Normal and Leukemic Hematopoiesis

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voor Papa en Mama

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LIST OF ABBREVIATIONS

ALL	Acute Lymphocytic Leukemia
Alpha MEM	Alpha Modified Eagle's Medium
ALT	Alternative Lengthening of Telomeres
AML	Acute Myeloid Leukemia
$ß_2$ $m^{-/-}$	$ß_2$-microglobulin$^{-/-}$
BFU-E	Burst Forming Unit- Erythroid
bHLH	Basic Helix Loop Helix
BM	Bone Marrow
BSA	Bovine Serum Albumin
CB	Cord Blood
CBF	C Binding Factor
CD	Cluster of Differentiation
CFU	Colony Forming Unit
CFU-GM	Colony Forming Unit-Granulocyte, Macrophage
CFU-Mk	Colony Forming Unit-Megakaryocyte
CIR	CBF-1 Interacting Protein
CML	Chronic Myeloid Leukemia
CR	Complete Remission
CRU	Competitive Repopulating Unit
DKC	Dyskeratosis Congenita
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DN-hTERT	Dominant Negative human Telomerase Reverse Transcriptase
DSL	Delta;Serrate;Lag2
EGF	Epidermal Growth Factor
FAB	French American British
FACS	Fluorescent Activating Cell Sorter
FCS	Fetal Calf Serum
FISH	Fluorescent In Situ Hybridization
FITC	Fluorescent Isothiocyanate
G-CSF	Granulocyte-Colony Stimulating Factor
GF	Growth Factor
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte and Monocyte-Colony Stimulating Factor
GO	Gemtuzumab Ozogamicin
HDAC	Histone Deacetylase
HERP	HES Related Repressor Protein

HES	Hairy Enhancer of Split
HFN	Hanks medium with 2% FCS and 0.1% sodium azide
hTERT	human Telomerase Reverse Transcriptase
IC	Intracellular Domain
IL	Interleukin
IMDM	Iscove's Modified Eagle's Medium
IRES	Internal Ribosomal Entry Site
ITAM	Immunoreceptor Tyrosine-based Activation Motifs
ITIM	Immunoreceptor Tyrosine-based Inhibition Motifs
J1	Jagged1
Lin	Lineage
LTC	Long Term Culture
LTC-IC	Long Term Culture-Initiating Cell
mAb	Monoclonal Antibody
MC	Methylcellulose
MDS	Myelodysplastic Syndrome
MSCV	Murine Stem Cell Virus
N/A	Not Applicable
NC	Nucleated Cells
ND	Not Done
NOD/SCID	Non-Obese Diabetic/Severe Combined Immunodeficient
NRARP	Notch Related Ankyrin Repeat Protein
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Population Doubling
PE	Phycoerythrin
PI	Propidium Iodide
Pts	Patients
RFS	Relapse Free Survival
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SC	Suspension Culture
SC-IC	Suspension Culture-Initiating Cell
SF	Steel Factor
SKIP	Ski-Interacting Protein
SL-IC	SCID Leukemia-Initiating Cell
TLE	Transducin-Like Enhancer of Split
TRAP	Telomeric Repeat Amplification Protocol
TS	Telomerase Substrate
UF	Unfractionated
WBC	White Blood Cell Count
WHO	World Health Organization
YAC	Yeast Artificial Chromosome

Chapter 1

Introduction

Chapter 1 Introduction

1.1 Acute Myeloid leukemia: the disease

Acute Myeloid Leukemia (AML) is a clonal myeloproliferative disease characterized by an uncontrolled proliferation and block in differentiation of myeloid committed blood cells in the bone marrow (BM). The diagnosis of AML is made when over 30% of myeloid blast cells are observed in the BM of the patient. The excessive amount of myeloid blast cells in the BM causes a lack of space for normal hematopoiesis to occur. This results in anemia, thrombocytopenia and granulocytopenia with symptoms as fatigue, dizziness, bleedings and infections. AML occurs as a result of genetic changes often evident as cytogenetic changes, which can be detected in the majority of patient samples. The type of cytogenetic abnormality is considered the most powerful independent risk factor for outcome and the various genetic changes can be classified in a favourable, intermediate or poor risk-group (Table 1 and Figure 1).[1] AML can also be classified according to its morphology using the French-American-British (FAB) classification (Table 2). Recently, a more clinical relevant classification has been proposed by the World Health Organization (WHO). This classification stratifies hematologic malignancies according to a combination of morphology, immunophenotype, genetic features and clinical syndromes.[2] According to the WHO classification the diagnosis of AML is made when over 20% instead of 30% blast cells are present in the BM. In Table 3, the WHO classification of AML is depicted.

The incidence of AML is 2.4/100,000 people per year in the United States and it occurs mainly in elderly patients with a median age of onset of 63-65 years.[3] AML patients under 50 years of age, treated with curative intent chemotherapy, have complete remission (CR) rates that average 70% with a median relapse free survival (RFS) of nearly 2 years.[4] In contrast, in the elderly AML patient, CR rates only average 50% with a median RFS in these patients of only 9 to 12 months.[5-8] The poorer outcome of AML in the elderly may result from adverse cytogenetics, expression of multi drug resistance proteins, reduced patient tolerance to chemotherapy or a combination of these factors.[3] However, despite age or biological differences, most patients eventually relapse and die of the disease.

CR or partial remission can be obtained after intensive chemotherapy currently often consisting of high/low dose cytarabine plus daunorubicin/idarubicin. After a CR is obtained, patients under 60 years have three main options: intensive chemotherapy, autologous stem cell transplantation or allogeneic stem cell transplantation. In general, the advantage of an allogeneic stem cell transplant is its graft versus leukemia effect with lower relapse rates. However, an HLA-matched sibling is available for only a minority of patients and the occurrence of graft-versus host disease necessitates long-lasting immunosuppressive medication. An autologous stem cell transplant, available for every patient in CR, does not have the advantage of the graft versus leukemia effect. In addition, Brenner et al. demonstrated that an autologous stem cell transplant can contribute to relapse of the disease.[9] Overall, transplantation with autologous or allogeneic blood or bone marrow derived stem cells carries substantial procedure-related mortality and

morbidity and the 5 year overall survival rates do not differ much between intensive chemotherapy and autologous or allogeneic stem cell transplants.[10,11] Recently, as an alternative to conventional stem cell transplantation, non-myeloablative stem cell transplantation has been performed and this strategy appears safe and minimally toxic while retaining its graft-versus-leukemia effect.[12,13] This novel strategy could provide transplantation opportunities for patients that are ineligible for conventional stem cell transplantation because of age or medical contraindications. In addition, some new therapies specifically targeting the AML cells have been used in clinical trials including antibody-targeted chemo- or radiotherapy.[14-16] The effect of those therapies on long-term survival is not yet known. Altogether we can conclude that although CR rates have dramatically improved in the last few decades, overall survival is still relatively short mainly due to relapse of the disease.

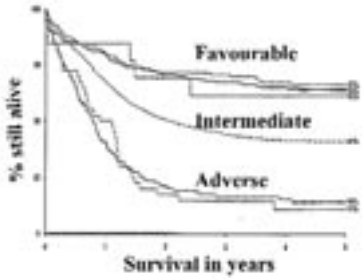

Figure 1: Overall survival of patients with favourable, intermediate and adverse cytogenetic abnormalities. Adapted from Grimwade et al. Blood. 1998;92:2322-2333.

