+), soluble
IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial
JAK2	Janus kinase 2
JAK3	Janus kinase 3
KDM6A	lysine (K)-specific demethylase 6A
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MLL	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila)
MPL	myeloproliferative leukemia virus oncogene
NPM1	nucleophosmin (nucleolar phosphoprotein B23, numatrin)
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
PPP1R8	protein phosphatase 1, regulatory subunit 8
PTEN	phosphatase and tensin homolog
PTPN11	protein tyrosine phosphatase, non-receptor type 11
PUF60	poly-U binding splicing factor 60KDa
RBM17	RNA binding motif protein 17
RBM22	RNA binding motif protein 22
RBM25	RNA binding motif protein 25
RBM5	RNA binding motif protein 5
RUNX1	runt-related transcription factor 1
SF1	splicing factor 1
SF3A1	splicing factor 3a, subunit 1, 120kDa
SF3B1	splicing factor 3b, subunit 1, 155kDa
SMNDC1	survival motor neuron domain containing 1
SRSF1	serine/arginine-rich splicing factor 1
SRSF2	serine/arginine-rich splicing factor 2
TET2	tet methylcytosine dioxygenase 2

TP53	tumor protein p53
U2AF1	U2 small nuclear RNA auxiliary factor 1
U2AF2	U2 small nuclear RNA auxiliary factor 2
WT1	Wilms tumor 1
YBX1	Y box binding protein 1
ZRSR2	zinc finger (CCCH type), RNA-binding motif and serine/arginine rich 2

Table 2.5 Genes included in Haloplex DNA sequencing

2.1.8 Nordic Myelodysplastic Syndrome Group Study of Azacitidine and Eltrombopag in High Risk Myelodysplastic Syndrome

2.1.8.1 Patient selection

Patients with MDS, aged 18 years or older, requiring treatment with Azacitidine (IPSS score Intermediate 2/high-risk, CMML with 10-29% bone marrow blasts, secondary AML with multi-lineage dysplasia and BM blasts (10-29%), with thrombocytopenia (platelet counts < 75 x 109/l)) were included. Major exclusion criteria were Eastern Cooperative Oncology Group (ECOG) performance status >2, previous treatment with any TPO-R agonist, life expectancy less than three months, previous treatment for cancer other than MDS/AML within two years from study start and east Asian ancestry (due to different routes of metabolism of Eltrombopag). Assessment at screening included evaluation of eligibility criteria, physical examination, laboratory assessment (including new blood and BM examinations) and a 12-lead Electrocardiogram. Site investigators enrolled patients on to the

trial and performed medical monitoring of the patients. Bone marrow morphology was centrally reviewed but cytogenetic analyses were performed locally.

The study was performed according to the International Conference on Harmonisation Guidelines for Good Clinical Practice (GCP) and the ethical principles outlined by the Declaration of Helsinki. Each patient provided written informed consent. The regional ethics committee and the Medical Product Agency of Sweden approved the protocol. An independent data monitoring committee (DMC) reviewed safety issues. An independent contract research organization (CRO Scandinavia) monitored the study. The study is registered at www.clinicaltrials.gov (NCT01481220).

2.1.8.2 Study design

This study was designed as a phase 1, non-randomised, single arm feasibility study. Four different doses of oral Eltrombopag (50mg, 100mg, 200mg and 300mg) were tested. A modified 3+3 patient cohort design was used so that no new patients were accepted to start on a higher dose without prior tolerance at the previous dose being confirmed. The DMC reviewed all data from previous cohorts before allowing opening of the next cohort. Azacitidine was administered, according to the recommendations by the Nordic Myelodysplastic Syndrome Group (NMDSG), 100 mg/m2 for 5 days in 28 days cycles given as subcutaneous injections. Eltrombopag was started one week before start of Azacitidine treatment as once daily tablets throughout three Azacitidine cycles. One dose escalation of Eltrombopag was done after five weeks for each patient. None of the patients were treated with Erythropoiesis-Stimulating Agents (ESA) or G-CSF.

Clinical and laboratory assessments included; weekly monitoring of peripheral blood (PB) values (including PB-blast count) and liver chemistry until week five and thereafter every four weeks, physical examination at week five and week 13 including assessment of bleeding

complications, transfusion dependency and drug interactions. BM morphology was assessed at screening, week five and 13.

2.1.8.3 Stem and Progenitor Cell Analysis

In total 12 patients entered the study. Flow cytometry stem/progenitor and cell cycle analysis was performed on BM samples from eight recruits (Patients 1-7 & 9) as detailed above in the flow cytometry analysis section. Due to insufficient bone marrow material, not all of the eight patients were analysed pre (baseline) and post Eltrombopag (week 5). See Chapter 5 for details.

2.2 Mouse Studies

C57BL/6 (CD45.2) and B6SJL (CD45.1) mice were bred at the Biomedical Sciences Centre, John Radcliffe Hospital, Oxford. All experiments were done with the approval of the UK Home Office, Project License 30/2570. All mice used were between 7-10 weeks of age.

2.2.1 Growth Factors and Chemotherapy

All growth factors and chemotherapeutic agents were purchased from the John Radcliffe Hospital Pharmacy, Oxford. Powdered Romiplostim (Nplate Amgen) was reconstituted in solvent for injection and frozen in aliquots at -20^{o}C. When required, individual sterile vials were thawed and diluted in PBS + 0.1% Bovine serum albumin (BSA) to give a final concentration of 20ug/ml for injection. Granulocyte colony stimulating factor (Zarzio Filgrastim) was purchased already reconstituted in pre-filled syringes (300ug in 0.5 ml) and was also diluted to a concentration of 20ug/ml. Cytarabine (Hospira) was purchased ready reconstituted (1g/10ml) and did not require dilution.

2.2.1.1 Growth Factor Administration

Growth factors (Romiplostim 100ug/kg or G-CSF 100-300ug/kg) were injected subcutaneously on day 1, 3 and 5 of the treatment regimen. No side effects were seen. Peripheral blood by cardiac puncture and bone marrow were harvested on day 7 for analysis and transplantation.

2.2.1.2 Phenotypic stem and progenitor cell analysis

Femur, tibia and crista were harvested from the treated mice and crushed in PBS+2% FCS to form a single cell suspension. Cells were then centrifuged and resuspended in Fc block

before being stained for FACS analysis. A list of antibodies used is provided below (**Table 2.6**).

Peripheral blood was aspirated at the same time point via cardiac puncture. An aliquot was taken for a full blood count which was analysed on a Sysmex KX-21N. Erythroid cells were then separated and lysed using dextran and ammonium chloride. Cells were then stained using the same antibodies as the bone marrow samples (**Table 2.6**).

Flow cytometry analysis was performed on the bone marrow and peripheral blood cells as identified by 7AAD exclusion after staining with the monoclonal antibodies. All experiments included fluorescent-minus-one (FMO) and single-stained controls. Cells were analysed on an LSR II flow cytometer (BD)

2.2.1.3 Mouse Cell Cycle analysis

Cell cycle analysis was performed on the bone marrow cells at the same time point as stem and progenitor analysis. Bone marrow cells were resuspended in Fc block and stained with cell surface antibodies (**Table 2.6**). The stained cells were then incubated in a fixation and permeabilisation solution (BD) and kept on ice for 30 minutes. After washing, the cells were stained with an anti-Ki67 antibody (BD) and left agitating overnight at room temperature in the dark. The following day samples were stained with DAPI and analysed on an LSRII.

Antigen	**Conjugate**	**Company**	**Clone**	**Staining Panel**
Sca 1	FITC	BD	E13-161.7	Stem Cell, BrdU and Cell Cycle
cKit	APCeF780	eBioscience	2B8	Stem Cell, BrdU and Cell Cycle
CD150	PECy7	Biolegend	TC15-12F12.2	Stem Cell, BrdU and Cell Cycle

CD48	APC	Biolegend	HM48-1	Stem Cell and Cell Cycle
CD5	PECy5	Biolegend	53-7.3	Stem Cell, BrdU and Cell Cycle
CD8	PECy5	Biolegend	53-6.7	Stem Cell, BrdU and Cell Cycle
B220	PECy5	Biolegend	RA3-6B2	Stem Cell, BrdU and Cell Cycle
GR1	PECy5	Biolegend	RB6-8C5	Stem Cell, BrdU and Cell Cycle
Ter119	PECy5	Biolegend	TER-119	Stem Cell, BrdU and Cell Cycle
DAPI				Stem Cell (viability) Cell Cycle (DNA stain)
Ki67	PE	BD	B56	Cell Cycle
CD48	PE	Biolegend	HM48-1	BrdU
BrdU	APC	BD	BU20A	BrdU

Table 2.6 FACS Antibody staining for mouse stem and progenitor, cell cycle and BrdU analysis

2.2.1.4 5-bromo-2-deoxyuridine (BrdU)

Mice were treated with Romiplostim (100ug/kg Day 1, 3, 5) or PBS. On day 5 and day 6, 2ug of BrdU was injected by intra-peritoneal injection. BrdU was administered on day 5 and 6 in order to determine the percentage of stem cells which were actively cycling at the time Cytarabine would be administered in the competitive repopulation experiments. Bone marrow was harvested for analysis on day 7 as described above. Fc blocked cells were stained with the antibodies (**Table 2.6**), prior to fixation and permeabilisation. DNAse was used to expose the incorporated BrdU. Intracellular staining with an anti-BrdU antibody was

then performed and cells were analysed on an LSRII. Mice treated with Romiplostim but not BrdU were used as a control for non-specific BrdU antibody staining.

2.2.1.5 Competitive Repopulation Assays

Donor CD45.1 mice were treated in 4 groups with either PBS (Day 1, 3, 5 and 6), Romiplostim (100ug/kg Day 1, 3, 5), Cytarabine (1g/kg or 3g/kg Day 5, 6) or Romiplostim (100ug/kg Day 1, 3, 5) followed by Cytarabine (1g/kg or 3g/kg Day 5, 6). Mice were culled on day 7, 24 hours after completion of treatment and bone marrow was harvested. Recipient CD45.2 mice were lethally irradiated (9 Gy) and injected with 5 million competitor (CD45.2) cells and the volume equivalent of 5 million donor cells. Cells from each donor were transplanted in to 2 mice. Long-term peripheral blood reconstitution was analysed at 16-17 weeks (**Table 2.7**).

The experimental design was exactly the same for the G-CSF (100-300ug kg D1, 3 5) competitive repopulation assays (**Figures 5.9** and **Figure 5.15**)

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

Table 2.7 FACS antibody panel for peripheral blood reconstitution

2.2.2 Myeloid progenitor assays

In order to investigate the stem/progenitor cell mobilisation ability of G-CSF and Romiplostim, colony forming cell (CFC-GM) assays were performed. Peripheral blood from mice treated with Romiplostim (100ug/kg D1, 3, 5), G-CSF (300ug/kg D1, 3, 5) and PBS (D1, 3, 5) was obtained by cardiac puncture on day 7. Erythroid cell separation and lysis was performed using dextran and ammonium chloride. 500,000 cells were plated in methycellulose (Stem Cell Technologies M3534 CFU GM only) and supplemented with the following cytokines: mGM-CSF 5ng/ml; hG-CSF 10ng/ml; mIL-3 5ng/ml and hFlt3 10ng/ml. Cells were cultured for 7 days at 37^0C 5% CO_2 in a humidified incubator. Colonies were then counted using an inverted light microscope.

2.2.3 Data Analysis

FlowJo (Version 8) analysis software (TreeStar) was used for all flow cytometry data analysis. Graph pad prism was used for all statistical analysis. Unless otherwise stated in the main text, statistical 2-way comparisons were made using the Mann Witney Test and multiple comparisons using the One Way Anova.

3. Phenotypic, functional and molecular characterisation of stem cells from low and intermediate-1 risk MDS patients

3.1 Introduction

The existence of cancer stem cells remains hugely controversial[37,38]. Broadly speaking, there are two models of how a cancer might propagate itself; the stochastic model and the hierarchical cancer stem cell model. A number of landmark studies have provided evidence for the existence of cancer stem cells in haematological and solid organ malignancies[41,43,46]. More recently, other studies have suggested that certain cancers do not require a rare and distinct population of cells for propagation and in fact numerous cell types may be able to sustain the cancer[48,52]. The key issue in the debate between these two models is the lack of a truly sensitive assay for determining cancer stem cell (CSC) activity.

Recently, elegant lineage tracing studies have been performed in mouse models of cancer, providing functional evidence for the existence of cancer stem cells in their natural environment[53-55]. Clearly such genetic labelling experiments are not possible in patients but provide crucial evidence for the existence of cancer stem cells. In order to definitively prove that a rare population of candidate cancer stem cells are absolutely required to maintain a cancer, we need to look beyond the limitations of the available *in vivo* and *in vitro* assays, go back to the patients and develop an assay which can be used *in situ*.

MDS is an ideal malignancy to investigate as previous studies already suggest that in patients with isolated del (5q) MDS, disease is very likely to driven by clonally involved MDS stem cells[43]. More recently, the majority of MDS patients in numerous studies have been found to have multiple recurrent DNA mutations[96,97,99]. However the majority of studies published have demonstrated the presence of such mutations in bulk bone marrow and peripheral blood cells but have not looked in purified stem and progenitor populations in order to establish where such mutations originate. If MDS follows the hierarchical cancer stem cell model, with the presence of rare MDS stem cells being absolutely required to sustain the

disease, then all stable DNA mutations must arise in such an MDS stem cell and be propagated to downstream progenitors and mature cells (**Figure 3.1a**). As downstream myeloid progenitors, such as Granulocyte Macrophage Progenitors (GMPs) or Megakaryocyte Erythroid Progenitors (MEPs), only have a short half-life, any genetic aberrations originating in these cells would not survive long term. However if the stochastic cancer model is correct, then mutations may arise in downstream progenitors, such as GMPs, or MEPs, which have gained self-renewal capacity and are therefore not reliant on upstream MDS stem cells for propagation (**Figure 3.1b**). *In vivo* tracking of DNA mutations in the stem and progenitor cells would provide unprecedented evidence for the existence and importance of cancer stem cells in patients with MDS if all genetic changes could be traced back to the MDS stem cell.

In this study we aimed to perform a detailed characterisation of the bone marrow stem and progenitor compartments in low/intermediate-1 risk MDS, beyond del (5q) MDS alone. Subsequently we then aimed to track all identifiable DNA mutations found in the bulk bone marrow, back to the distinct stem and progenitor cells, in order to determine the likely cell of origin for all genetic changes.

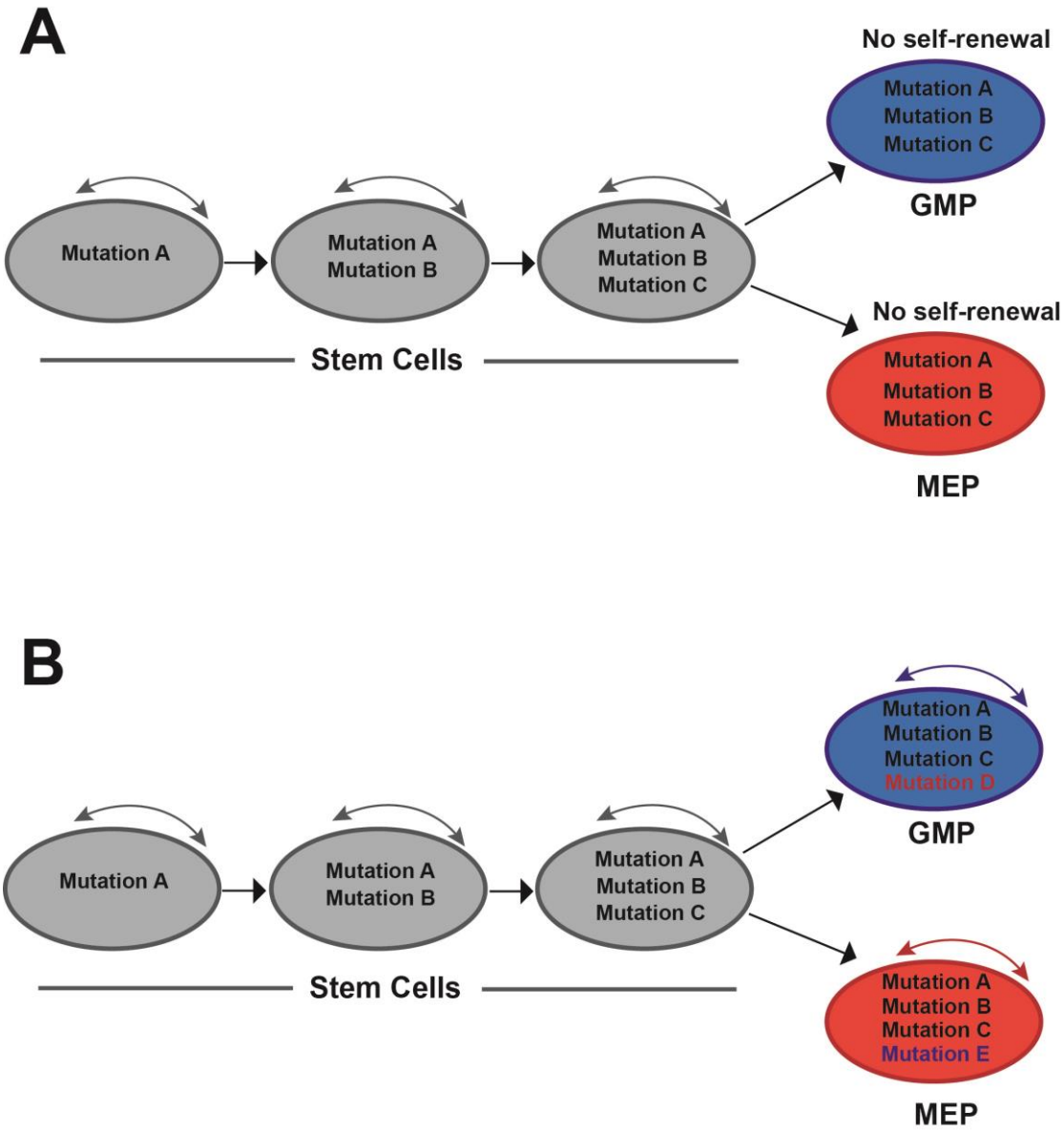


Figure 3.1 Models of mapping somatic mutations to the MDS stem cell compartment

A and B are alternative models for sequential acquisition of genetic aberrations in the stem cell compartment. **A** If the MDS stem cell is the only cell with self-renewal potential, all stable mutations found in the bulk cells and progenitors will also be present in the stem cell compartment. **B** If the MDS progenitors have acquired aberrant self-renewal ability, stable mutations may arise in such progenitors and be sustained. These mutations would not be found in up-stream stem cells but may be detected in bulk bone marrow. Curved arrows indicate self-renewal.

3.2 Materials and Methods

Patients and normal volunteers. 29 patients with low and intermediate-1 risk MDS were selected from Lund University Sweden; Karolinska Institutet, Sweden; Oslo University Hospital Norway; Aarhus University Hospital, Denmark and Hopital Saint Louis, Paris, France (**Table 3.1**). Normal age matched bone marrow was provided from volunteers recruited at Lund University Sweden; Karolinska Institutet, Sweden and Oslo University Hospital Norway. All patients and normal volunteers provided written informed consent and the study was approved by the relevant local ethics committees. Due to limitations in the number of cells available from certain patients, we were unable to perform all assays on all patients and were required to prioritise certain experiments in particular patients.

Flow cytometry. Stem/progenitor analysis/isolation of BM mononuclear was performed as described in Chapter 2 Methods.

***In-vitro* assays**. Colony-forming-cell (CFC) and long-term-culture-CFC (LTC-CFC) assays were performed as described in Chapter 2 Methods.

Gene expression analysis. Gene expression was analysed by the Fluidigm Dynamic Array as described in Chapter 2 Methods.

Xenograft transplantation. Cells were transplanted into irradiated NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice, analysed as described in Chapter 2 Methods.

Mutational analysis. Targeted DNA mutation screening of 54 genes known to be of importance in myeloid malignancies or other cancers (see Chapter 2 Methods) was performed on bone marrow mononuclear cells, purified populations and LTC-CFC colonies of 13 patients. DNA sequencing reads from bulk tumour material were compared to normal T cells to determine if a somatic mutation was present.

Statistical analysis. Statistical analysis was performed using Graphpad Prism 6 software.

Pat ID	Sex/Age	WHO Diagnosis (IPSS)	Months after diagnosis	Hb (g/L)	WCC/ANC (10^9/L)	Platelets (10^9/L)	% BM Blasts	Karyotype/FISH	On-going treatment
1	F/76	RCMD (Int-1)	6	101	2.8/1.2	104	<5	46,XX,del(5)(q14q34),t(11;16)(q13q23)del(13)(q13q21)[24]/46,XX[1]	Lenalidomide
2	F/77	RCMD-RS (Int-1)	12	114	6.3/2.3	327	<5	46,XX,del(5)(q13q15),del(11)(q14)[12]/46,XX[10]	ESA
3	M/66	RCMD (Int-1)	8	87	5.7/3.3	85	<5	61% del(5q) by FISH	Lenalidomide
4	F/71	Isol 5q (Low)	~60	101	4.0/1.8	307	<5	46,XX,del(5)(q14q33)[20]	None
5	M/83	Isol 5q MDS/MPD (Low)	0	87	22/16	877	<5	46,XY, del(5)(q21q34)	None
6	M/58	Isol 5q (Low)	12	82	4.8/2.1	166	5	46,XY,del(5)(q23q24)[4]/46,XY[20]	Lenalidomide
7	F/64	Isol 5q (Low)	0	66	4.7/2.6	235	<5	46,XX,del(5)(q13q33)[19]/46,XX[4]	None
8	F/79	Isol 5q	96	124	5.0	178	<5	46,XX,del(5)(q13	ESA

		(Low)						q33)[11]/46,XX[13]	
9	F/64	Isol 5q (Low)	0	77	14.0/10.0	487	<5	46,XX,del(5)(q14q34)[25]	None
10	F/73	RCMD-RS (Int-1)	0	91	4.9/1.6	183	<5	+8	ESA
11	M/72	RCMD-RS (Int-1)	0	103	2.4/1.5	226	<5	+8	None
12	M/62	RCMD (Int-1)	12	91	1.2/0.22	167	<5	46,XY	ESA
13	M/35	RAEB-II (Int-2)	0	110	18/8.0	36	15.5	46,XY	None
14	F/66	RCMD (Int-1)	12	105	1.9/0.4	259	<5	+8	ESA
15	F/59	Isol 5q (Low)	0	83	2.5/1.2	460	<5	46,XX,del(5)(q14 q34)[24]/46,XX[1]	None
16	F/64	RCMD (Int-1)	18	107	3.0/0.5	20	<5	46,XX,del(5)(q14q34),del(1 1)(q21)[8]/46,idem,del(12) (p12; p13)[2] /46,XX[13]	Lenalidomide

17	M/74	RAEB-1 (Int-1)	0	91	NA	50	9	46, XY	ESA
18	M/82	RCMD (Int-1)	0	112	3.1/1.9	272	<5	Del(5q), +21	Azacitidine
19	F/78	RCMD (Int-1)	0	82	4.3/2.6	192	<5	+8	None
20	F/83	Isol 5q (Low)	0	113	6.6/4.7	174	<5	46,XX,del(5)(q13q33) [14]/46,XX [11]	ESA
21	F/79	RAEB-1 (Int-1)	0	86	4.1/2.4	217	5	46,XX,del(5)(q13q33)[19]/ 46,XX[1]	ESA
22	F/63	RCMD (Int-1)	0	98	3.2	209	<5	del(5)(q31),+21[13]/46,XX[2]	None
23	F/53	Isol 5q (Low)	0	115	5.8/2.4	819	<5	46,XX,del(5)(q13q33)[17]/ 46,XX[9]	None
24	F/71	RAEB-1 (Int-1)	0	101	9.5/7.1	297	6.5	46,XX,del(5)(q12q33)[25]	None
25	M/69	RCMD	0	119	3.6/3.0	77	2.8	46 XY del(11q)	None

		(Int-1)							
26	M/61	RCMD (Int-1)	0	78	5.4/3.4	509	3	46,XY,del(5)(q13)[18]/46,idem,+21[5]/46,XY[2]	Lenalidomide
27	F/65	RCMD (Int 1)	0	102	8.7/4.4	309	<5	46, XX	None
28	M/	MDS/MPN (Int-1)	12	101	2.0/0.9	386	<5	46, XY	None
29	F/70	RCMD (Int-1)	12	118	4.4	289	<5	46,XX,del(5)(q13q33)[5]/47,XX,+8[6]/46,XX[12]	Lenalidomide

Table 3.1 Clinical and Laboratory Parameters of Low and Intermediate Risk-1 Patients

Abbreviations used: IPSS, International Prognostic Scoring System; Hb, haemoglobin; WCC, white cell count; ANC absolute neutrophil count; Plts, platelets; F, female; M, male; RCMD, refractory cytopenia with multi-lineage dysplasia; Isol 5q, isolated 5q- syndrome; RCMD-RS, refractory cytopenia with multi-lineage dysplasia with ringed sideroblasts; RAEB, refractory anaemia with excess blasts; MPD, myeloproliferative disease; Int, intermediate; ESA Erythropoiesis stimulating agent; NA, Not available.

3.3 Results

3.3.1 Low and intermediate-1 risk MDS patients retain distinct stem and progenitor populations

Stem and progenitor cells isolated from bone marrow mononuclear cells were analysed by flow cytometry and, although the profiles of some of the patients were slightly disrupted, all patients still had a full complement of identifiable stem and progenitor (CMP, GMP and MEP) populations (**Figure 3.2**). The compartments remained normal in size, similar to what was seen in the normal controls. The frequencies of stem and progenitor cells as a fraction of total mononuclear cells in the patients were not significantly different to normal age-matched controls (**Figure 3.3**). This is in sharp contrast to more aggressive myeloid malignancies, such as AML, where leukaemic progenitors will often constitute the majority of the bone marrow mononuclear cells, resulting in the inability to phenotypically distinguish all the stem and progenitor populations[34].

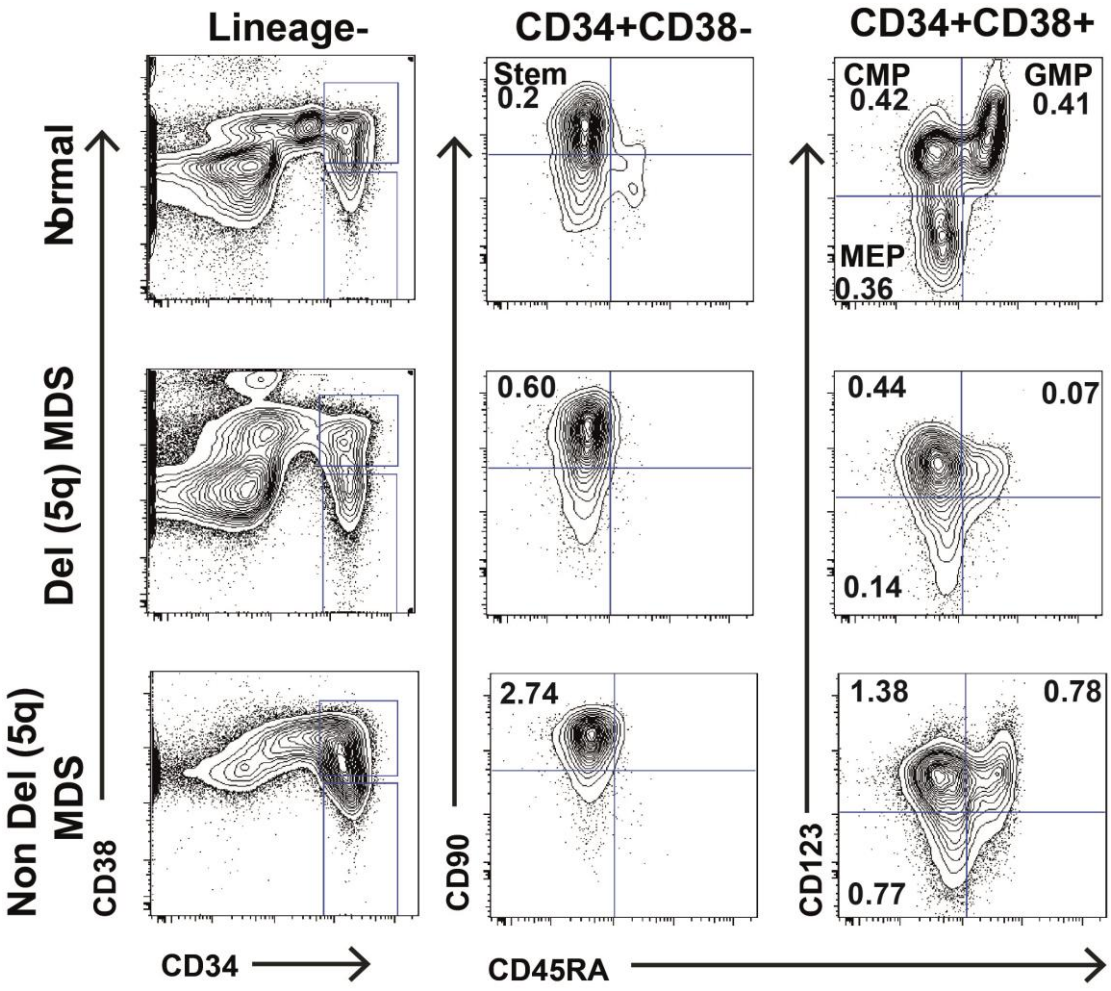


Figure 3.2 Preservation of the haematopoietic stem and progenitor hierarchy in low and intermediate-1 risk MDS patients

Representative FACS profiles of stem and progenitor populations from a healthy age-matched bone marrow (upper panel), del (5q) MDS patient (middle panel) and MDS with normal cytogenetics (lower panel). Numbers within quadrants indicate the frequency of the population in total BM.

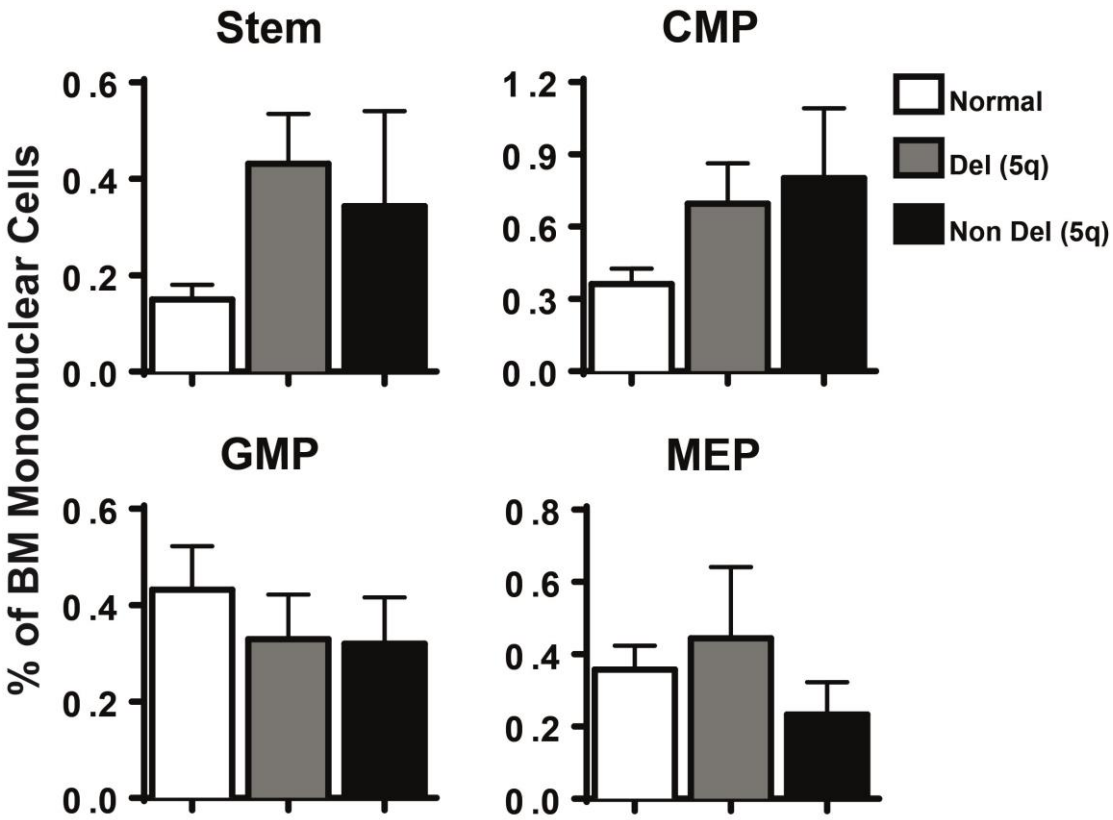


Figure 3.3 Frequencies of stem and progenitor cells in MDS patients

Mean (SEM) frequencies of populations as a percentage of bone marrow mononuclear cells, as determined by flow cytometry. There was no significant difference (student t-test) between normal (n=7), del (5q) (n=18) and non-del (5q) patients (n=11) for any of the populations.

3.3.2 MDS stem and progenitor cells are clonally involved

Having demonstrated by flow cytometry that phenotypic stem and progenitor populations were preserved in MDS patients, it was essential to demonstrate that they were clonally involved, and not composed of residual normal haematopoietic cells. Stem and progenitor cells were FACS sorted and fluorescence *in situ* hybridisation (FISH) was performed for del (5q) and trisomy 8. This demonstrated that the vast majority of stem and progenitors from del (5q) and trisomy 8 patients were clonally involved (**Figure 3.4**), confirming that the majority of these cells were indeed part of the malignant clone.

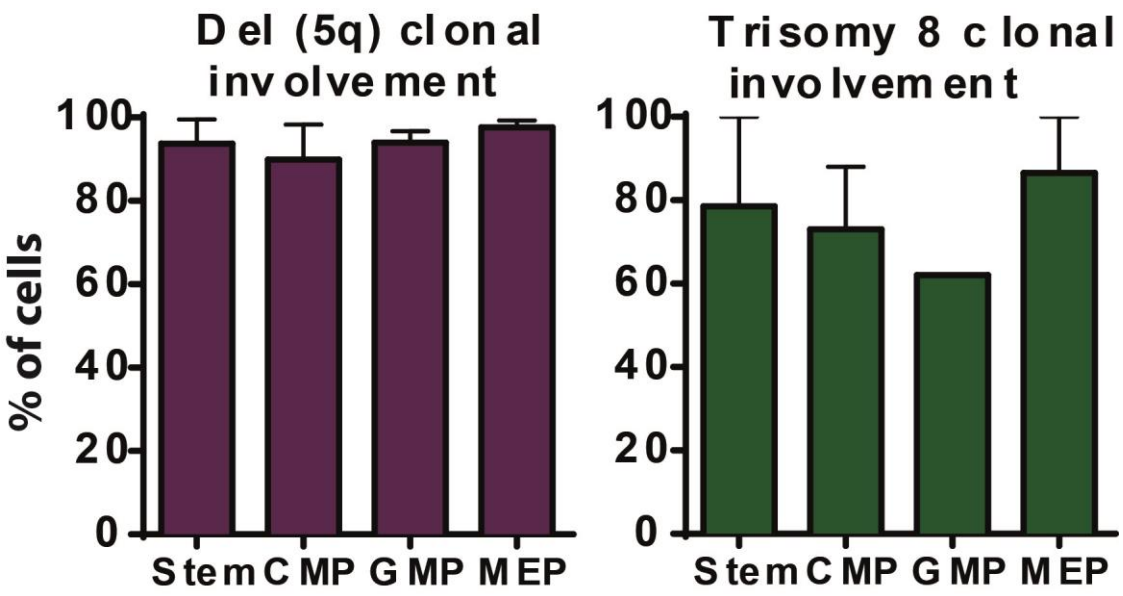


Figure 3.4 Clonal involvement of stem and progenitor populations

Mean (SEM) del (5q) (n=3) and trisomy 8 (n=3) involvement of FACS purified populations determined by FISH. Cells were centrifuged onto slides and hybridised with a specific probe against the 5q31 region or the centromeric region of chromosome 8. At least 100 cells were counted per population when available.

3.3.3 MDS progenitor cells have distinct gene expression profiles

In order to determine if these phenotypically distinct populations were molecularly distinct, as is the case for normal stem and progenitors, quantitative gene expression analysis was performed. The GMP and MEP population were chosen for this analysis, rather than the CMP as these two populations have clearly defined gene expression patterns that are very different from each other. In all the MDS patients studied (n=10) GMPs retained a myeloid gene expression signature and MEPs an erythroid gene expression signature (**Figure 3.5**). This provides supportive evidence that the flow cytometry cell surface markers (CD34, CD38, CD45RA and CD123) used to identify different progenitor populations were sufficient to isolate distinct progenitor populations. Interestingly, the four non del (5q) MDS patients included in this part of the study seemed to have markedly lower expression of almost all the myeloid and erythroid genes analysed. However the number of patients analysed was small and the patients were composed of a heterogeneous group of low/int-1 risk group. It would be valuable to extend this analysis to a larger group of non del (5q) patients to see if any further associations, such as specific cytogenetic aberrations, could be drawn.

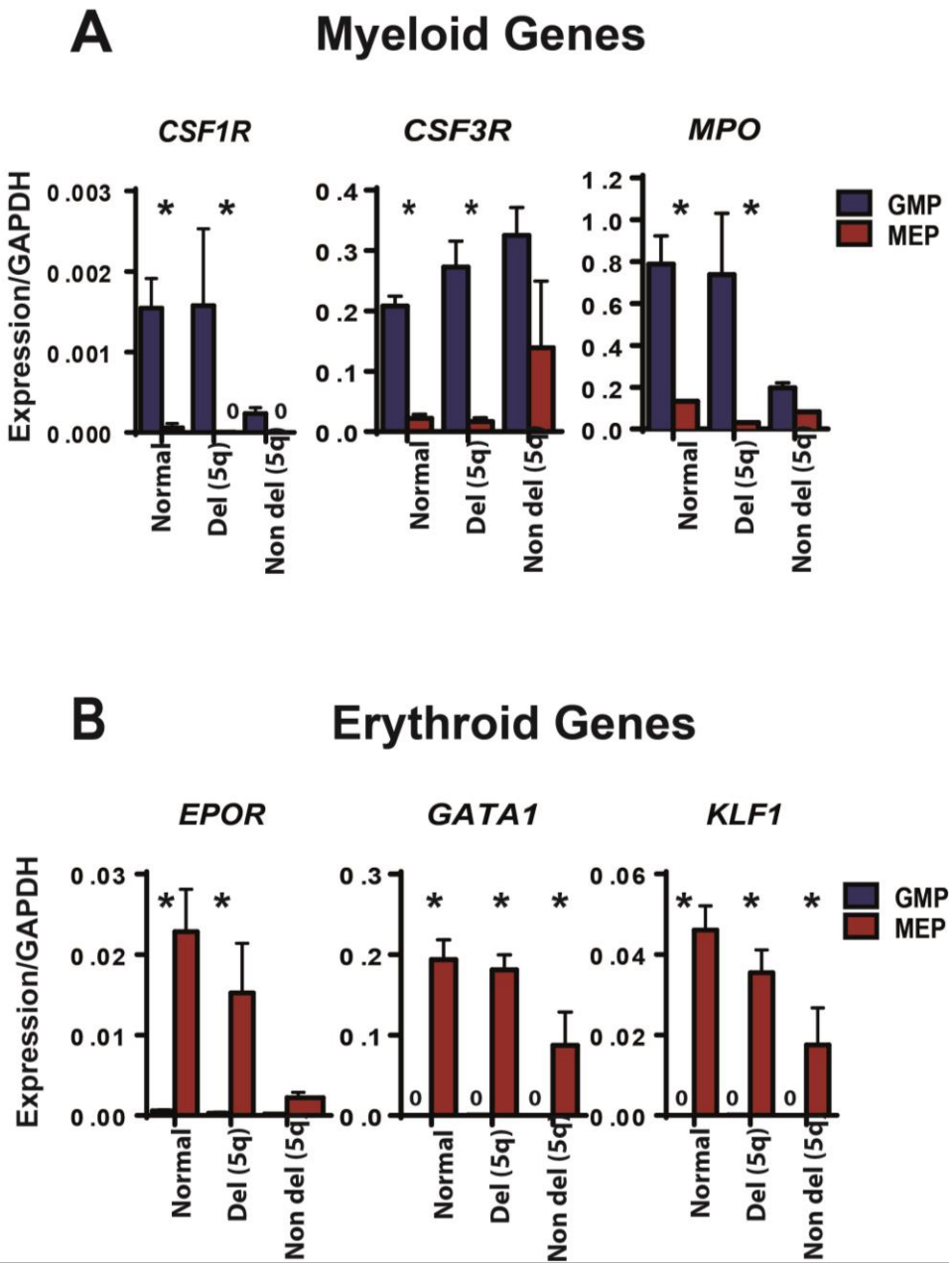


Figure 3.5 Molecularly distinct progenitors in MDS patients

Mean (SEM) expression of myeloid and erythroid transcripts within GMPs and MEPs from normal BM (n=7), del (5q) MDS (n=6) and non-del (5q) MDS (n=4). (*p<0.05 by Mann-Whitney test). 50-100 cells per population per patients were FACS sorted in 2 replicates and underwent multiplexed quantitative PCR, using the BioMark 48.48 Dynamic Array platform (Fluidigm).

3.3.4 MDS progenitors are functionally distinct *in vitro*

In order to determine the myeloid and erythroid lineage potentials of the purified GMPs and MEPs, methylcellulose colony forming cell (CFC) assays were performed. When CFC assays were performed on FACS sorted GMPs and MEPs from MDS patients (n=19), GMPs only gave rise to myeloid colonies and the MEPs predominantly gave rise to erythroid colonies (**Fig 3.6**). This is what was seen when normal bone marrow GMPs and MEPs were plated, confirming that these MDS progenitors retain their expected functional capacity. These experiments all confirmed that MDS progenitors are phenotypically, molecularly and functionally distinct from each other.

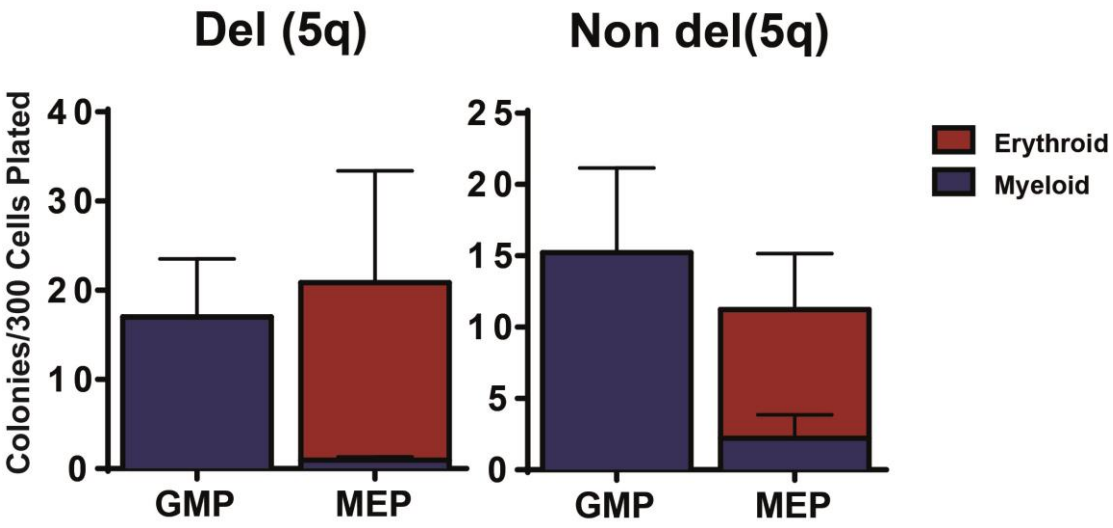


Figure 3.6 Functionally distinct progenitors in MDS

Mean (SEM) myeloid and erythroid colony formation of FACS sorted GMPs and MEPs from del (5q) MDS (n=12) and non-del (5q) MDS (n=7) patients. 150-300 sorted cells per population per patient were plated in 2 replicates in methylcellulose + cytokines (see Chapter 2 methods). Cells were cultured for 14 days and colonies formed were enumerated and characterised as myeloid or erythroid based on morphology and cytospins.

3.3.5 MDS stem cells are molecularly distinct and the only cells with self-renewal ability

Having clearly demonstrated that the GMPs and MEPs are lineage restricted, we next wanted to investigate whether they had acquired aberrant self-renewal potential. Alongside this we also wanted to determine if purified phenotypically defined stem cells (CD34+CD38-CD90+CD45RA-) were also molecularly and functionally distinct from the downstream progenitors, as would be expected for a candidate MDS stem cell. Gene expression analysis of established stem cell genes revealed that MDS stem cells retained a similar signature to stem cells from healthy controls, which was distinct from the signature of progenitors (**Figure 3.7**).

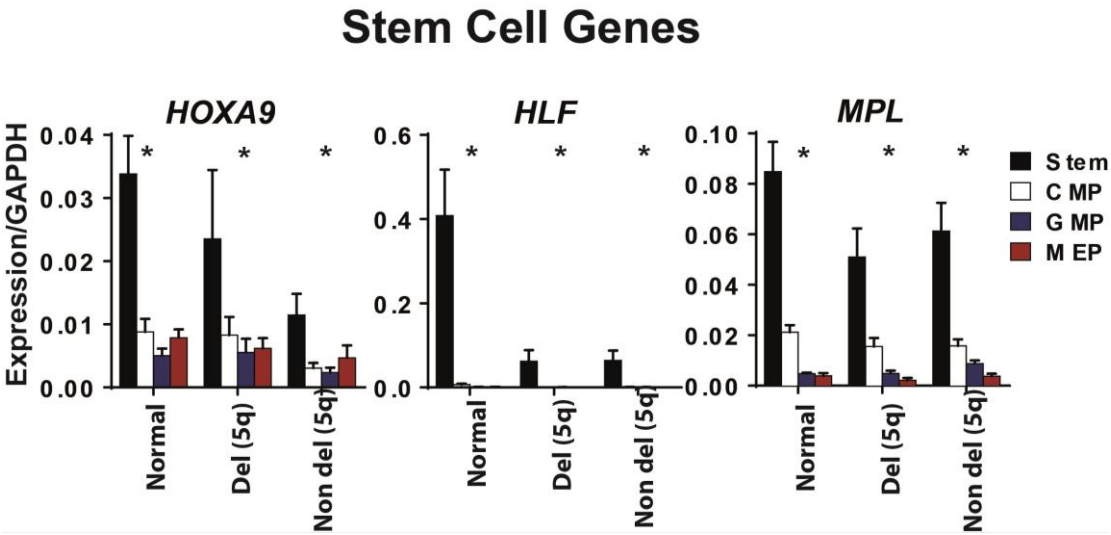


Figure 3.7 Molecularly distinct CD34+CD38-CD90+CD45RA+ stem cells in MDS

Mean (SEM) expression of transcripts of three stem cell genes (*HOXA9, HLF* and *MPL*) in healthy age-matched controls (n=7), del (5q) MDS (n=6) and non-del (5q) MDS (n=4) stem and progenitor cells. (*p<0.05 refers to pair wise comparisons between the stem/cmp, stem/gmp and stem/mep using the Mann-Whitney test). 50-100 cells per population per

patient were FACS sorted in 2 replicates and underwent multiplexed quantitative PCR, using the BioMark 48.48 Dynamic Array platform (Fluidigm).

One of the absolutely key defining features of a stem cell is its unique ability to self-renew long term. In order to examine this feature we first used the LTC-CFC assay. After 6 weeks of culture, only the purified phenotypically defined stem cells were able to produce myeloid colonies, confirming that they were the only population with long-term self-renewal ability (**Figure 3.8**). Importantly none of the MDS progenitor populations had gained self-renewal capacity.

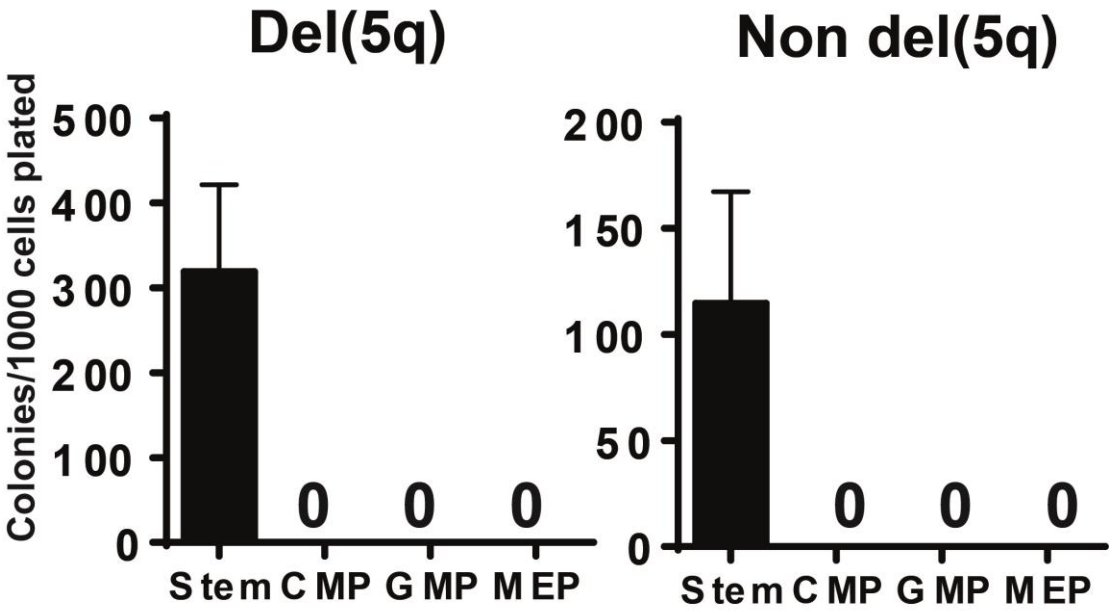


Figure 3.8 Functionally distinct CD34+CD38-CD90+CD45RA+ stem cells in MDS

Mean (SEM) LTC-CFC formation of purified stem and progenitor cells from del (5q) MDS (n=15) and non-del (5q) MDS (n=11). 500-2000 cells per population per patient were co-cultured in 2-3 replicates on a stromal feeder cell layer for 6 weeks. A 50% media change was performed weekly and at 6 weeks cells were transferred to methylcellulose, supplemented with cytokines. After 14 days in methylcellulose, colonies were enumerated. Individual colonies were also picked for FISH and DNA mutation analysis.

3.3.6 MDS bone marrow rarely reconstitutes NSG mice

To further establish which populations were capable of disease propagation xenotransplantation of MDS patient bone marrow cells was performed. Initially unfractionated CD34+ enriched cells from 19 MDS patients, where sufficient material was available, were transplanted into sub-lethally irradiated NSG mice. Engraftment was defined using the presence of human CD45 as a pan-human leucocyte marker. Only 2 patients engrafted at greater than 0.1% human CD45 as a percentage of total CD45 following bulk transplantation (**Figure 3.9**). This low engraftment rate is in keeping with previous studies of MDS patients, although a recent study did achieve improved levels of engraftment from patients with MDS with monosomy 7[109,110].

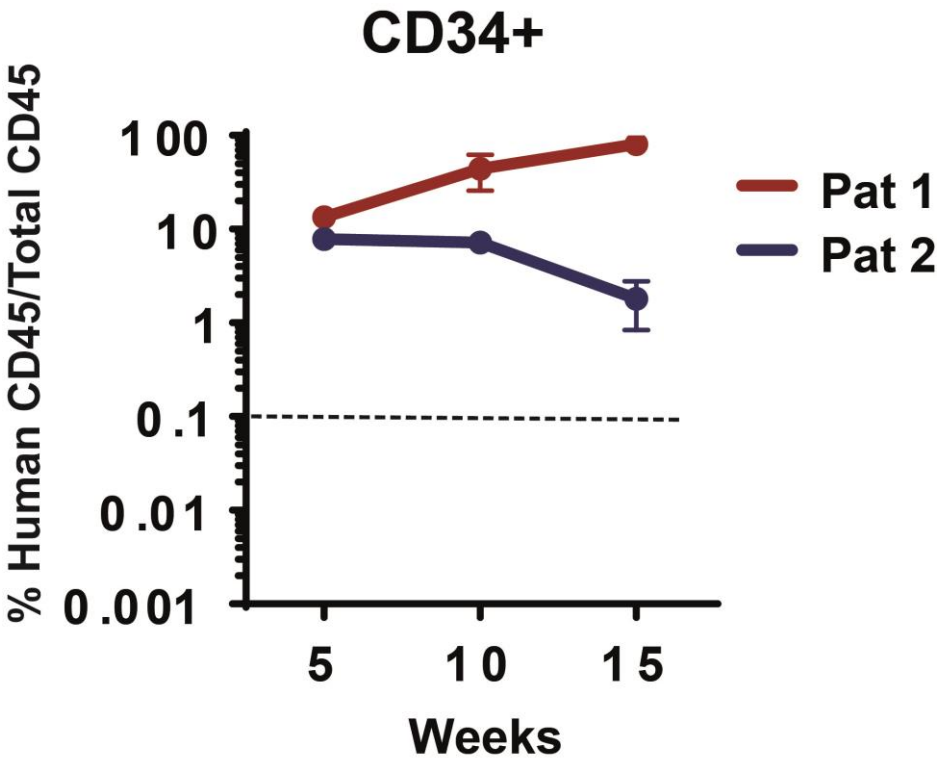


Figure 3.9 Human engraftment in the bone marrow of NSG mice

Mean (SEM) engraftment. Mice were injected with CD34 enriched bone marrow from patients 1 and 2. Bone marrow aspirations were performed 5, 10 and 15 weeks post transplantation (2-5 mice per sample). Human engraftment was determined using flow cytometry and was defined as >0.1% hCD45+ cells as percentage of total CD45 (mouse + human CD45).

3.3.7 MDS stem cells are the only purified population with reconstitution ability

When purified stem and progenitor populations were transplanted from the two engrafting patients, only the CD34+ CD38- CD90+ CD45RA- stem cells and not the downstream progenitors engrafted (**Figure 3.10**) as is the case with normal age matched bone marrow. This suggests that the MDS stem cells are the likely candidate population with disease propagating ability. These results are also consistent with those from the LTC-CFC assay, where only the stem cells gave rise to colonies (**Figure 3.8**).

When stem cells from normal donors engraft in NSG mice, B lymphopoiesis dominates. However, as B cells are severely suppressed in MDS patients[111], mice engrafted with MDS stem cells showed myeloid biased reconstitution (**Figure 3.11**).

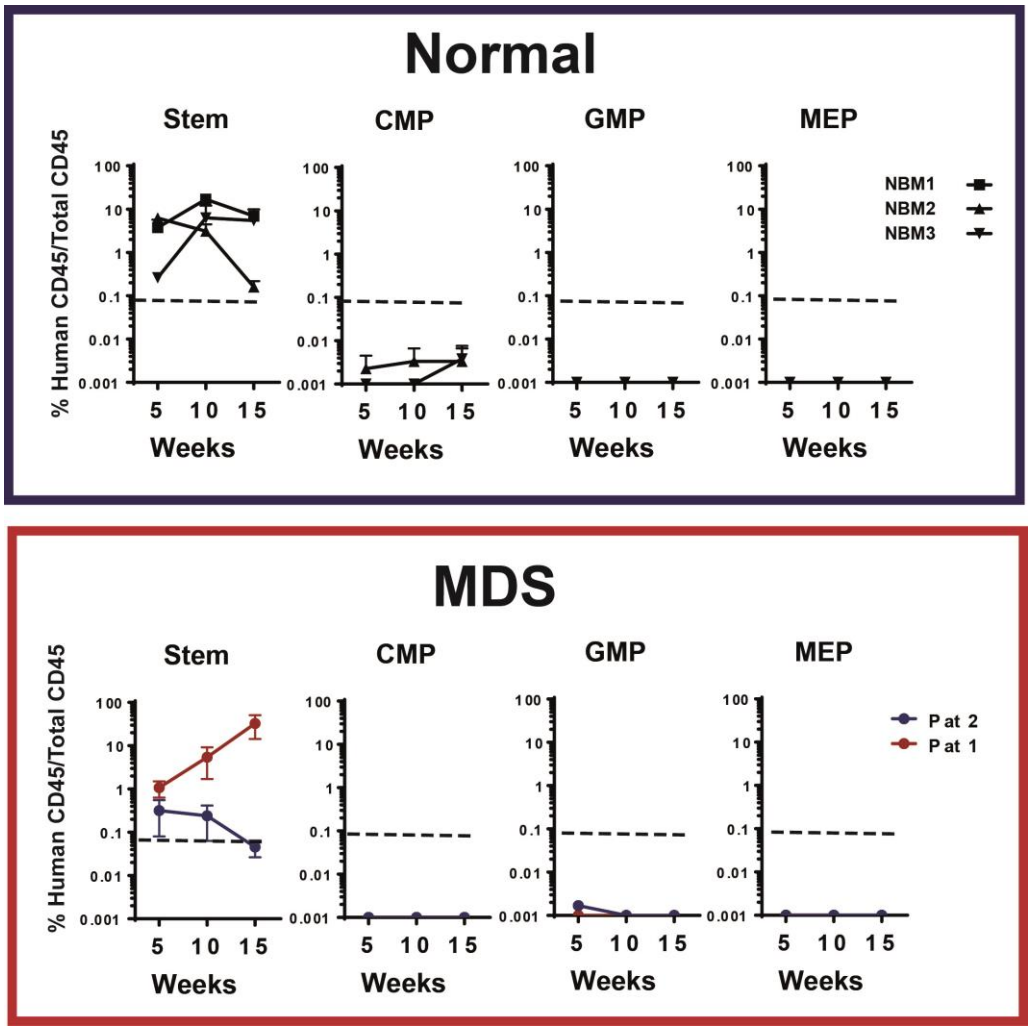


Figure 3.10 Engraftment in NSG mice following transplantation of purified stem and progenitor cells

Mean (SEM) human (hCD45) engraftment in the bone marrow of NSG mice (2-5 mice per cell population) transplanted with purified stem/progenitor cells from normal age-matched controls (upper panel) and del (5q) MDS (Patient 1 and 2), examined by bone marrow aspiration at 5, 10 and 15 weeks post transplantation.

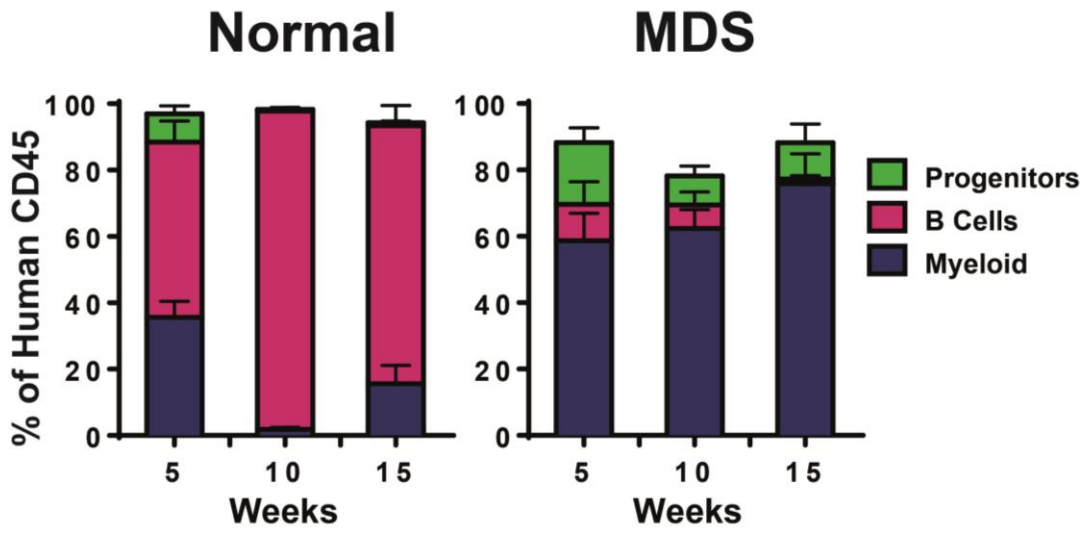


Figure 3.11 Lineage biased engraftment in normal and MDS patients

Mean (SEM) lineage distribution (Myeloid CD15+CD33+CD66b+; Lymphoid CD15-CD33-CD66b-CD19+; Progenitors CD34+) of the engrafted hCD45+ cells examined by bone marrow aspiration at 5, 10 and 15 weeks post transplantation of stem cells from age matched normal controls (n=3) and MDS patients (Patients 1 and 2).

3.3.8 Clonally involved MDS stem and progenitor cells are hierarchically arranged

FACS profiles of the NSG mice transplanted with purified stem cells from patient 1, demonstrated self-renewal of human stem cells and replenishment of the downstream progenitors (**Figure 3.12**). Over 90% of the stem and progenitors present in these mice were part of the MDS clone, as evidenced by presence of the 5q deletion by FISH (**Figure 3.13**). As only stem cells and not progenitors were able to engraft and reconstitute all stem and progenitor populations, this provided definitive *in vivo* evidence for a hierarchical relationship, with stem cells residing at the top.

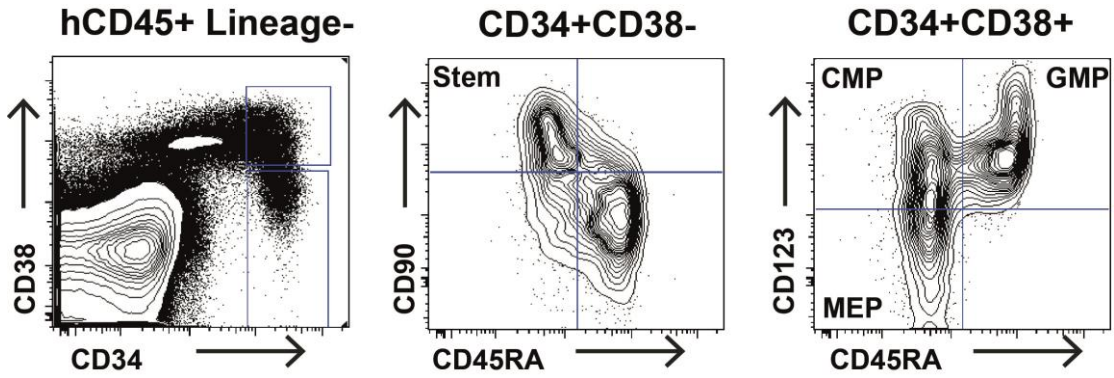


Figure 3.12 Hierarchical replenishment of human stem and progenitor cells in NSG mice

Representative stem and progenitor FACS profile of bone marrow mononuclear cells isolated from an NSG mouse engrafted with stem cells from patient 1.

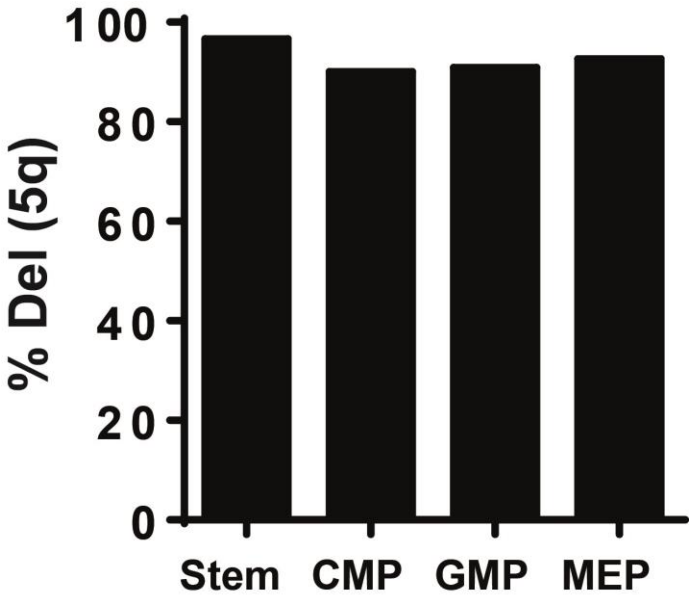


Figure 3.13 Clonal involvement of stem and progenitor cells isolated from an NSG mouse engrafted with stem cells from Patient 1

Del 5q involvement, determined by FISH, of purified populations isolated from NSG bone marrow following transplantation of stem cells from patient 1.

In order to see if MDS stem cells are capable of replenishing molecularly distinct stem cells and myelo-erythroid progenitor cells in NSG mice, gene expression analysis was performed on stem cells, GMPs and MEPs from NSG mice engrafted with stem cells from Patient 1 (**Fig 3.14**). The results confirmed the presence of stem and progenitor populations with the same lineage restricted gene expression signatures in the reconstituted mice as in the patient (**Figure 3.5** and **Figure 3.7**).

These key experiments clearly show that not only are CD34+CD38-CD90+CD45RA- MDS stem cells the only population able to reconstitute mice, they also give rise to CMPs, GMPs and MEPs. This confirms a distinct hierarchical relationship between the MDS stem cell and the downstream progenitors. Secondary transplants from primary mice transplanted with MDS stem cells were then performed and demonstrated multi-lineage engraftment, confirming self-renewal (data not shown).

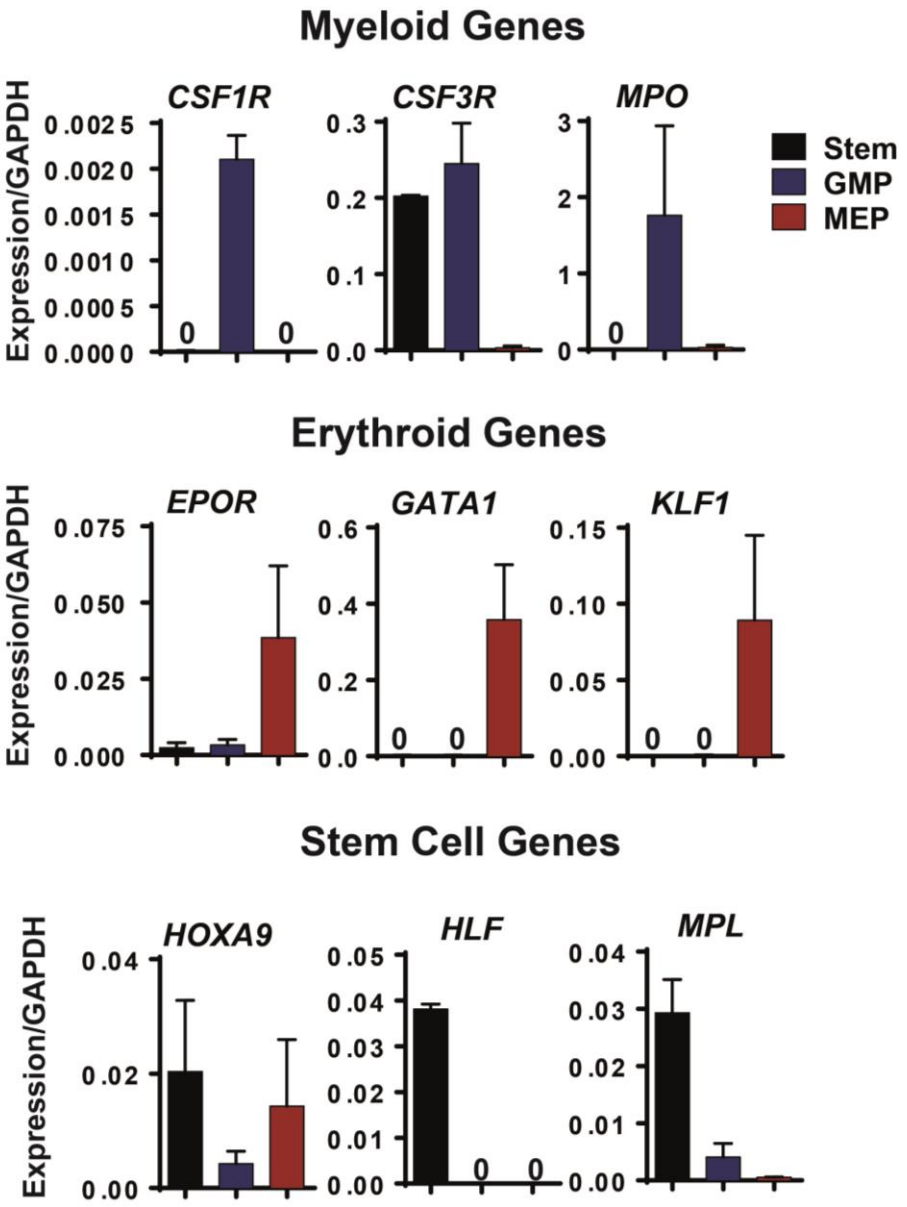


Figure 3.14 Replenishment of molecularly distinct stem and myelo-erythroid progenitor cells in NSG mice engrafted with stem cells from Patient 1

Mean (SEM) gene expression in stem/progenitor cells purified from the bone marrow cells of NSG mice transplanted with purified MDS stem cells from patient 1 (n=3). Bone marrow cells were harvested from the engrafted mice and human stem and progenitor cells were FACS sorted before undergoing quantitative PCR.

In summary, these results confirm that only CD34+CD38-CD90+CD45RA- MDS stem cells are able to self-renew and give rise to distinct downstream progenitors, thus supporting the hypothesis that such MDS stem cells are the origin of disease, essential for its propagation and are likely to require eradication to achieve cure. This supports the hierarchical cancer stem cell model and refutes the stochastic model in low and intermediate risk MDS.

3.3.9 DNA mutations originate exclusively in MDS stem cells

13 patients had sufficient bone marrow material to undergo targeted DNA mutation screening. In total 30 somatic genetic aberrations, including del (5q), were detected in the bulk bone marrow of these patients (**Table 3.2**). Mutations in epigenetic regulators (ASXL1, TET2) transcription factors (RUNX1, ETV6), apoptosis regulators (TP53), signalling molecules (JAK2, CSF3R) and splice factors (SF3B1, SFRS2, U2AF2 and SRSF6) were detected. All the patients who were known to have a del (5q) by standard karyotyping, also had a detectable del (5q) by Haloplex gene analysis, using the presence of recurrent SNPs on the commonly deleted region (CDR) of chromosome 5 (see Chapter 2 Methods). The most common mutation was in *JAK2* (5/13). The most frequent combination of events was del (5q) plus *JAK2* V617F (4/13). The majority of patients had 2 or more genetic changes (10/13).

		Pat 1	Pat 3	Pat 4	Pat 5	Pat 6	Pat 7	Pat 8	Pat 9	Pat 10	Pat 11	Pat 12	Pat 13	Pat 14
	Del(5q)													
Cytokine Signalling	JAK2	V617F	V617F	V617F	V617F						N1108S			
	CSF3R					Q754*								
Epigenetic Regulators	TET2									R544*	L34F			
	ASXL1					R634fs	I641L							R417*
	ETV6					R398C								
Transcription Factors	RUNX1									P425L	L95S			
	TP53		V274F	S241P										
Splice Factors	SF3B1							K700E		R625C	K700E			
	SRSF2											P95H		
	U2AF2												S79P	
	SRSF6												T185K	

Table 3.2 DNA mutation screening of bulk bone marrow cells from 13 MDS patients

Bone marrow mononuclear cells (500,000-1,000,000 per patient) underwent targeted Haloplex DNA sequencing of selected recurrently mutated genes (see Chapter 2 Methods). Peripheral blood or bone marrow FACS sorted T cells (CD11b-CD14-CD19-CD34-CD3+CD4+/CD8+) were used as somatic controls. Genetic changes were defined as somatic mutations if the mutated read frequency was <5% in the T cell control material and present at least a 5 times higher frequency in the bulk bone marrow. For detection of deletion of chromosome 5q, frequent SNPs within the common deleted region (CDR) were used.

In the 13 patients analysed, *all* the mutations detected in the bulk bone marrow could be traced back to the MDS stem cell. In addition to being detected in the FACS purified stem cells, all mutations could be detected in at least one of the LTC-CFC colonies derived from single FACS purified stem cells. In the patient 1 stem cell engrafted NSG mice, the genetic aberrations seen in the patient could also be found in the myeloid cells of the mice.

These data collectively confirmed that the origin of all the mutations was the MDS stem cell and all mutations were then inherited by the lineage restricted progenitors, as would be expected in a hierarchically organised disorder.

I will now focus on three very interesting case studies to illustrate some additional novel findings.

3.3.9.1 Patient 1

A 76 year old female, presented with anaemia. Following a bone marrow biopsy, she was diagnosed with MDS and classified as Refractory Cytopenia with Multi-lineage Dysplasia (RCMD) with an IPSS score of Int-1 (**Table 3.1**). She was commenced on Lenalidomide but did not respond to treatment and was taken off Lenalidomide after just 4 months of treatment. Following this she was managed with best supportive care and remained transfusion dependent. She later died of an unknown cause, which was thought not to be related to her diagnosis of MDS.

Bone marrow cells isolated prior to treatment with Lenalidomide were screened for DNA mutations (**Figure 3.15**). T cells were FACS sorted from bone marrow at the same time point. The del (5q) cytogenetic aberration, as well as being seen by FISH (**Figure 3.4**), was also evident via DNA mutation screening. In addition to the known del (5q), a *JAK2* V617F mutation was evident in 25% of the reads in the bulk marrow.

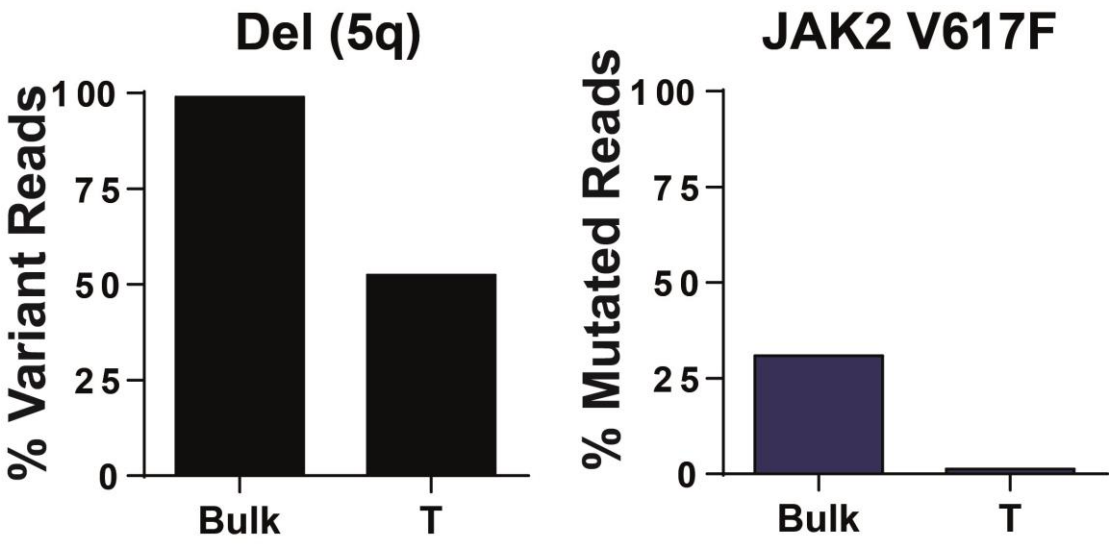


Figure 3.15 Haloplex mutation screening of bulk bone marrow and T cells from Patient 1

Bone marrow mononuclear cells underwent targeted Haloplex DNA sequencing of selected recurrently mutated genes (see Chapter 2 Methods). Bone marrow FACS sorted T cells (CD11b-CD14-CD19-CD34-CD3+CD4+/CD8+) were used as somatic controls. Y-axis numbers indicate % mutated reads within reported genes

Stem and progenitor cells were then FACS purified from bone marrow mononuclear cells from the same time point. The loss of heterozygosity for the 5q CDR and the *JAK2* V617F mutation could be detected in the purified GMPs, MEPs and MDS stem cells (**Figure 3.16**).

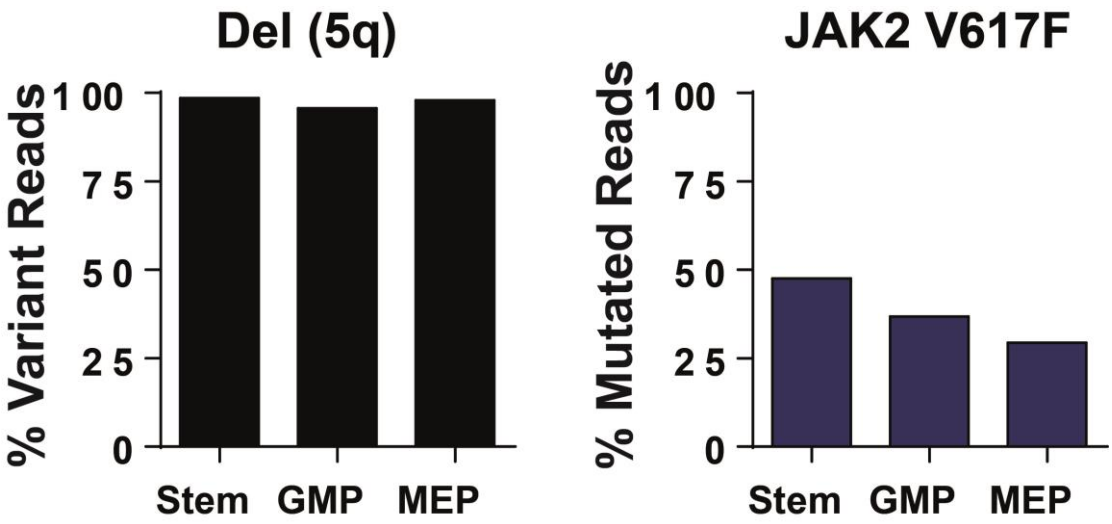


Figure 3.16 Haloplex mutation screening of stem/progenitor cells in Patient 1.

Purified populations underwent the same targeted Haloplex DNA sequencing of selected recurrently mutated genes as the bulk bone marrow. 100 cells were sorted per population in two replicates for sequencing.

Purified stem cells (200-1000 per well) were plated into LTC-CFC assays and cultured for 6 weeks (See Chapter 2 Methods). At 6 weeks, each well was then examined by light microscopy before transferring all the cells from one well to one methylcellulose dish (H4100). Cells were then incubated at 37^{o}C 5% CO_2 for 14 days. At this 8 week time-point, only clones derived from functionally defined stem cells would be present. Importantly all clones derived from one methycellulose dish may be derived from the same stem cell and

cannot be viewed as independent. Therefore, only clones derived from separate methycellulose dishes can be defined as having arisen from independent stem cells.

Four individual stem cell derived clones then underwent the same haloplex DNA mutation screening as the purified stem and progenitor populations. As each clone is derived from a single stem cell, any genetic changes were scored as present (+) if the variant read frequency was greater than 25% or absent (-) if the variant frequency was <5%. Any colonies with a read frequency of 5-25% were scored as indeterminate.

Both the del (5q) and *JAK2* V617F mutation were present in all 4 clones sequenced (**Figure 3.17**). This strongly supports our hypothesis that the 5q deletion and JAK2 mutation arose in the stem cell which then allowed the propagation of the clone into downstream progenitors. However it was not possible to tell what the likely order of acquisition of the del (5q) or the *JAK2* V617 mutation was, as the two genetic events were both present in all populations and LTC-CFC clones. Analysis of more clones may reveal more heterogeneity.