Table 1: *Riskgroups of cytogenetic abnormalities observed in AML patients*

Risk group	Abnormality
Favourable *	t(8;21)
	t(15;17)
	Inv(16)
Intermediate	normal
	cytogenetic abnormality not classified as Favourable or Adverse
Unfavourable *	-5
	-7
	Del(5q)
	Del(7q)
	t(6;9)
	t(9;22)
	abnormal 11q
	abnormal 3q
	complex •

* Whether alone or in conjunction with other abnormalities
• Presence of a clone with ≥ 5 unrelated cytogenetic abnormalities

Table 2: *FAB Classification of AML*

FAB classificiation	Morphology
M0	Acute Myeloblastic Leukemia with minimal differentiation
M1	Acute Myeloblastic Leukemia without maturation
M2	Acute Myeloblastic Leukemia with maturation
M3	Acute Promyelocytic Leukemia
M4	Acute Myelomonocytic Leukemia
M4eo	Acute Myelomonocytic Leukemia with abnormal eosinophils
M5a	Acute Monoblastic Leukemia
M5b	Acute Monocytic Leukemia
M6	Acute Erythroleukemia
M7	Acute Megakaryocytic leukaemia

1.2 Acute Myeloid leukemia: the biology

The hematopoietic system is a complex and tightly regulated process in which pluripotent stem cells undergo proliferation and differentiation to produce mature blood cells of the various lineages while maintaining a compartment of uncommitted cells. As mentioned above AML occurs as a result of genetic changes. Such changes occur in a primitive hematopoietic cell, which gives rise to the malignant clone. Despite the lack of mature cells derived from the leukemic clone in the majority of AML patients, there is considerable heterogeneity among the leukemic blasts even within individual patients, particularly with respect to their capacity to proliferate and or self-renew.[17-19] This suggests that subsequent to transformation and prior to the maturation arrest some differentiation of AML blasts occurs, which creates a hierarchy of AML progenitors just as is seen in normal hematopoiesis. The ability to phenotypically and functionally characterize the AML stem cell, which is thought to be responsible for maintaining the disease, is not only essential for a better understanding of the disease, but can also contribute to new therapies in which these AML stem cells are specifically targeted.

Table 3: *WHO Classification of AML*

WHO classificiation	Definition
I	AML with recurrent cytogenetic translocations
II	AML with myelodyplasia-related features
III	Therapy-related AML and MDS
IV	AML not otherwise categorized

Over the years several *in vitro* assays have been developed to detect normal hematopoietic progenitors. Lineage committed progenitor cells can be detected in the colony forming cell assay (CFU assay), in which hematopoietic cells are plated in semi-solid media complemented with growth factors (GF).[20,21] More primitive progenitors can be assayed in the long-term culture assay (LTC).[22] In this assay, cells are cultured for 5 to 8 weeks on murine stromal feeders producing human GFs and after 5 to 8 weeks the cells are harvested and plated in semi-solid media upon which the LTC-initiating cell (LTC-IC) content can be determined. It is thought that the most primitive pluripotent stem cells can only be detected *in vivo*. Multi-lineage engraftment of human cells can be detected in the non-obese diabetic severe combined immunodeficient (NOD/SCID) mice, which have defects in DNA double-strand break repair and in V(D)J recombination, creating B and T cell deficiencies, and a defect in their natve killer (NK) cells and antigen presenting cells.[21,23,24]

AML progenitors and stem cells can also be assayed using the same detection assays used for normal hematopoietic cells. A subpopulation of the malignant cells from an AML patient gives rise to clusters (10-20 cells) or colonies (>20 cells) of terminally differentiated blasts (CFU-blasts) when plated in a CFU assay.[25,26] More primitive progenitors can be detected *in vitro* using the LTC assay.[27] More recently, the suspension culture (SC) assay described by McCulloch et al.[28] was adapted in our laboratory by using recombinant GFs, serum free media and extension of the culture period to 8 weeks.[17] The cells capable of producing CFU-blasts after 2 to 8 weeks in SC as quantitated by limiting dilution analysis, are called SC-IC and are thought to have the same biological properties as LTC-IC. For the evaluation of AML progenitors *in vivo*, NOD/SCID mice can be utilized.[19,29-31] About 70% of the AML patient samples can successfully engraft NOD/SCID mice, with the cells responsible for engraftment called SCID leukemia-initiating cells (SL-IC).[29] The frequency of SL-IC in a study by Ailles et al. was reported to be 0.7-45 per 10^7 cells [29], while a higher frequency of 20-1000 SL-IC per 10^7 cells had been reported in an earlier study.[19] Each SL-IC was shown to produce more than 10^6 leukemic blasts as well as many AML CFU and AML LTC-IC as detected 8 weeks post-injection into mice in support of the proposed hierarchy in AML stem and progenitor cells.[29] Ailles study also showed that higher levels of engraftment were obtained with AML samples with cytogenetic changes associated with a poor clinical prognosis compared to those with a good prognosis.[29] Furthermore, cells from FAB subtype M3 and to a lesser extent, M2 engrafted more poorly.[29] To obtain more consistent engraftment and engraftment with more patient samples variants of the NOD/SCID model were tested.[32] NOD/SCID mice transgenic for the human GFs Steel Factor, GM-CSF and IL-3, did not reveal a major improvement in engraftment of AML cells.[32] Another model tested is the ß2-microglobulin deficient NOD/SCID mice, which have a reduced innate immunity due to a lack of NK cell activity.[33] These mice revealed an 11-fold increase in NOD/SCID repopulating cells (SRC) frequency compared to the standard NOD/SCID mice. In addition, they showed improved detection of progenitors with myeloid restricted and short-term lympho/myeloid repopulating potential when transplanted with normal human cord blood (CB) cells.[34,35] Engraftment using this model allowed detection of some AML progenitors which were not

detectable in the NOD/SCID mouse model, but the added benefit of either of these models seemed modest.[32]

The phenotype of primitive AML progenitors was studied by sorting cells into phenotypic subpopulations, which were then assayed by their long-term growth *in vitro* (CFU, SC-IC or LTC-IC) or by their growth in the immunocompromised NOD/SCID mice.[19,30,31] As in the normal hematopoietic system, LTC-IC and SL-IC represent a very rare population in AML cells. Primitive AML progenitors express CD34 and lack the expression of CD38, CD71 and HLA-DR[18] just like their normal counterparts[36,37] and can be enriched 2~3 logs by sorting for this phenotype.[18] AML progenitors express only low levels of c-kit (CD117)[38], which may differ from the expression on normal progenitor cells.[39,40] Recently, a normal $CD34^-$ stem cell has been proposed[41,42] and a $CD34^-$ AML SL-IC has also been described.[43] Despite the similarities Blair et al. showed that CD90 (Thy.1), which is expressed on normal LTC-IC[44], is not expressed on primitive AML progenitors, giving this marker a possible role in future positive selection strategies for autologous transplants.[24] Interestingly, crosslinking of CD90 on normal hematopoietic cells inhibited their proliferation, suggesting that loss of CD90 could release AML cells from such inhibiting constraints.[45]

1.3 CD133 (AC133)

In search of novel cell surface markers on hematopoietic stem cells to improve stem cell enrichment, Yin et al. produced a novel monoclonal antibody (MoAb), AC133.[46] This antibody recognizes a 5-transmembrane antigen (CD133), which is the human orthologue of the mouse prominin.[47] CD133 is a heavily glycosylated 120kDa protein, containing five transmembrane domains and two large extracellular loops.[48] Recently, novel isoforms of human CD133 and murine prominin have been described, which both differ from the originally described proteins by a 9-amino acid deletion in the N-terminal extracellular.[49,50] Initially, CD133 expression appeared to be restricted to hematopoietic progenitors.[46,48] However, more recent studies showed CD133 expression in epithelial[51], endothelial progenitor cells[52] and neuronal stem cells.[53] A characteristic feature of the CD133 protein is its rapid down regulation upon cell differentiation. This property makes it a unique cell surface marker for the identification and isolation of stem cells and progenitor cells.[47,49,54] The ability of CD133 isoforms to localize to the plasma membrane suggests a possible role for CD133 in cell-cell interactions or ligand-receptor interactions.[47,48] Prominin has been shown to specifically interact with membrane cholesterol implying a role in membrane organization and membrane-to-membrane interactions for this protein.[55] However, to date no ligands have been identified for CD133 and its precise function remains to be elucidated.