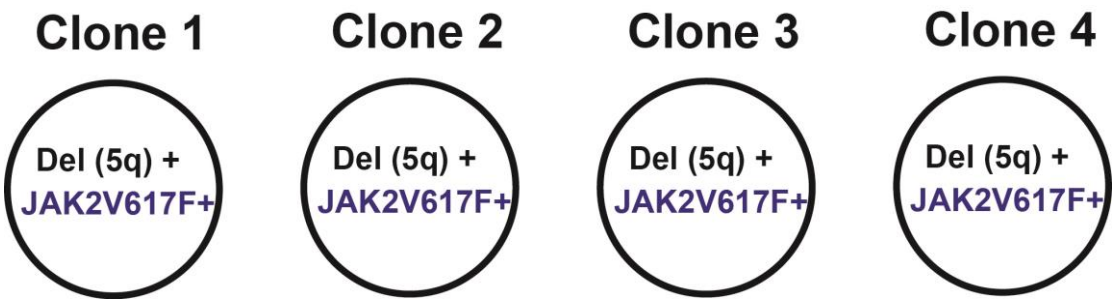


Figure 3.17 Clonal involvement of LTC-CFCs in Patient 1

Individually picked LTC-CFCs underwent Haloplex mutation screening and were scored as positive (+) if the variant read frequency was >25% or absent (-) if the variant frequency was <5%. Any colonies with a read frequency of 5-25% were scored as indeterminate.

As patient 1 was one of the patients which were able to reconstitute NSG mice (**Figure 3.9**), FACS sorted myeloid cells from the mice transplanted with purified MDS stem cells were also screened for the del (5q) and *JAK2* V617F mutation (**Figure 3.18**). Both aberrations were clearly evident by haloplex DNA screening in the human myeloid cells derived from the engrafted mice.

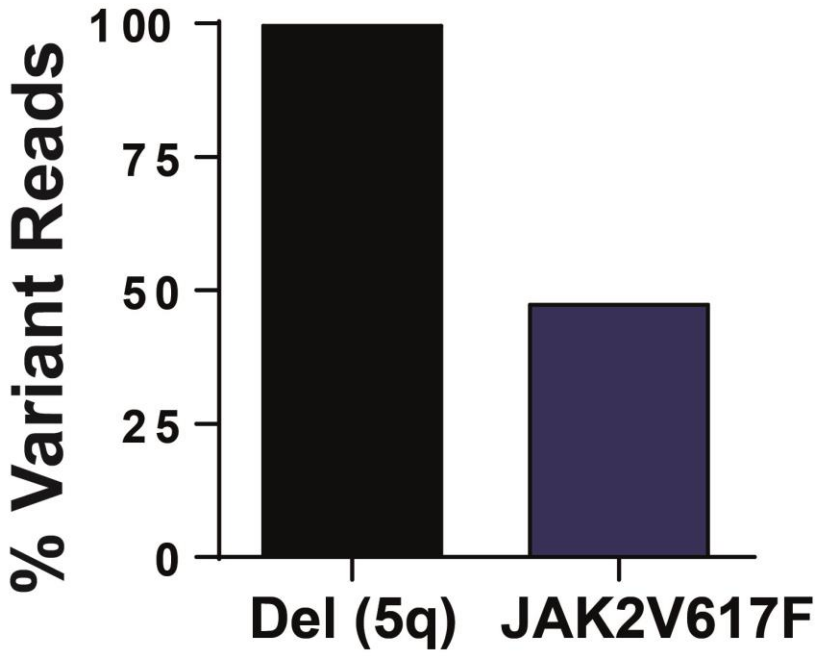


Figure 3.18 Clonal involvement of del (5q) and JAK2 V617F in purified human myeloid cells, engrafted in NSG mouse

Human myeloid cells (hCD45+CD15+CD33+CD66b+) were FACS purified from mice engrafted with stem cells from patient 1, 16 weeks after transplantation. Myeloid cells underwent the same targeted Haloplex DNA sequencing of selected recurrently mutated genes.

3.3.9.2 Patient 6

A 58 year old male, presented with anaemia and was diagnosed with isolated del (5q) syndrome. He was started on Lenalidomide and responded initially with a complete clinical and cytogenetic remission. One year later he became progressively anaemic and was deemed to have lost response to Lenalidomide. He then progressed to RAEB-I (blast count 5%) and was commenced on Azacitidine to which he did not respond. He quickly progressed to RAEB-II (blast count 17%) within a few months and was treated with AML induction chemotherapy, before undergoing allogeneic stem cell transplantation. DNA mutation analysis was performed on bone marrow cells obtained whilst he was losing response to Lenalidomide but prior to commencing Azacitidine. DNA mutation analysis of bulk bone marrow revealed a mutation in ASXL1, ETV6 and the CSF3-R. As with the previous patient all genetic aberrations could be traced back to all the stem and progenitor populations (**Figure 3.19**).

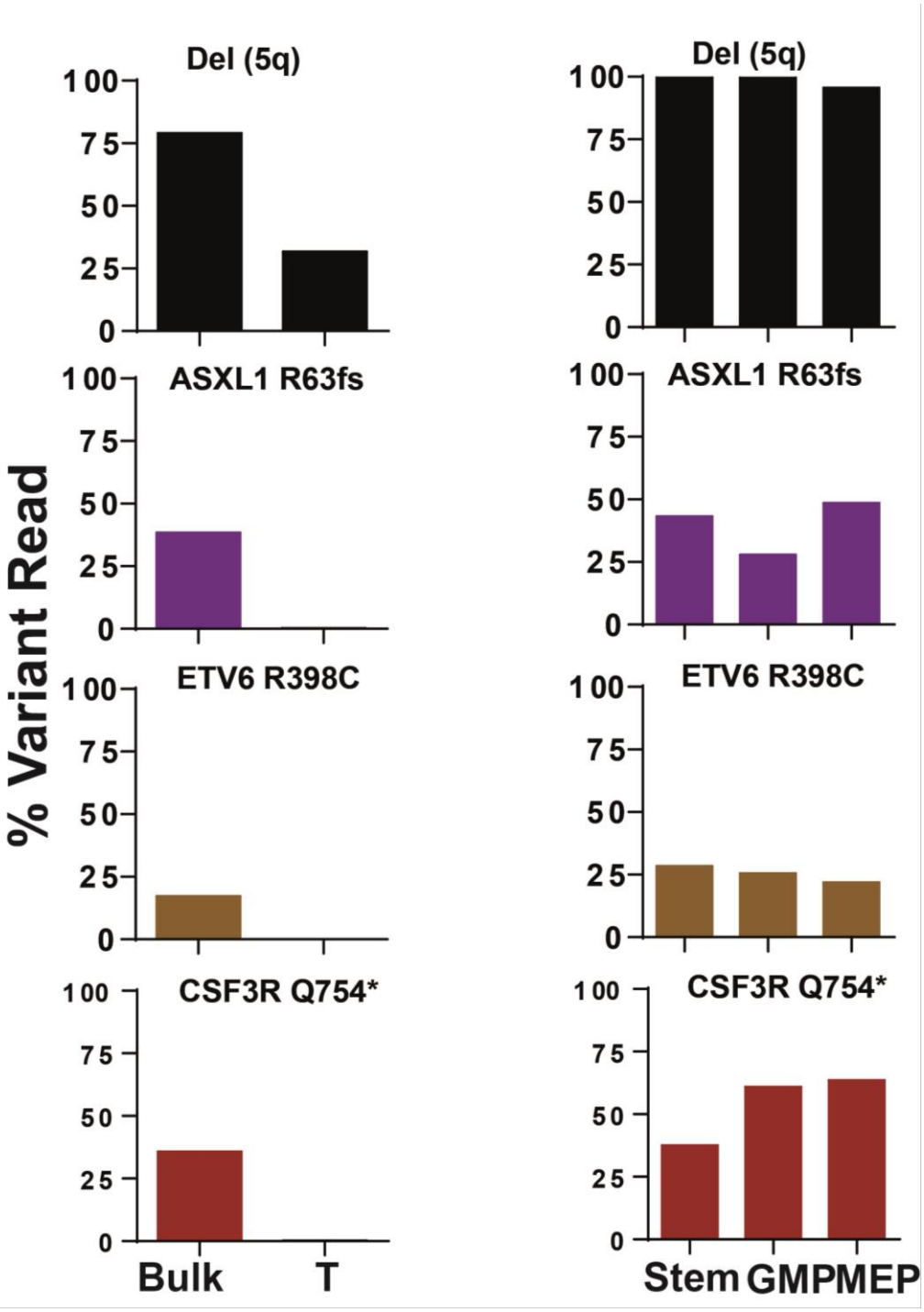


Figure 3.19 DNA mutation screening from Patient 6

Bone marrow bulk mononuclear cells and FACS sorted populations underwent targeted Haloplex DNA sequencing of selected recurrently mutated genes (see Chapter 2 Methods). Bone marrow FACS sorted T cells (CD11b-CD14-CD19-CD34-CD3+CD4+/CD8+) were used as somatic controls. Y-axis numbers indicate % mutated reads within reported genes.

Interestingly, when LTC-CFC colonies were screened for aberrations, the del(5q) was evident in all 4 picked clones but the ASXL1, ETV6 and CSF3-R mutations were evident in only 3/4 colonies (**Figure 3.20a**). This suggests that the del (5q) may have originated before the three additional mutations in the stem cells (**Figure 3.20b**). However, a larger number of colonies would need to be screened in order to draw more statistically robust conclusions.

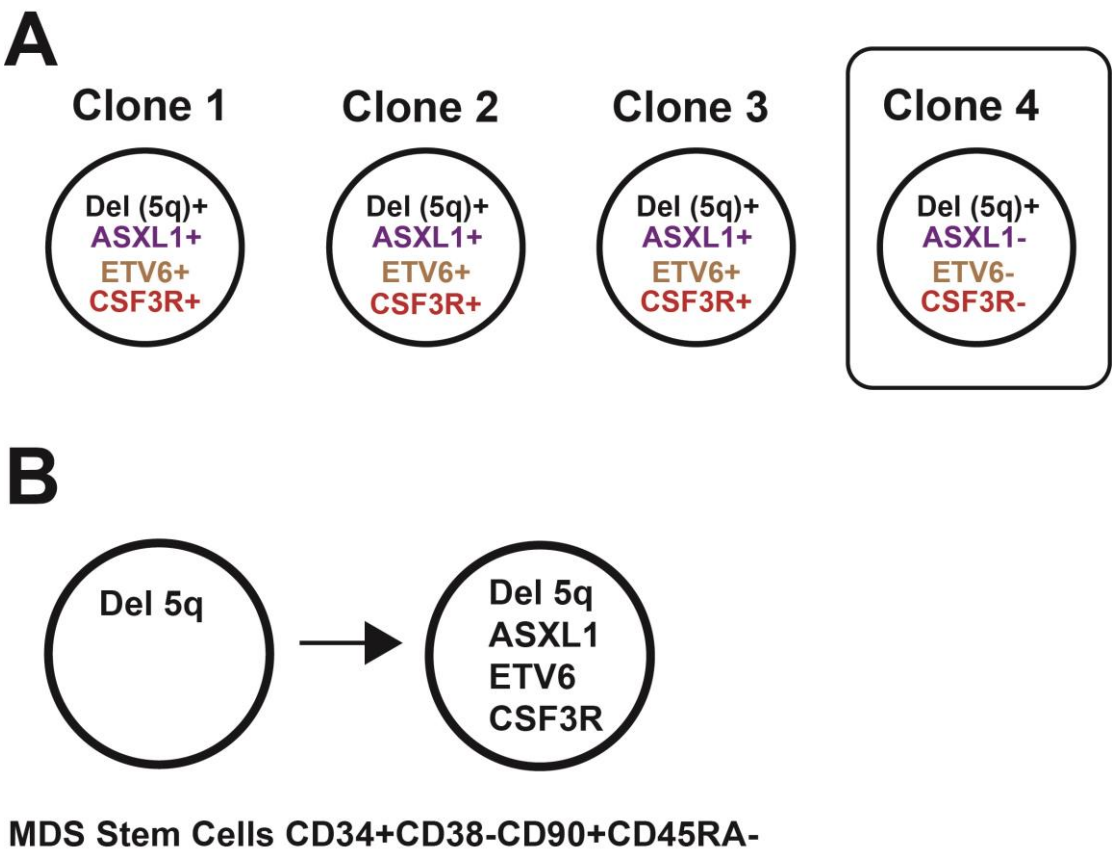


Figure 3.20 Del (5q) precedes all other somatic mutations in Patient 6

A. Individually picked LTC-CFCs derived from single stem cells were scored as positive (+) if the variant read frequency was >25% or absent (-) if the variant frequency was <5%. Any colonies with a read frequency of 5-25% were scored as indeterminate. **B** Model of acquisition of genetic changes based on clonal LTC-CFC results suggesting the del (5q) precedes the acquisition of the additional mutations in the MDS stem cell.

3.3.9.3 Patient 10

Patient 10, a 53 year old female, was diagnosed with Refractory Cytopenia with Multi-lineage Dysplasia and Ring Sideroblasts (RCMD-RS). Karyotyping revealed a trisomy 8 aberration but no del (5q). She was enrolled in a clinical trial investigating the role of Azacitidine in low and intermediate risk MDS patients. She was one of the few patients who achieved an excellent clinical response, becoming transfusion independent, although there was no cytogenetic response. She remains well and on Azacitidine to date. DNA mutation screening revealed three mutations in *TET2*, *RUNX1* and *SF3B1*, a splice factor known to be recurrently mutated in patients with MDS and ring sideroblasts (**Figure 3.21**). All mutations were detected in the stem and progenitor cells analysed. Unfortunately there was not adequate coverage of the *RUNX1* gene in two of the four colonies. Therefore we can only conclude that all the mutations were present in 2 colonies.

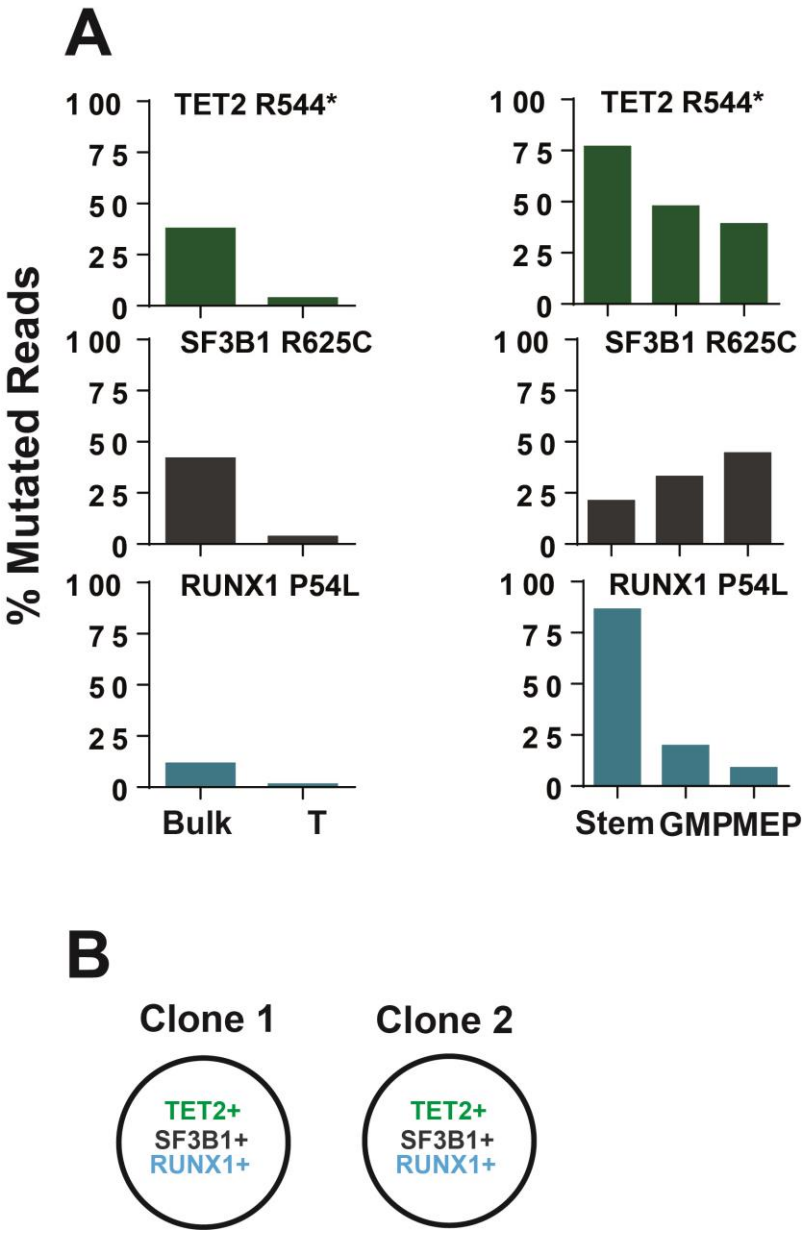


Figure 3.21 DNA mutation screening from patient 10

A Bone marrow bulk mononuclear cells and FACS sorted populations underwent targeted DNA sequencing of selected recurrently mutated genes (see Chapter 2 Methods). Bone marrow FACS sorted T cells (CD11b-CD14-CD19-CD34-CD3+CD4+/CD8+) were used as somatic controls. Y-axis numbers indicate % mutated reads within reported genes. **B** Two individually picked LTC-CFCs were analysed and scored as positive (+) if the variant read frequency was >25% or absent (-) if the variant frequency was <5%. Any colonies with a read frequency of 5-25% were scored as indeterminate.

3.4 Discussion

In order to develop effective new anti-cancer agents, identification of the propagating cells in a particular tumour is of paramount importance. Moreover, establishing whether cancer cells are hierarchically or stochastically arranged provides key information regarding which cells should be targeted. If only rare well-defined cancer stem cells at the top of a hierarchy are able to self-renew and replenish downstream progeny then the ability to cure disease is dependent on elimination of these particular cells. In contrast, if cancer cells are not hierarchically arranged, then cure may be reliant on the elimination of numerous cell types. We hypothesised that the former is most likely to be true in low/intermediate-1 risk MDS.

It has previously been demonstrated that the cell of origin in del (5q) MDS is likely to be a CD34+ CD38- CD90+ multi-potent stem cell[43]. These del (5q) MDS stem cells also appeared to be clinically important, as they persisted in patients who achieved a complete clinical and cytogenetic response to Lenalidomide[103]. Over time the persistence of the del (5q) MDS stem cells enabled clonal expansion and replacement of normal haematopoiesis. This selective resistance of del (5q) MDS stem cells to Lenalidomide is likely to be the cause of eventual treatment failure in patients previously responsive to therapy. However these previous studies were limited to del (5q) MDS patients and the assays used to detect MDS cancer stem cell potential have lacked sensitivity, resulting in the inability to definitively say that these CD34+ CD38- CD90+ cells really are the *only* cells with MDS propagating ability. This emphasised the need for an improved cancer stem cell assay which could be used in patients.

We hypothesised that tracing DNA mutations detected in MDS bulk bone marrow back to the candidate MDS stem, could be used to provide definitive evidence for the existence and identity of MDS stem cells in patients. However this method of mutation tracking was

predicated on the existence of a hierarchical arrangement between the candidate MDS stem cell and the downstream progenitors. In order to confirm such a relationship, we began by using flow cytometry to demonstrate that both del (5q) and non-del (5q) low and intermediate-1 risk MDS patients, retain phenotypically distinct stem and progenitor compartments, not dissimilar to the structure found in normal haematopoiesis (**Figure 3.2**). However, as the profiles in the MDS patients resembled those of the normal controls, it was important to show that these stem and progenitor cells were clonally involved MDS cells and not just residual normal haematopoietic cells. Fluorescence *in situ* hybridisation demonstrated that the purified MDS stem and progenitors were indeed part of the clone (**Figure 3.4**). Secondly, as cell surface markers are often aberrantly expressed in malignant disorders, it was important to be able to distinguish these populations molecularly and functionally. If these phenotypically purified populations were truly distinct from each other, then they should behave differently in molecular and functional assays, as is the case with normal stem and progenitors. The gene expression signatures of the MDS stem cells, GMPs and MEPs were very similar to those of the corresponding normal control populations (**Figure 3.5** and **Figure 3.7**). Functional *in vitro* colony forming assays also confirmed that the GMPs and MEPs were distinct from each other, with the GMPs giving rise to myeloid colonies and the MEPs to erythroid colonies, again as would be expected for normal progenitors. If MDS stem and progenitors were hierarchically arranged, with the stem cells sitting at the apex of the hierarchy, then by definition the MDS stem cells would be the only cells capable of self-renewal and giving rise to the progenitors. Progenitors would not be able to give rise to stem cells. Using the LTC-CFC assay and xenotransplantation in NSG mice, this was shown to be the case in all investigated patients. Only the CD34+ CD38- CD90+ CD45RA- stem cells had the capacity to self-renew and give rise to LTC-CFCs (**Figure 3.8**) and only the CD34+ CD38- CD90+ CD45RA- stem cells were able to reconstitute immunocompromised mice (**Figure 3.10**). Importantly there was no evidence that the

downstream progenitors had acquired self-renewal potential. These data confirm that in low and intermediate-1 risk MDS patients the most likely candidate MDS stem cell is CD34+ CD38- CD90+ CD45RA-. However these data were limited by the lack of sensitivity of the *in vitro* and *in vivo* assays which were utilised.

In order to overcome these limitations, we hypothesised that if the MDS stem cell is truly the only cell which can propagate and sustain disease, all stable genetic changes seen in the patients must have arisen in such a stem cell and would then be inherited by downstream progenitors. In the 13 patients on whom we performed detailed DNA mutation analysis, all mutations detected in bulk bone marrow could indeed be traced back to the stem cell compartment and were also present in all the progenitors. In conjunction with the supporting *in vitro* and *in vivo* data, this supports a hierarchical arrangement within MDS haematopoiesis, with the MDS stem cell at the top. As all stem cells were highly clonally involved, this also provides compelling evidence that these mutated MDS stem cells have a significant competitive advantage over their normal counterparts. Interestingly, even in patients with multiple mutations, these genetic changes did not confer self-renewal capacity to the downstream myeloid restricted progenitors. However our study was limited by looking at only one time point so it is possible that later analysis may have revealed alterations in self renewal capacity, perhaps as patients progressed. We did not analyse the CD34+CD38-CD90- populations, which may also have harboured self-renewal potential.

Patient 1 was a particularly informative patient in our study, being one of the patients transplanted to engraft in NSG mice. Although only a small number of patients engrafted, key information was obtained regarding the clear hierarchy between the self-renewing MDS stem cells and the unique ability of the stem cells to regenerate downstream progenitors *in vivo*. Factors which may have been important regarding the ability of patient 1 to engraft, include a complex karyotype and Lenalidomide resistance. Both of these suggest that she

may have had more aggressive disease than many other patients. Patient 1 also had the commonest combination of somatic genetic events seen in this cohort - a deletion of chromosome 5 and a *JAK2* V617F mutation. As such a high proportion of the patients studied had a *JAK2* mutation, which is far more commonly detected in patients with a myeloproliferative disorder (MPD), we went back through the clinical histories of our patients to ensure they fulfilled criteria for MDS. This was found to be the case, although it is worth noting that Patient 5 was later diagnosed as MDS/MPD due to a high platelet and white cell count alongside clear bone marrow dysplasia and an isolated del (5q) aberration (**Table 3.1**). A number of studies have found that the prevalence of the *JAK2* V617F mutation in patients with del (5q) MDS is 5-10% [112,113]. A small study of highly selected Chinese patients found the *JAK2* V671F mutation at a far higher frequency of 27% (3/11 patients)[114]. In patients with classical isolated del (5q) syndrome, this mutation does not seem to impact on long-term survival or the risk of leukaemic transformation, with the caveat that the number of cases reported and length of follow up has been limited.

Patient 6 also provided some unique insights, particularly into the order of genetic event acquisition in MDS stem cells. At the time of bone marrow sampling patient 6 was already losing response to Lenalidomide, having been on treatment for less than one year. At this point, mutations in *ASXL1*, *ETV6* and *CSF3R* were detected in the stem and progenitor cells. It is worth noting that mutations in the *CSF3R* are extremely rare in MDS. As they are more common in congenital neutropaenia, chronic neutrophilic leukaemia and atypical chronic myeloid leukaemia[115] we looked at whether patient 6 should actually be reclassified but there was no clinical or laboratory evidence supporting any of these three diagnoses.

Although the del (5q) and three mutations were present in all the stem and progenitor cell populations, when 4 single stem cell derived colonies were analysed, the del (5q) was detectable in all 4 colonies but the remaining 3 mutations were only present in 3 of the 4

colonies. This strongly suggested that the ancestral clone contained the del (5q) alone. This clone is then likely to have acquired the additional genetic changes resulting in a daughter stem cell clone with all 4 aberrations. This also supports the already large body of evidence concluding that acquisition of a del (5q) is likely to a very early event in MDS, which precedes all other mutations. This patient also demonstrates the value of examining single cells in determining the order of genetic changes in a malignancy. As all mutations were found in the same clone, the mutations are likely to have been acquired in a sequential linear fashion.

Patient 10 was diagnosed with RCMD-RS. She did not have a del (5q) but did have a detectable trisomy 8 in both bulk bone marrow and stem and progenitor populations (**Figure 3.4**). She was entered into a trial investigating the utility of Azacitidine therapy in low and intermediate risk MDS patients. She had a particularly interesting clinical course as she was one of 4/24 patients in the trial who had a sustained response to Azacitidine and remains on therapy. In multivariate analysis, trisomy 8 was significantly (p=0.01) associated with response in this trial[116]. However the study did not perform mutation analysis so it would be interesting to investigate whether any of the mutations in *TET2*, *RUNX1* or *SF3B1*, all found in patient 10, were also associated with a response to Azacitidine. Patient 11 was also one of the four responders in this trial and had a trisomy 8 and the same complement of mutated genes, although the exact mutations in the genes were not the same (**Table 3.2**). These findings demonstrate how mutation analysis could potentially be used not only to risk stratify patients but to also direct therapy.

It has previously been demonstrated that del (5q) MDS is likely to originate in and be driven by a malignant stem cell within the limitations of the available *in vitro* and murine *in vivo* assays available at the time[43,102]. We have now used DNA mutation tracking of stem and progenitor cells in patients to confirm that low risk MDS, beyond isolated del (5q), is indeed a stem cell disease and hierarchically organised. We have also shown that tracking of DNA

mutations in order to elucidate cancer stem cell potential is a valuable and effective tool which can importantly be used in patients. The use of DNA mutation tracking could enable the monitoring of MDS stem cells in patients with normal karyotypes who previously would have had no detectable clonal marker. These findings also have extensive implications for improving our understanding of cancer biology and the development of new targeted therapies in MDS as well as other malignant disorders.

4. Characterisation of CMML stem and progenitor cells

4.1 Introduction

The vast majority of published data on MDS stem cells has focused on low risk MDS and even more specifically on del (5q) MDS. We decided to investigate a group of patients at the opposite end of the prognostic spectrum with chronic myelomonocytic leukaemia (CMML). CMML is a clonal myelodysplastic / myeloproliferative neoplasm characterised by a persistent peripheral blood monocytosis (>1x10*9/L), bone marrow myelodysplasia and less than 20% blasts. Patient also often present with splenomegaly and systemic symptoms, further distinguishing them from low risk MDS. The prognosis for patients is extremely poor, with 30-50% transforming to acute leukaemia and a median survival of 5-20 months only[117]. This is in sharp contrast to low risk MDS where the majority of patients do not transform to leukaemia and the median survival can be up to 97 months in those that are transfusion independent.[94] Some studies have shown some efficacy with demethylating agents such as Azacitidine but currently the only therapy which offers a durable response is allogeneic stem cell transplantation and even following this intensive treatment, many still relapse[118,119].

No studies have conclusively investigated the cellular origin of CMML as definitively as has now been done in low/intermediate-1 risk MDS. Although until recently only limited clonal markers were available, one study could demonstrate del (20q) involvement of multiple myeloid lineages in one patient with CMML, although the lymphoid lineage was not investigated[120]. The limited functional studies performed on CMML bone marrow suggest that cells with stem cell potential can read out in *in vitro* and *in vivo* assays[121,122]. However the exact identity of the cells which initiate and propagate CMML still remains to be elucidated. It is also key to remember that unlike patients with low/intermediate-1 risk MDS, patients with CMML are very heterogeneous with regards to clinical features, response to therapy and subsequent disease prognosis. This may suggest that as is the case in other

aggressive myeloid disorders such as AML, patients may differ from each other significantly, as might the identity of the disease propagating cell[34].

One of the key issues limiting the investigation of clonal involvement of stem and progenitor populations has been the lack of clonal markers as 60-80% of patients have a normal karyotype. However, as in MDS, next generation sequencing has recently identified numerous mutations in CMML patients, which now can be used as clonal markers. Certain mutations in genes such as *TET2*, *CBL* and *ASXL1*, previously found in numerous myeloid diseases, are particularly common in CMML and potentially of prognostic significance[104,123]. As well as aiding the understanding of the molecular pathogenesis of CMML, these mutations now provide an increasing number of clonal markers to delineate the cellular hierarchy.

Even more importantly, it has become clear that specific mutations in the RNA splicing machinery are prevalent, particularly in CMML patients[97,105,124]. Highly conserved mutations in the serine/arginine-rich splicing factor 2 gene (*SRSF2*) have been found in 28-47% of CMML patients, with almost all mutated patients having a single amino acid substitution at position 95. SRSF2 is a component of the early RNA splicing machinery and is involved in recognition of the 3' splice site. Spliceosome mutations may conceivably affect splicing in a number of ways. Dysregulation of alternative splicing, intron retention or altered splice site recognition and have been proposed to have a role in the pathogenesis of many cancers[125]. There is very limited data on the functional impact of *SRSF2* mutations to date, in either bulk bone marrow or sorted stem and progenitor populations. However due to the high prevalence of this preserved mutation in patients with CMML, it seems very likely that understanding the resultant impact on splicing is likely to be crucial to understanding disease pathology.

Within the limits of the available *in vitro* and *in vivo* assays, we have previously demonstrated that low/intermediate-1 risk MDS originates in the phenotypic (CD34+ CD38- CD90+ CD45RA-) stem cell, and only this population retains self-renewal capacity and the ability to differentiate in to all downstream populations. Prior to commencing this study, the preservation of phenotypically, molecularly and functionally distinct stem and progenitor populations in CMML had not been demonstrated and the identity of the CMML propagating cell was unknown.

4.2 Materials and Methods

Patients. 10 patients with CMML were selected from Lund University, Sweden, Karolinska Institutet Sweden, Oslo University Hospital Norway and Aarhus University Hospital, Denmark (**Table 4.1**). All patients provided written informed consent and the study was approved by the relevant local ethics committees. Due to limitations in the number of cells available from certain patients, we were unable to perform all assays on all patients and were required to prioritise certain experiments in particular patients.

Flow cytometry. Stem/progenitor analysis/isolation of BM mononuclear cells was performed as described in Chapter 2 Methods.

***In-vitro* assays**. Colony-forming-cell (CFC) and long-term-culture-CFC (LTC-CFC) assays were performed as described in Chapter 2 Methods.

Gene expression analysis. Gene expression was analysed by the Fluidigm Dynamic Array as described in Chapter 2 Methods.

Cell cycle analysis. Cells were fixed and permeabilised with 3.2% paraformaldehyde and 90% ice cold methanol before being stained with surface markers CD19, CD34, CD38, CD90 and intracellular markers, Ki67 and 4', 6-diamidine-2-phenylidole dihydrochloride (DAPI). Further description in Chapter 2 Methods.

Xenograft transplantation. Cells were transplanted into irradiated NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice, analysed as described in Chapter 2 Methods.

Mutational analysis. Targeted DNA mutation screening of 54 genes known to be of importance in myeloid malignancies or other cancers (See Chapter 2 Methods) was performed on bone marrow mononuclear cells, purified populations and LTC-CFC colonies.

DNA sequence reads from bulk tumour material were compared to normal T cells to determine if a somatic mutation was present.

Statistical analysis. Statistical analysis was performed using Graphpad Prism 6 software.

Pat ID	Sex/Age	WHO Diagnosis	Hb (g/L)	WCC/ANC (10^9/L)	Plts (10^9/L)	Monocytes (10^9/L)	% BM Blasts	Karyotype	SRSF2	On-going treatment
1	M/81	CMML 1	121	14.1	59	1.1	9	12q-	Mutated	ESA
2	M/69	CMML 1	94	45	198	5.4	3	Normal	Mutated	Nil
3	M/74	CMML 1	131	15.2	223	2	6	Normal	Wild type	ESA
4	F/56	CMML 1	114	42.4	36	4	6	Normal	Wild type	Nil
5	M/44	CMML 1	119	4.5	54	2.2	<5	Normal	Mutated	Nil
6	M/86	CMML 1	85	40	114	16.8	<5	Normal	Mutated	Nil
7	M/82	CMML 1	75	33	79	6	<5	Normal	Mutated	Nil
8	M/82	CMML 2	116	10.9	25	5.1	17	Normal	Mutated	Nil
9	M/70	CMML 2	121	64	60	19.4	17	Normal	Mutated	Nil

10	M/76	CMML 2	131	12.1	96	1.4	16	Normal	Wild type	Nil

Table 4.1 Clinical and Laboratory Parameters of CMML Patients

Abbreviations used: CMML, chronic myelomonocytic leukaemia; Hb, haemoglobin; WCC, white cell count; ANC; Plts, platelets; F, female; M, male; ESA Erythropoiesis stimulating agent

4.3 Results

The majority of the patients were classified as CMML-1 (7/10) with up to 9% blasts, rather than the more advanced CMML-2 (3/10) with up to 17% blasts (**Table 4.1**). The median age was 75 (Range 56-86) and 9/10 patients were male. All patients were untreated at the time of sampling, apart from patient 1 and patient 3 who were on an Erythropoiesis Stimulating Agent (ESA). All patients except for patient 1 had a normal karyotype and interestingly the prevalence of the *SRSF2* P95 mutation in our cohort was higher than previously reported for CMML (70%.).

4.3.1 CMML patients retain phenotypically distinct stem and progenitor compartments

CMML-1 and CMML-2 patients, like low/int-1 risk MDS (Chapter 3), retained discernible phenotypic stem (CD34+CD38-CD90-) and progenitor compartments (CD34+CD38+) (**Figure 4.1a**). The compartments remained normal in size which is similar to what was seen in the low/int-1 risk MDS patients. The frequencies of stem and progenitor cells as a fraction of total mononuclear cells in the patients were not significantly different to normal age-matched controls (**Figure 4.1b**).

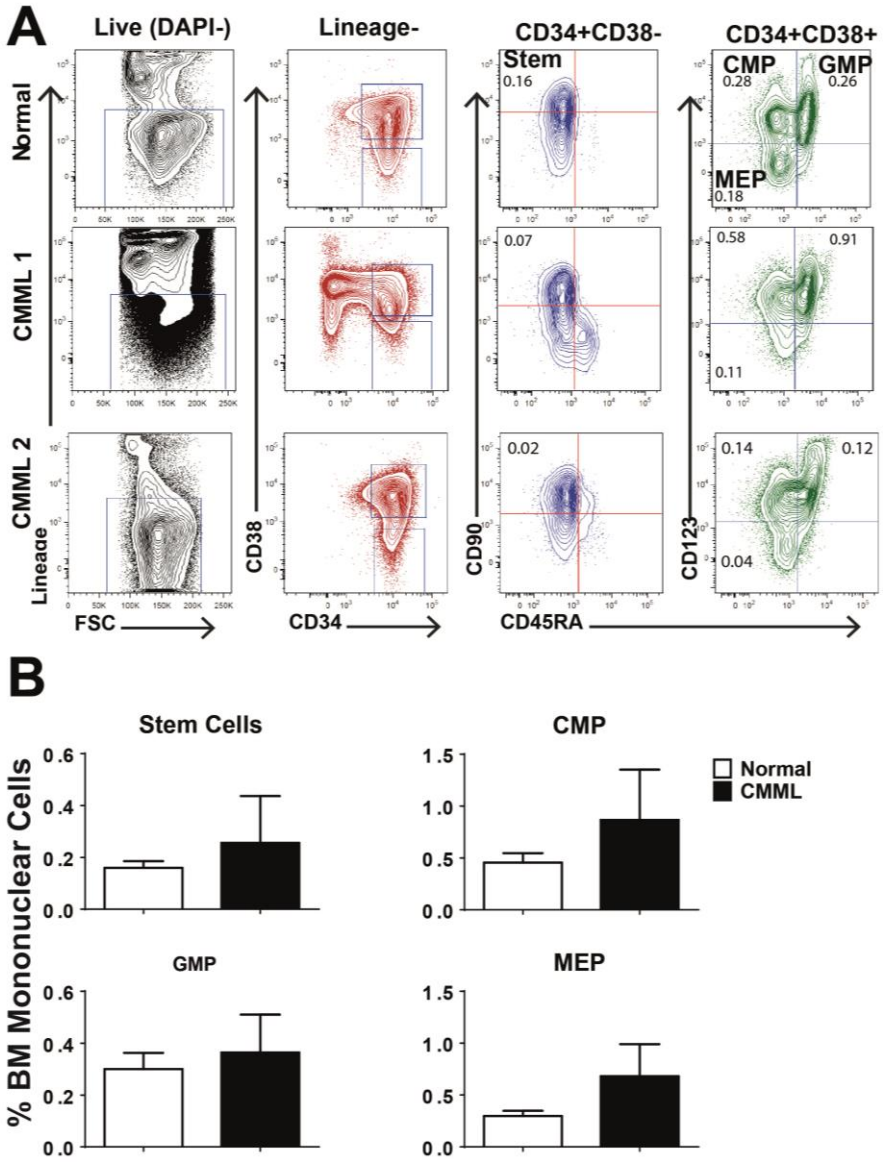


Figure 4.1 CMML patients retain phenotypically distinct stem and progenitor populations

A Representative FACS profiles of a normal age matched control, CMML-1 (Pat 3) and CMML-2 (Pat 9) patients. Numbers within quadrants indicate the frequency of the population within live BM MNCs. **B** Mean (SEM) frequency of phenotypic stem and progenitor populations of normal age matched controls (n=4) and CMML patients (n=10). There was no significant difference between the frequencies of any of the populations between normal and CMML patients (student t test).

4.3.2 CMML stem and progenitors are highly clonally involved

In the absence of cytogenetic aberrations which could be tracked by FISH (9/10 CMML patients had a normal karyotype), clonal involvement of CMML stem and progenitor compartments were investigated by targeted mutation analysis. As the most frequent genetic aberration was the *SRSF2* P95 mutation, this was used as a clonal marker (**Figure 4.2**). In the 7 patients with a *SRSF2* P95 mutation, the mean (SEM) frequency of mutated *SRSF2* P95 reads as a proportion of total reads was: Stem cells 33.04% (10.01); GMP 29.71% (9.99) and MEP 39.72% (14.73). As the DNA used for the stem and progenitor analysis was amplified due to low cell numbers, these proportions might not accurately reflect the clonal involvement. However, as the *SRSF2* mutation is known to be heterozygous in CMML patients, mutated read frequencies of around 30-40% would be consistent with the majority of cells sequenced being heterozygous for the mutation. In 6/7 patients the mutation could be tracked back to the purified stem cell population.

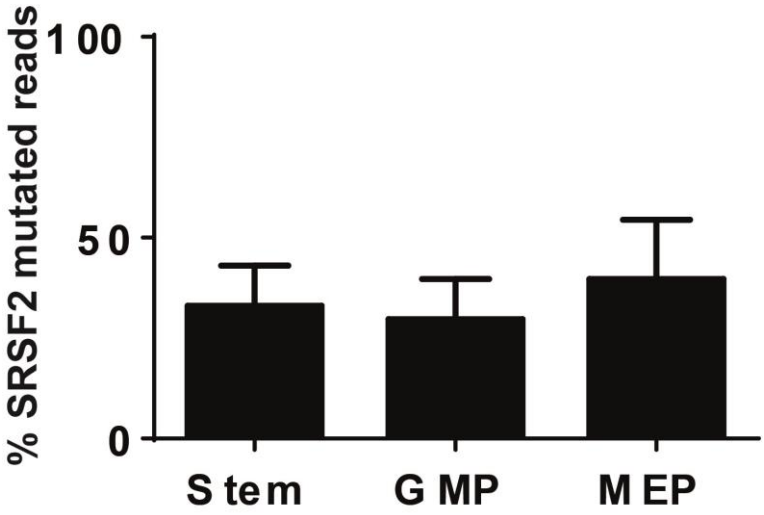


Figure 4.2 Clonal involvement of CMML stem and progenitor cells

Mean (SEM) frequency of mutated reads. DNA was isolated from purified populations (100 cells each) which underwent targeted Haloplex DNA sequencing of the *SRSF2* gene (n=7).

4.3.3 CMML progenitors are molecularly distinct

To confirm that the phenotypically purified progenitors (GMPs and MEPs) were molecularly distinct, quantitative gene expression analysis was performed (**Figure 4.3**). The GMPs in all patients analysed (n=3) retained a myeloid gene expression signature and had low levels of expression of erythroid genes. The MEPs from the same patients retained an erythroid gene expression signature.

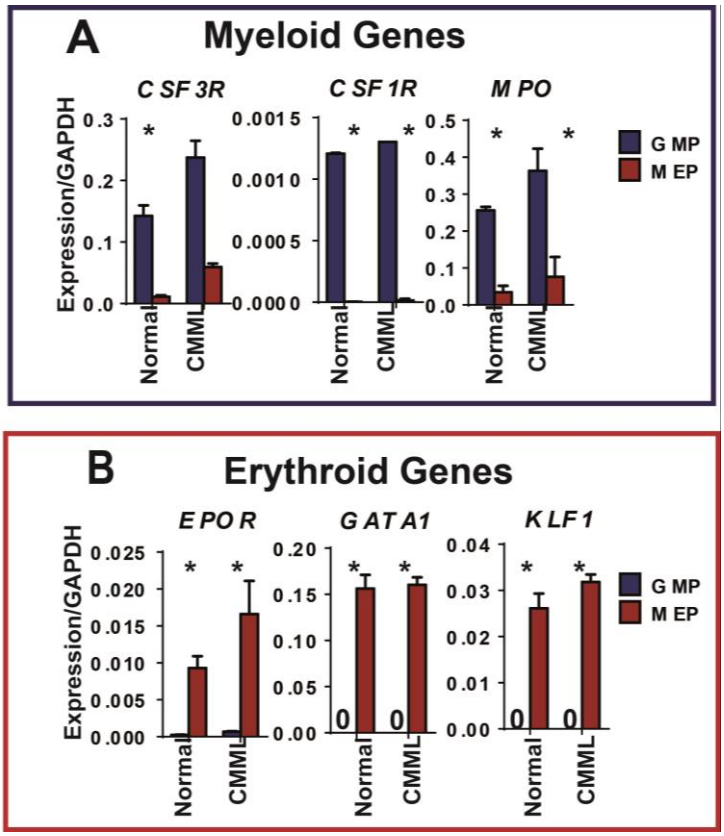


Figure 4.3 Lineage affiliated gene expression analysis of purified GMPs and MEPs

Quantitative PCR was performed on sorted progenitor populations (50 cells per patient per population) from normal controls (n=3) and CMML patients (n=3). **A** Mean (SEM) expression of myeloid genes (*CSF3R*, *CSF1R* and *MPO*). **B** Mean (SEM) expression of erythroid genes (*GATA1*, *KLF1* and *EPOR*). (* $p<0.05$ Mann-Whitney test).

4.3.4 CMML progenitors are functionally distinct *in vitro*

As gene expression data suggested CMML GMPs and MEPs are molecularly distinct and compatible with restriction to myeloid and erythroid lineages respectively, we next investigated the functional lineage potential of these populations *in vitro*. When plated in standard methylcellulose colony forming cell assays, supporting both myeloid and erythroid differentiation, FACS sorted GMPs (n=7) gave rise to myeloid colonies and MEPs (n=7) to erythoid colonies as would be expected for their normal counterparts (**Figure 4.4**). The molecular and functional data both provide compelling evidence that GMPs and MEPs isolated from CMML patients are distinct and behave as lineage restricted progenitors.

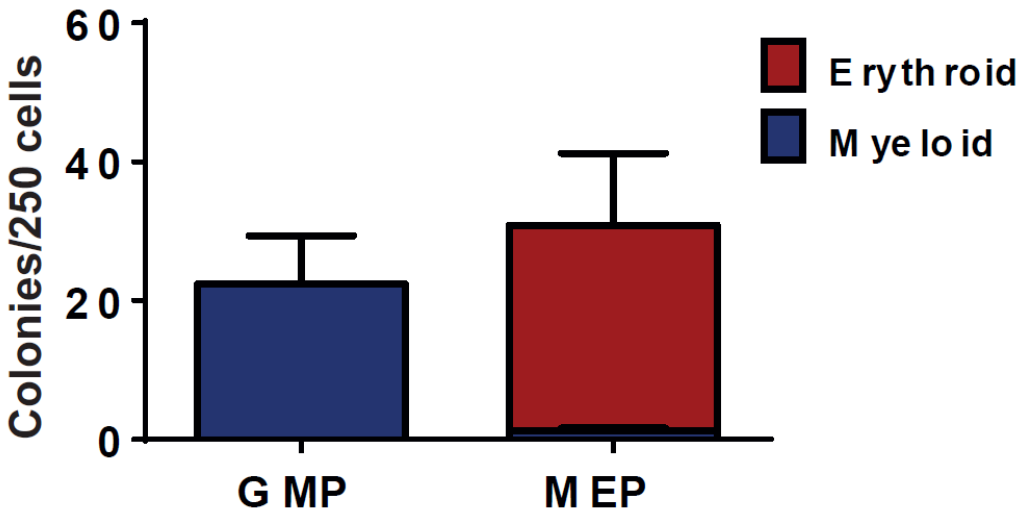


Figure 4.4 Functionally distinct GMPs and MEPs in CMML

Mean (SEM) number of colonies formed. FACS sorted GMPs and MEPs were purified from CMML patients (n=7) and plated in to methylcellulose with cytokines. 100-350 cells per population per patient were plated in 2 replicates. Colonies were read out after 14 days of incubation. Colonies were scored using an inverted light microscope as myeloid or erythroid based on morphology and haemoglobinisation.

4.3.5 CMML stem cells are molecularly distinct from progenitors and the only population with self-renewal capacity *in vitro*

In order to ascertain whether progenitors had aberrantly acquired evidence of self-renewal capacity at the molecular level, quantitative PCR for genes which are known to be highly expressed in normal human stem cells was performed. Increased expression of such genes may indicate that the GMPs and MEPs had a propensity for self-renewal. In the CMML patients (n=3) and the normal controls (n=3) the expression of *MPL, HLF and HOXA9 was* significantly higher in the stem cells compared to the progenitor populations, as was evident in the normal controls (**Figure 4.5a**). There was no evidence of stem cell signature in the purified progenitors.

A key feature of a cancer stem cell is the ability to self-renew and thus an ability to sustain long-term cultures *in vitro*. In order to confirm that the progenitors had not acquired aberrant self-renewal potential and that the phenotypic stem cells were the only cells capable of this, LTC-CFC assays were performed. Purified stem cells, CMPs, GMPs and MEPs were plated in to LTC-CFC assays (**Figure 4.5b**). As myeloid progenitors are short-lived, any colony forming potential sustained after 6 weeks of culture, must originate from a cell with self-renewal ability. Of the 10 patients, 8 read out in the LTC-CFC assay. Cultures from patient 5 were contaminated and discarded and patient 4 did not generate any LTC-CFCs. In the remaining patients only the purified stem cells were able to sustain myeloid colony potential. No LTC-CFCs were generated by the downstream progenitors. This provides substantial evidence that the phenotypic stem cells are likely to represent candidate CMML stem cells.

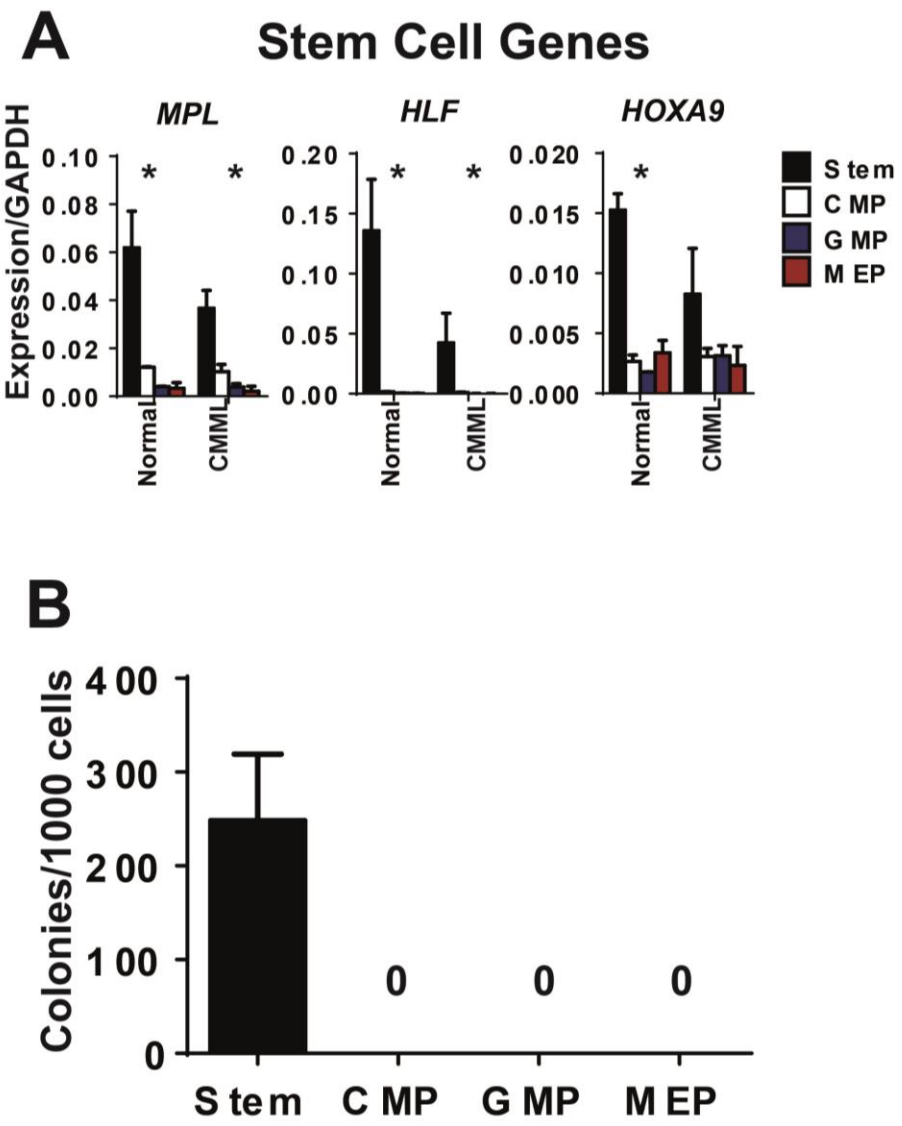


Figure 4.5 Self-renewal potential of CMML stem and progenitor cells

A Mean (SEM) expression of transcripts of three stem cell genes (*HOXA9*, *HLF* and *MPL*) in healthy age-matched controls (n=3) and CMML (n=3) stem and progenitor cells (*p<0.05 refers to pair wise comparisons between the stem/cmp, stem/gmp and stem/mep using the Mann-Whitney test). **B** Mean (SEM) LTC-CFC formation of purified stem and progenitor cells (n=8). 500-2000 cells per population per patient were co-cultured in 2-3 replicates for 6 weeks. Cells were then transferred to methylcellulose. After 14 days in methylcellulose, colonies were enumerated. Individual colonies were also picked for DNA mutation analysis.

4.3.6 CMML stem cells are highly quiescent

Another key feature of functional stem cells is the high proportion of quiescent cells residing in G_0, relative to actively cycling downstream progenitors. In order to see if candidate CMML stem cells also retained this property and that it had not been aberrantly acquired by the progenitors, cell cycle flow cytometry analysis was performed. In all the CMML patients analysed (n=4), greater than 75% of the stem cells were in G_0 whereas only 10% of the progenitors were in G_0 (**Figure 4.6** and **Figure 4.7**). This is similar to what has been found in low risk MDS[103].

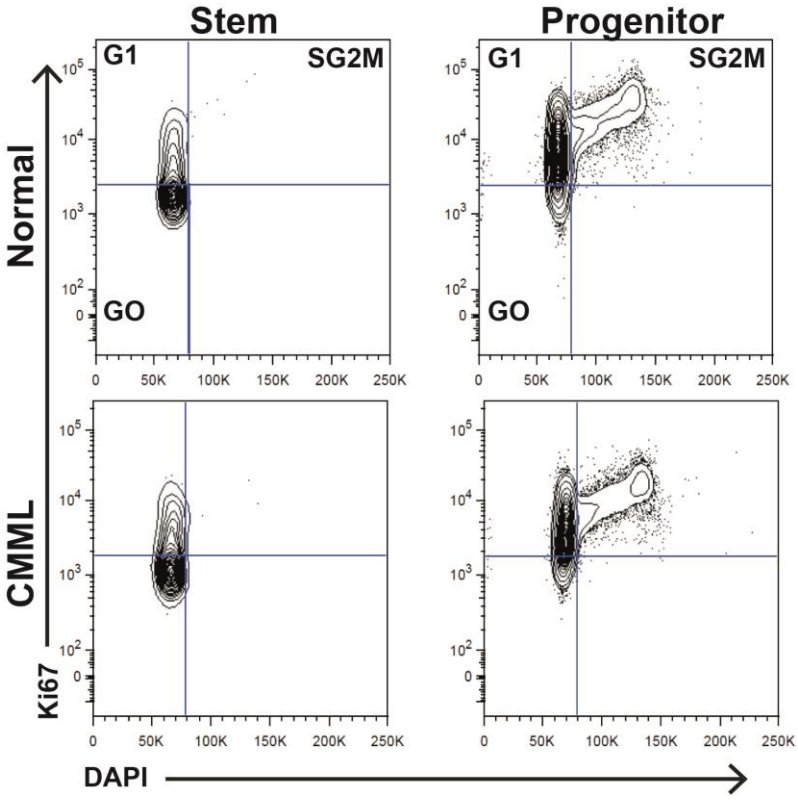


Figure 4.6 CMML stem cells are highly quiescent

Representative FACS profiles of a normal control and a CMML patient (Patient 3). Cell cycle status of purified stem (CD34+CD38-CD90+) and progenitor cells (CD34+CD38+) as determined by Ki67 and 4',6 diamidine-2-phenylidole dihydrochloride (DAPI).

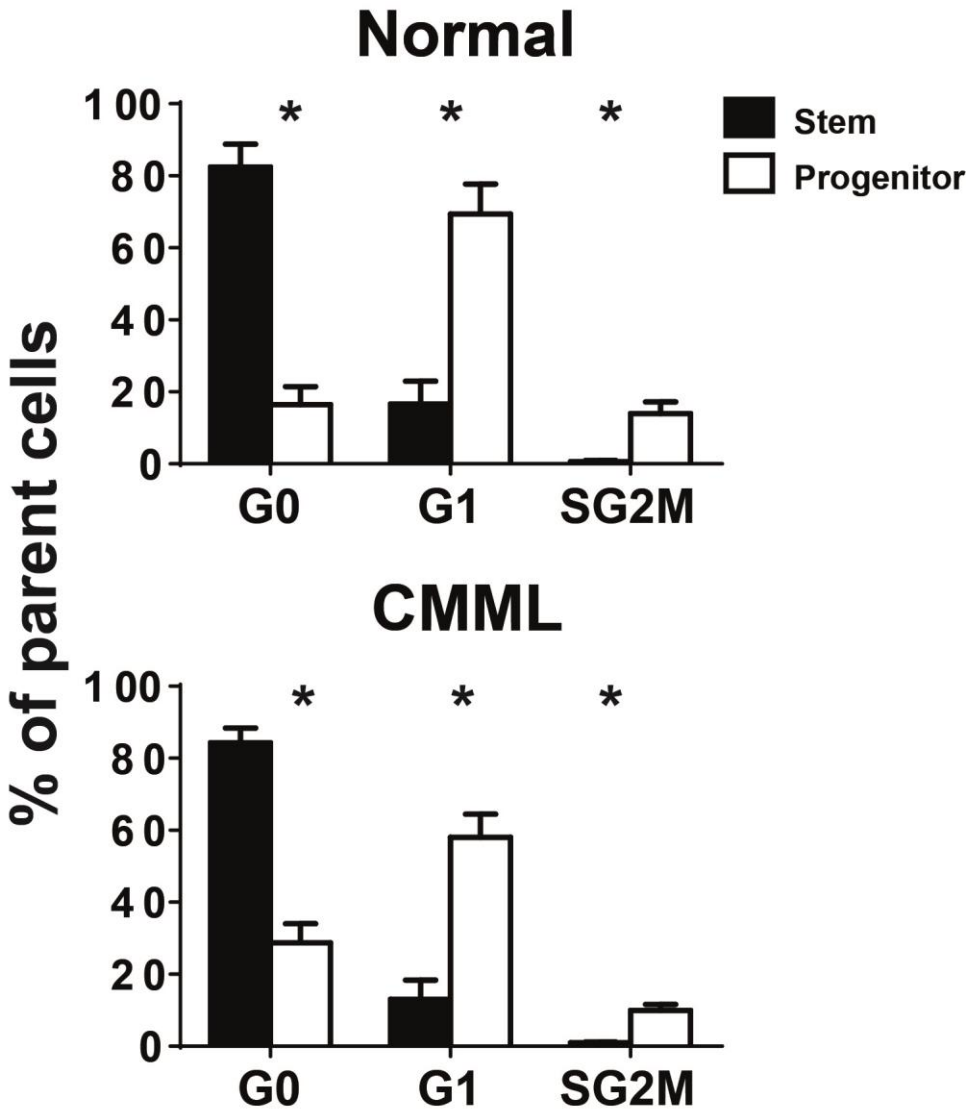


Figure 4.7 Frequencies of quiescent and cycling stem and progenitor cells in CMML

Bar chart shows mean (SEM) percentages of stem (CD34+ CD38- CD90+) and progenitor (CD34+ CD38+) cells in G_0 (Ki67−DAPI−), G_1 (Ki67+DAPI−), and S/G_2/M (Ki67+DAPI+) (Normal n=2 CMML n=4) (*p<0.05 Mann-Whitney test).

4.3.7 NSG Transplantation

As was the case with the del (5q) MDS patients, the *in vitro* assays suggested that the most likely candidate CMML stem cells were the phenotypic CD34+ CD38- CD90+ stem cells. In order to investigate the disease propagating ability of this population and the downstream progenitors *in vivo*, xenotransplantation of purified populations was performed. Due to a large number of cells being required for transplantation, this was performed on a limited number of patients (n=3), where adequate cell numbers were available. Bone marrow stem and progenitor populations were FACS purified and injected in to NSG mice. Serial bone marrow aspirations were then performed to detect any human engraftment. Engraftment was defined as >0.1% hCD45+ cells as percentage of total CD45.

4.3.7.1 Patient 1

Patient 1, aged 81 years, presented with thrombocytopenia and a persistent mild monocytosis. A bone marrow biopsy revealed granulocytic and megakaryocytic dysplasia, a blast count of 9% and a del (12) (q13.1) (**Table 4.1**). A diagnosis of CMML-1 was made. The patient was already on an erythropoiesis stimulating agent (ESA) following a diagnosis of chronic kidney disease of unknown cause. No additional treatment was given and he was monitored closely in clinic.

At 5, 10 and 16 weeks, the only population which gave persistent engraftment was the stem cell (**Figure 4.8a**). There was no persistent engraftment from mice transplanted with CMPs GMPs or MEPs. When the lineage distribution of the engrafting human cells in the stem cell transplanted mice was analysed at 5, 10 and 16 weeks, an interesting pattern was seen. At 5 weeks the engrafting cells in both stem cell transplanted mice were predominantly myeloid with mouse #1 demonstrating 85.4% myeloid and mouse #2, 75.7% myeloid cells (**Figure**

4.8b). However at the 10 and 16 week analysis, the lineage distribution in both mice changed becoming increasingly B cell biased. One explanation for this phenomenon could be that the initial engraftment was derived from CMML cells but over time residual normal human engraftment from patient 1 took over. An alternative explanation could be that all engraftment was normal but the lymphoid cells simply took longer to be regenerate. To investigate whether the human or CMML haematopoiesis at 16 weeks was derived from clonal CMML cells, myeloid (CD15+CD33+CD66b+) cells and B cells (CD15-CD33-CD66b-CD19+) were FACS purified from the NSG bone marrow. As the patient was known to have an *SRSF2* P95L mutation in the stem cells, the myeloid and B lymphoid cells purified from the NSG mice were also screened but the mutation was not detectable in these populations, supporting the fact that these human cells were not part of the CMML clone. The mutated read frequency of the *SRSF2* P95L was 21% in the injected purified stem cell sample, which suggests that around 42% of the stem cells transplanted were mutated. Therefore if engraftment was CMML rather than normal haematopoiesis, it would be expected that around half of the myeloid cells engrafted should have been mutated which was not the case.

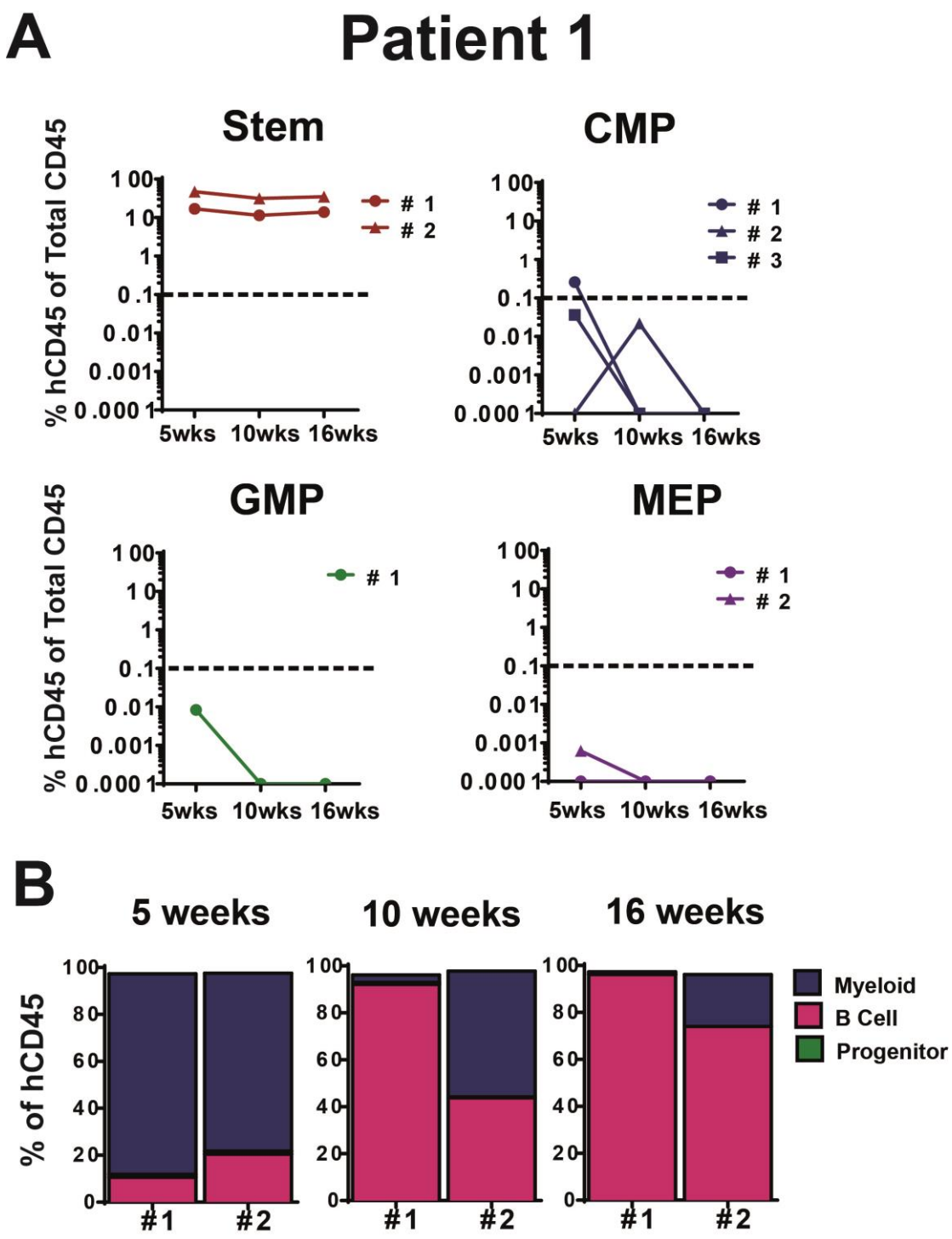

A
Patient 1
Stem
CMP
GMP
MEP
% hCD45 of Total CD45
100
10
1
0.1
0.01
0.001
0.0001
5wks 10wks 16wks
1
2
3
B
5 weeks
10 weeks
16 weeks
% of hCD45
100
80
60
40
20
0
#1 #2
Myeloid
B Cell
Progenitor

Figure 4.8 NSG transplantation of stem and progenitor populations from patient 1

A Human (hCD45) engraftment in the bone marrow of individual NSG mice (1-3 mice per cell population) transplanted with purified populations. This was examined by bone marrow aspiration at 5, 10 and 16 weeks post transplantation. Cell numbers transplanted per mouse were: Stem 5,500; CMP 25,000; GMP 4000 and MEP 4,000.

B Lineage distribution of bone marrow cells aspirated from stem cell engrafted mice at 5, 10 and 16 weeks post transplantation. Myeloid cells (CD15+CD33+CD66b+), B cells (CD15-CD33-CD66b-CD19+) Progenitors (CD15-CD33-CD66b-CD19-CD34+).

4.3.7.2 Patient 2

Patient 2, a 61 year old male, presented with a marked monocytosis and neutrophilia. Bone marrow examination revealed bilineage dysplasia and a blast count of 3%. Due to systemic symptoms and a persistent leucocytosis, he commenced Azacitidine. After 4 cycles of treatment a repeat bone marrow aspiration revealed a blast count of 16%. He then started intensive chemotherapy, achieving a clinical remission. However 4 months later he progressed to AML (28% blasts) and died.

Purified stem and progenitor populations were transplanted in to NSG mice in order to identify the CMML propagating population. 5 weeks post transplantation, low level engraftment was seen in 3 of the mice transplanted with purified stem cells (#1 #3 and #4) but this declined over the next two time points and became undetectable (**Figure 4.9a**). The fourth mouse, injected with stem cells (#2) maintained stable low level engraftment at 5 and 16 weeks (0.13 and 0.27% hCD45 respectively). No data was available for 10 weeks from this mouse as the aspiration did not yield an adequate number of cells. There was very limited engraftment with purified CMPs at 5-10 weeks but this also became undetectable by 16 weeks. When lineage distribution of the engrafting cells was examined at 5 weeks, almost all the cells were myeloid, suggesting CMML engraftment rather than residual normal human engraftment (**Figure 4.9b**). Lineage distribution at 10 weeks was very similar.

This data suggests that the population with the highest engraftment potential was the stem cell. However the engraftment levels were still extremely low at 5 weeks in the four mice, ranging from 0.1-1%. This low level engraftment then declined further, becoming undetectable. In the case of patient 2, xenotransplantation did not provide conclusive evidence regarding the identity of the CMML propagating cell. Like patient 1, the peak level

of myeloid engraftment was seen at the early time point, suggesting again that the NSG model may not be ideal for looking at long term CMML engraftment.

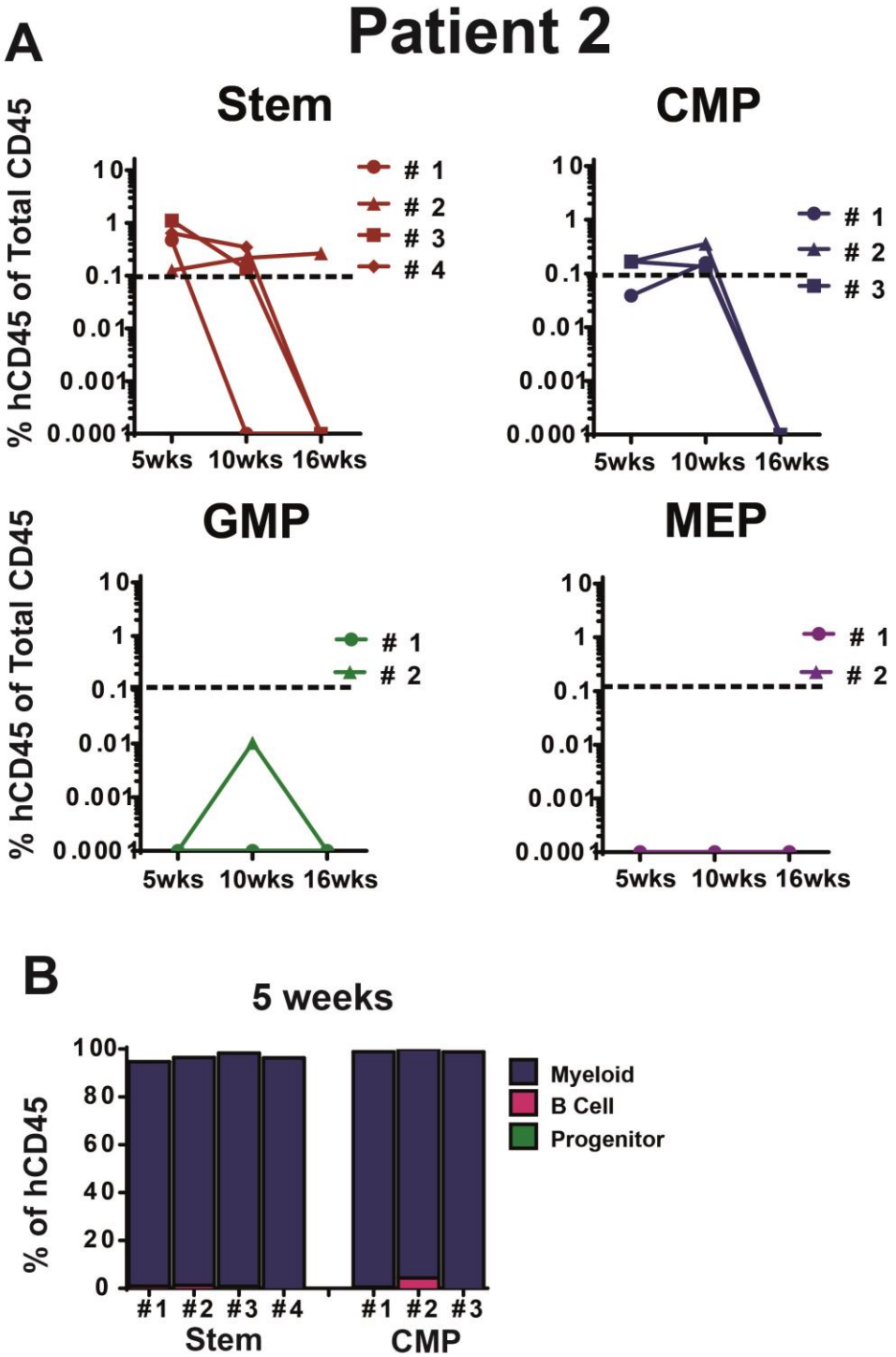


Figure 4.9 NSG transplantation of stem and progenitor populations from patient 2

A Human (hCD45) engraftment in the bone marrow of individual NSG mice (2-4 mice per cell population), examined by bone marrow aspiration at 5, 10 and 16 weeks post transplantation. Bone marrow aspiration of stem cell mouse #2 failed at 10 weeks. Cell numbers transplanted per mouse: Stem 15,000; CMP 22,000; GMP 6,000 and MEP 7,500.

B Lineage distribution of bone marrow cells aspirated from stem cell and CMP engrafted mice at 5 weeks. Myeloid cells (CD15+CD33+CD66b+), B cells (CD15-CD33-CD66b-CD19+) Progenitors (CD15-CD33-CD66b-CD19-CD34+).

4.3.7.3 Patient 8

Patient 8, an 82 year old male, presented with severe thrombocytopenia and splenomegaly. He was also noted to have a persistent monocytosis. A bone marrow aspiration revealed trilineage dysplasia and 17% blasts supporting a diagnosis of CMML 2. He rapidly became red cell and platelet transfusion dependent, developing intractable bleeding. He received one cycle of Azacitidine but did not respond and died shortly afterwards.

When purified bone marrow stem and progenitor populations were transplanted into NSG mice, 5 weeks after transplantation, engraftment was detected from the stem cell (0.1-10% hCD45), CMP (4.2-6.6% hCD45) and GMP (0.7-1.4% hCD45) injected mice (**Figure 4.10a**). At 10 and 16 weeks, engraftment fell below the threshold of 0.1% hCD45 in all mice. The lineage distribution of the engrafting cells at 5 weeks was >98% myeloid, supporting CMML rather than normal reconstitution. However, as in patient 2, engraftment was not sustained (**Figure 4.10b**), confirming that NSG reconstitution could not be used to determine long term repopulating ability.

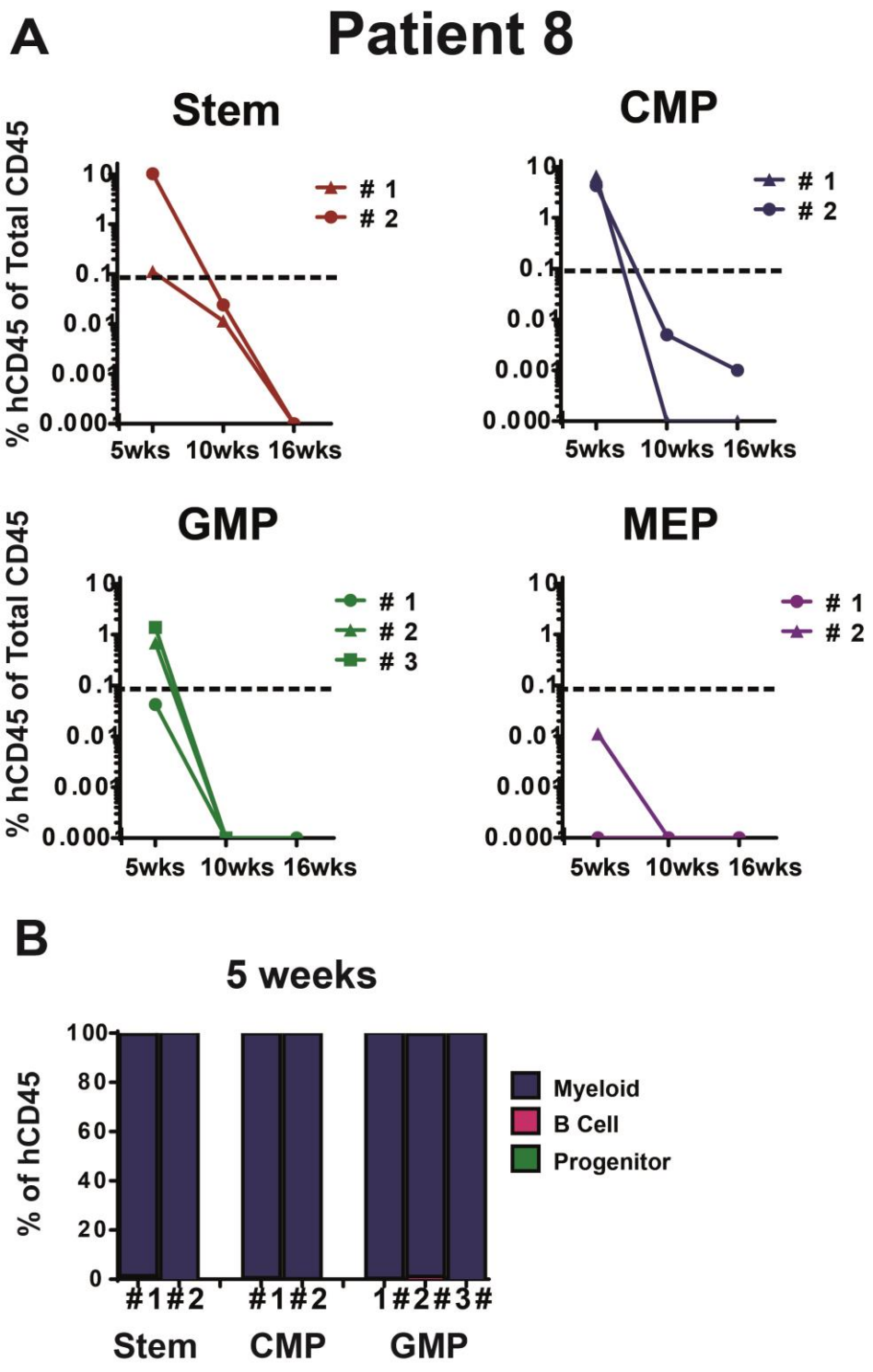

A
Patient 8
Stem
CMP
GMP
MEP
% hCD45 of Total CD45
10
1
0.1
0.01
0.001
0.000
5wks 10wks 16wks
1
2
3
B
5 weeks
% of hCD45
100
80
60
40
20
0
#1#2
#1#2
1#2#3#
Stem
CMP
GMP
Myeloid
B Cell
Progenitor

Figure 4.10 NSG transplantation of stem and progenitor populations from patient 8.

A Human (hCD45) engraftment in the bone marrow of individual NSG mice (2-3 mice per cell population), examined by bone marrow aspiration at 5, 10 and 16 weeks post transplantation. Cell numbers transplanted per mouse: Stem 2,500; CMP 17,000; GMP 50,000 and MEP 12,000.

B Lineage distribution of bone marrow cells aspirated from stem cell, CMP and GMP engrafted mice at 5 weeks. Myeloid cells (CD15+CD33+CD66b+), B cells (CD15-CD33-CD66b-CD19+), Progenitors (CD15-CD33-CD66b-CD19-CD34+).

4.3.8 Mutation profiling of CMML stem and progenitor cells

In order to investigate the mutational composition of CMML patients and track the identified mutations back to cellular compartments, as was demonstrated for low/int-1 risk MDS patients (Chapter 3), all 10 patients in this study underwent DNA mutation sequencing of bulk bone marrow. Following on from this, patients where adequate material was available were selected for targeted mutation tracking back to purified stem and progenitor populations.

20 different somatic mutations were found distributed amongst 9 genes. At least one mutation was detected in the bulk bone marrow of all of the patients (n=10). 3 patients had 1 detectable mutation, 2 patients had 2 mutations, 3 patients had 3 mutations and 2 patients had 4 mutations (**Table 4.2**).

Mutations were found in splice factors (SRSF2, ZRSR2), epigenetic regulators (TET2, ASXL1 and EZH2), transcription factors (RUNX1, GATA2) and cell signalling molecules (CBL, NRAS). What was perhaps most striking regarding the bulk mutation analysis was the high prevalence of the *SRSF2* P95 mutation (n=7). Five of the *SRSF2* mutated patients had the most commonly reported P95H substitution. *TET2* (6/10) and *ASXL1* (3/10) were the second and third most recurrently mutated genes. This has been further supported by other studies which have found that these three genes are mutated in a high proportion of CMML patients[105]. Interestingly two patients with an *SRSF2* mutation were also found to have a mutation in *EZH2*. These two mutations have previously been thought to be mutually exclusive in CMML[105,124].

Targeted DNA sequencing of stem and progenitor cells was then performed on 4 of the 10 patients (Patients 1, 2, 8 and 9). Three of these patients (Patients 1, 2 and 8) were the same patients used for xenotransplantation.