As mentioned above, CD133 was first identified as a hematopoietic stem cell antigen. Expression of CD133 is found on a subset (30-90%) of the $CD34^+$ cells in the human bone marrow (BM), fetal liver, umbilical cord blood and growth-factor primed peripheral blood (PB).[46,56] Although it was initially thought that CD133 expression was restricted to the $CD34^+$ subset[57], later studies demonstrated that CD133 is also expressed on a rare population of $CD34^-$ cells.[58]

In vitro colony assays using normal adult BM and CB cells have shown that the CD133$^+$ population contains CFU-GM, BFU-E, CFU-Mix (containing granulocytic and erythroid cells) and CFU-Mk (megakaryocytic).[46,56,59] In addition, the majority of LTC-IC were detected in the CD34$^+$CD133$^+$ subset as were the NOD/SCID repopulating cells. CD34$^+$CD133$^+$ cells also successfully engrafted primary and secondary recipients using the fetal sheep model.[46] Recent evidence suggests that cells with stem cell properties are enriched in a rare subpopulation of CD34$^-$lineage$^-$ cells and that these CD34$^-$ cells are the precursors of the CD34$^+$ cells.[42,60] The CD34$^-$ cells with stem cell properties express CD133, suggesting that CD133 is an excellent stem cell marker and useful to purify normal hematopoietic stem cells.[58]

It was of interest to see whether CD133 expression could also be detected on leukemic cells. First Miraglia et al. showed that CD133 was co-expressed with CD34 in 4/5 AML and 3/6 acute lymphocytic leukemia (ALL) samples, while CD133 expression could not be detected in one CD34$^-$ AML sample.[48] Later reports confirmed this observation with the majority of CD34$^+$ AML samples expressing CD133.[61,62] However, CD133$^+$ AML blasts that lacked CD34 expression could be identified in ± 10% of primary AML samples.[62,63] A correlation between CD133 expression and FAB type has been reported with high levels of the CD133 cell surface marker on M4 and M5 FAB leukemias[48] and on FAB M0 leukemias.[63] CD133 expression on FAB M3 cases has been low to absent in all reports and this is consistent with an absence of CD34 expression on those samples. No correlation between CD133 expression and cytogenetics or prognosis has been reported.

CD133 expression could also be observed on CD34$^+$ cells of patients with myelodysplastic syndrome, but not on CD34$^-$ cells.[63] In chronic myeloid leukemia (CML) CD133$^+$ expression could be detected in both Philadelphia chromosome (genetic marker for CML) positive and negative populations suggesting that CD133 selection is not useful as a purging strategy for autologous transplants in CML patients.[64] On the other hand, in childhood B-cell precursor ALL no CD133 expression could be detected on the leukemic blast cells of 40% of the samples and therefore could be a potential marker to separate residual normal from leukemic cells in the subgroup of CD133 negative B-cell precursor ALL.[65,66]

Overall, CD133 is expressed in a substantial number of leukemic samples especially CD34$^+$ samples. Larger scale studies are necessary to determine whether CD133 could be a useful selection marker for the purging of autologous transplants in some samples of the various types of leukemias.

1.4 CD33

CD33 is a cell surface antigen that was originally identified on human myeloid cells by a panel of monoclonal antibodies.[67,68] CD33 is a 67kD type I transmembrane glycoprotein that belongs to the sialo-adhesion family of cellular interaction molecules also referred to as the siglecs (sialic acid binding Ig-related lectins).[69] CD33 is the smallest member of the family and contains only two Ig-like extracellular domains: one V-set Ig-like domain that

is conserved within the siglecs and is essential for the binding of sialic acid and one C2-set Ig-like domain.[69] The cytoplasmic tail of CD33 has two tyrosine-based motifs, which are immunoreceptor tyrosine-based inhibition motifs (ITIM).[70-72] Although the function of CD33 is still largely unknown, the fact that CD33 contains ITIMs has suggested an inhibitory function for CD33 through the recruitment of the Src homology (SH) 2 domain-containing protein tyrosine phosphatases (SHP-1 and SHP-2).[70-72] Most of the inhibitory receptors characterized to date are efficient at suppressing signals generated by immunoreceptor tyrosine-based activation motifs (ITAM) containing receptor systems.[73] Indeed, it has been shown that CD33 can inhibit activation mediated by CD64, an ITAM containing receptor, *in vitro*.[72] However, the physiological function of CD33 and its possible inhibitory effect on CD64 remains to be elucidated.

CD33 expression is restricted to the myeloid lineage.[74] Its expression is low on the earliest pluripotent progenitor cells[75-77], but expression levels are higher on early myeloid progenitors. During myelomonocytic differentiation CD33 remains present until it is downregulated on granulocytes, but is retained on monocytes. CD33 is expressed in 75%-90% of the primary AML samples at diagnosis.[78-80] Although a few groups have found a correlation between better overall survival[80,81] or superior complete remission rates[80,82] and the expression of CD33, others have failed to do so[79,83,84], suggesting that CD33 is not a valuable prognostic factor in AML. Because of its myeloid-specific expression levels and high expression levels in AML, CD33 mAbs are extensively used in the immunodiagnosis of AML. Furthermore, it has been shown that CD33 is expressed on short-term clonogenic leukemic cells[85-87] and mAbs against CD33 have been used for purging in AML.[68,88] More recently, mAbs against CD33 have been used to target myeloid leukemia cells specifically. Treatment of patients with HuM195, a mAb against CD33, produced rare CRs in patients with relapsed or refractory myeloid leukemia[89,90] and showed activity against minimal residual disease in patients with acute promyelocytic leukemia.[91] Later, *in vitro* studies demonstrated that addition of mAbs against CD33 to cultures of normal and chronic myeloid leukemic cells resulted in an inhibition of proliferation.[92] Those mAbs also induced apoptosis in cultures with primary AML cells emphasizing the possible important role of CD33 targeting in the treatment of myeloid leukemias.[93] In the meanwhile, a variety of immunotoxins using anti-CD33 mAbs were produced. Radio-active particles were conjugated to anti-CD33 mAbs and these immunotoxins could kill the myeloid leukemia cell line HL-60 *in vitro* and appeared to be relatively safe in both mouse and men.[15,16,94] In May 2000, the United States Food and Drug Administration approved gemtuzumab ozogamicin (GO or Mylotarg®) for the treatment of relapsed $CD33^+$ AML in patients older than 60 years. GO consists of a humanized anti-CD33 mAb (IgG_4) conjugated to calicheamicin, a highly potent anti-tumor antibiotic that cleaves double-stranded DNA by the abstraction of specific hydrogen atoms.[95] GO selectively targets $CD33^+$ cells and after binding to the CD33 receptor rapid internalization occurs with subsequent induction of cell death.[96] In *in vitro* studies GO killed the myeloid leukemia cell line HL-60 and clonogenic progenitors from primary AML samples[96] and targeted HL-60 human xenograft tumors *in vivo*.[97] A phase I study demonstrated that leukemic cells were eliminated from the peripheral blood in 20% of patients with relapsed or refractory $CD33^+$

AML and GO was reasonably well tolerated.[14] Phase II studies showed that approximately 30% of CD33+ AML patients in first relapse obtained a CR after treatment with GO.[98,99] Response rates were similar in younger and older patients and in patients with favorable, intermediate and poor risk cytogenetics. Interestingly, increased CD33 expression did not seem to improve response or survival, suggesting that a threshold level of CD33 molecules is sufficient to induce the full response.[96,98] In a recent study, the exact number of CD33 antigens on the cell surface was quantified but again no relation with the response to GO was found.[100] Side effects of GO treatment were relatively mild when compared with classical multi-agent chemotherapy and consisted of infusion related symptoms like fever, nausea and hypotension, infections due to neutropenia and thrombocytopenia.[98,99] However, in a few cases severe liver toxicity (hepatic sinusoidal obstruction syndrome) has been reported.[101] There were no occurrences of cardiac or cerebellar toxicity. Despite the promising clinical results with GO there is still heterogeneity of response among different patients. One reason for this heterogeneity of response could be the activity of multi-drug resistance proteins (MDR) resulting in the efflux of calicheamicin following internalization of the mAb conjugate and dissociation of calicheamicin from the CD33 mAb. Interestingly, the efflux of calicheamicin appears to be correlated with pump activity, but not always with the expression of MDR proteins like MDR1/Pgp, MRP1 and LRP.[102-104] Addition of inhibitors of the MDR pumps rarely resulted in full restoration of the cytotoxicity of GO, suggesting that other mechanisms play a role in GO resistance.[102-104] The long-term effects of GO are not yet known. In general, patients, who responded to GO therapy had a longer median survival compared to patients that did not respond, but relapse still appears a major problem.[98,99]

1.5 Telomerase

Normal human somatic cells have a limited life span and telomeres and telomerase play an important role in the regulation of the lifespan and senescence of human cells.[105] Telomeres are DNA tandem repeats of $(TTAGGG/CCCTAA)_n$ that cap the ends of eukaryotic linear chromosomes and prevent chromosome fusion and genetic instability.[106,107] Depending on the age of the donor, the cell type and the number of cell divisions of the cell, the length of telomeres in humans varies between 2-15 kilobase pairs.[108] With every cell division telomeric DNA is lost, presumably because of the inability of DNA polymerase to fully replicate the ends of a linear DNA template (end replication problem) and because of the lack of telomerase activity.[105,109-111] The average rate of telomere shortening in a cell that lacks telomerase is 50-150 base pairs per population doubling.[105,109,111] When telomeres reach a critical length, the cell will undergo cell cycle arrest and enter a non-dividing state known as replicative senescence. [112] Telomerase is a DNA polymerase that serves to maintain the telomeric ends of chromosomes.[113,114] It contains an RNA template, the catalytic subunit human telomerase reverse transcriptase (hTERT) and proteins involved in telomerase assembly, nuclear localization and stability.[107,115-117] Telomerase is capable to extend the 3' ends of telomeres. Most adult human cells, including fibroblasts, neurons, kidney cells, endothelial cells and mammary epithelial cells, have undetectable telomerase activity and these cells will eventually

senescence.[111,118,119] Ectopic overexpression of the hTERT gene in human fibroblasts, endothelial cells and retinal pigment epithelial cells resulted in telomere elongation and an extended replicative life span, suggesting that the absence of telomerase plays an important role in the senescence of these cells.[120,121]

In contrast to most cell types, low telomerase levels can be detected in various hematopoietic cells. For example, T lymphocytes have low telomerase activity, which is upregulated during T cell development and during T cell activation.[122] Primitive hematopoietic progenitors also exhibit low telomerase activity.[123-126] Despite telomerase activity in the hematopoietic progenitors, telomere shortening occurs with age. Furthermore, although telomerase activity is up-regulated during *in vitro* expansion of hematopoietic progenitors, telomere length decreases.[127] This suggests that telomerase activity alone is not sufficient to maintain telomere length in these cells.

Malignant cells have developed mechanisms with which to avoid senescence and to gain unlimited proliferative ability. One of these mechanisms is the ability to maintain telomere length. Telomerase appears responsible for telomere length maintenance in about 90% of human cancers.[118] In the remainder, telomeres appear to be maintained via a recombination-mediated process named ALT (alternative lengthening of telomeres).[118] To investigate whether inhibition of telomerase could inhibit cancer growth, Zhang et al. introduced a dominant negative form of human telomerase into cancer cells.[128] This resulted in telomerase shortening followed by proliferation arrest and cell death, suggesting that the inhibition of telomerase could play an important role in the treatment of cancer. More recently, Damm et al demonstrated that potent and selective telomerase inhibitors could arrest the growth of human cancer cell lines of breast, prostate, lung and fibrosarcoma.[129]

Telomerase activity in AML cell lines and total and $CD34^+$ hematopoietic cells of AML samples was higher compared to total and $CD34^+$ cells of normal individuals, suggesting that telomerase might be a critical factor in the unlimited proliferation of AML cells.[124,125,130-132] The literature is not clear regarding whether $CD34^+$ or $CD34^-$ AML cells express higher levels of telomerase.[130,132] Higher telomerase activity appeared to be associated with and abnormal karyotype and FAB type in AML samples.[132] Furthermore, patients with high telomerase activity had a significantly poorer prognosis compared to those with low telomerase activity, indicating that telomerase activity may be a prognostic factor in AML.[133,134] However, Seol et al. found that higher telomerase levels in AML samples were correlated with an increase in CR.[130] Overall, most studies thus far showed that an increase in telomerase levels was correlated with disease progression with higher levels of telomerase upon relapse and lower levels after chemotherapy-induced remissions.[128,131,132] Interestingly, while telomerase activity is higher in AML, telomere length is on average 1.2 kb shorter in AML cells compared to their normal counterparts.[131,135,136] These shorter telomeres probably result from the increased turn-over in AML cells and telomerase activity could be required to maintain critically short telomeres in AML stem cells. Furthermore, short telomeres can contribute to genomic instability and therefore to disease progression. Telomerase activity is upregulated upon *in*

vitro culture of normal hematopoietic progenitors in contrast to AML cells that downregulate telomerase activity upon culture.[131] Down-regulation of telomerase could be one of the reasons why it is difficult to expand AML progenitors over normal progenitors in *ex-vivo* cultures. A highly significant correlation was observed between high telomerase activity and the expression levels of hTERT, the catalytic subunit of telomerase in AML. A slight increase in mRNA levels of the RNA template could also be detected in AML samples but no increase in the telomere associated protein (TP1).[132] This suggests that hTERT may be a rate-limiting component in the regulation of telomerase activity. Recently, Delhommeau et al. overexpressed a dominant-negative hTERT (DN-hTERT) mutant in two different AML cell lines (UT-7 and U937).[137] Clones expressing the transgene showed a drastic shortening of telomere length, cytogenetic instability and an inhibition of the proliferation after two weeks in culture.[137] However, 45-55% of the DN-hTERT clones showed a reactivation of telomerase activity after 5-8 weeks in culture, resulting in a recovery of the proliferation similar to control clones. Of note is that some translocations in acute leukemias contain interstitial telomere repeats emphasizing the important role of telomeres in leukemias.[138,139]

1.6 Notch

The Notch superfamily comprises a group of highly conserved proteins, which has been shown to influence the cell fate in progenitors of numerous cell types in a wide range of organisms.[140,141] In humans four Notch receptors have been identified, Notch1-4. They consist of an extracellular domain, containing tandem EGF repeats and 3 Notch repeats, a transmembrane domain and an intracellular domain (IC), containing 6 cdc10/ankyrin repeats, putative nuclear localization signals and a C-terminal OPA/EST region.[140] Notch activation occurs through binding of the receptor to one of its ligands, Jagged1, 2 and Delta1, 3 or 4.[142,143] These ligands share a Delta:Serrate:Lag2 (DSL) domain required for Notch binding and activation. In general, upon ligation of Notch to its receptor, a member of the ADAM metalloproteases cleaves the Notch receptor extracellularly resulting in the shedding of the ectodomain.[144,145] This is followed by a presenilin-mediated proteolysis within the transmembrane domain, releasing the Notch-IC (Figure 2).[146,147] Notch-IC can then either bind the Numb protein in the cytosol, which prevents Notch translocation or it can bind to the Deltex protein which results in Notch translocation to the nucleus.[148,149] In the nucleus, Notch-IC can bind to various nuclear proteins like NRARP and Mastermind.[150,151] The best characterized Notch signalling pathway is mediated by binding to the C Binding Factor (CBF)-1 with subsequently transcription of Hairy Enhancer of Split (HES) genes or HES-related Repressor Protein (HERP) genes.[152] HES and HERP are Basic Helix Loop Helix (bHLH) genes, which act as transcriptional repressors.[152] A Notch pathway independent of CBF1 has also been described.[153,154] Notch activation is modulated by a variety of other proteins (Figure 2). For example, the Fringe protein family, Radical, Lunatic and Manic Fringe, are glycosyl-transferases which modify the EGF repeats on the Notch receptor, thereby modifying the binding specificity of the Notch receptor to its ligands.[155] With the availability of four Notch receptors and five ligands in humans it is difficult to sort out the extent of redundancy which might exist in Notch

functions, particularly since Notch1-4 all activate HES genes. It is known that Notch1 null and Jagged1 null mice are embryo lethal.[156,157] Jagged1 and Delta1 null mice have a phenotype which is not identical, suggesting non-overlapping functions for Notch genes and ligands, at least during development.[156,158]