		Pat 1	Pat 2	Pat 3	Pat 4	Pat 5	Pat 6	Pat 7	Pat 8	Pat 9	Pat 10
Splice Factors	SRSF2	P95L	P95H			P95R	P95H	P95H	P95H	P95H	
	ZRSR2			R290*							
Epigenetic Regulators	TET2				P363L	Y1255*	D1343*	Q1627*	Q325*	C1271Y	
	ASXL1		L775*		P622fs			I641L			
	EZH2		C543W			D185Q					
Transcription Factors	RUNX1										P68fs
	GATA2					A164T					
Cell Signalling	CBL		I383M								
	NRAS								G12D	G13D	

Table 4.2 DNA mutation screening of bulk bone marrow cells from 10 CMML patients

Bone marrow mononuclear cells (500,000-1,000,000 per patient) underwent targeted Haloplex DNA sequencing of selected recurrently mutated genes (see Methods). Peripheral blood or bone marrow FACS sorted T cells (CD11b-CD14-CD19-CD34-CD3+CD4+/CD8+) were used as somatic controls when available. Genetic changes were defined as somatic mutations if the mutated read frequency was <5% in the T cell control material and present at least a 5 times higher frequency in the bulk bone marrow. T cell controls were not available for patient 1 and patient 8 so only previously reported recurrent mutations were accepted as somatic changes.

4.3.8.1 Patient 1

Patient 1 was an 81 year old diagnosed with CMML-1 (see previous NSG transplantation section for further clinical details). Bulk DNA mutation screening revealed a lone *SRSF2* P95L mutation. No T cells were available, but as this specific mutation is known to have a prevalence of 28%-47% in CMML patients, it was regarded as a somatic change. The mutation was detected in the purified stem cells, GMPs and MEPs (**Figure 4.11a**). It was also present in all of the LTC-CFCs analysed (**Figure 4.11b**). This supports the hypothesis that in this patient the most likely candidate CMML propagating stem cell is the CD34+ CD38- CD90+ stem cell.

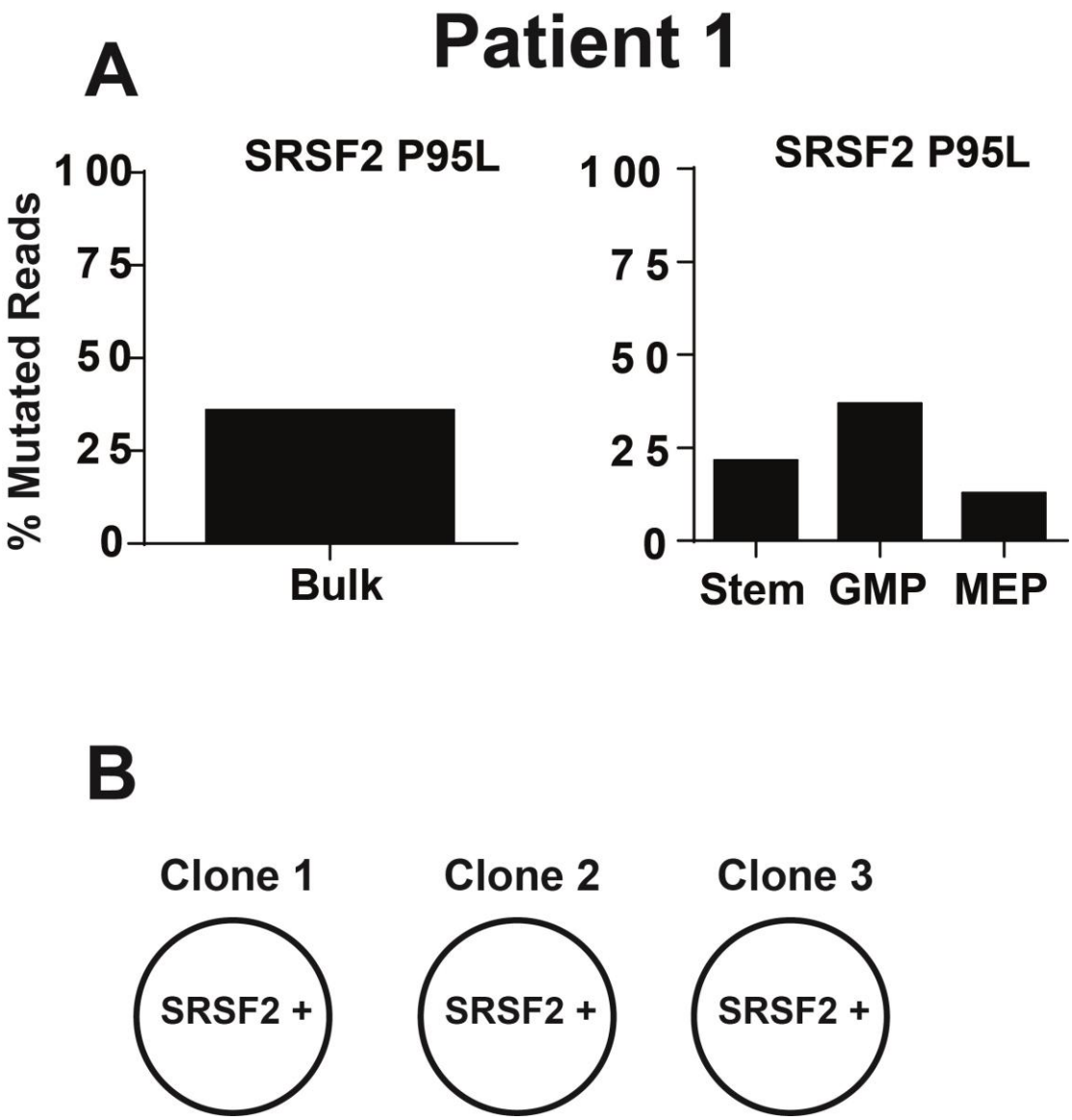


Figure 4.11 Tracking of DNA mutations in Patient 1

A: Detection of the *SRSF2* P95L mutation from the bulk sample and the stem and progenitor populations. Bone marrow mononuclear cells underwent targeted Haloplex DNA sequencing of selected recurrently mutated genes. No T cell control was available so only mutations known to be recurrently mutated in CMML were called somatic changes. Y-axis numbers indicate % mutated reads within *SRSF2*. **B:** Individually picked LTC-CFCs underwent Haloplex mutation screening and were scored as positive (+) if the variant read frequency was >25% or absent (-) if the variant frequency was <5%. Any colonies with a read frequency of 5-25% were scored as indeterminate.

4.3.8.2 Patient 2

Patient 2, a 61 year old, was diagnosed with CMML-1 (see previous NSG transplantation section for further clinical details). Bulk mutation analysis revealed 4 mutations in *SRSF2*, *TET2*, *EZH2* and *CBL*, all genes recurrently mutated in CMML. All the mutations detected in the bulk bone marrow could also be detected in the stem and progenitor populations and LTC-CFCs (**Figure 4.12**). Interestingly, the *CBL* mutation appeared to be homozygous in the LTC-CFCs, with almost >99% of the reads being mutated (data not shown). Although only a limited number of colonies were analysed, this may suggest that they have a competitive advantage in the LTC-CFC assay, resulting in a selection for homozygous *CBL* mutated LTC-CFCs. More colonies would need to be analysed to confirm this hypothesis.

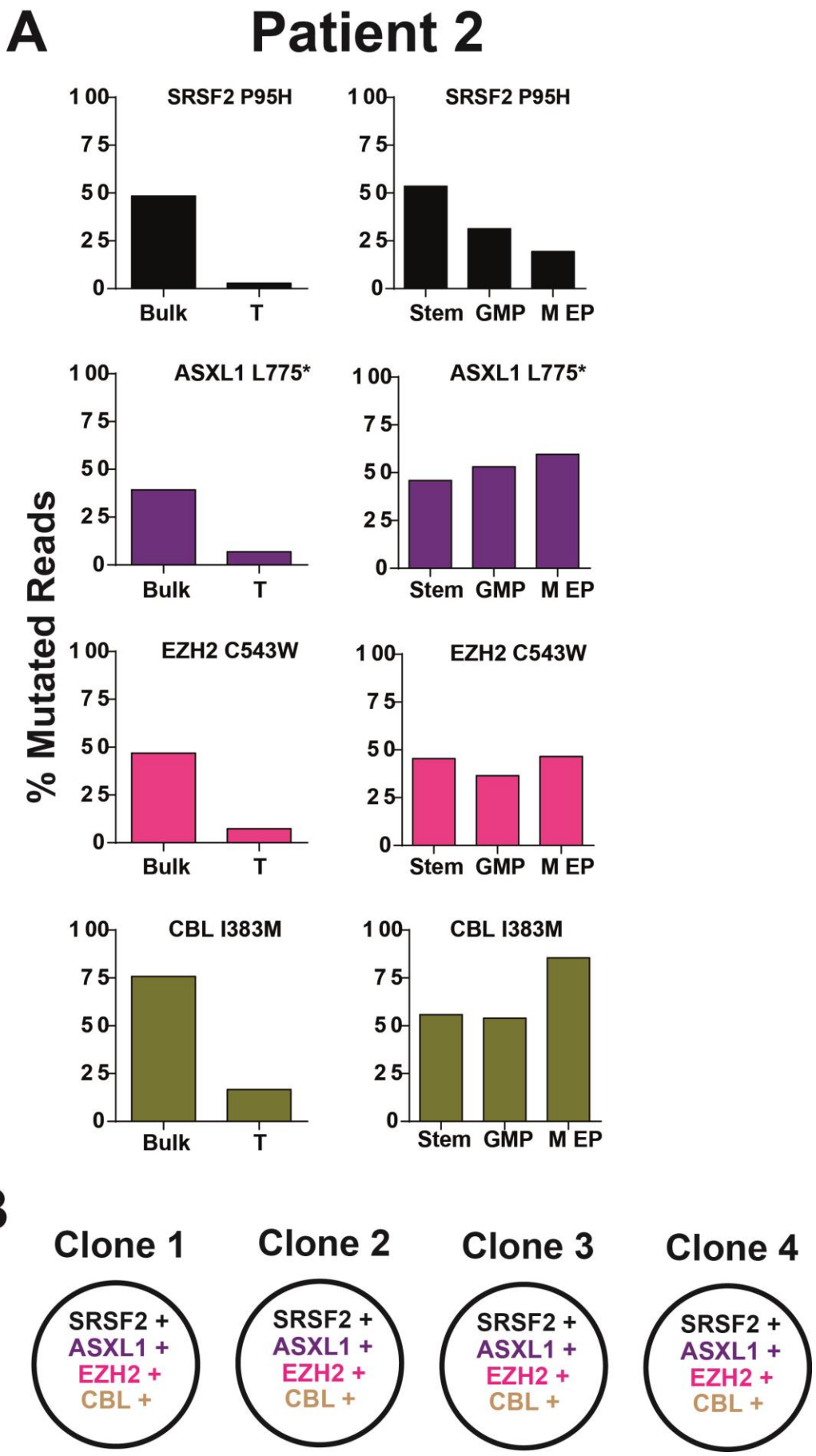

A
Patient 2
% Mutated Reads
SRSF2 P95H
ASXL1 L775*
EZH2 C543W
CBL I383M
100
75
50
25
0
Bulk
T
Stem
GMP
M EP
B
Clone 1
Clone 2
Clone 3
Clone 4
SRSF2 +
ASXL1 +
EZH2 +
CBL +

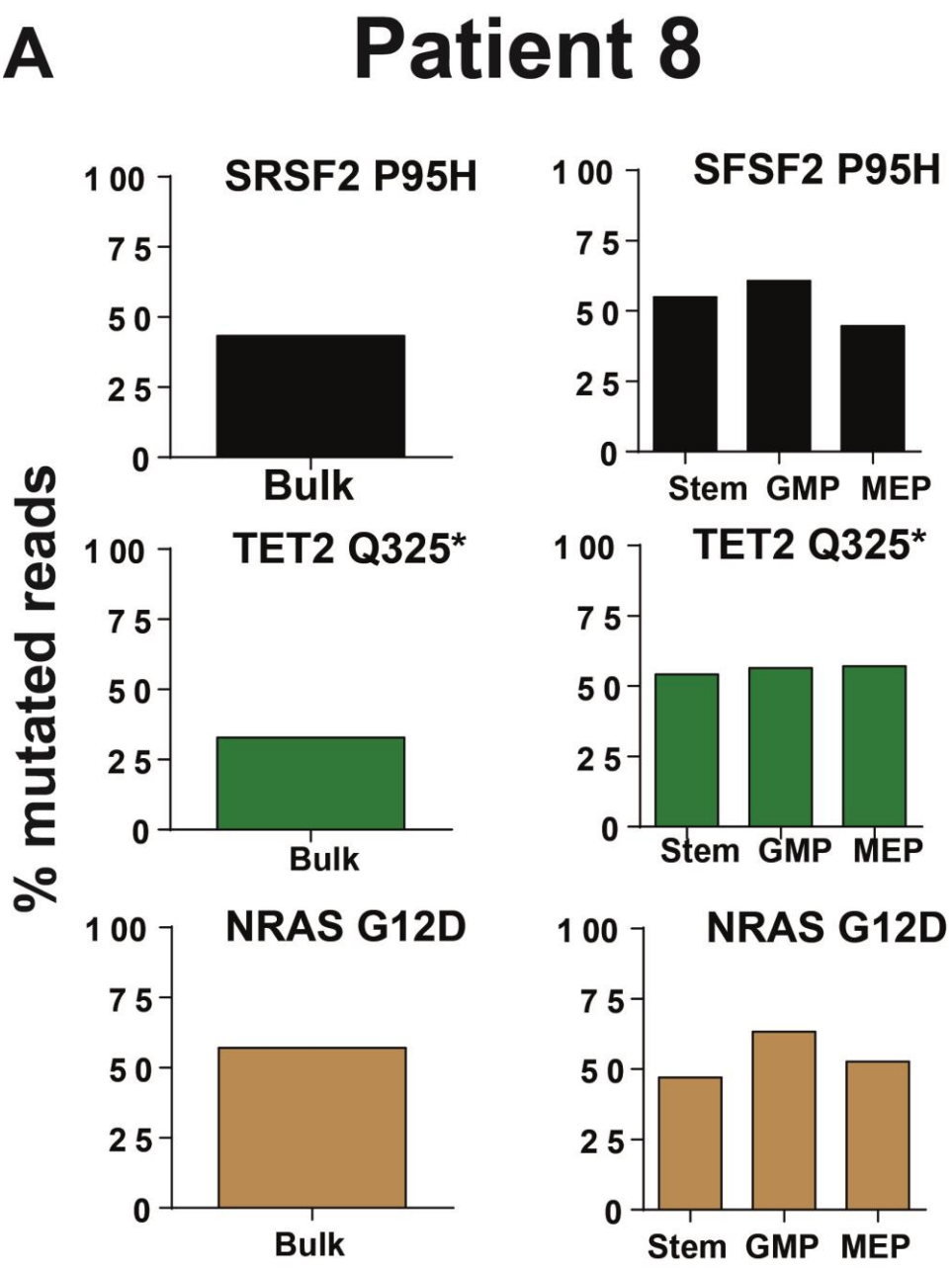
Figure 4.12 Tracking of DNA mutations in Patient 2

A: Detection of DNA mutations from the bulk sample and stem and progenitor populations. Bone marrow mononuclear cells, T cells and purified stem cells, GMPs and MEPs, underwent targeted Haloplex DNA sequencing of selected recurrently mutated genes. Y-axis numbers indicate % mutated reads within genes.

B: Individually picked LTC-CFCs underwent Haloplex mutation screening and were scored as positive (+) if the variant read frequency was >25% or absent (-) if the variant frequency was <5%. Any colonies with a read frequency of 5-25% were scored as indeterminate.

4.3.8.3 Patient 8

Patient 8 was diagnosed with CMML-2 and commenced treatment with Azacitidine soon after diagnosis (see previous NSG transplantation section for further clinical details). Mutation profiling of the pre-treatment bone marrow revealed mutations in *SRSF2*, *TET2* and *NRAS* (**Table 4.2**). All 3 mutations were detected in purified stem and progenitor populations (**Figure 4.13a**). However of the three LTC-CFC colonies analysed, one did not harbour any detectable mutations whereas all three mutations could be detected in the remaining two colonies (**Figure 4.13b**). This is in keeping with the first colony arising from a normal or pre-CMML stem cell clone, and the other two colonies being derived from malignant CMML stem cells. However, in order to draw more definitive conclusions, many more colonies would need to be analysed.


A
Patient 8
% mutated reads
SRSF2 P95H
SFSF2 P95H
TET2 Q325*
TET2 Q325*
NRAS G12D
NRAS G12D
100
75
50
25
0
Bulk
Stem GMP MEP

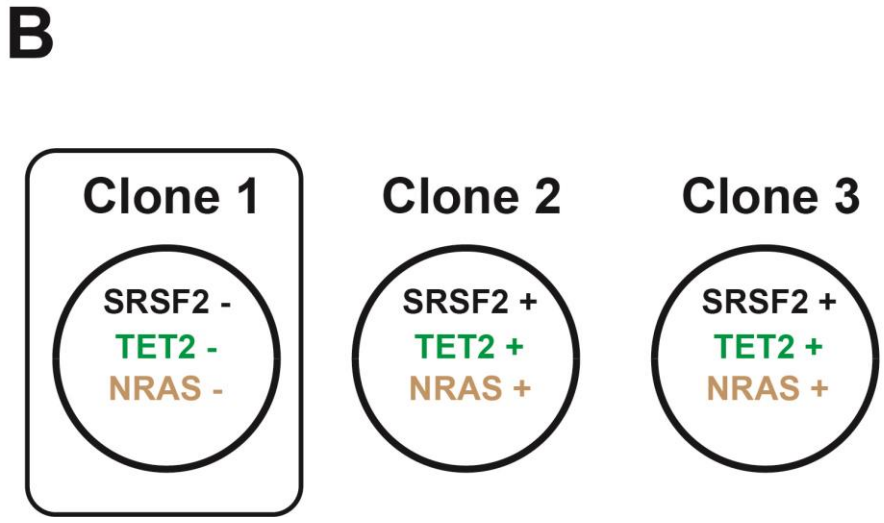

B
Clone 1
SRSF2 -
TET2 -
NRAS -
Clone 2
SRSF2 +
TET2 +
NRAS +
Clone 3
SRSF2 +
TET2 +
NRAS +

Figure 4.13 Tracking of DNA mutations in Patient 8

A Detection of DNA mutations from the bulk sample and the stem and progenitor populations. Bone marrow mononuclear cells, purified stem cells, GMPs and MEPs were analysed by targeted Haloplex DNA sequencing of selected recurrently mutated genes. No T cell control was available so only mutations known to be recurrently mutated in CMML were called somatic changes. Y-axis numbers indicate % mutated reads within the selected genes**.**

B Individually picked LTC-CFCs underwent Haloplex mutation screening and were scored as positive (+) if the variant read frequency was >25% or absent (-) if the variant frequency was <5%. Any colonies with a read frequency of 5-25% were scored as indeterminate.

4.3.8.4 Patient 9

Patient 9, a 70 year old male, presented with a marked leucocytosis, monocytosis and thrombocytopenia. A bone marrow aspirate revealed a blast count of 17% and a diagnosis of CMML-2 was made (**Table 4.1**). He was treated with one course of Azacitidine but rapidly progressed to AML (22% blasts) and died.

The diagnostic bone marrow sample was screened for DNA mutations, revealing somatic mutations in *SRSF2*, *TET2* and *NRAS* (**Table 4.2**). Although the mutated genes were the same as in patient 8, the pattern of distribution among the stem and progenitor cells was very different. In this patient, the SRSF2 P95H mutation was detectable at very low frequencies in the bulk bone marrow but was *not* detected in the purified stem cells. The mutation was also not detectable in the MEPs but was present at a frequency of 36.8% in the purified GMPs. In this patient we were also able to isolate B cell progenitors (Pro B cells) from the bulk bone marrow. If a mutation were to be detected in the B cell lineage, it would provide additional evidence that the mutation arose in an early multi-potent progenitor rather than a lineage restricted myeloid progenitor. However, the *SRSF2* P95H mutation was not detectable in the purified Pro B cells (**Figure 4.14a**). The *SRSF2* P95H mutation was also not detected in any of the 9 LTC-CFCs analysed (**Figure 4.14b**). However it is important to note that only a small number of colonies were analysed and all 9 could have been derived from the same stem cell. Although we were not able to detect the mutation in any stem cell or stem cell derived colony, our data does not definitively prove that the mutation did not originate in a stem cell. It may have been present but below the level of detection of the assays and analysis employed.

In contrast the *TET2* C1271Y mutation was detected in the bulk bone marrow, stem and progenitors cells, including the Pro B cells and all 9 LTC-CFC colonies (**Figure 4.14**). This

would be consistent with the *TET2* mutation being a very early event, originating in the stem cell. The *NRAS* G13D mutation was present in the bulk bone marrow, stem cells, GMPs, MEPs and ProB cells but was *not* detected in any of the 9 LTC-CFC colonies (**Figure 4.13b**). As it was detected in the stem cells and the Pro B cells, this provides evidence for it being present in a multi-potent stem cell. However its absence from the stem cell derived LTC-CFCs could be consistent with the stem cell clone containing the *NRAS* mutation being at a competitive disadvantage in the LTC-CFC assay when compared to the ancestral *TET2*-mutated stem cell clone. Alternatively it could be consistent with the *NRAS*-containing stem cell clone being at a much lower frequency compared to the *TET2* only mutated clone, resulting in a need to screen many more LTC-CFCs before the *NRAS* mutation would be found. The mutated read frequencies are were also very informative in this patient, as the *NRAS* variant reads are at a much lower frequency than the *TET2* variant reads in the bulk and purified populations, suggesting they are part of a smaller clone.

This pattern of mutation prevalence in the stem and progenitor populations may be consistent with the *TET2* C1271Y being an early mutation, acquired in the stem cell. This may have been followed by the acquisition of the *NRAS* G13D in the stem cell. The data does not confirm the acquisition of the *SRSF2* mutation in the stem cell. This may be due to a lack of sensitivity in our assays or alternatively the SRSF2 mutation may have been acquired in a cell downstream of the stem cell (**Figure 4.15**). It is possible that it could have been acquired in the downstream GMP but in order for this mutation to be sustained, the GMP would have to acquire self-renewal potential. GMP self-renewal ability is not supported by the LTC-CFC results. Alternatively, it may have occurred in a self-renewing progenitor downstream of the stem cell that then gave rise to mutated GMPS, such as a multi-potent progenitor (MPP).

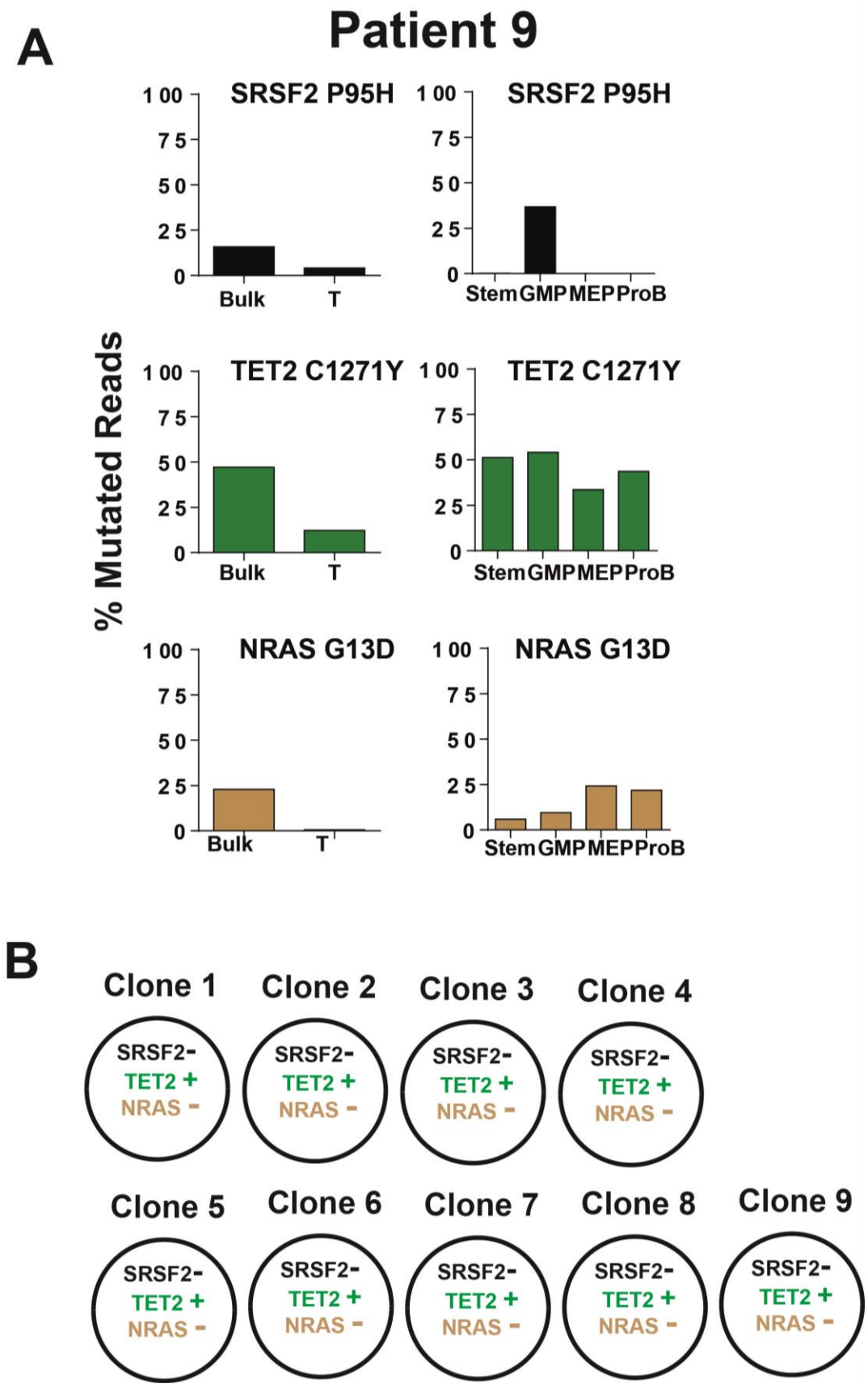

A
Patient 9
% Mutated Reads
SRSF2 P95H
TET2 C1271Y
NRAS G13D
100
75
50
25
0
Bulk
T
Stem
GMP
MEP
ProB
B
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
Clone 6
Clone 7
Clone 8
Clone 9
SRSF2-
TET2 +
NRAS -

Figure 4.14 Tracking of DNA mutations in Patient 9

A Detection of DNA mutations from the bulk sample and stem and progenitor populations. Bone marrow mononuclear cells, T cells and purified stem cells, GMPs and MEPs, underwent targeted Haloplex DNA sequencing of selected recurrently mutated genes. Y-axis numbers indicate % mutated reads within genes.

B Individually picked LTC-CFCs underwent Haloplex mutation screening and were scored as positive (+) if the variant read frequency was >25% or absent (-) if the variant frequency was <5%. Any colonies with a read frequency of 5-25% were scored as indeterminate.

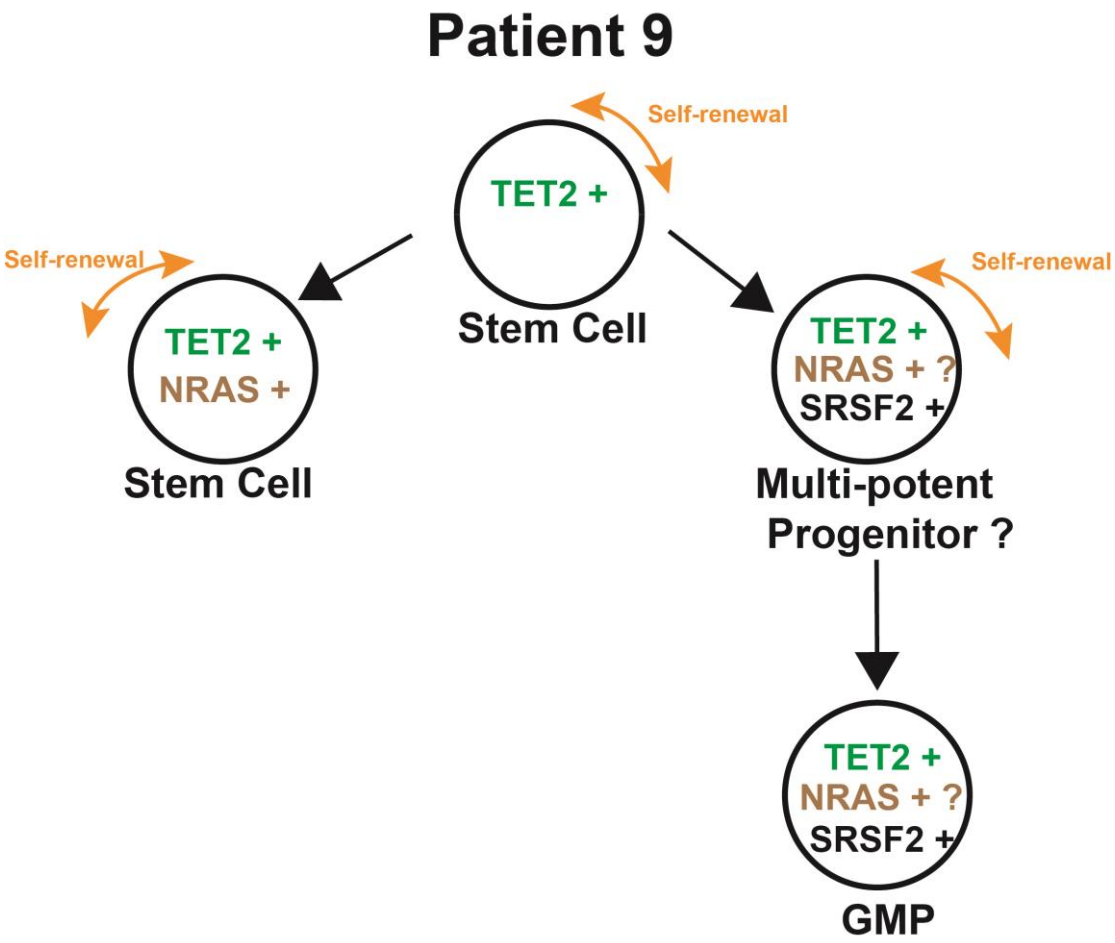


Figure 4.15 Model of mutation acquisition in stem and progenitor cells in Patient 9

The ancestral clone may have contained the *TET2* C1271Y mutation alone. A subset of this clone may then have acquired the NRAS G13D mutation, demonstrating a linear acquisition of mutations. The *SRSF2* P95H may have occurred outside of the stem cell compartment, in a downstream progenitor, which has acquired self-renewal potential. Alternatively, the SRSF2 P95H mutation may have occurred in the stem cell but be present at a frequency below the detection threshold of our assays.

4.4 Discussion

Previous studies have reported that del (5q) MDS originates in the CD34+ CD38- CD90+ multi-potent stem cell. In the previous chapter of this thesis it was demonstrated that this is also the case for low/int-1 risk MDS patients beyond del (5q). We used tracking of mutations in patients to show definitively that this population was the only population with functional MDS stem cell activity. However, low/int-1 risk MDS patients only comprise a fraction of all MDS patients and may not model how more advanced disorders behave at the cellular or molecular level. Therefore a key question in the field is what is the driving cancer stem cell population in other more advanced subtypes of MDS and other myeloid malignancies?

We chose to study patients with the MDS/MPD overlap disorder CMML, a disease at the opposite end of the prognostic spectrum compared to del (5q) MDS with a median survival of only 5-20 months. Prior to commencing this study, very little was known about the cellular origin or clonal architecture of CMML. An additional important reason for making CMML such an exciting disease to study, was the fact that the potentially key genetic drivers of the disease were being elucidated at the time this study started.

A number of studies have shown that patients with CMML have a striking 28-47% prevalence of a highly conserved somatic mutation in the *SRSF2* gene[97,99,105,124]. A single point mutation produced an amino acid change at position 95, resulting in a substitution of proline for histidine, in the majority of patients. The remaining patients almost all had a single base change at the same position resulting in substitution of proline for leucine or arginine.

With such a high prevalence, the *SRSF2* P95 mutation is not only likely to be a key driver of CMML, but for the purposes of understanding the cellular structure of CMML, it provided a clonal marker.

In order to assess whether tracking of SRSF2 and additional mutations would prove to be a useful tool for identifying the CMML stem cell in patients, it was essential to establish whether CMML haematopoiesis was hierarchically arranged and retained identifiably distinct stem and progenitor cells. Using the same cell surface markers as were used to identify stem and progenitor cells in the low/int-1 MDS patients, it was clear that patients with CMML did retain distinct stem and progenitor compartments (**Figure 4.2**). Gene expression and *in vitro* functional assays clearly demonstrated that the GMPs and MEPs isolated from CMML patients retained distinct myeloid and erythroid signatures (**Figure 4.3** and **Figure 4.4**). Importantly the progenitors had not acquired aberrant self-renewal potential, as can be seen in other myeloid malignancies (**Figure 4.5**).

The phenotypic CD34+ CD38- CD90+ stem cells retained a stem cell like gene expression signature, were the only population to produce colonies in the LTC-CFC assay and remained predominantly quiescent. All these features were highly suggestive of the fact that as in low/int-1 MDS, the most likely candidate cancer stem cell in CMML was the phenotypic CD34+ CD38- CD90+ stem cell. Overall, these data provided evidence that the haematopoietic hierarchy was preserved with the stem cells likely to be residing above the downstream progenitors but limited conclusions could be drawn from this data alone, as it was all generated *in vitro.*

In order to investigate CMML propagating ability *in vivo,* stem cells, CMPs, GMPs and MEPs were purified from three patients and injected in to NSG mice. The results clearly demonstrated some of the shortcomings of relying on this technique to establish long-term

repopulating ability. Only transplantation of stem cells from patient 1 resulted in any long-term engraftment but there was no evidence to suggest that this was derived from clonal CMML cells. These experiments confirmed the lack of sensitivity of xenotransplantation, as a reliable human cancer stem cell assay. However, the LTC-CFC assays performed suggested that the CD34+ CD38- CD90+ stem cell was the most probable candidate CMML stem cell.

As CMML patients have a multitude of somatic mutations, these mutations could be used to aid identification of the cell population driving disease in an individual patient. Any stable mutation would need to originate in a cell with long-term self-renewal capacity in order to be propagated throughout the clone. Therefore all such somatic changes should be detected in the candidate CMML stem cell in a given patient.

All patients included in the study had at least one detectable somatic mutation (**Table 4.2**). All mutations apart from the *SRSF2* P95H mutation in patient 9 could be traced back to the CD34+ CD38- CD90+ stem cell. Along with the fact that all functional assays strongly supported that this was the only cell with self-renewal capacity, this genetic fate mapping approach suggests that the phenotypic stem cell is the most likely candidate CMML stem cell.

Patient 9 provided the most unique and interesting data. Mutations in *SRSF2*, *TET2* and *NRAS* were detected in the bulk sample. The *TET2* mutation was present in all purified populations, including Pro B cells (CD34+ CD19+) and all stem cell derived LTC-CFCs, providing strong evidence that this genetic change occurred in the stem cell. The allelic burden of this mutation was also higher than the remaining two mutations. The *NRAS* mutation was also present in all the purified populations, including Pro B cells but was not detected in any of the 9 LTC-CFC colonies screened. However detection in the stem cell and Pro B cells supported a stem cell origin of this mutation. This could be consistent with the

NRAS mutation occurring in a stem cell which had already acquired the *TET2* mutation, resulting in a new *TET2* and *NRAS* mutated sub-clone, which did not read out in the LTC-CFCs analysed so far. Finally the *SRSF2* mutation was only detected in the bulk bone marrow and purified GMPs. It was not detectable in the stem cells, MEPs, Pro B cells or LTC-CFCs. Our data do not definitively demonstrate that this mutation did not occur in the stem cell. It may simply have been that our assays were not sensitive enough to detect the mutation. Although it was not detected in any of the 9 single stem cell derived clones analysed, it is important to note that all of these clones could have been derived from only one stem cell. It would be very informative to perform targeted mutation analysis of FACS purified single stem cells. Purified single stem cells could also be put in to liquid culture and the derived colonies could also be sequenced[126]. A combination of these techniques would enable screening of a large number of phenotypically defined single stem cells.

An alternative explanation may be that this mutation arose in a GMP but in order for it to be maintained the mutated GMPs would have to acquire self-renewal potential. This seems unlikely as the LTC-CFC assay did not uncover any self-renewal potential in the GMPs but these *in vitro* results do not rule out GMP self-renewal capacity in the patient. Alternatively, the SRSF2 mutation may have occurred in a progenitor downstream of the stem cell, such as a multi-potent progenitor (MPP). Such a cell would be required to have self-renewal ability but may have limited differentiation capacity, only giving rise to *SRSF2*-mutated GMPs, rather than MEPs or lymphoid cells. In order to see if the GMP was the most likely origin of the SRSF2 mutation, it would be very informative to analysed single GMPs or single GMP-derived colonies, to reveal their clonal composition. As neither LTC-CFCs or NSG transplantation demonstrated GMP self-renewal ability, serial replating of GMP-derived CFCs may be helpful. Functional and genetic analysis of populations upstream of the GMP

such as the CMP (CD34+ CD38+ CD45RA-CD123+) and MPP (CD34+ CD38- CD90- CD45RA-) may help elicit if an alternative progenitor is the source of the mutation.

Overall the data from this cohort of CMML patients suggest that *in vitro* assays and DNA mutation tracking can in combination be powerful tools for uncovering the identity of cancer stem cells.

4.4.1 Future Directions

Since this project began, a competing group led by Eric Solary have published a detailed study of the clonal architecture of CMML[126]. They utilised detailed mutation analysis of single cell derived colonies to determine the clonal composition of malignant haematopoiesis in 28 CMML patients. Initially CD14+ peripheral blood cells were screened for mutations in 18 genes commonly mutated in myeloid malignancies. The majority of patients had at least 2 mutations. Next, stem and progenitor populations (HSC CD34+ CD38- CD90+; MPP CD34+ CD38- CD90-; CMP CD34+ CD38+ CD45RA- CD123 +; GMP CD34+ CD38+ CD45RA+ CD123+) were FACS sorted from the bone marrow of 11 of the 28 patients. Phenotypic profiles and *in vitro* colony forming capacity of progenitors was reported as not being significantly different to the normal controls, as was the case for the patients in our study. With regards to the functional properties of the HSCs and MPPs, serial re-plating (not LTC-CFCs) were performed, but this was not performed on the CMPs or GMPs to rule out aberrant self-renewal capacity. Single cells were then sorted from the four subpopulations and cultured for 12 days. Clones were then picked and screened for mutations. At least 1 mutation was detected in >75% of HSC clones. In all but one patient, all the mutations found in the bulk material could be traced back to the CD34+ CD38- CD90+ HSCs. In patients where CD3+ T cells were sorted, 7 of the 16 mutations detected in the CD14+ bulk cells, were also

detected in the CD3+ T cells. In combination, these data led the authors to conclude that the CMML clone originated in an early multi-potent CD34+ CD38- cell.

One limitation of this comprehensive study was that culturing single cells could potentially cause skewing in the prevalence of certain mutations and may not be entirely representative of the mutational composition of the original sample. Another important limitation was the fact that they did not look at functionally defined stem cells but relied on phenotypic markers. Interestingly, although our data also strongly suggests that the CMML clone originates in an early multi-potent stem cell, we did not find a large number of mutations with a high frequency of mutated reads in the T cells. This discrepancy between the studies could be due to the fact that we sorted using a much stricter mature T cell phenotype (CD11b-CD34-CD19-CD3+CD4+/8+) resulting in a lower likelihood of sorting non-T cells. Alternatively, our study could be limited as we used prevalence of mutations in the T cells as an indicator of a germline event and may have excluded mutations which would have been included in the Solary study.