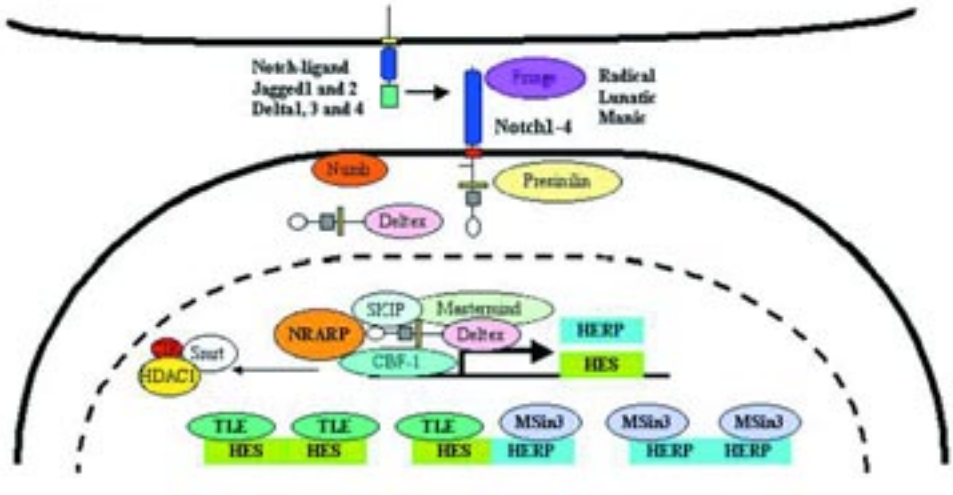

Figure 2: A simplified diagram of the Notch signalling pathway. Upon Notch binding to the ligand, Presinilin will cleave the receptor and the IC is released in the cytoplasm. In the cytoplasm Notch can bind to Numb which prevents nuclear localization or Deltex after which Notch-IC is translocated to the nucleus. In the nucleus Deltex-Notch-IC will displace the Smrt corepressor complex from CBF-1. SKIP is not displaced with the complex and is thought to have a role in converting CBF-1 from a repressor to an activator. Mastermind and NRARP are recruited and transcription of the repressors HES and/or HERP occurs. HES and HERP can each form homodimers or heterodimers with each other. The corepressor TLE will bind to HES whereas HERP recruits the mSin3 corepressor complex. Not all proteins,which can interact with Notch are depicted here.
NRARP Notch Regulated Ankyrin Repeat protein; SKIP Ski-Interacting Protein; HES Hairy Enhancer of Split; TLE Transducin-like Enhancer of Split; HERP HES Related Repressor Protein; HDAC Histone Deacetylase; Smrt Silencing Mediator of retinoic acid and thyroid hormone receptors; CIR CBF-1 Interacting Corepressor.

Notch1 was first identified in humans as the (7;9)(q34;q34.3) translocation, observed in a subset of T cell ALL. This translocation, which involved the Notch1 gene, causes overexpression of a truncated form of the Notch1 protein.[159] In the adult human hematopoietic system Notch1 was shown to be expressed in high levels on $CD34^{+}Lin^{-}$ and $CD34^{+}Lin^{+}$ human marrow cells and in lower levels on $CD34^{-}$ marrow cells.[160] A later study using single cell RT-PCR on human hematopoietic cells revealed high Notch1 expression in macrophage and macrophage progenitors. Notch4 was expressed in macrophages and Notch3, Delta1 and Jagged1 and the Fringe genes were expressed in

multiple lineages. Further studies showed that primary human BM stromal cells, HUVEC cells as well as FACS sorted $CD34^+CD38^-Lin^-$, $CD34^+CD38^+Lin^-$, myeloid, T and B cells expressed Notch1 and 2 and also Jagged1 although all to somewhat variable degrees.[161] In addition, RT-PCR on human FACS purified populations demonstrated Notch1, Notch2, and Delta1 and the metalloproteinase Kuzbanian expression in both $CD34^+CD38^+$ and $CD34^+CD38^-Lin^-$ progenitors, and in myeloid, T and B cells. Delta4 was expressed in $CD34^+CD38^+Lin^-$ but not $CD34^+CD38^-$ progenitors and in myeloid cells but not T or B cells and Delta3 was not expressed in any of the cell populations.[162]

Functional studies revealed that Notch1-Jagged1 ligation promoted the survival and expansion of primitive progenitors in both human and murine hematopoiesis *in vitro*.[161,163-167] Furthermore, $CD34^+CD38^-Lin^-$ human CB stem cells cultured for up to 15 days with or without soluble hJagged1 and transplanted into NOD/SCID mice showed higher engraftment frequency and level of engraftment in the hJagged1 treated cells.[161] Soluble hDelta1 and hDelta4 expanded CFU and $CD34^+38^-$ cells *in vitro* while only hDelta1 seemed to increase repopulating stem cells detected in NOD/SCID mice *in vivo*.[162,168]

In the murine system, studies using the IC domain of Notch have been limited by the development of T cell leukemias usually ~ 3 weeks following transplantation of transduced marrow.[169] Recently, a study using Notch1-IC transduced BM cells from RAG-1-deficient mice demonstrated that Notch1-IC transduced BM cells had fewer CFU as compared to controls but the colonies present were larger.[170] Furthermore, LTC-IC quantified by limiting dilution were more frequent in the Notch1-IC cells and generated larger colonies after secondary replating.[170] Notch1-IC and control transduced cells competitively transplanted into mice revealed a reduction in differentiated myeloid and erythroid cells and an increase in primitive progenitors upon FACS analysis of the BM of the mice. In secondary transplantation experiments primitive cells further expanded in the Notch1-IC mice while control mice had a marked decline in stem cell numbers.[170]

In myeloid cell line studies, initial reports showed that Notch1 and Notch2 can inhibit myeloid differentiation in the murine myeloid progenitor cell line 32D in a cytokine-specific manner: Notch1 inhibited G-CSF induced myeloid differentiation of 32D cells, whereas Notch2 inhibited GM-CSF induced myeloid differentiation.[171,172] However, a more recent study demonstrated that Notch activation in the 32D cell line resulted in induction of differentiation and reduction of proliferation.[173] The reason for these conflicting results is not yet clear, however a dose effect or co-regulating molecules can alter the response to Notch stimulation. Overexpression of Notch1 in the K562 cell line resulted in suppression of erythroid and an increased megakaryocytic differentiation.[174] The role of Notch and its ligands in myeloid leukemias has not been well studied thus far. One study reported a new human leukemia cell line derived from blast cells of a patient with de novo acute myeloblastic leukemia with tri-lineage myeloplasia.[175] This cell line expressed Notch1 and 2 and Jagged1 but lacked the expression of Delta1 (other Notch receptors and ligands were not investigated). The proliferation of the AML cell line increased significantly upon stimulation with Delta1 whereas a slight increase was observed upon stimulation with Jagged1.[175]

As mentioned above expression of constitutively active Notch1 in human and murine hematopoietic cells leads to the development of a leukemic proliferation of $CD4^+CD8^+$ T cells in the BM.[139,159,176 169] Similarly, Delta4 transduced Lin^- murine BM produced a marked but non-transplantable proliferation of circulating $CD4^+CD8^+$ cells, which are normally found only in the thymus, while the number of total and mature B cells was markedly reduced.[177,178] When mice were transplanted with BM of the Rag-$2^{-/-}$ mice (no VDJ recombination and a lack in pre-TCR signalling) transduced with constitutive active Notch1 the $CD4^+CD8^+$ population failed to develop but precursor T cells were seen. When the lack of pre-TCR signalling was restored, the cells went on to proliferate and underwent leukemic transformation.[179] This suggests that Notch controls the initial T/B decision but that subsequent proliferation depends on T cell specific signals.[179] Activating Notch signalling by co-culture with the stromal cell line S17 expressing Delta1 similarly inhibited B cell differentiation, while promoting cells with a T cell/NK cell precursor phenotype. Jagged1 expressing S17 cells did not alter B, T or NK cell development, suggesting alternative signalling pathways for different Notch ligands.[180] Further evidence that Notch1 is important to control the T versus B cell developmental decision was derived from Cre-LoxP conditional Notch1 knockout experiments. These mice had a block in early T cell development in the thymus at the $CD4^-CD8^-TCR^-$ precursor stage while B cells ($B220^+$) accumulated in the thymus.[181] Further studies demonstrated that the block in T cell differentiation is not due to a failure of Notch$1^{-/-}$ precursors to home to the thymus, but rather to the adoption of a B cell fate by the common lymphoid precursor in the absence of Notch1.[182] Moreover, ectopically expressed Lunatic Fringe also induced the lymphoid progenitor to develop into B cells in the thymus.[183] Early reports suggested Notch directed the decision between αβ versus γδ T lineage with reduced Notch activity favoring the gd T cell fate.[184] It was also suggested that activated Notch1 favors an increase in CD8 with a decrease in CD4 cells,[185] however subsequent reports could not confirm this.[186]

Notch2 and Notch3 have also been shown to cause T cell tumors in humans and/or animal models.[159] Feline Leukemia Virus induced thymic lymphomas in cats resulted in overexpression of a truncated Notch2 protein.[187] Mice transgenic for Notch3-IC developed aggressive multicentric T cell lymphomas and these lymphoma cells expressed high levels of NF-κB.[188]

The transforming ability of Notch genes is not restricted to T cells. Notch1 expression in Hodgkin's Reed Sternberg cells and in tumor cells from T cell derived anaplastic large cell lymphoma was elevated and stimulation by Jagged1 increased the growth and inhibited apoptosis of these cells. Notch2 was overexpressed in B-CLL and contributed to the overexpression of one of its target genes CD23.[189] Notch4 was first identified as the int-3 oncogene associated with primary mouse mammary tumors.[190] Deregulation of the Notch/CBF1 signalling pathway has been associated with Epstein-Barr Virus induced B cell malignancies.[191,192] In addition, the Notch pathway has been shown to interact with various signalling pathways involved in oncogenesis. The tumorigenic activity of Notch4-IC in murine mammary tumor cell lines required active signals from the Erk/MAP kinase and PI-3 kinase pathways downstream of Ras.[193] Notch1 was essential to maintain the neoplastic phenotype in Ras-transformed human cells *in vitro* and *in vivo*.[194] Provirus

insertion of the oncogene c-Myc occurs in close proximity to the Notch1 gene resulted in high expression of the Notch1-IC in $CD4^+CD8^+$ T cell tumors in $MMTV^D$/myc transgenic mice .[195] Notch can function as a negative regulator of NF-κB, by preventing binding to its DNA binding sites.[196] In contrast, NF-κB2 is a putative target gene of activated Notch1 via the CBF1/RBP-Jκ pathway.[197] One report showed that the Rel/NF-κB pathway upregulated the expression of the Notch ligand, Jagged1, thereby triggering the Notch signalling pathway.[198] Members of the p53 tumor supressor gene family, p63 and p73, have also been shown to upregulate Jagged1 and 2.[199] Notch1 accelerated lymphoid oncogenesis in E2A-PBX1 transgenic mice, suggesting a synergistic interaction between the Notch and E2A-PBX1 pathways.[200 201] Interestingly, a recent report suggested a tumor suppressor role for Notch1 in mouse skin tumours.[202] These studies suggest a complex but interesting connection between Notch pathways and pathways implicated in oncogenesis. [203-205]

Project Overview

The work presented in this thesis can be divided in two sections:

1. Characterization and purification of AML stem cells based on the phenotype of AML stem cells (CD133 and CD33).

2. Studies on genetic pathways and enzymes capable of modulating AML growth (telomerase and Notch).

Work in both areas is complementary and typical of modern leukemia research combining modern tools of cell biology, cell culture, xenotransplantation, flow cytometry and cell sorting with increased knowledge of genetic pathways and information about the molecular abnormalities that characterize the leukemic stem cell genome. Undoubtably, these approaches will result in a beter understanding of the disease and hopefully resulting in cures.

Hypothesis

I. Within the AML blasts, a rare population of leukemic progenitors exists, responsible for maintaining the disease.

II. The hematopoietic developmental program, controlled by sequential changes in gene expression, is dysregulated in AML.

III. Overexpression of telomerase in AML stem cells contributes to the longeveity of AML stem cells and targeting telomerase expression could result in senescence of these cells.

IV. The Notch gene family is involved in the maintenance and differentiation of hematopoietic progenitors and therefore might play a role in the pathogenesis of AML.

Objectives

1. Phenotypically and biologically characterize primitive AML progenitors

   a) Determine whether CD133, a novel stem cell marker, is differentially expressed on primitive AML versus normal hematopoietic progenitors.

   b) Study whether CD33, a cell surface antigen used for specific delivery of toxic agents to AML cells, is expressed on primitive AML progenitors.

2. Investigate genetic pathways involved in the maintenance of AML stem cells.

   a) Determine whether inhibition of telomerase can affect the growth of AML cells.

   b) Study the expression of the four Notch genes in the hematopoietic system and in AML cells.

   c) Compare the function of Notch1 and *4* in normal human hematopoiesis.

   d) Compare the effects of Notch signalling on normal and leukemic hematopoietic progenitors.

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Chapter 2

CD133 (AC133) Expression on Acute Myeloid Leukemia Cells and Progenitors

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Abstract

Background. Acute myeloid leukemia (AML) blasts differ in their functional capability, creating a hierarchy of progenitors. CD133 (AC133) is a newly described transmembrane protein expressed on $CD34^+$ and $CD34^-$ normal progenitors. We characterised the prognostic significance of CD133 expression in AML and expression of CD133 on AML progenitors thought to be responsible for maintaining this disease.

Methods. AML cells of 102 patients were analyzed for CD133 and CD34 expression and correlated with outcome in 92 treated patients. AML cells were also FACS sorted into $CD34^+CD133^+$, $CD34^+CD133^-$, $CD34^-CD133^+$ and $CD34^-CD133^-$ subfractions, and assayed *in vitro* in colony forming assay (CFU) and in suspension culture (SC) assay for up to 8 weeks, and *in vivo* in NOD/SCID mice to determine the phenotype of progenitors detected in these assays.

Results. CD133 expression was not correlated with event-free or overall survival, FAB subtype, cytogenetic abnormality or white blood count but was correlated with CD34 expression. Primary AML CFU were present in all four sorted fractions. After an increasing period of time in SC a higher proportion of cells capable of forming leukemic CFU were found in the $CD34^+CD133^+$ subfraction. Cells capable of producing leukemic engraftment in NOD/SCID mice were found in all subfractions including the $CD34^-CD133^-$ subfraction in many patients.

Discussion. CD133 is not useful as a prognostic marker in AML. CD133 is expressed with CD34 on most primitive leukemic progenitors detected *in vitro*, however, *in vivo* progenitors could not be purified using CD133 in these patients.