Analysis of the cultured single cell colonies was then used to ascertain the order and pattern of mutation acquisition in patients with more than one mutation. In most patients the colony data supported a linear acquisition of mutations. *TET2* and *ASXL1* mutations appeared to precede all other mutations in most cases. Although the majority of mutations were acquired in a linear fashion, analysis of large numbers of colonies also enabled them to demonstrate that certain patients developed subclones with loss of heterozygosity of particular mutations. Interestingly, the most common gene in which loss of heterozygosity was detected was *CBL*. In our study, patient 2 also demonstrated a loss of heterozygosity in *CBL* in the LTC-CFCs.

Despite its limitations, Itzykson *et al* elegantly showed that in a large group of patients, the CMML clone arises in an early multi-potent CD34+CD38- cell. They also provide compelling evidence that in the majority of patients, genetic aberrations are acquired in a linear fashion.

Although the clonal architecture of CMML has now been thoroughly investigated, the Solary study did not focus on the role of the *SRSF2* mutation in CMML pathogenesis. To date, very little is known about the impact of splicing mutations on cancer development. In order to investigate this we are currently working on three approaches.

Firstly in collaboration with a group at the Karolinska Institute, Stockholm, led by Rikard Sandberg, we are developing a method for performing RNA sequencing on small cell numbers. This will enable the examination of the impact of the *SRSF2* mutation on splicing in the stem and progenitor cells of our patients. Secondly, I have designed an *SRSF2* P95H lentiviral vector construct. This will enable gene delivery into human cells in order to study the effect of this specific mutation on stem and progenitor cells *in vitro* and *in vivo* in NSG mice. Finally, another member of our lab has designed an *SRSF2* P95H knock-in mouse model which will enable us to look at the mechanism by which the *SRSF2* P95H mutation may exert a phenotype *in vivo*. These three avenues will provide unique tools for examining the impact of splice factor mutations on malignant haematopoiesis.

5. Activation and therapeutic targeting of stem cells

5.1 Introduction

CD34+ CD38- CD90+ MDS stem cells are clinically important as they appear to be selectively resistant to treatments such as Lenalidomide, which otherwise efficiently eliminate progenitors and mature cells. This appears to be due to MDS stem cells escaping therapeutic elimination[103]. One likely explanation for stem cell resistance to therapy is the highly quiescent nature of MDS stem cells.

Activation of thrombopoietin (TPO) signalling through its receptor MPL is a vital regulator of normal stem cell maintenance, proliferation and cycling in mouse and man[78,79]. This makes TPO an attractive candidate growth factor to specifically target true stem cells in order to enable more effective therapeutic targeting.

A recombinant human TPO (Megakaryocyte Growth and Development Factor) was developed and entered into clinical trials, primarily to improve platelet yield from apheresis in normal volunteers. However, this product was highly immunogenic and rapidly removed from clinical practice due to the development of cross reactive anti-TPO antibodies resulting in severe immune thrombocytopenia in a number of patients[127]. More recently, two new thrombopoietin receptor agonists have been developed and licensed for use in patients with severe thrombocytopenia, most commonly immune mediated thrombocytopaenic purpura (ITP) refractory to standard treatment[128,129]. Clinical trials have demonstrated that Romiplostim and Eltrombopag are able to increase platelet count successfully but no effect on purified stem cells has been published.

Romiplostim is a subcutaneously administered ‘peptibody’. It is composed of the Fc fragment of IgG1, to which four 14-amino acid peptides are bound via glycine bridges at the carboxy terminus of each IgG heavy chain[130]. The amino acid component has no sequence

homology with TPO, making the development of cross-reactive antibodies less likely. It is able to bind the extracellular domain of human and mouse MPL, mimicking thrombopoietin. Treatment of BAF3-MPL cells with Romiplostim resulted in phosphorylation of downstream targets; MPL, JAK2 and STAT5 in a manner very similar to TPO stimulation[130]. Overall *in vitro* data suggests that Romiplostim is a very good TPO mimetic.

Eltrombopag is an orally administered non-peptide small molecule, with a lipophilic end, an acidic end and chelator backbone[131,132]. In contrast to romiplostim, it does not mimic TPO when activating the receptor, as it binds to the trans-membrane portion of MPL rather than the extra-cellular domain. There are significant differences in signalling between Eltrombopag and TPO, including reduced STAT3 and Akt phosphorylation. It is not cross-reactive with mouse MPL[131,132]. Eltrombopag also has a cytotoxic effect on AML cell lines which do not express the TPO receptor and MDS/AML patients samples, strongly suggesting that Eltrombopag may have considerable effects beyond its ability to activate the TPO signalling pathway[133,134].

Although there is some controversy regarding the impact of TPO on stem cell cycling, we hypothesised that TPO receptor agonists would be excellent drugs to enhance cycling of dormant stem cells and enhance their susceptibility to therapeutic targeting. We chose to focus our studies on the impact of Romiplostim rather than Eltrombopag for a number of reasons. As our hypothesis is based on the known importance of TPO in stem cell biology, it made more sense to study Romiplostim as *in vitro* and *in vivo* data both suggest that it mimics the action of TPO more closely than Eltrombopag. Romiplostim is also known to cross react with mouse MPL whereas Eltrombopag does not.

5.2 Materials and Methods

Mice. C57BL6 (CD45.2) and B6SJL (CD45.1) were bred at the Biomedical Sciences Centre, John Radcliffe Hospital, Oxford. All mice used were between 7-10 weeks of age. (See Chapter 2 Methods for detailed experimental design and methods)

Growth factors and cytotoxic agents. Romiplostim (Nplate), G-CSF (Zarzio) and Cytarabine were all patient grade drugs and purchased from the pharmacy at the John Radcliffe Hospital, Oxford

Gene expression analysis. Gene expression was analysed by the Fluidigm Dynamic Array as described in Chapter 2 Methods.

Flow cytometry. Stem/progenitor analysis/isolation of BM mononuclear cells was performed as described in Chapter 2 Methods.

Cell cycle analysis. Cells were fixed and permeabilised with a commercially available kit (BD cell cycle Fix and Perm) before being stained with surface markers and intracellular markers, Ki67 and 4', 6-diamidine-2-phenylidole dihydrochloride (DAPI). Flow cytometry was then performed. Further details are described in Chapter 2 Methods

BrdU analysis Cells were fixed and permeabilised with a commercially available kit (BD Fix and Perm for BrdU analysis). Cells were stained with surface markers prior to DNAse treatment and anti-BrdU staining. Further details are described in Chapter 2 Methods.

***In vitro* assays**. Colony-forming-cell (CFC) assays were performed as described in Chapter 2 Methods.

Competitive transplantation. Donor B6SJL (CD45.1) mice were treated with PBS, growth factors (Romiplostim or G-CSF), Cytarabine or growth factor + Cytarabine. Donor bone

marrow cells plus 5x10*6 C57BL/6 (CD45.2) competitor cells were transplanted into irradiated (9Gy) C57Bl/6 recipients. Lineage reconstitution was performed 3 and 16-17 weeks post transplantation. Further details are described in Chapter 2 Methods.

Xenograft transplantation. Cells were transplanted into irradiated NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice, analysed as described in Chapter 2 Methods.

Single cell tracking. Human HSCs were incubated with different cytokine cocktails in terasaki wells. Cell division was tracked every 24 hours until 96 hours post plating as described in Chapter 2 Methods.

5.3 Results

5.3.1 High expression of the thrombopoietin receptor MPL on MDS stem cells

We hypothesised that Romiplostim would enhance cycling of stem cells by binding the thrombopoietin receptor MPL and subsequently activating the thrombopoietin pathway. It is well established that normal human HSCs express high levels of MPL at both the transcript and protein levels. To investigate whether MDS stem cells also express similar levels of *MPL,* quantitative RT-PCR was performed on the stem and progenitor cells of low/int-1 MDS patients (n=4). As in normal HSCs, the expression of *MPL* was significantly higher on CD34+ CD38- CD90+ stem cells compared to progenitors (CD34+ CD38+) in the MDS patients ($p<0.05$) (**Figure 5.1**).

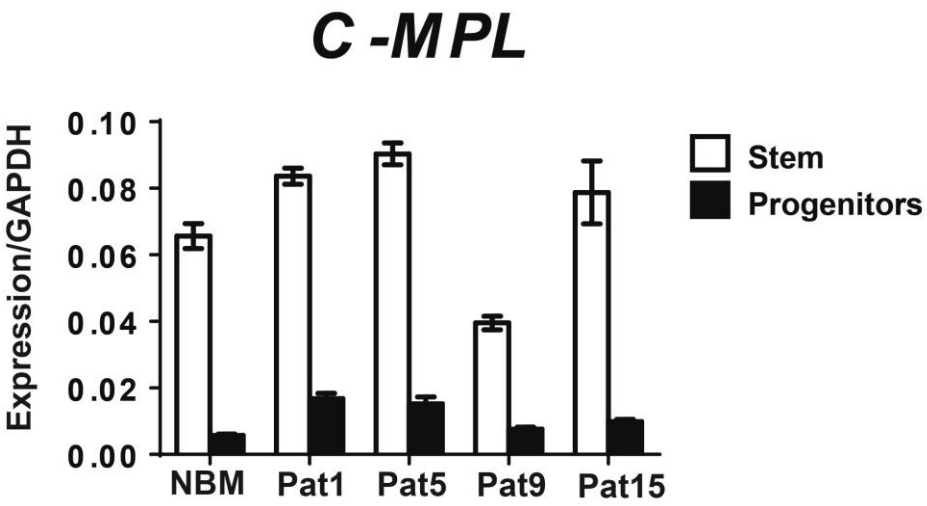


Figure 5.1 Expression of *C-MPL* on MDS stem and progenitor cells

Quantitative RT-PCR was used to assess relative expression of *C-MPL* on sorted stem cells (CD34+CD38- CD90+) and progenitors (CD34+ CD38+) from MDS patient bone marrow (n=4) and a normal control (n=1). 50 cells were FACS purified in 2 replicates per patient per population. Patients shown are from the low/int-1 risk study (Chapter 3 **Table 3.1**).

5.3.2 Romiplostim increases the number of peripheral blood platelets and bone marrow stem cells in mice

Having demonstrated that MDS stem cells expressed high levels of *MPL,* we turned our attention to investigating the impact of Romiplostim at the stem cell level. As patients are currently only infrequently treated with Romiplostim, *in vivo* investigations were pursued by studying the impact of Romiplostim on stem cells in wild type mice.

Mice were injected subcutaneously with Romiplostim (100ug/kg) on alternate days for 5 days (Day 1, 3 and 5). In order to demonstrate that this regimen of Romiplostim was working in the mice, peripheral blood was analysed on day 7. Treatment of mice with Romiplostim resulted in a significant increase in the number of platelets (2.7 fold; p<0.0001) but no expansion in the number of white cells and red cells was seen (**Figure 5.2**).

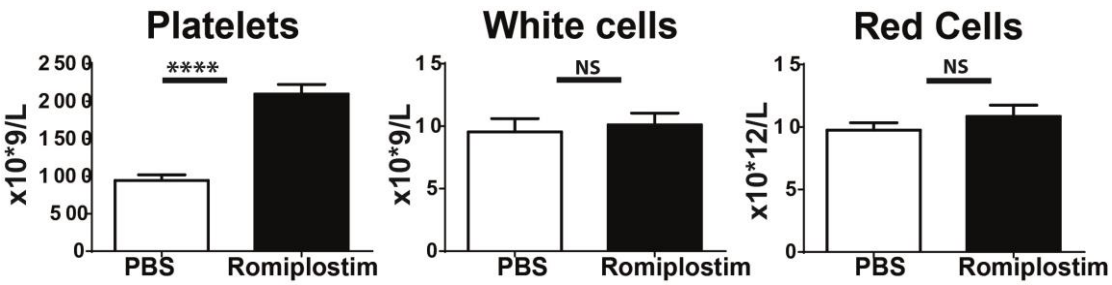


Figure 5.2 Peripheral blood counts post Romiplostim

Mean (SEM) platelet, white cell and red cell counts (**** p<0.0001 student t test). Mice were injected subcutaneously with Romiplostim (n=12) or PBS (n=12) on alternate days for 5 days (100ug/kg Day 1, 3 and 5) before analysing peripheral blood on day 7 using a Sysmex KX-21N analyser. Data from 4 separate experiments is shown.

There was also a clear expansion of the LSK (Lineage- Sca1+ Kit+) compartment in the bone marrow ($p<0.0001$) after Romiplostim. This is a population enriched for mouse stem and progenitor cells. On further sub-fractionation of the LSK compartment, there was a significantly expanded population of phenotypic HSCs (LSK Flt3- CD150+ CD48-; $p<0.001$). There was also a marked expansion of the MPPs (CD150-CD48+) and the CD150+CD48+ compartment ($p<0.001$) (**Figure 5.3** and **Figure 5.4**). However, the lymphoid-primed multi-potent progenitors (LMPPs; LSK Flt3hi) which do not express the *Mpl* receptor at high levels[135], were not increased in number, supporting the fact that Romiplostim is likely to be acting via the thrombopoietin signalling pathway.

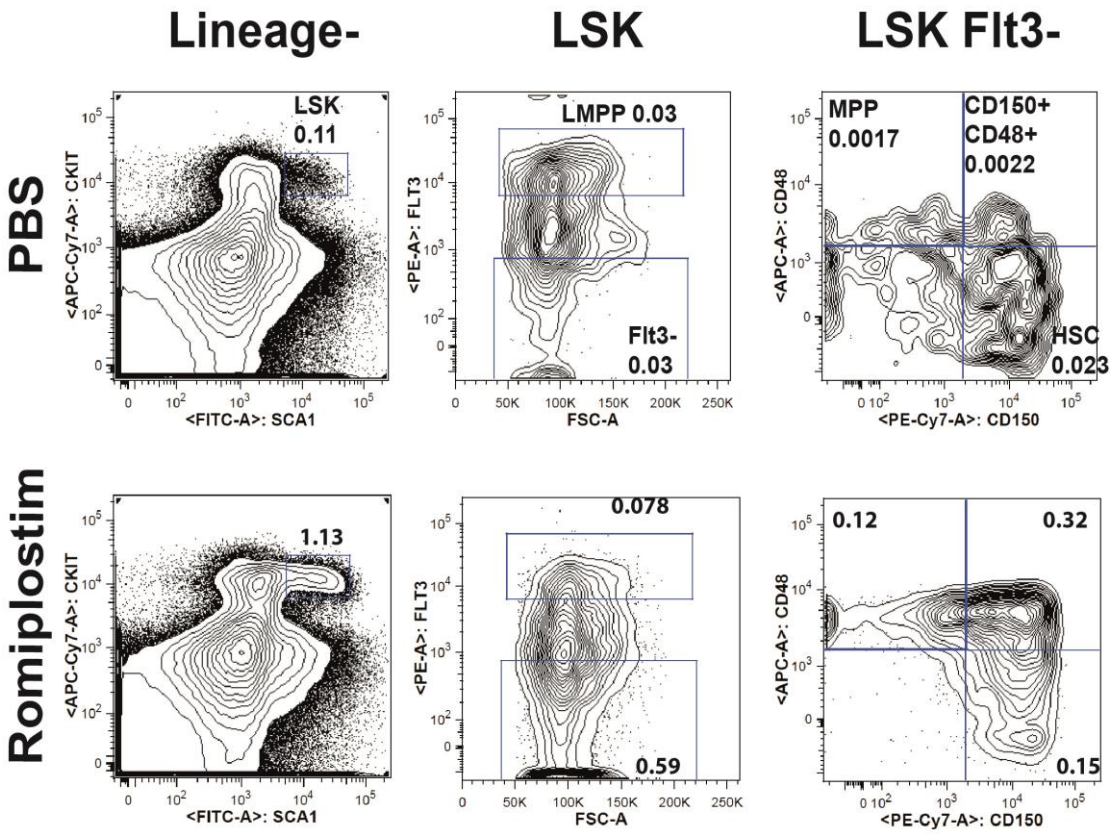


Figure 5.3 FACS profiles of the bone marrow of mice injected with Romiplostim

Representative FACS profile of a mouse treated with PBS (upper panel) and Romiplostim (lower panel). Numbers in quadrants are frequencies of the population as a fraction of the total bone marrow. Mice were treated with Romiplostim or PBS as previously described and bone marrow was harvested for FACS analysis on day 7.

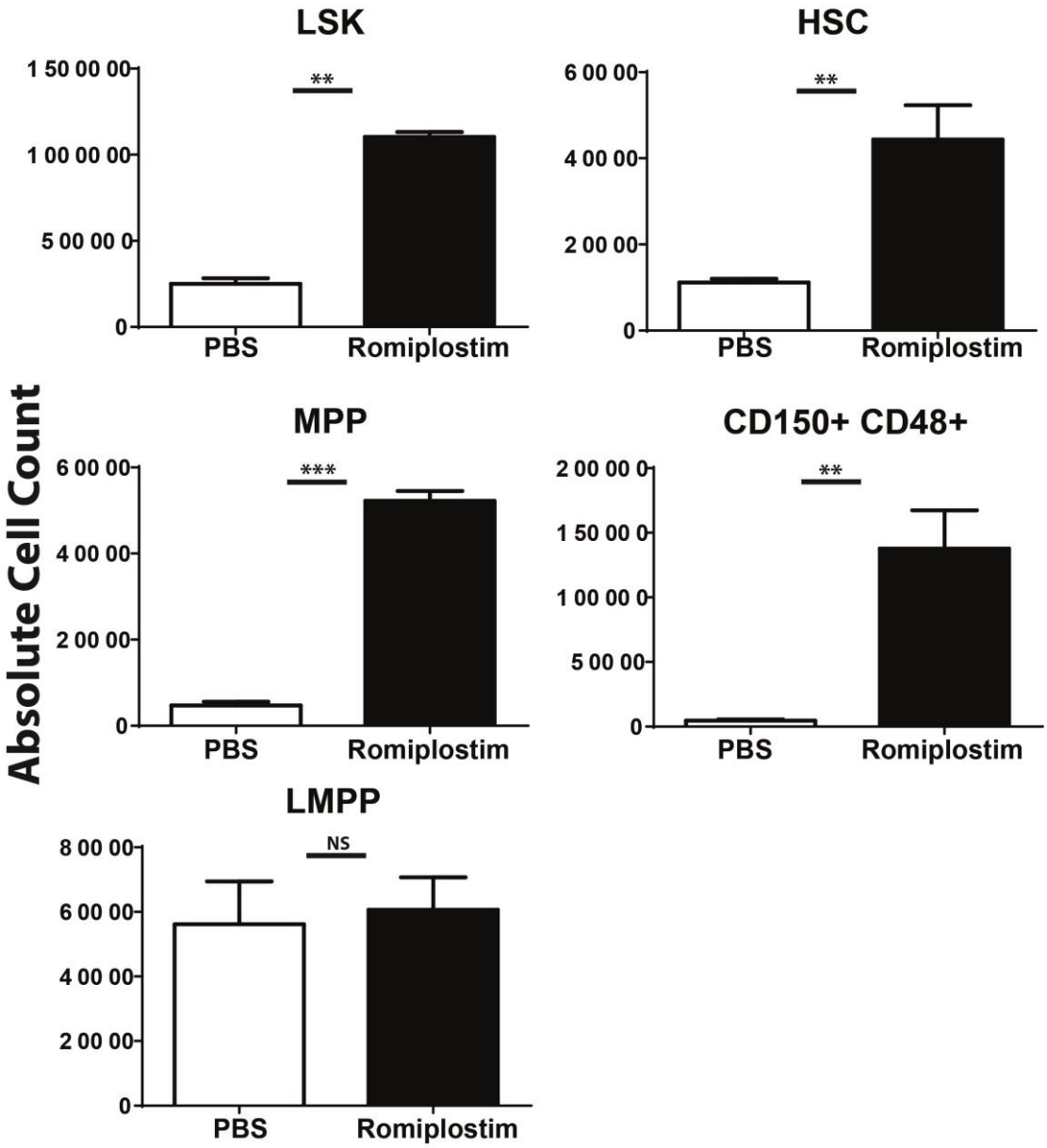


Figure 5.4 Impact of Romiplostim on bone marrow stem and progenitor cell numbers

Mean (SEM) absolute number of bone marrow stem and progenitor cells. Mice were treated with Romiplostim or PBS as previously described. Bone marrow was taken on day 7 of the treatment schedule from Romiplostim (n=8) and PBS (n=8) treated mice. Absolute cell counts were calculated based on total bone marrow cellularity (analysed on a Sysmex KX-21N) and the frequency of the population by flow cytometry (** $p<0.01$, ***$p<0.001$).

5.3.3 Romiplostim increases the cycling of HSCs

Cell cycle analysis by flow cytometry was performed on the bone marrow HSCs at the same time point as the stem and progenitor cell analysis. Romiplostim treatment resulted in a significant reduction of cells in G_0 and a corresponding rise in the number of cells in G_1. This was compatible with Romiplostim potently activating HSCs following injection (**Figure 5.5**). However there was no significant rise in the number of cells in SG_2M.

Phenotypic cell cycle flow cytometry is limited by the fact that it only catches cells at one particular time point but cell cycling is a dynamic process. It is not able to capture cells which have already divided but are now back in the G_0 phase of cell cycle. To further analyse whether cells had entered active synthetic phase at any time point after the final Romiplostim injection, short term BrdU incorporation was performed. BrdU is a thymidine analogue and will only incorporate in to newly synthesised DNA, which can then be stained for and analysed by flow cytometry. Cells which have incorporated the BrdU will retain staining even if they subsequently become more quiescent[59]. Mice were treated with Romiplostim (n=2) or PBS (n=2) as before but on day 5 and day 6 the mice were also given 2mg of BrdU intra-peritoneally. On day 7 the bone marrow was analysed using flow cytometry and although just a small number of mice were analysed, there was a 2.5 fold increase in the frequency of HSCs that had incorporated BrdU post Romiplostim (PBS 16.7% v Rom 39.0%) (**Figure 5.6**). These results confirm that Romiplostim induced over twice as many stem cells to actively proliferate and enter the DNA synthetic phase of cell cycle. This was not uncovered by phenotypic FACS analysis using DAPI and Ki67.

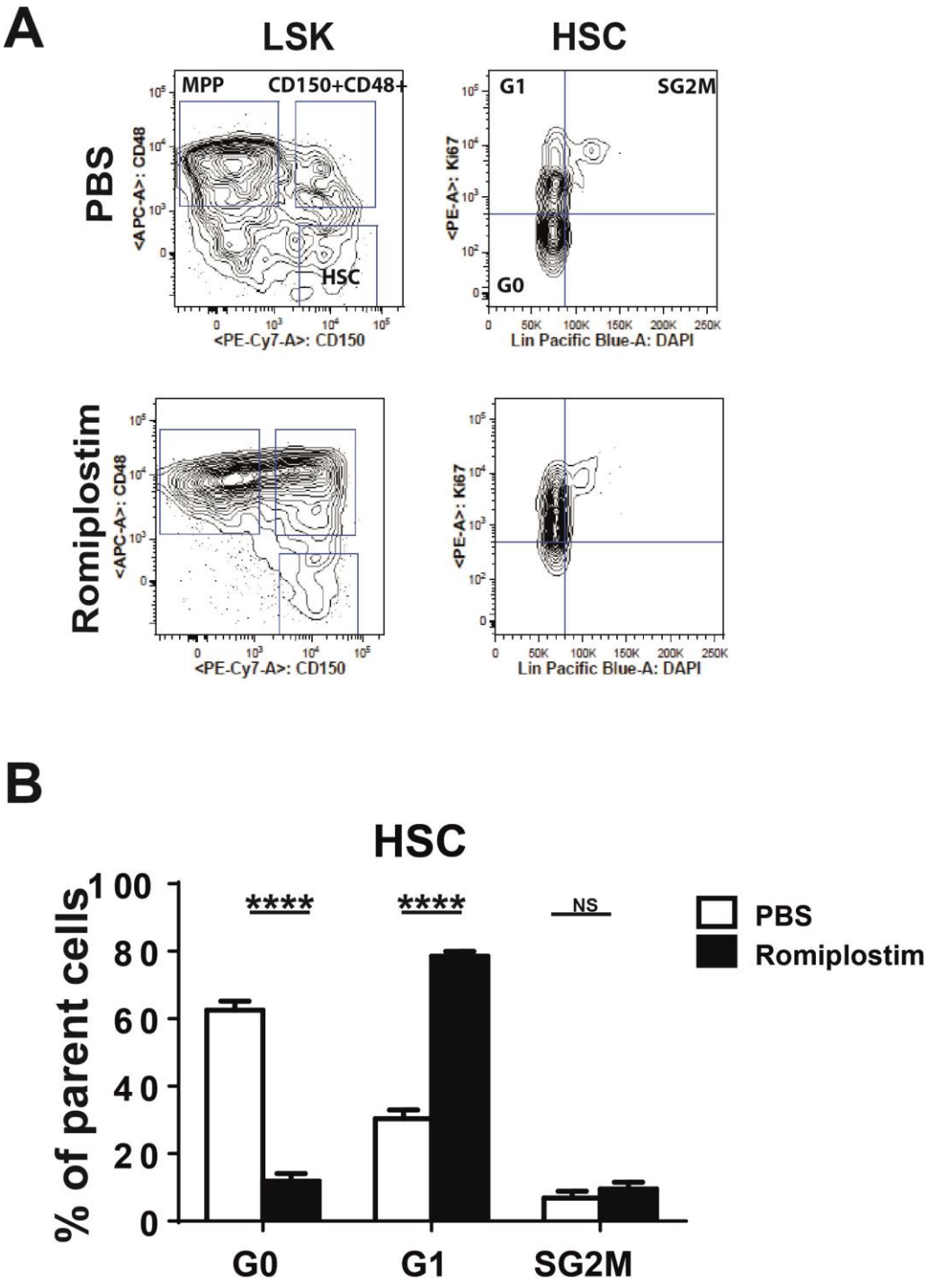


Figure 5.5 Impact of Romiplostim on the cell cycle status of HSCs

A Representative cell cycle FACS profile of PBS and Romiplostim treated mice. **B** Mean (SEM) number of HSCs in G_0 (DAPI-Ki67-) G_1 (DAPI-Ki67+) and SG_2M (DAPI+Ki67+) post PBS (n=8) or Romiplostim treatment (n=8). (**** $p<0.0001$) Data from three independent is experiments shown.

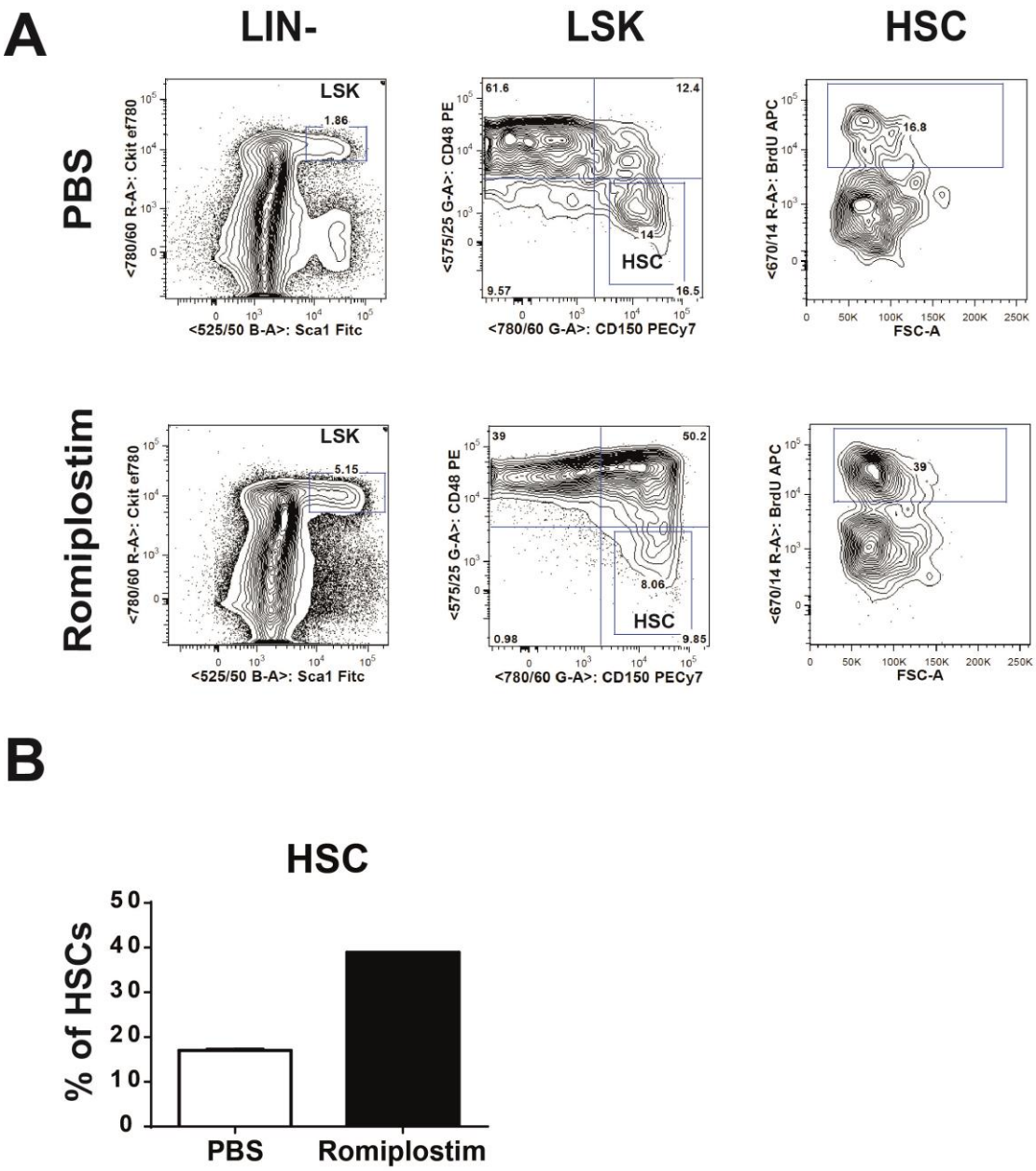


Figure 5.6 BrdU incorporation of HSCs post Romiplostim

A Representative FACS plots of BrdU incorporation in the stem cells of mice treated with PBS or Romiplostim. **B** Mean (SEM) frequency of HSCs which incorporated BrdU. Mice were injected with PBS or Romiplostim on day 1, 3 and 5. On day 5 and day 6 mice were injected with BrdU (2mg IP). Bone marrow was analysed on day 7 for BrdU incorporation by flow cytometry.

5.3.4 Romiplostim mobilises stem and progenitor cells to the spleen and peripheral blood

Another feature of growth factors utilised in clinical practice, is their ability to mobilise bone marrow stem and progenitor cells to the peripheral blood. As this had not previously been demonstrated with Romiplostim, phenotypic stem and progenitor cell analysis was performed on peripheral blood and spleen cells. There was a 9-fold increase in the number of LSK cells ($p<0.001$) and a 6-fold increase in the number of HSCs ($p<0.01$) post Romiplostim compared to PBS in the blood. In the spleen, there was a similar fold increase of LSK cells ($p<0.001$) and HSCs ($p<0.001$) (**Figure 5.7**). In order to quantify mobilisation of functional progenitor cells to the peripheral blood, colony assays were performed. The peripheral blood mononuclear cells from mice treated with PBS (n=3) did not give rise to any colonies. However when the same numbers of cells were plated from the peripheral blood of mice treated with Romiplostim, a mean of 21 colonies were formed (**Figure 5.8**). These data clearly show that Romiplostim is a very efficient stem cell mobilising agent.

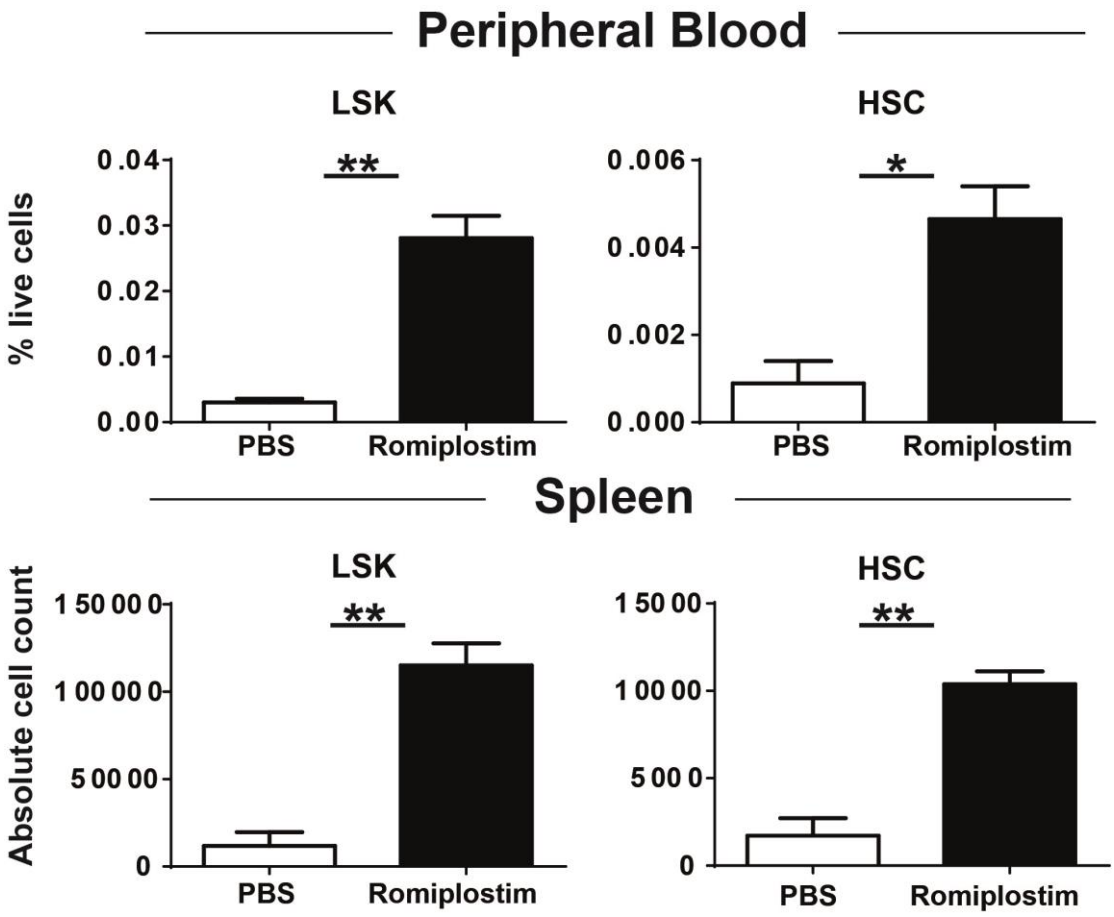


Figure 5.7 Mobilisation of bone marrow LSK cells and HSCs to the peripheral blood and spleen

Mean (SEM) number of LSKs (Lin-Sca1+cKit+) and HSCs (LSK CD150+ CD48-) in the peripheral blood and spleen of mice after treatment with PBS (n=3) or Romiplostim (n=3) as per the treatment regimen previously described. Peripheral blood cells were plotted as the frequency of live mononuclear cells by flow cytometry. Spleen cell counts were plotted as absolute cell counts, calculated based on total bone spleen cellularity and the frequency of the population by flow cytometry (*p<0.05, **p<0.01).

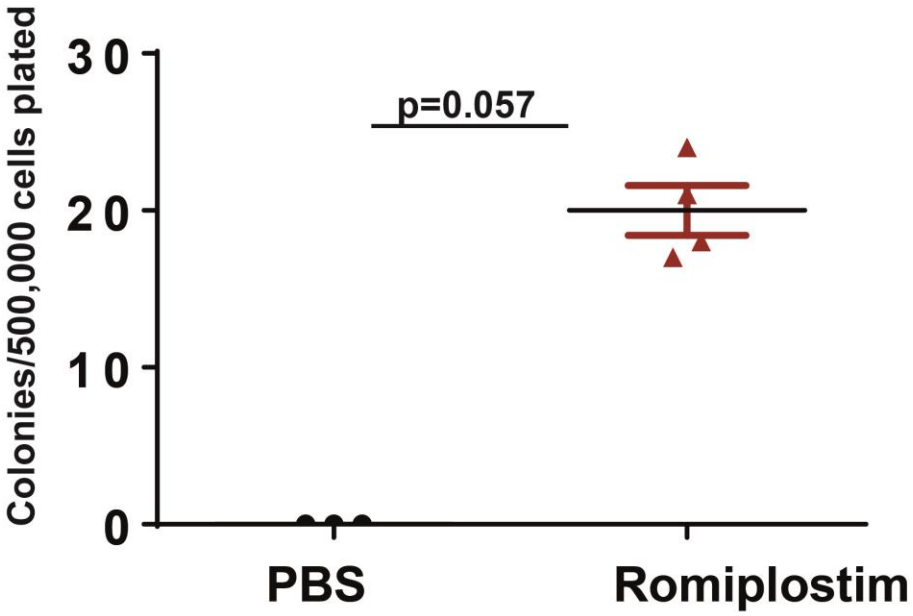


Figure 5.8 Myeloid colony forming ability of peripheral blood cells post Romiplostim

Mean (SEM) number of colonies formed after plating 500,000 peripheral blood mononuclear cells in to methylcellulose + cytokines (see Chapter 2 Methods) post PBS (n=3) and post Romiplostim (n=5). Thick black horizontal lines represent the mean and the horizontal red lines are the SEM. Each symbol represents an individual mouse (p=0.057 Mann Witney Test).

5.3.5 Romiplostim enhances chemotherapeutic elimination of normal mouse stem cells

In order to assess whether Romiplostim induced HSC activation could enhance susceptibility to chemotherapy, mice treated with Romiplostim (100ug/kg, days 1, 3, 5) were treated with Cytarabine chemotherapy (1g/kg IP) on days 5 and 6. It was important to contrast HSC elimination with what could be achieved with Cytarabine alone, so a control arm was treated with PBS (Day 1, 3 and 5) and Cytarabine (Day 5 and 6). Additional control mice were also treated with PBS throughout or single agent Romiplostim (Day 1, 3 and 5). To assess the impact of these regimens on the elimination of functional stem cells, long-term competitive repopulation assays were performed. The treated donor mice were culled on day 7 and bone marrow mononuclear cells transplanted alongside competitor cells into lethally irradiated recipients (**Figure 5.9**). In order to assess long-term donor multi-lineage reconstitution, peripheral blood analysis was then performed at 16 weeks. Secondary transplants were then performed to assess on-going self-renewal capacity of the residual donor cells.