Keywords: Acute myeloid leukemia-CD133-CD34-NOD/SCID mice- prognosis

Introduction

The use of monoclonal antibodies to target malignant cells in a background of normal cells has been useful for the diagnosis of minimal disease, for the specific delivery of radioisotopes or toxins, and for purging prior to autologous transplantation. The application of these approaches in AML has been complicated both by the similarity of antigen expression between normal stem cells and AML cells, and by the recent observation that the heterogeneity of antigen expression between AML blasts from the same patient reflects also a heterogeneity in the function of these cells. Only a small minority of AML blasts are capable of extensive long-term proliferation *in vitro* and engraftment into NOD/SCID mice.[1-4] These cells have a phenotype unlike bulk blasts but similar in some respects to normal primitive progenitors in that most are CD34$^+$, lack expression of CD71, HLA-DR and CD38,[2-4] however, they differ from normal progenitors in their lack of expression of Thy.1 (CD90)[5] and their lower c-kit (CD117) expression.[6] These AML *in vitro* and *in vivo* proliferating populations share the same phenotype and are likely also related to the cells capable of sustaining AML in the patient. It thus seems likely that only the most primitive AML cells are responsible for maintaining the disease and consequently are the most relevant cells to target for disease therapy. Human CD133 is a novel 5-transmembrane molecule, which is expressed on primitive normal hematopoietic progenitors.[7,8] CD133 is of unknown function but is felt to be the human homologue of mouse prominin.[9] In the hematopoietic system, CD133 is expressed on a subset (30-70%) of the CD34$^+$ cells in the human bone marrow (BM), fetal liver, umbilical cord blood and growth-factor primed peripheral blood (PB).[8,10] *In vitro* colony assays using normal adult BM, have shown that the majority of CFU-GM are contained in the CD34$^+$CD133$^+$fraction, while the majority of BFU-E and CFU-Mix are in the CD34$^+$CD133$^-$ fraction.[10] However, when clonogenic assays were performed using cord blood, the majority of CFU-GM as well as the majority of BFU-E and CFU-Mix were detected in the CD34$^+$CD133$^+$ subset.[8] CD34$^+$CD133$^+$ cells successfully engraft primary and secondary recipients using the fetal sheep model[10] and only CD34$^+$CD133$^+$, but not CD34$^+$CD133$^-$ cord blood cells are capable of sustaining hematopoiesis long-term in the presence of stroma (LTC-IC) and of repopulating NOD/SCID mice.[8] These studies suggest that CD133 can be useful as an additional marker to CD34 for the purification of primitive hematopoietic progenitors. Furthermore, recent evidence suggests that cells with stem cell properties are a rare subpopulation of CD34$^-$lineage$^-$ cells,[11,12] and these CD34$^-$ cells also express CD133, allowing their purification.[13]

While the expression of CD133 on primitive AML cells is unknown, it has been reported that CD133 is expressed on the majority of bulk CD34$^+$ AML cells, whereas CD133 expression on CD34$^-$ AML cells is low to absent in most,[7,14-16] but not all,[14] cases. There have been conflicting results regarding a correlation between CD133 expression and FAB type, with increased CD133^{bright} cells in the M4 and M5 FAB subtypes[7] and in FAB M0[17] reported. CD133 expression on FAB M3 cases has been low to absent in all reports and this is consistent with an absence of CD34 expression on those samples. No correlation between CD133 expression and cytogenetics or prognosis has been reported.

To investigate whether CD133 selection is useful as a purging strategy in AML, CD133 expression on primitive AML cells capable of long-term *in vitro* and *in vivo* proliferation was determined. Furthermore, a possible correlation between CD133 expression and CD34 expression and clinical parameters was studied.

Materials and Methods

Patient Cells

Bone marrow (BM) cells from normal healthy donors and peripheral blood (PB) cells from 102 patients at diagnosis of AML were obtained after informed consent and with approval of the Clinical Research Ethics Board of the University of British Columbia. The mean number of blasts in the samples was 63 ± 26%. Patients with FAB M3 type were excluded because of a different treatment strategy given to M3 patients. Blood cells were Ficoll separated to obtain a mononuclear cell population then frozen in Dulbecco's modified Eagle's medium (DMEM) (StemCell Technologies Inc., Vancouver, B.C.) with 50% fetal calf serum (FCS) (StemCell Technologies Inc.) and 10% dimethylsulphoxide (DMSO) and stored at -135°C.

Patient Therapy

Of the 102 patients (pts) assessed, 92 patients received treatment with induction chemotherapy and were analyzed for event-free and overall survival. Ten patients were not treated as they were too ill and died prior to treatment initiation (2 pts) or treatment was precluded due to age or medical problems (8 pts). The median age of the 92 patients was 51 (range 18-78) and there were 47 men and 45 women. Sixty-six patients received high-dose cytarabine plus daunorubicin (29 pts) or mitoxantrone and etoposide (37 pts). Twenty-four patients received low-dose cytarabine plus daunorubicin (7+3) and 2 patients received other. Sixty-eight patients (74%) achieved a complete remission. Forty-five patients went on to consolidation chemotherapy with high-dose (32 pts) or low-dose (13 pts) cytarabine plus daunorubicin (30 pts), mitoxantrone and etoposide (12 pts) or carboplatin (3 pts). Twenty-four patients had a second cycle of consolidation chemotherapy. Thirty-two patients underwent an allogeneic (25 pts) or autologous (7 pts) transplantation after receiving high dose therapy.

AML Cell Phenotyping and Sorting

Prior to sorting, thawed AML cells were suspended in HFN (Hanks medium with 2% FCS and 0.1% sodium azide) at 10^7 cells/ml. Cells were stained for 30 minutes on ice with monoclonal antibodies CD34-FITC (Dr. Peter Lansdorp, Terry Fox Laboratory) used at 4 µg/ml and CD133-PE (Miltenyi Biotec Inc., Auburn, California) used according to manufacturer's instruction. Separate aliquots were stained with an irrelevant mouse IgG1_FITC antibody (Becton Dickinson, San Jose, California) and mouse IgG1_PE antibody

(Becton Dickinson) as an isotype control. Cells were then washed twice in HFN at 4°C, propridium iodide (PI) at 2 µg/ml was added to the cells prior to the second wash, and the cells were maintained on ice prior to sorting. Cells were analyzed and sorted on a dual laser FACStarplus (Becton Dickinson) on the basis of fluorescence intensity after gating out non-viable (PI^+) cells. Gates were set to exclude 99.5% of isotype control stained cells (Figure 1). Fractions were sorted into DMEM with 50% FCS in microcentrifuge tubes at 4oC. Sorted fractions were washed, resuspended at known cell concentrations and used to initiate colony forming assays (CFU) and suspension culture (SC) assays or for injection into NOD/SCID mice.

Primary CFU Assays

Immediately after FACS sorting, a known number of sorted and control unsorted PB cells were plated in methylcellulose culture medium, (Methocult H4330, StemCell Technologies Inc.), containing 20 ng/ml rhIL-3 (Sandoz, Basel, Switzerland), 20 ng/ml rhIL-6 (Terry Fox Laboratory), 20 ng/ml rhG-CSF (Amgen Canada Inc., Mississauga, Ontario), 20 ng/ml rhGM-CSF (Sandoz) and 50 ng/ml rhSteel Factor (SF) (Terry Fox Laboratory). After 10 days of incubation at 37°C in a 5% CO_2 humidified incubator, AML blast clusters (10-20 cells) or colonies (> 20 cells) were counted and the numbers were pooled to obtain CFU counts.

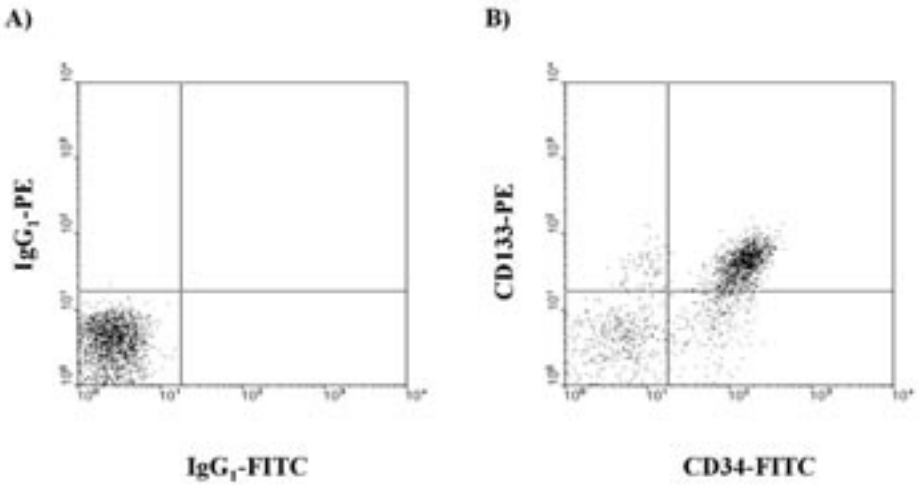

Figure 1: FACS profiles demonstrating gates for sorting of CD34-FITC and CD133-PE subpopulations. A) PB of AML patient 319 was stained with IgG1_FITC and IgG1_PE as isotype controls and analyzed by FACS. Sorting gates were set to exclude ≥ 99.5% of isotype stained control cells. B) PB cells from the same patient were also stained with CD34-FITC and CD133-PE and sorted according to the gates set using the isotype controls. Gates were set for each patient using the patient specific isotype control.