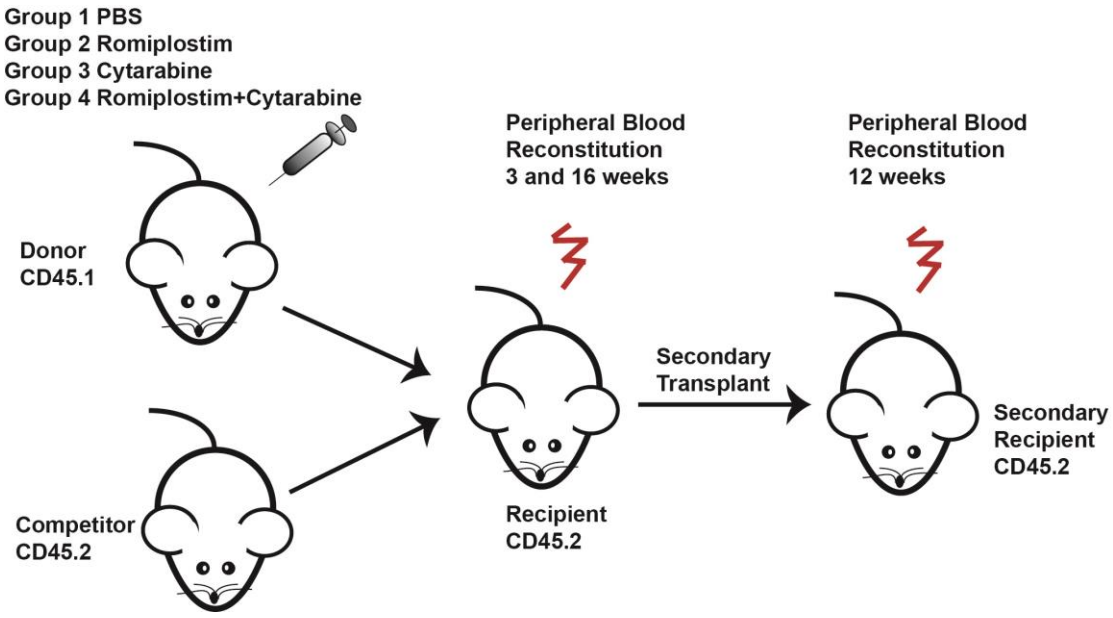
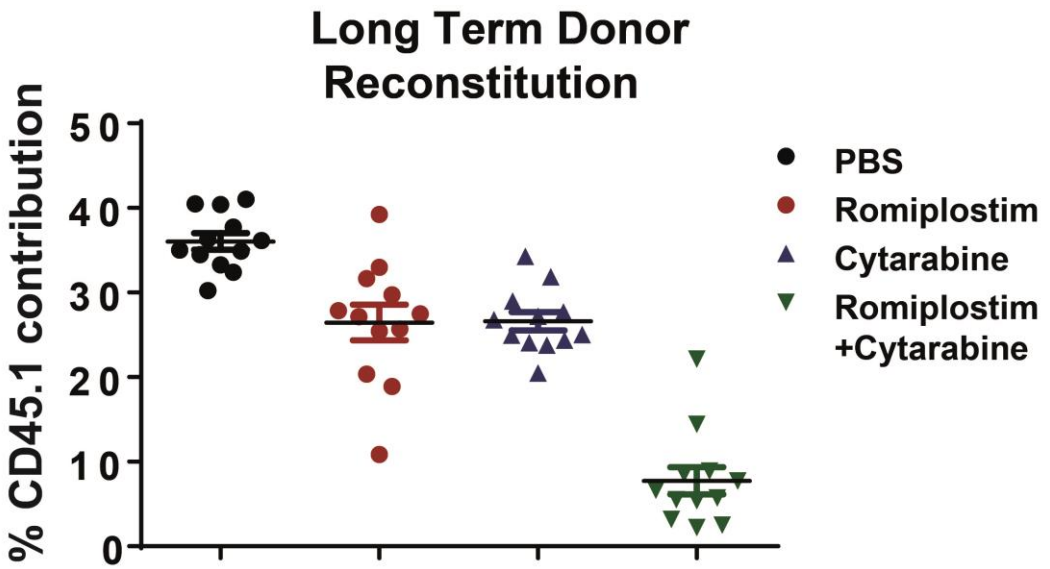


Figure 5.9 Experimental design of competitive transplantation

Donor B6SJL CD45.1 mice were treated in 4 groups with PBS (n=6), Romiplostim (n=6), Cytarabine (n=6) and Romiplostim followed by Cytarabine (n=6). Mice were culled on day 7, 24 hours after completion of treatment and bone marrow was harvested. Recipient C57BL/6 CD45.2 mice were lethally irradiated (9 Gy) and injected with 5 million competitor (CD45.2) and the volume equivalent of 5 million donor cells. Cells from each donor were transplanted into 2 mice (n=24). Peripheral blood reconstitution was analysed and secondary transplantations performed at 16 weeks.

Romiplostim alone had a minimal impact on donor reconstitution compared to PBS treatment, resulting in a 27% reduction in donor reconstitution (PBS 36.0%, Romiplostim 26.45%). Cytarabine alone also resulted in a very similar 26% reduction in CD45.1 contribution (PBS 36%, Cytarabine 26.61%). However, Romiplostim followed by Cytarabine, resulted in a dramatic 80% reduction in donor reconstitution (PBS 36.03%, Romiplostim + Cytarabine 7.75%) (**Figure 5.10**).

Lineage distribution analysis of the donor-derived cells demonstrated a reduction in all lineages, supporting the hypothesis that a multi-potent cell had been eliminated by the dual drug treatment regimen (**Figure 5.11**). As mature T cells and B cells can be long lived, they might not represent cells originating from long-term HSCs. However, myeloid cells are short lived and therefore their depletion offers a more reliable readout with regard to long-term HSCs.



Bonferonni's Multiple Comparisons Test	Mean Diff.	95% CI of diff.	Significant?	Summary
PBS vs. Romiplostim	9.589	3.661 to 15.52	Yes	***
PBS vs. Cytarabine	9.427	3.498 to 15.35	Yes	***
PBS vs. Romiplostim+Cytarabine	28.29	22.36 to 34.21	Yes	****
Romiplostim vs. Cytarabine	-0.1626	-6.091 to 5.766	No	ns
Romiplostim vs. Romiplostim+Cytarabine	18.7	12.77 to 24.62	Yes	****
Cytarabine vs. Romiplostim+Cytarabine	18.86	12.93 to 24.79	Yes	****

Figure 5.10 Impact of Romiplostim on the susceptibility of functional stem cells to chemotherapy

Mean (SEM) contribution of donor CD45.1 cells to recipient haematopoiesis. Each coloured symbol represents an individual recipient mouse. Mice were treated as per the experimental design in **Figure 5.9**. Peripheral blood was taken from the primary recipient mice (n=48) 16 weeks post transplantation and flow cytometry performed to assess contribution of donor (CD45.1) and competitor (CD45.2) cells. (One way ANOVA ***p<0.001 ****p<0.0001).

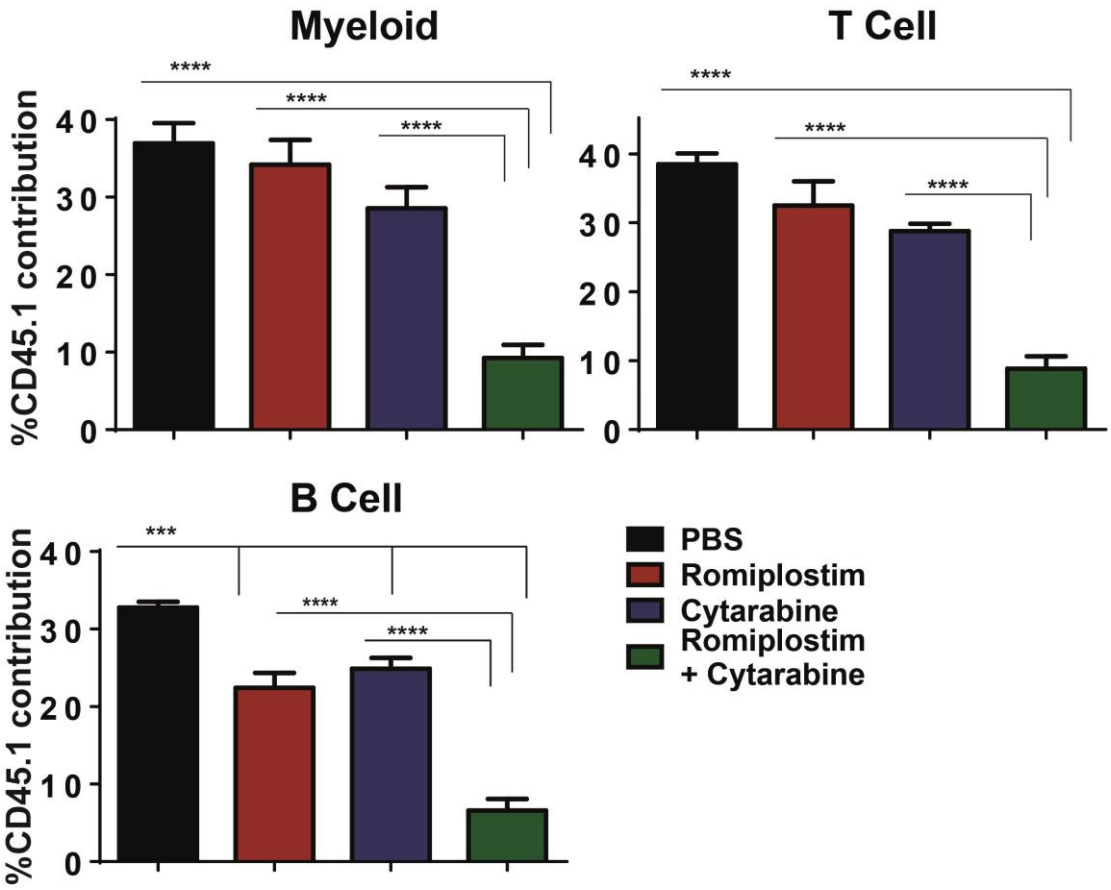


Figure 5.11 Impact of Romiplostim on specific mature lineage reconstitution

Mean (SEM) contribution of donor CD45.1 to the different lineages. Mice were treated as per the experimental design in **Figure 5.9**. Peripheral blood was taken from the primary recipient mice (n=48) 16 weeks post transplantation. Flow cytometry was performed to assess contribution of donor (CD45.1) cells to myeloid cells (Mac1+ Gr1+), T cells (Mac1- Gr1- CD4+/CD8+) and B cells (Mac 1- Gr1- CD19+). All significant changes are shown in the figure. All other differences between the 4 groups were not significant (One way Anova ***p<0.001 ****p<0.0001). Data from two independent experiments are shown.

To investigate reduction in self-renewal capacity of the residual donor derived cells, secondary transplantation of whole bone marrow derived from 3 primary recipients in each treatment arm was performed (**Figure 5.12**). There was a clear reduction in CD45.1 reconstitution in the recipients of mice treated with Romiplostim plus Cytarabine compared to Cytarabine alone, although this did not reach statistical significance due to the limited number of mice transplanted ($p=0.154$). Further secondary transplantations are on-going.

These data show that as previously reported, a large number of HSCs evade chemotherapeutic targeting. However combination treatment with Romiplostim followed by Cytarabine significantly increased the sensitivity of the HSCs to chemotherapy. Overall this clearly demonstrates that combination therapy with Romiplostim and Cytarabine results in enhanced elimination of mouse stem cells as demonstrated by markedly reduced multi-lineage reconstitution in a competitive transplantation and reduced self-renewal in a subsequent secondary transplant.

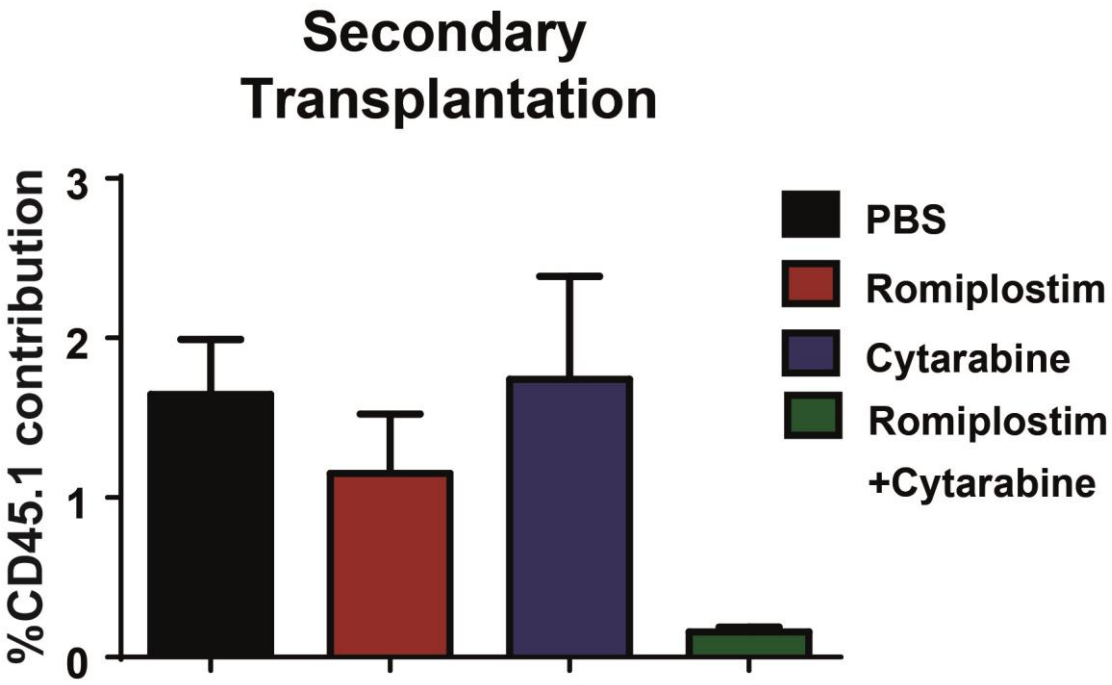
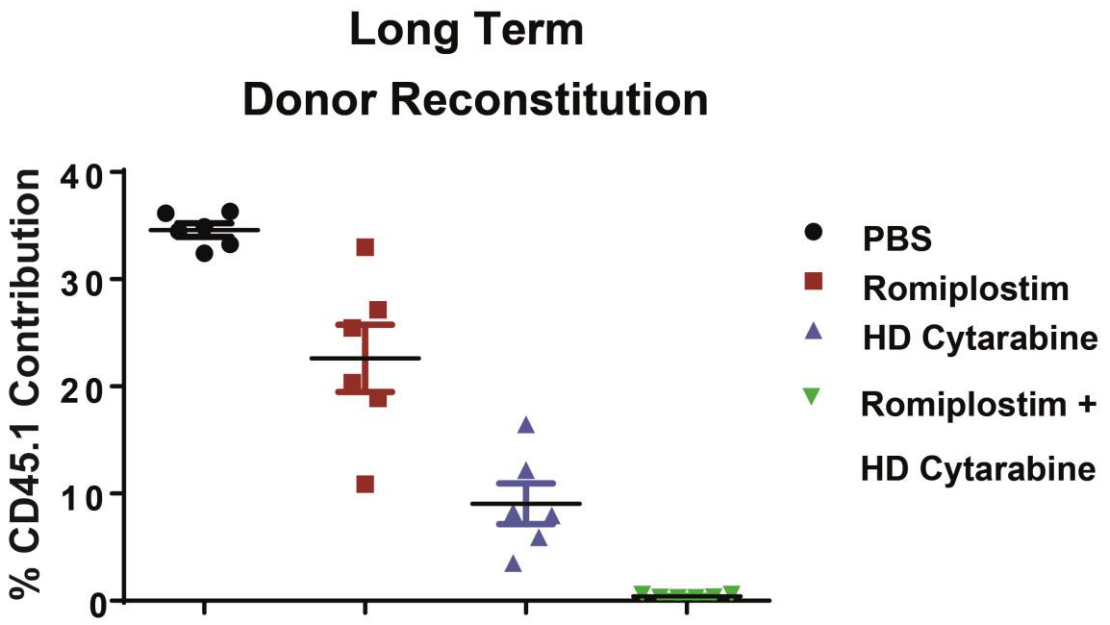


Figure 5.12 Romiplostim priming results in reduced self-renewal capacity of stem cells.

Bone marrow was taken from half of the competitive transplantation recipients from each treatment arm (n=3 per group) and transplanted into one CD45.2 secondary recipient each (n=12) Peripheral blood was then analysed for donor (CD45.1) contribution at 12 weeks. None of the changes seen reached statistical significance (One way Anova).

5.3.6 Increasing the dose of Cytarabine eliminates almost all HSCs

Although combining Romiplostim and Cytarabine treatment can enhance HSC kill, 7.75% of stem cells still remain in the recipients in a competitive transplantation setting and are able to repopulate the recipient (**Figure 5.10**). If this combination were to be used as part of a conditioning regimen in the pre-allogeneic stem cell transplantation setting, it would be important to see if stem cell elimination could be enhanced further, to aid full ablation of the bone marrow. In order to see if this was possible, mice were treated with the same dose of Romiplostim (100ug/kg Day 1, 3 and 5) but given higher doses of Cytarabine (3g/kg Day 5 and 6), a dose used in AML induction regimens. At 17 weeks post competitive transplantation high dose Cytarabine (3g/kg) eliminated more HSCs than the low dose (1g/kg) alone. However there was an even more dramatic 88% reduction in CD45.1 reconstitution between the high dose Cytarabine alone (Mean 9.05%) and high dose Cytarabine plus Romiplostim (Mean 0.40%) (**Figure 5.13**).



Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
PBS vs. Romiplstim	11.96	4.294 to 19.63	Yes	**
PBS vs. HD Cytarabine	25.53	17.86 to 33.20	Yes	****
PBS vs. Romiplostim+HD Cytarabine	34.18	26.51 to 41.85	Yes	****
Romiplostim vs. HD Cytarabine	13.57	5.896 to 21.24	Yes	***
Romiplostim vs. Romiplostim+HD Cytarabine	22.22	14.55 to 29.89	Yes	****
HD Cytarabine vs. Romiplostim+HD Cytarabine	8.649	0.9788 to 16.32	Yes	*

Figure 5.13 Impact of increasing the dose of Cytarabine (3g/kg) on stem cell elimination

Mean (SEM) contribution of donor CD45.1 cells to recipient haematopoiesis. Each coloured symbol represents an individual recipient mouse. Mice were treated as per the experimental design in **Figure 5.9** but the dose of Cytarabine was increased to 3g/kg. Peripheral blood was taken from the primary recipient mice (n=24) 17 weeks post transplantation and flow cytometry performed to assess contribution of donor (CD45.1) and competitor (CD45.2) cells (One way Anova *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001)

5.3.7 G-CSF does *not* cycle HSCs or improve subsequent therapeutic targeting

Although the majority of the published literature suggests that the key impact of G-CSF is to mobilise stem cells, rather than put them in to cell cycle[68,136,137], it was important to establish that the cycling effects seen with Romiplostim could not be recapitulated if Romiplostim was substituted with G-CSF. Mice were injected subcutaneously with PBS, G-CSF (100ug/kg) or G-CSF (300ug/kg) on alternate days for 5 days (Day 1, 3 and 5). The doses of G-CSF chosen were based on the current literature where 100-250ug/kg is the dose used in mouse peripheral blood mobilisation studies. This is much higher than the standard 10ug/kg dose used in patients to mobilise CD34+ cells. However, in case 100ug/kg was simply too low to cycle stem cells, a supra-normal dose of 300ug/kg of G-CSF was also tested.

In contrast to Romiplostim, cell cycle analysis on day 7 post G-CSF revealed that the HSCs of the mice treated with G-CSF did not cycle more actively than those treated with PBS as evidenced by no significant change in the percentage of HSCs residing in G_0 (68.96 vs 64.81 p=0.548), G_1 (25.19 vs 31.66 p=0.339) or SG_2M (5.37 vs 3.45 p=0.233) (**Figure 5.14**).

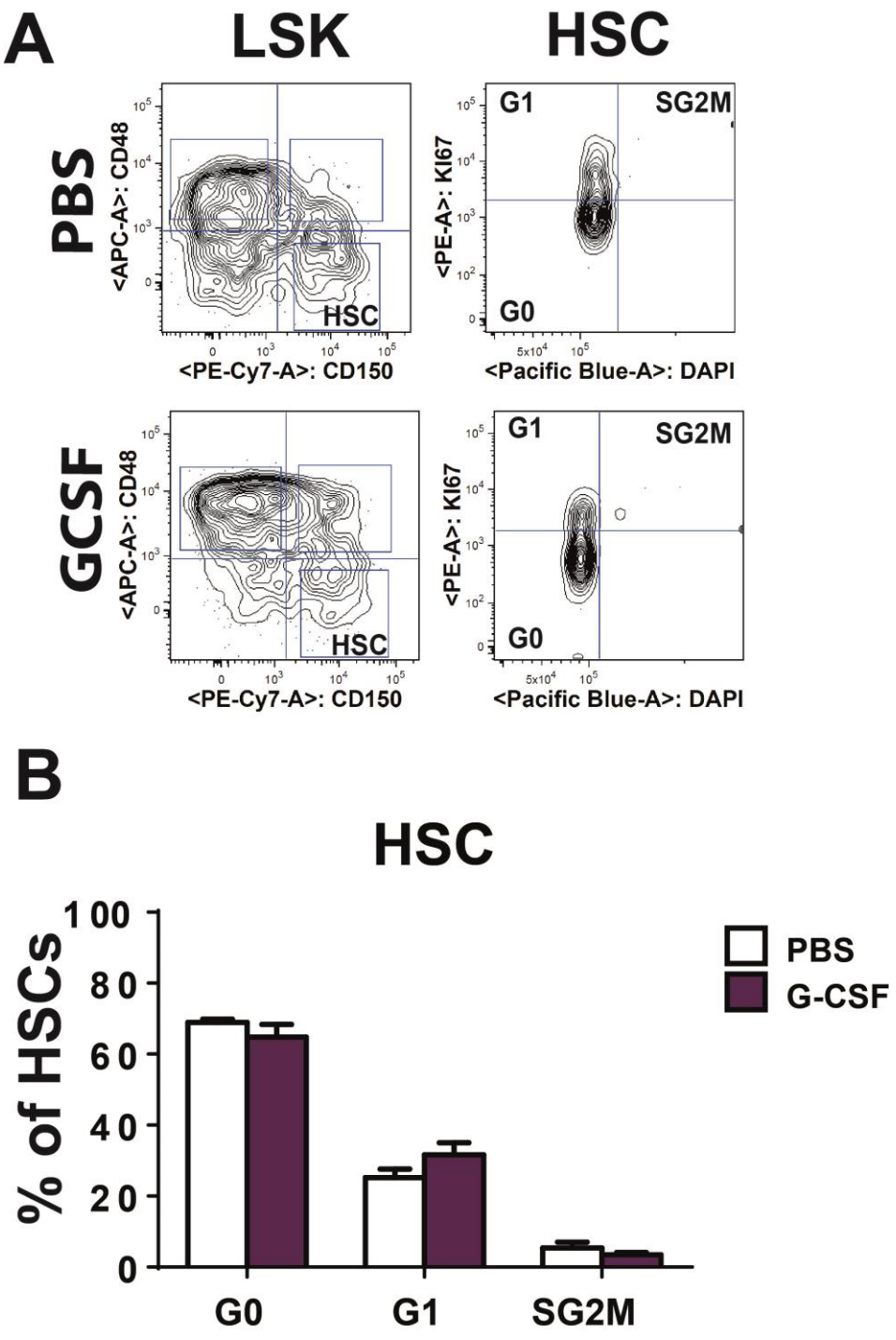


Figure 5.14 Impact of G-CSF on the cell cycle status of HSCs (LSK CD150+ CD48-)

A Representative cell cycle FACS profile of a PBS and G-CSF treated mouse. **B** Mean (SEM) number of HSCs in G_0 (DAPI-Ki67-) G_1 (DAPI-Ki67+) and SG_2M (DAPI+Ki67+) post PBS (n=2) or G-CSF treatment (n=6). Results were combined for G-CSF 100ug/kg and 300ug/kg as there was no significant difference between the two doses (student t test).

In order to assess the impact of G-CSF on stem cell elimination in response to Cytarabine, a similar competitive transplantation experimental design as described in detail above for Romiplostim, was instituted (**Figure 5.15**). G-CSF (100 or 300ug/kg) or PBS was administered on days 1, 3 and 5 subcutaneously. On day 5 and 6 Cytarabine or PBS was given intra-peritoneally. The mice were sacrificed on day 7. Bone marrow mononuclear cells were transplanted along with competitor cells in to lethally irradiated recipients. Peripheral blood reconstitution analysis was then performed at 17 weeks to assess long-term donor CD45.1 contribution to the recipient haematopoiesis.

At 17 weeks post competitive transplantation there was no significant impact of G-CSF alone on donor CD45.1 reconstitution. Most importantly Cytarabine had a clear impact on donor reconstitution but this was not augmented by the addition of G-CSF at either 100ug/kg or 300ug/kg (**Figure 5.16**).

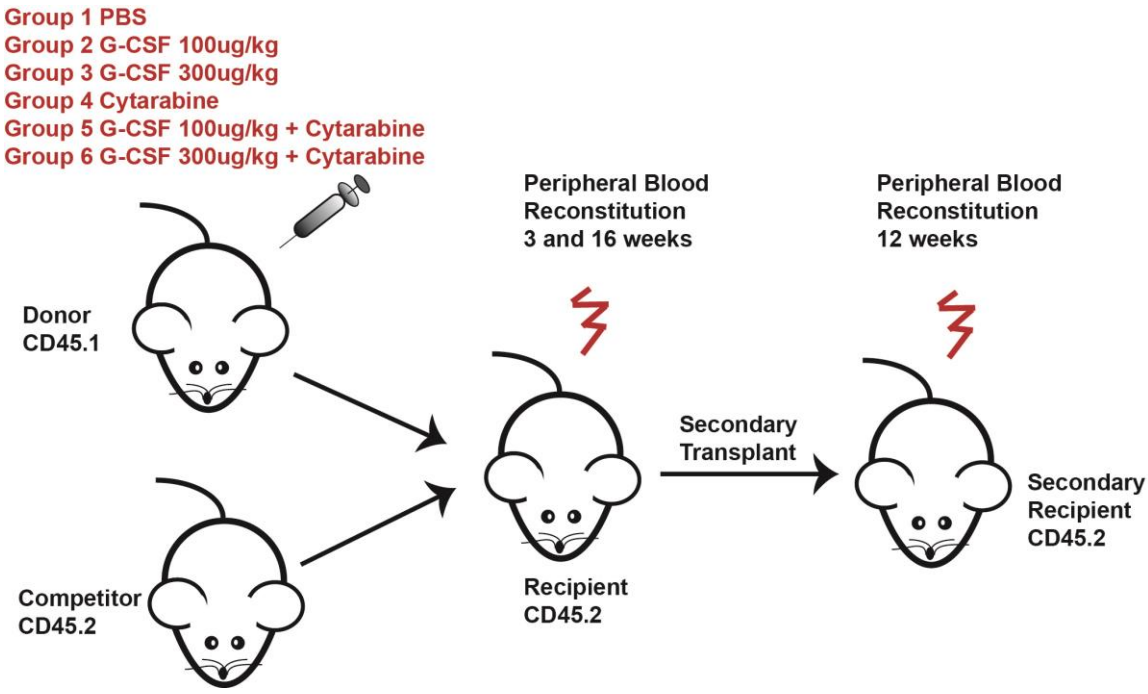


Figure 5.15 Experimental design of G-CSF competitive transplantation

Donor CD45.1 mice were treated in 6 groups with PBS (n=5) G-CSF 100ug/kg (n=6), G-CSF 300ug/kg (n=6) Cytarabine 1g/kg (n=6) G-CSF 100ug/kg + Cytarabine (n=6) and G-CSF 300ug/kg + Cytarabine (n=6). Mice were culled on day 7, 24 hours after completion of treatment and bone marrow was harvested. Recipient CD45.2 mice were lethally irradiated (9 Gy) and injected with 5 million competitor (CD45.2) and the volume equivalent of 5 million donor (CD45.1) cells. Cells from each donor were transplanted into 2 mice (n= 34).

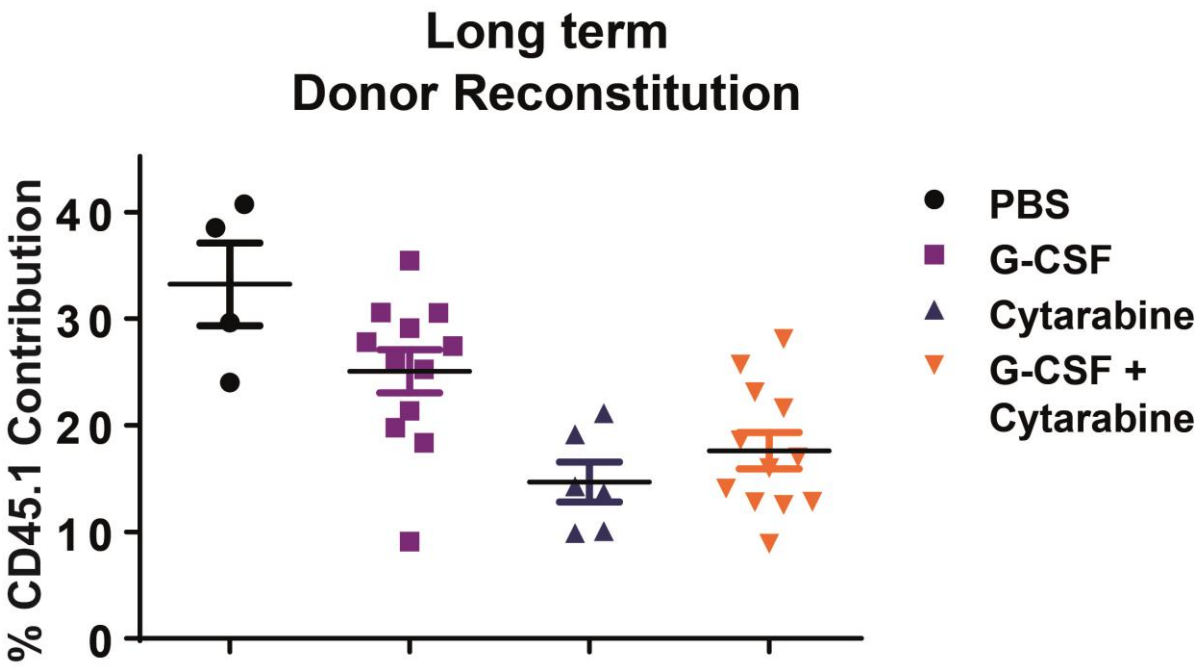


Figure 5.16 Impact of G-CSF on the susceptibility of functional stem cells to chemotherapy. Mean (SEM) contribution of donor CD45.1 cells to recipient haematopoiesis. Each coloured symbol represents an individual recipient mouse. Mice were treated as per the experimental design in **Figure 5.15**. Peripheral blood was taken from the recipient mice (n=34) 17 weeks post transplantation and flow cytometry performed to assess contribution of donor (CD45.1) cells.

As the experiments so far demonstrated no significant difference between G-CSF and PBS with regards to cell cycle, it was important to confirm that the G-CSF was actually working. As it is a well established stem and progenitor cell mobilising agent, peripheral blood from mice treated with PBS and G-CSF (300ug/kg Day 1, 3 and 5) was taken and methylcellulose colony forming assays (CFU-GM) were performed. On plating of 500,000 PB MNCs, no colonies formed from the PBS treated mice. However in the G-CSF treated mice there was an increase in CFU-GM. (Mean 7 SEM+/- 1.08), confirming that the G-CSF was mobilising progenitors from the bone marrow and therefore the doses used were not sub-therapeutic. In combination these studies confirm that G-CSF is able to mobilise but not cycle stem cells.

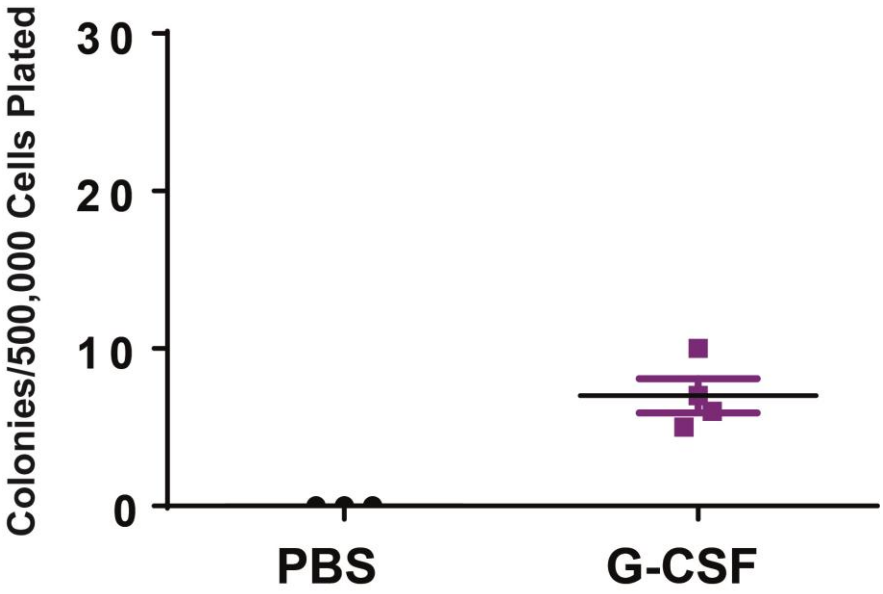


Figure 5.17 Myeloid colony forming ability of peripheral blood cells post G-CSF

Mean (SEM) number of colonies formed after plating 500,000 peripheral blood mononuclear cells in to methylcellulose + cytokines (See Chapter 2 methods) Mice were treated with PBS (n=3) or 300 ug/kg G-CSF on day 1,3 and 5 (n=4). Peripheral blood was taken on day 7. Each symbol represents an individual mouse. NB Scale is the same as in **Figure 5.9** showing colony formation after Romiplostim is far greater.

5.3.8 Romiplostim enhances the rate of human HSC division

Having demonstrated that Romiplostim was clearly able to cycle and target mouse stem cells, the next step was to examine the impact on human stem cells. In order to see if Romiplostim could induce human stem cells to cycle and divide more rapidly, single CD34+CD38-CD90+ normal bone marrow HSCs were sorted and incubated in Terasaki wells containing media supplemented with either stem cell factor (SCF 100ng/ml), SCF (100ng/ml)+ rhTPO (100ng/ml) or SCF (100ng/ml) + Romiplostim (100ng/ml). SCF was used to support stem cell viability as the cells were then incubated for 96 hours and would otherwise fail to survive. Having established that each well only contained one cell at baseline (24 hours post sort), cell division was assessed by counting the number of viable cells per well at 48, 72 and 96 hours. By 96 hours almost all cells incubated with Romiplostim had divided, whereas the vast majority of those incubated in SCF alone had not divided (Divided cells: SCF 12.5%, TPO 93.75%, Romiplostim 87.5%). This confirms that Romiplostim, as is already recognised with rhTPO[43], enhances the rate of cell division of human stem cells (**Figure 5.18a**).

In order to investigate whether activation of stem cells with Romiplostim would enhance the susceptibility to chemotherapy *in vitro*, purified HSCs were sorted and incubated in either SCF or SCF + Romiplostim for 48 hours in Terasaki wells (10-20 cells per well). After 48 hours of incubation, Cytarabine was added to the cells at varying concentrations (16nM-128nM). Viable cells were counted prior to the addition of Cytarabine and then at 72 hours and 96 hours, i.e. 24 hours and 48 hours post Cytarabine. Addition of Cytarabine enhanced the rate of elimination of HSCs in both arms but the impact was greater in the cells treated with Romiplostim + SCF rather than SCF alone by 72 hours, although this was not significant (student t test) (**Figure 5.18b**).

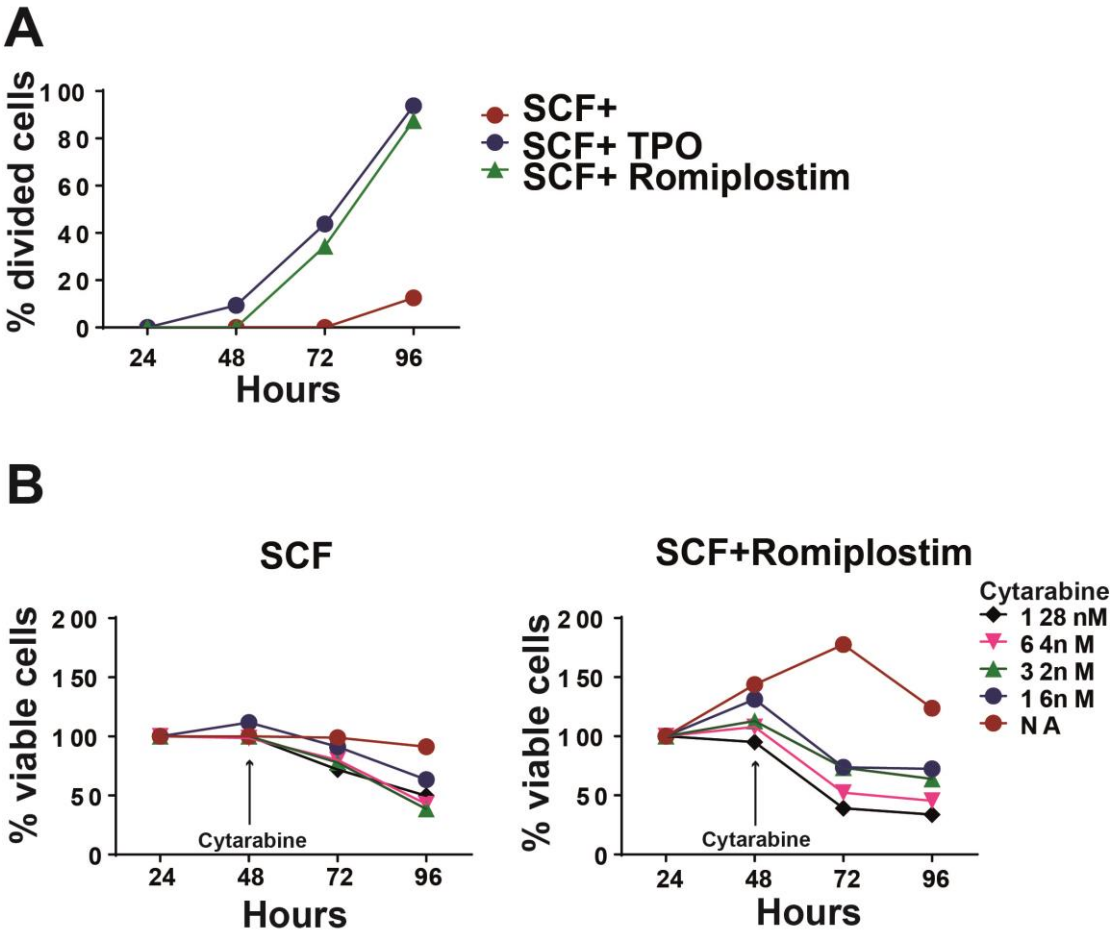


Figure 5.18 Impact of Romiplostim and Cytarabine on human HSC cell division and viability

A Normal bone marrow single HSCs (CD34+CD38-CD90+) were sorted and incubated in either SCF (100ng/ml), SCF (100ng/ml) + rhTPO (100ng/ml) or SCF (100ng/ml) + Romiplostim (100ng/ml). Cell division was tracked by counting the number of viable cells per well at 24, 48, 72 and 96 hours. **B** HSCs from the same normal bone marrow (10-20 per terasaki well) were sorted and incubated in SCF (100ng/ml) or SCF (100ng/ml) + Romiplostim (100ng/ml) for 48 hours. After 48 hours Cytarabine was added (16nM-128nM). A 'no addition of Cytarabine' control (NA) was included. 3-5 replicates were plated per condition. Viable cells were counted at 24, 48 (prior to Cytarabine), 72 and 96 hours (post Cytarabine). The viable cell count at 24 hours was used as a baseline (100%). Percentage increase or reduction in the number of viable cells is then plotted on the y axis.

5.3.9 Romiplostim enhances cycling and chemotherapeutic elimination of human cells engrafted in to NSG mice

In order to examine the impact of Romiplostim on human HSCs *in vivo*, NSG mice were reconstituted with human cord blood cells and then treated with Romiplostim. Mice were injected intravenously with 100,000-200,000 CD34 enriched cord blood cells. Bone marrow aspirations were performed 4 weeks post transplantation in order to check reconstitution levels. All mice had greater than 50% human CD45 engraftment. 6 weeks post injection, 3 mice were treated with Romiplostim (100ug/kg sc Day 1, 3 and 5). Bone marrow was then harvested and cell cycle FACS analysis performed on untreated control mice (n=2) and on the Romiplostim treated cohort on day 6. Romiplostim resulted in a dramatic reduction in the number of CD34+CD38-cells residing in G_0 (p=0.0033) and a significant increase in the number of cells in G_1 (p=0.0064), supporting enhanced cell cycling (**Figure 5.19**). There was a trend towards an increased number of cells in SG_2M but this was not significant (p=0.115). The cord blood engrafted mice had a very small CD34+CD38-CD90+ population, therefore only the CD34+CD38- stem cell enriched population was analysed.

To then demonstrate enhanced susceptibility to Cytarabine post Romiplostim, a further cohort of cord blood engrafted mice were treated with PBS or Romiplostim (100ug/kg D1, 3 and 5) (n=4), followed by PBS or Cytarabine (1g/kg Day 5 and 6) (n=3). Baseline bone marrow aspirations were performed prior to treatment (week 0) and then 15 weeks after completing Cytarabine (week 15). Flow cytometry for human lineage reconstitution was performed. There was a clear drop in human reconstitution post treatment in the Cytarabine and Romiplostim + Cytarabine groups. Although the drop was greater in mice who received dual therapy, this did not reach statistical significance (p=0.10) (**Figure 5.20**). This

demonstrates that Romiplostim enhances the susceptibility of human stem cells to chemotherapy *in vivo*.

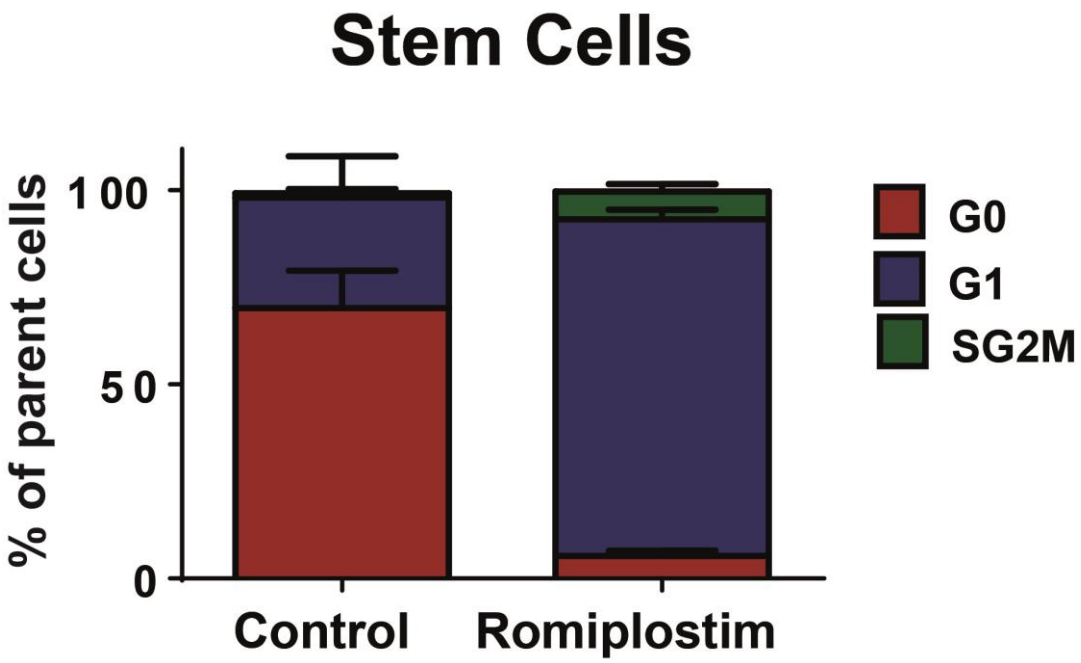


Figure 5.19 Romiplostim enhances the cycling of human stem cells engrafted in NSG mice

Mean (SEM) percentage of stem cells in G_0 G_1 and SG_2M. NSG mice were engrafted with CD34 enriched human cord blood cells (100-200,000 cells per mouse) resulting in >50% human reconstitution (data not shown). Engrafted mice (n=3) were treated with Romiplostim (100ug/kg Day 1, 3 and 5). Bone marrow was harvested on day 6 from the mice and cell cycle analysis performed on CD34+CD38- stem cells. Untreated human engrafted mice were used as controls (n=2).

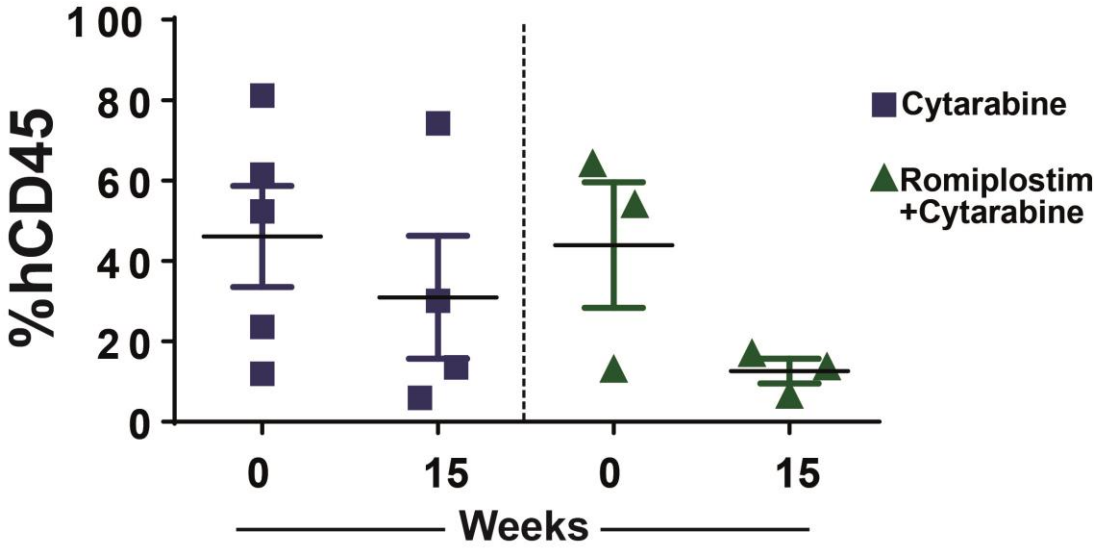


Figure 5.20 Romiplostim enhances Cytarabine mediated elimination of human reconstituting cells

Mean (SEM) hCD45 engraftment post Cytarabine or Romiplostim + Cytarabine in cord blood reconstituted NSG mice. Symbols represent individual mice. NSG mice reconstituted with cord blood were treated with Romiplostim (100ug/kg D1, 3 and 5) followed by Cytarabine (1g/kg D5 and 6) or Cytarabine alone (1g/kg D5 and 6). Bone marrow aspirations were performed prior to treatment (week 0) and 15 weeks post treatment, to assess remaining human engraftment.

5.3.10 Nordic MDS Group study of Eltrombopag and Azacitidine in high risk MDS

Having demonstrated that Romiplostim was able to activate and enhance susceptibility of normal human stem cells, the next step was to investigate the impact of Romiplostim in patients. This proved to be problematic with regards to Romiplostim treatment of MDS patients, as a phase 2 international clinical trial in low/int risk MDS patients was terminated early, due to concerns regarding a rise in blast count in some Romiplostim treated patients. Since then the one year follow up has shown no difference in the rate of transformation to AML in the Romiplostim and placebo treated groups but the license to treat MDS patients with Romiplostim has been withdrawn[138].

The Nordic MDS Group (NMDSG) ran a pilot phase one study using an alternative TPO receptor agonist Eltrombopag, in patients with int-2/high risk MDS eligible for treatment with Azacitidine. The primary endpoint of the study was to demonstrate that Eltrombopag could safely be given to patients with this subtype of MDS and a platelet count $<75x10^9$/L and to discern the most appropriate dose. As the impact of TPO receptor agonists on stem cells in patients with MDS was unknown, this trial provided a great opportunity to examine the bone marrow compartments in patients before and after treatment with an alternative TPO receptor agonist. However as mentioned in the introduction, Eltrombopag does not appear to mimic TPO as closely as Romiplostim does.

5.3.10.1 Trial Design

A 3+3 cohort design was employed using 4 different doses of Eltrombopag (cohort 1: 50mg, cohort 2: 100mg, cohort 3:200mg and cohort 4: 300mg). Eltrombopag was given daily, commencing one week prior to the first course of Azacitidine (100mg sc daily for 5 days every 28 days). Bone marrow aspirations were performed prior to starting therapy (week 0)

and after cycle 1 of Azacitidine (week 5). 12 patients were recruited to the study but only 9 had sufficient bone marrow material bio banked for stem and progenitor cell cycle analysis (**Table5.1**).

Pat ID	Sex/Age	WHO Diagnosis	IPSS	Hb (g/L)	WCC (10^9/L)	Plts (10^9/L)	% BM Blasts	Karyotype	Eltrombopag dose (daily)
1	F/64	CMML-II	Int 2	11.2	6.4	35	10	46XX	50mg
2	F/78	CMML-II	Int 2	10.5	12.5	26	14	46XX	50mg
3	F74	RAEB-I	Int 2	11.2	4.4	54	9	Complex	50mg
4	M/53	RAEB-II	Int 2	9.1	0.9	25	17	46XY	100mg
5	F/77	RAEB-II	Int 1	11.8	2.5	67	15	46XX	100mg
6	M/60	RCMD	Int 2	12.8	3.8	Aggregates	6	Del11q	100mg
7	M/69	RAEB-II	Int 2	10.1	1.6	58	12	46XY	200mg
9	M/78	RAEB-II	High	7.9	NA	25	0.2	Complex	200mg

Table 5.1 Baseline clinical characteristics of patients from the NMDSG10A study

At this timepoint, patients were not on Eltrombopag but started the dose stated 1-2 weeks later. Abbreviations used: IPSS, International Prognostic Scoring System; Int 1, intermediate 1 risk; Int 2, intermediate 2 risk; Hb, hemoglobin; WCC, white cell count; Plts, platelets; F, female; M, male; CMML, Chronic myelomonocytic leukaemia; RCMD, refractory cytopenia with multi-lineage dysplasia; RAEB, refractory anemia with excess blasts; NA, not available.

5.3.11 Eltrombopag does *not* enhance cycling of stem cells in patients with Int-2/High risk MDS

Of the eight patients bio banked, only 4 had adequate bone marrow cell numbers for cell cycle analysis pre and post Eltrombopag + Azacitidine. As is often the case in higher risk MDS and AML, CD90 expression was very low on the stem and progenitor cells of these patients. Therefore the CD34+CD38- (stem) and CD34+CD38+ (progenitor) populations were compared. On comparing the cell cycle status of these patients (n=4) pre and post Eltrombopag +Azacitidine, there was no significant difference in the number of stem cells in G_0, G_1 or SG_2M before and after treatment (**Figure 5.21**). Despite the lack of a pre-treatment comparator, the post treatment stem cells from the remaining four patients analysed demonstrated no expansion of cells in G_1 or SG_2M (data not shown). This confirmed that Eltrombopag +Azacitidine did not activate stem cells in any of the eight patients.

This may seem somewhat surprising, given the dramatic effects seen with Romiplostim *in vivo* in mice and in normal human bone marrow *in vitro.* However as previously mentioned , there is a significant body of evidence suggesting that Eltrombopag is structurally and functionally very different to thrombopoietin which may explain the discrepancies seen between the two drugs[131,133].

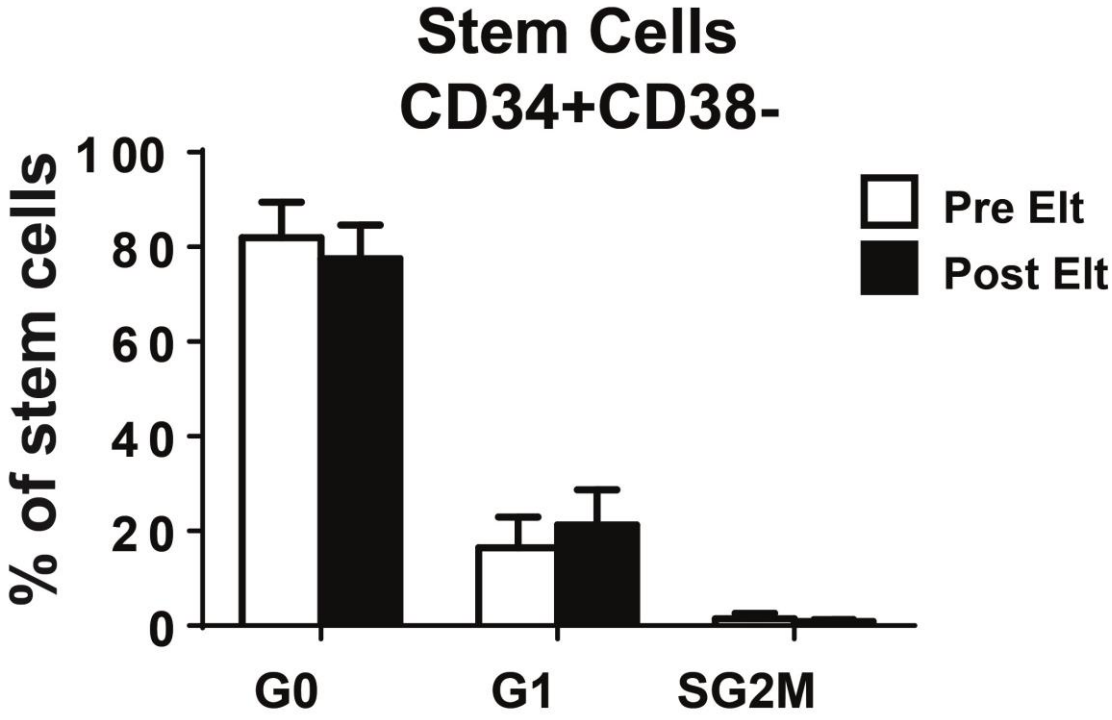


Figure 5.21 Cell cycle analysis of CD34+CD38- stem cells pre and post Eltrombopag (Elt)

Bar chart shows mean (SEM) percentages of stem cells (CD34+CD38-) cells pre and post Eltrombopag in G_0 (Ki67−DAPI−), G_1 (Ki67+DAPI−), and SG_2M (Ki67+DAPI+). There were no statistically significant differences in the number of cells in any compartment (n=4 student t test)

5.4 Discussion

Cancer stem cell resistance to therapeutic targeting is an extremely significant clinical problem, likely to contribute to the inability to cure a large number of patients. This has been clearly demonstrated in del (5q) MDS where the MDS stem cells are selectively resistant to Lenalidomide[103]. Cancer stem cell quiescence has been proposed as key mechanism of resistance[58]. As such, studies have endeavoured to utilise growth factors such as G-CSF and IFN-alpha to enhance stem cell cycling in order to improve subsequent therapeutic targeting[62,63,70,71]. To date these approaches have not been successful in patients. We hypothesised that TPO receptor agonists would be better drugs to activate dormant stem cells.

5.4.1 Mouse HSCs

In our studies, Romiplostim administration prior to chemotherapy resulted in a markedly enhanced elimination of donor stem cells. Phenotypic cell cycle analysis post Romiplostim demonstrated a reduction in the number of cells in G0 and a significant increase in the G1 fraction. Such phenotypic cell cycle analysis did not result in a significant rise in the percentage of stem cells in SG2M, but BrdU incorporation did demonstrate a 2.5 fold increase in stem cells entering S phase (**Figure 5.6**), although this was only analysed in a very limited number of mice (n=2). These findings are supportive of Romiplostim inducing cycling of mouse stem cells. Although a number of studies support the ability of TPO and TPO receptor agonists to cycle stem cells[84,85], other studies have suggested that TPO may in fact induce quiescence[79,86] and a more recent study suggested no effect at all[87]. There are a number of possible reasons for these conflicting results. The different effects may be due to diverse TPO dosing and scheduling regimens and the timing of subsequent cell cycle

analysis. In support of this is the fact that one of studies demonstrated only a transient expansion of quiescent stem cells (2 days after injection of 100ug/kg TPO) but analysis 6 days after administration revealed no significant difference when compared to non-injected controls[86]. De Laval an colleagues reported no alterations in stem cell cycling post injection of TPO but a much lower dose (8ug/kg) was used in this study, supporting a potential dose dependent effect[87].

Recent studies have confirmed the existence of platelet-primed stem cells which are crucially dependent on TPO[66]. In this study, after antibody mediated platelet depletion and resultant increased levels of TPO, only the platelet-primed stem cell subgroup were induced to cycle. It may therefore be that these platelet-primed stem cells are particularly sensitive to thrombopoietin and only this subgroup of stem cells responds to high levels of TPO, by cycling actively.

Yoshihara and colleagues suggested that TPO/MPL signalling plays a crucial role in the interaction between stem cells and the bone marrow niche[86]. It may therefore be that changes in the bone marrow microenvironment in these different studies may explain the different effects seen on cell cycling.

Cell cycle analysis was performed in mice treated with the same Romiplostim dose and scheduling and all were examined at the same time point post treatment. It would be valuable to analyse cell cycle status over time, in order to gain an understanding of the response dynamics. Cytarabine is a cytosine analogue, which is thought target dividing cells. Therefore finding the time point where the majority of stem cells are cycling post Romiplostim, may help to identify the optimum time for subsequent chemotherapy administration, when translating these murine studies to clinical trials. The HSC cycling ability of Romiplostim has also been demonstrated by another group using CFSE (5(6)-carboxyfluorescein diacetate N-succinimidyl ester) tracking[85].

In the mice, a large proportion of HSCs evaded chemotherapeutic targeting when treated with Cytarabine alone but pre-treatment with Romiplostim followed by Cytarabine significantly increased the sensitivity of the HSCs to chemotherapy (**Figure 5.10**). However, not all the donor reconstitution was eliminated. In certain clinical settings such as myeloablative allogeneic stem cell transplantation, elimination of all recipient stem cells is the goal. This is not possible in certain groups of patients such as those with multiple co-morbidities, as the risk of mortality outweighs the benefit of a possible cure. If it were possible to fully ablate the bone marrow with a combination of Romiplostim and a tolerable dose of chemotherapy, curative full intensity allogeneic stem cell transplantation, could be an approach open to many more patients. Romiplostim followed by a higher dose of Cytarabine (3g/kg) resulted in elimination of almost all of the donor stem cells in mice, leaving a residual CD45.1 contribution of only 0.85% (**Figure 5.13**). This suggests that Romiplostim in combination with reduced intensity non-myeloablative conditioning regimens, may enhance stem cell kill making such a regimen fully myeloablative, without intolerable toxicity.

Equivalent stem cell activation and targeting could *not* be achieved with G-CSF. If G-CSF were able to cycle and enhance stem cell elimination in a similar manner, this would be of concern when trying to translate this approach to clinical practice, as large trials have shown that G-CSF does not appear to improve remission rates or reduce relapse rates in AML[71]. Interestingly Romiplostim mobilised stem/progenitor cells to the peripheral blood more effectively than G-CSF **(Figure 5.8** and **Figure 5.17**). This suggests that Romiplostim could be a very useful peripheral blood mobilising agent in patients who do not mobilise successfully after G-CSF and warrants further investigation in clinical studies.

5.4.2 Human HSCs

In vitro experiments confirmed that Romiplostim could activate and potentially enhance elimination of normal human stem cells (**Figure 5.18**). In order to look at the impact of Romiplostim on functional stem cells *in vitro*, the next step would be to incubate cells in Romiplostim followed by Cytarabine before plating in to LTC-CFCs. This would enable analysis of the impact of Romiplostim and Cytarabine on the ability of single stem cells to self-renew. In an alternative attempt to study the impact of Romiplostim on functional human stem cells *in vivo*, cord blood engrafted NSG mice were treated. As was seen in the wild type mice, Romiplostim was able to cycle stem cells (**Figure 5.19**) and enhance susceptibility to chemotherapy (**Figure 5.20**). It would have been ideal to translate this by looking at the impact on engrafted MDS patient samples, but this was not possible due to the limited number of MDS or CMML patients reconstituting NSG mice (see Chapters 3 and 4).

5.4.3 Eltrombopag

As very limited numbers of MDS patients are now treated with Romiplostim, bone marrow from patients receiving Eltrombopag was analysed instead. In the patients investigated, there was no significant change in the cycling of CD34+CD38- stem cells (**Figure 5.21**) or CD34+CD38-CD90+ stem cells. There are a number of reasons why Eltrombopag may not appear to be activating stem cells in a manner similar to Romiplostim. A simple issue may have been that the dose and scheduling of Eltrombopag may not have been optimal to show an effect on stem cell cycling in these patients. The patient group was clinically extremely heterogeneous and stem and progenitor analysis demonstrated wide variations in the FACS profiles, which is not seen in low risk MDS patients. Our study is predicated on the knowledge that low risk MDS stem cells are quiescent and express high levels of MPL

relative to progenitors. It is not known whether patients with int-2/high risk MDS have high levels of MPL expression on their stem cells. If MPL expression was not high, this could indicate that the TPO signalling may not be so critical for maintaining and cycling stem cells in these patients. RT-PCR for *MPL* expression would be informative and could be performed on sorted populations from patients with remaining frozen viable cells. The fact that patients were concurrently given Azacitidine may also have impacted on the cell cycle results as the impact of Azacitidine on cell cycle is unknown. We are currently working in collaboration with an alternative clinical trial, where Eltrombopag is being given to low and intermediate risk MDS patients as single agent therapy to overcome some of these pitfalls.

Eltrombopag does not mimic TPO as closely as Romiplostim. It is a small molecule, with no structural similarity to TPO and activates TPO signalling via the trans-membrane domain of the MPL receptor. It appears to be dependent on the presence of a histidine reside at position 499[132]. Studies have also demonstrated that in platelets and megakaryocytes, Eltrombopag activates downstream signalling differently to TPO. It does not activate the JAK-STAT pathway to the same degree with reduced phosphorylation of STAT3 and does not appear to phosphorylate AKT at all[131]. Eltrombopag also appears to have additional effects on cell biology, independent of the MPL receptor, such as direct cytotoxicity to leukaemic blasts in AML and an iron chelating effect[133,134]. These features confirm differences between TPO and Eltrombopag and may explain the differences seen between Romiplostim and Eltrombopag in our studies. However one study does provide compelling evidence for an effect of Eltrombopag on the haematopoietic stem cells of patients with refractory severe aplastic anaemia. Single agent Eltrombopag resulted in multi-lineage clinical responses in 11/25 patients[139]. Although this suggests that Eltrombopag may have a role in expanding normal stem and progenitor cells, as increasing evidence is accumulating regarding the

cytotoxic impact of Eltrombopag, it is possible that Eltrombopag may actually be eliminating the dominant malignant clone, allowing the resurgence of residual normal haematopoiesis.

5.4.4 Future Directions

5.4.4.1 Immune mediate thrombocytopenia (ITP)

The largest group of patients being treated with TPO-R agonists are those with ITP. Collaborations have been initiated with a number of groups to obtain bone marrow samples before and during treatment with Romiplostim. This will enable us to see if Romiplostim has such a clear cycling effect on normal human stem cells as is suggested by our mouse data. In addition to performing phenotypic stem, progenitor and cell cycle analysis, I would also like to treat samples from patients' pre and post commencing Romiplostim, with Cytarabine *in vitro* and then perform LTC-CFC assays. This will give an indication as to whether *in vivo* treatment with Romiplostim increases the sensitivity of functional stem cells to *in vitro* chemotherapy.

5.4.4.2 Mouse models of malignant disease

MDS is an ideal disease to study the effects of Romiplostim on the stem cell as it has previously been shown that thrombopoietin activates MDS stem cells and we have now shown that human MDS stem cells express MPL at high levels. However there are no good mouse models that fully recapitulate MDS and xenotransplantation is extremely inefficient. An alternative approach would be to examine the impact of Romiplostim on stem cells in a different malignant disorder as a proof of principle. A mouse model is potentially an excellent way of establishing whether Romiplostim may selectively cycle malignant stem cells more effectively than normal stem cells. I am currently planning to extend the experiments performed on wild type mice presented in this thesis, to a conditional knock-in *JAK2* V617F mouse model[140]. The *JAK2* V617F mutation is highly relevant for both MDS and

myeloproliferative disorders and is known to originate in the stem cell. This may also enable me to look at the differential impact of Romiplostim on malignant and non-malignant stem cells in an *in vivo* setting.

6. Discussion

6.1.1 Disease modelling

The first aim of this thesis was to fully characterise the bone marrow stem and progenitor compartments of patients with low/intermediate-1 risk MDS and CMML. Using phenotypic, functional and genetic analysis, we have shown that both diseases are hierarchically arranged. More importantly we have shown that low/intermediate-1 risk MDS and CMML are driven by CD34+CD38-CD90+ stem cells. These findings have wider implications for the fields of haematology and cancer biology.

Heterogeneity has been noted within cancer cells for many years. How this heterogeneity arises and what its significance is has not always been clear. In low/intermediate-1 risk MDS and CMML, we have shown that this heterogeneity is due to a hierarchy existing between the clonally involved cells. MDS and CMML are similar to normal haematopoiesis in this regard, with the stem cell residing at the apex. Broadly speaking, there are two methods by which a cancer stem cell might arise. On the one hand a normal tissue specific stem cell might acquire genetic or epigenetic changes which enable it to outcompete its normal counterpart, whilst retaining its ability to self-renew and give rise to down-stream cells. Alternatively normal down-stream progenitors may acquire aberrant self renewal capacity and outcompete the normal tissue specific stem cells. Using many techniques, we have shown beyond reasonable doubt that in the myeloid malignancies, MDS and CMML, the former is the case. Although previous studies have supported that del (5q) MDS was driven by transformation of a normal HSC, there was no such evidence is CMML. The evidence in acute myeloid or lymphoid leukaemias very much supports the hypothesis that committed progenitors are capable of transforming into leukaemia stem cells[34,141]. As CMML is much more aggressive than del (5q) MDS, with perhaps more clinical similarity to AML, it was particularly illuminating to discover that CMML did not appear to be driven by malignant

transformation of a progenitor. This demonstrates clear differences in the modelling of acute and chronic haematological malignancies.

The biology behind transformation of disorders such as MDS and CMML to acute leukaemia is yet to be elucidated. In Chapter 4 of this thesis we reported an interesting patient (Patient 9) who gave some important insights in to possible mechanisms of transformation, which may have broader implications. Patient 9 had a particularly aggressive clinical course, presenting with CMML-2, transforming to AML within a short period of time and then dying shortly after commencing Azacitidine. Targeted mutation screening revealed that the earliest mutation was likely to be a *TET2* C1271Y mutation, followed by an *NRAS* G13D mutation. Both were present in the stem cells. Interestingly the *SRSF2* P95H mutation detected in the bulk sample was detected in the purified GMPs but not in the stem cells. This strongly suggests that it was acquired later and did not arise in the stem cell but in a downstream progenitor. It is well recognised that AML with a GMP-like leukaemic stem cell may arise de-novo or secondary to CML in blast crisis[34,50]. In our patient, subsequent transformation to AML may have been driven by the acquisition of mutations outside of the stem cell, which may have endowed self-renewal capacity on progenitors. This has broader implications regarding the understanding of transformation in MDS and CMML at the cellular level.

6.1.2 Minimal Residual Disease Monitoring

Currently all response to treatment criteria in MDS and CMML are based entirely on morphological, blood count and cytogenetic criteria[142]. However the majority of patients who achieve a complete remission following hypomethylating agents, chemotherapy or stem cell transplantation (SCT), still relapse. A complete clinical remission is important but

not sufficient for long term disease control or cure. It would seem advantageous to be able to quantify the depth of remission in these patients using a much more sensitive method.

The on-going persistence of cancer stem cells has been proposed as an explanation for why patients, who initially respond very well to chemotherapy or radiotherapy, relapse with time. If this is the case would phenotypic and molecular monitoring of candidate CSCs enable prediction of imminent relapse? Could it be used as a more sensitive method for monitoring minimal residual disease (MRD) and perhaps enable changes in treatment to be made in a more timely fashion?

Molecular MRD monitoring of bulk bone marrow is the standard of care in patients with chronic myeloid leukaemia (CML) and acute pro-myelocytic leukaemia (APL) and is routinely used to guide therapy. In children with acute lymphoblastic leukaemia (ALL), molecular MRD monitoring has been shown to be the most sensitive and specific predictive test for relapse. It has been successfully used to de-escalate treatment in children who are predicted to be at low risk of relapse[143]. However one concern when looking at bulk MRD may be that differential expression of molecular markers in the cancer stem cells and down-stream cells is likely to be overlooked although this heterogeneity maybe clinically important.

In myeloid disorders such as MDS, the question more commonly asked is should treatment be escalated, perhaps post treatment with Azacitidine or allogeneic stem cell transplantation? Currently in the UK, newly diagnosed patients with MDS do not routinely have mutation screening performed, although this may change in certain hospitals over the next few years. It might then be possible to integrate a pathway involving FACS sorting of populations and targeted mutation screening, as we have performed in our study. This may allow a personalised characterisation of an individual's cancer stem and progenitor cell profile.

This approach would need to be validated in a pilot clinical trial and has many limitations. From a pragmatic point of view, CSC characterisation would need to be performed at a centre with expertise in FACS and molecular diagnostics. The additional cost of sorting serial samples will not be insubstantial. Although the cost of DNA mutation analysis is falling, this would also need to be taken in to consideration. With current MRD strategies, there is often concern regarding standardisation of techniques and inter-centre variability but there may be huge potential benefits in looking at the genetic changes in the cell population driving the disease rather than the bulk bone marrow.

6.1.3 Drug Design

There has been a huge amount of interest in developing therapeutic agents which specifically target cancer stem cells but progress has been relatively slow. In MDS and CMML, targeting all cells expressing the phenotypic MDS stem cell signature, would not select for malignant stem cells as these markers also define normal HSCs. It is therefore important to unravel the molecular targets within MDS and CMML stem cells which could be potential therapeutic targets. Perhaps more detailed phenotypic characterisation, using markers not expressed on normal HSCs would reveal cell surface molecules unique to MDS stem cells. This route has shown potential in acute myeloid leukaemia, where the antigen CD47 has been shown to be preferentially expressed on leukaemic rather than normal stem cells[144]. Clinical trials are underway with anti-CD47 monoclonal blocking antibodies.

6.1.4 Activation and therapeutic targeting

It is known that del (5q) MDS stem cells are resistant to therapy. Stem cell quiescence has long been proposed as a mechanism of cancer stem cell therapeutic resistance. Activation of normal and leukaemic stem and progenitor cells with growth factors such as G-CSF and IFN-alpha, prior to chemotherapy, has resulted in mixed success. In Chapter 5, we were able to significantly enhance cytotoxic elimination of normal HSCs in mice by pre-treating with the TPO-R agonist, Romiplostim. Targeting of resistant stem cells is likely to be of crucial importance in many malignancies, beyond MDS. It is likely that Romiplostim would be effective in any haematological disorder driven by the stem cell where TPO signalling was an integral part of stem cell function. Further to demonstrating the enhanced cytotoxic elimination of normal stem cells, the next step is to examine the impact on patient stem cells, as mentioned in the discussion for Chapter 5, before moving on to clinical trials.

6.1.4.1 Intermediate and High Risk MDS patients

In the studies reported in this thesis, Cytarabine was used as the cytotoxic agent to eliminate stem cells post Romiplostim. This was a relevant drug to test as it is the mainstay of treatment for patients with MDS who require intensive chemotherapy. However many more MDS patients are treated with the DNA hypomethylating agent Azacitidine. Other than allogeneic stem cell transplantation, Azacitidine is the only therapy which has been found to improve overall survival in high risk MDS[145]. However one major drawback of this agent is that it is not curative. *In vitro* Azacitidine is cytotoxic at high doses whereas it induces hypomethylation and differentiation at lower doses. It is not clear what the mechanism of action is in MDS patients.

In the Nordic MDS Group study of Azacitidine and Eltrombopag in intermediate-2/high risk MDS (Chapter 5), no enhanced stem cell cycling was seen in patients post treatment with Azacitidine and Eltrombopag. However as already discussed Eltrombopag may not be as good a TPO mimetic as Romiplostim, which may explain why we see no cycling in these patients. There are of course other possible reasons including insufficient dosing and scheduling of Eltrombopag. It would have been very informative to perform stem cell analysis on stem and progenitors in the one week window after starting Eltrombopag, before patients started Azacitidine.

However, our findings in these patients do not rule out the possibility that Romiplostim, rather than Eltrombopag may activate stem cells in high risk MDS patients. Currently around 50% of MDS patients respond to Azacitidine. It is possible that pre-treating patients with Romiplostim and then starting Azacitidine may improve the therapeutic efficacy of Azacitidine, enhancing the remission rate.

If we are able to demonstrate that Romiplostim aids the elimination of malignant rather than just normal stem cell using *in vitro* or *in vivo* models (Chapter 5 discussion), the next step would be to design a pilot study in patients. Data already suggests that Romiplostim may improve platelet counts in low/intermediate-1 risk MDS patients and was well tolerated[146,147]. However a subsequent phase 2 study closed prematurely due to initial concerns regarding enhanced transformation to leukaemia in the Romiplostim arm. More mature data has identified no significant difference between Romiplostim and placebo treatment[138]. No published clinical trials have looked at the impact of Romiplostim on high risk MDS patients. The ideal MDS patient population for our study would probably be patients who are already eligible for Azacitidine. Ideally patients would be pre-treated with Romiplostim, followed by standard Azacitidine therapy (75 mg/m^2 for 7 days per 28 day cycle). Romiplostim would precede Azacitidine for each cycle of treatment. In order to

assess whether Romiplostim improved remission rates, a control arm of matched patients treated with just Azacitidine would be required. Blinding to Romiplostim therapy would not be possible as it is delivered by subcutaneous injection. To monitor response, bone marrow sampling would ideally be performed at enrolment (week 0), post the Romiplostim priming phase (week 1) and after the first Azacitidine cycle (week 5). On-going bone marrow sampling would be required as it can take many months for Azacitidine to induce a response. Stem/progenitor and cell cycle FACS analysis would be required. DNA mutation screening of populations would also enable an assessment of the clonality of the residual cells, providing information regarding whether Romiplostim was selectively priming normal or malignant cells.

One valid concern might be that if Romiplostim does not have a differential impact on malignant and normal stem cells, all normal haematopoietic regenerative capacity may be eliminated by combination of Romiplostim cycling all stem cells, followed by Azacitidine. Romiplostim has the potential to make things much worse, prolonging life-threatening cytopenias after Azacitidine or chemotherapy if normal stem cells are not able to repopulate the bone marrow. As a result, this approach may require very careful dose titration or be viewed as a method for prolonging remission in patients, rather than aiming to cure.

6.1.4.2 Allogeneic Stem Cell Transplantation

One setting where elimination of all stem cells, both normal and malignant is less of a concern and often the main aim is allogeneic stem cell transplantation (SCT). Currently this is thought to be one of the only treatment options that can cure numerous haematological malignancies, including CMML and MDS. However, most patients are not considered candidates for an allogeneic SCT as the conditioning regimens are too toxic, resulting in the risk of mortality outweighing the possibility of cure. If Romiplostim were able to cycle stem

cells in these patients, it may enable lower doses of chemo/radiotherapy to myeloablate the transplant recipient's bone marrow. This may make an allogeneic SCT more tolerable for elderly patients or patients with significant comorbidities, opening it up as a potentially curative option to more patients.

As an initial clinical study, MDS patients suitable for a reduced intensity conditioning (RIC) allogeneic SCT could be offered the opportunity to have Romiplostim prior to the conditioning regimen, to enhance the elimination of stem cells. To monitor response, bone marrow sampling would need to be performed at enrolment (week 0), ideally post Romiplostim (week 1) and one month after donor stem cell infusion (week 6-7). As the numbers of MDS patients receiving an allogeneic SCT are still rather low, an initial study would probably need to be non-randomised.

This approach may also have utility beyond haematological malignancies for example in inherited or autoimmune conditions requiring an allogeneic SCT.

6.1.4.3 Autologous Stem Cell Transplantation

High dose chemotherapy followed by autologous stem cell rescue results in improved remission rates compared to chemotherapy alone in patients with myeloma and relapsed lymphoma[148]. Stem/progenitor cell mobilising agents like G-CSF and Plerixafor are vital components of these regimens. However as many patients receiving autologous transplantations have had multiple rounds of myelotoxic chemotherapy, mobilising autologous stem/progenitor cells can be problematic and sometimes impossible. During our studies, it was incidentally found that Romiplostim may be a far better mobilising agent than G-CSF, as demonstrated by a three-fold increase in peripheral blood colony formation. Romiplostim is not currently licensed for peripheral blood mobilisation but it would be valuable to investigate the mobilising capacity of this agent in patients. Interestingly, studies

have also shown that a key predictive factor for poor autologous stem cell mobilisation is a low platelet count, which may also be ameliorated by Romiplostim[149].

6.1.4.4 Chronic myeloid leukaemia

Chronic myeloid leukaemia (CML) is characterised by the reciprocal translocation of the *ABL* gene on chromosome 9 and the *BCR* gene on chromosome 22 (t(9;22)) resulting in the *BCR-ABL* fusion oncogene[150]. It is characterised by an expansion of myeloid progenitors and mature cells resulting in marked myeloproliferation. Most patients rapidly achieve a clinical and cytogenetic response on commencing a targeted BCR/ABL tyrosine kinase inhibitor such as Imatinib and remain well[151]. However, even in responding patients the *BCR-ABL* transcript can still be detected in many of these patients, suggesting that some CML cells are resistant to therapy and Imatinib alone is rarely curative[152]. Importantly a number of studies have reported that when Imatinib is stopped in patients in a clinical and cytogenetic remission, the disease usually relapses demonstrating that the malignant clone is able to overtake normal haematopoiesis[74,153]. Improvements in therapeutic targeting are needed in order to enable the transition of CML from a chronic to a readily curable disease and allow patients to stop tyrosine kinase inhibitor therapy.

The *BCR-ABL* fusion gene has been detected in the phenotypic CD34+CD38-CD90+ stem cell compartment in CML patients pre and post treatment with Imatinib, demonstrating that they are resistant to therapy[154,155]. CML stem cells quiescence is thought to be a highly likely mechanism of resistance as is the case with normal and MDS stem cells[156].

G-CSF has been found to enhance sensitivity to Imatinib *in vitro* but a subsequent clinical trial did not find any benefit[63,157]. This suggests that in principle the approach might work but an alternative growth factor may improve efficacy. There are a number of lines of

investigation which can be followed to test this hypothesis but there are also many limitations.

A combination of *in vitro* and *in vivo* approaches may be valuable. With regards to *in vitro* studies, I have already shown in this thesis (chapter 5), that Romiplostim enhances cell division of normal human stem cells. Similar tracking of CML samples post incubation with Romiplostim may be informative. Following on from this, I am planning on looking at the ability of Romiplostim to enhance chemotherapy induced elimination of MDS LTC-CFCs. A similar approach could be taken by examining LTC-CFC capacity following pre-treatment of CML stem cells with Romiplostim followed by Imatinib, compared to Imatinib alone.

In this thesis I have reported that Romiplostim enhanced the susceptibility of normal human stem cells to chemotherapy *in vivo*, using a cord blood engrafted mouse model. A similar approach could be taken by treating NGS mice reconstituted with CML cells. Diagnostic CML samples would be preferable but sufficient numbers of engrafting patients may be challenging to obtain. Studies also suggest that as Imatinib treated patient samples are enriched for *BCR-ABL* positive CML stem cells, they tend to reconstitute mice more efficiently[152]. Alternatively treatment of a CML mouse model could be attempted, although choosing an appropriate model would be very important to ensure clinical relevance. A number of retroviral and transgenic models have demonstrated a high penetrance of disease but a recent *BCR-ABL* knock-in mouse developed no phenotype[158].

6.1.5 Conclusions

MDS and CMML are hierarchically arranged and driven by malignant stem cells. These data demonstrate how *in vitro* assays and DNA mutation tracking can in combination be powerful tools for uncovering the identity of cancer stem cells. These data also provide broader insights in to mutations which are likely to be initiating and those that are perhaps more important for disease progression, beyond just MDS and CMML. Elimination of these malignant stem cells is likely to be an absolute requirement for cure.

Romiplostim dramatically enhances the chemotherapeutic sensitivity of normal stem cells to Cytarabine chemotherapy. If Romiplostim is able to activate malignant stem cells, it is likely to potentiate subsequent chemotherapeutic elimination. This approach may enable improved cure rates in any number of diseases driven by cancer stem cells which are otherwise resistant to therapy.

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