Suspension Culture Assays (SC)

Suspension cultures were initiated with a known number of sorted and unsorted cells and maintained generally, as previously described,[4,5] in 1.0 ml of serum free medium containing the growth factor cocktail described above and 41.5 µg/ml low density lipoprotein (Sigma-Alderich, Oakville, Ontario). Cultures were maintained at 37°C in a 5% CO_2 humidified incubator and 0.5 ml of fresh medium with growth factors was added weekly. Every second week half the cells and volume of the SC were removed, the cells were washed in DMEM with 10% FCS and cultured in methylcellulose to determine the CFU content of the SC. SC were maintained for 8 weeks then the entire contents of the wells were harvested and assessed for CFU content. To allow comparisons between experiments the proportion of progenitors derived from each sorted fraction at each time point was determined by comparison to a progenitor recovery of 100% from all fractions at that time point. Using PB cells of the AML patients no formation of feeder layers in the SC was observed.

Transplantation of Leukemic Cells into NOD/SCID Mice

Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Jackson Laboratory, Bar Harbour, ME) were bred and maintained in the animal facility of the British Columbia Cancer Research Centre (Vancouver, B.C., Canada) under sterile conditions in sterile micro-isolator cages and were provided exclusively with autoclaved food and water containing 100 mg/l ciprofloxacin and HCl. Twenty-four hours prior to transplantation, mice were irradiated with 3.5 Gy γ-irradiation from a ^{137}Cs source at a dose rate of 1.22 cGy/min. Unsorted AML PB cells and sorted subfractions were suspended in 0.3 ml Alpha minimal essential medium (Alpha MEM)(StemCell Technologies Inc.) with 5% FCS and injected intravenously into the lateral tail vein of 6-8 week old NOD/SCID mice. Whenever possible, various cell concentrations were injected as a semi-quantitative assessment of the number of cells required for engraftment. Normal BM cells were given 15 Gy irradiation using 250kVp X-rays (Philips RT-250, HVL 1.5 mm Cu) at a dose rate of 5.1 Gy/min. In each case, 2×10^6 irradiated BM cells were co-injected with the unsorted cells and sorted subfractions to enhance engraftment potential. Eight weeks post-injection the mice were sacrificed by CO_2 inhalation. BM was removed from the femurs by flushing with Alpha MEM with 5% FCS.

Flow Cytometry Analysis of Murine Tissues

Cell suspensions from femoral BM were lysed in ammonium chloride (StemCell Technologies Inc.) for 20 minutes and then washed in HFN with 5% human serum for blocking of human Fc receptors and prepared for flow cytometric analysis as described.[4,5] Briefly, cells were incubated on ice with an anti-mouse IgG Fc receptor monoclonal antibody (2.4G2) (Dr. P. Lansdorp, Terry Fox Laboratory, Vancouver). Cells were then stained with a human pan leukocyte antibody, 9.4 (anti CD45)-FITC (Dr. P. Lansdorp) for 30 minutes on ice. Separate aliquots were stained with an irrelevant IgG_1-FITC antibody (Becton Dickinson) as an isotype control. Aliquots of normal human PB cells and BM cells from a non-injected NOD/SCID mouse were also stained with CD45-FITC

and the isotype control to serve as positive and negative controls for antibody specificity. Samples were analyzed using a FACScan (Becton Dickinson), gate settings which excluded ≥ 99.9% of the cells in the matched isotype control were used to determine the CD45 expression of each sample. Any positive value (i.e. ≤ 0.1%) for the isotype control was then subtracted from the percentage positive in the CD45 stained samples. We defined human engraftment as expression of ≥ 0.1% $CD45^+$ cells in a sample. Whenever possible, the $CD45^+$ cells derived from murine BM were sorted and plated in methylcellulose with recombinant growth factors. The derived colonies were plucked onto slides for subsequent cytogenetic analysis. Alternatively, cytospin preparations were made from flow-sorted $CD45^+$ cells and the slides were stained for morphological analysis with May-Grünwald-Giemsa.

Cytogenetic Analysis

Colonies from primary CFU or from CFU which were derived from SC were evaluated for the leukemia specific cytogenetic change by either standard cytogenetics or by fluorescence in situ hybridisation (FISH) whenever possible. Likewise, sorted $CD45^+$ cells from BM removed from NOD/SCID mice and CFU derived from these cells were evaluated for the leukemic transformation. For analysis, single colonies were plucked and plated onto slides after colony synchronisation with colcemid.[18] Probes specific for centromeric repeat sequences were used to detect cytogenetic abnormalities in 5 patients. These included D8Z2 (ATCC, Rockville, MD) to detect the +8 abnormality, the Y probe[19] pRY3.4, and a yeast artificial chromosome (YAC) clone 909g3 (Research Genetics) to detect the +13 abnormality. Total plasmid DNAs containing the centromeric specific DNA were labelled with digoxigenin (Boehringer-Mannheim, Mannheim, Germany) by nick translation. The +8 probe was hybridised in Hybrisol VI (Oncor). The signal was amplified by rabbit anti-sheep FITC (Jackson Immuno Research Laboratories, West Grove PA) and detection by sheep anti-digoxigenin-FITC (Boehringer Mannheim, Quebec, Canada). Counterstaining was in PI at 200 ng/ml in Vectashield (Vector Laboratories, Inc. Burlingome, CA). Colonies were defined as positive whenever ≥ 60% of cells contained the respective leukemic karyotype of that patient. Colonies containing only 40-60% of cells with the leukemic change were classed as undetermined and those with < 40% leukemic cells were defined as negative. Whenever both metaphase and interphase cells could be scored approximately 200 cells (mean ± SE, 226 ± 11 cells) were counted per colony.

Calculations and statistical analysis

The Cox regression model was used to study the correlation of event-free and overall survival with CD133 or CD34 expression, FAB subtypes, cytogenetic abnormality or white blood cell count (WBC) in 92 pts. For the correlation between CD133 expression and CD34 expression, FAB type, cytogenetic abnormality or WBC a Wilcoxon Rank Sum Test was performed. To study the correlation between CD133 or CD34 expression and NOD/SCID engrafting ability a Chi square test was performed. For statistical analysis CD133 and CD34 were considered positive when more than 10% of the blasts expressed

the surface marker and high when more than 40% of blasts expressed the marker. FAB types were divided in a favourable group (FAB M1, M2 and M4) or unfavourable (FAB M0, M5, M6 or AML with prior MDS). Cytogenetic abnormalities were divided into a favourable, intermediate or poor groups according to the guidelines set by Grimwade et al.[20] The white blood cell count (WBC) was grouped into WBC under or over 50 x 10^9 cells/L. For all the tests a significant level of 0.05 was chosen.

RESULTS

Expression of CD34 and CD133 on AML blasts

Peripheral blood mononuclear cells from 102 AML patients were evaluated for co-expression of CD34 and CD133 (Figure 2). These patients were FAB M0 (n=8), M1 (n=18), M2 (n=16), M4 (n=23), M4e (n=7), M5 (n=13), M5a (n=7), M5b (n=2). Four patients had myelodysplastic syndrome (MDS) before developing AML and 4 patients had an undetermined (UD) subtype. Expression of CD133 was heterogeneous in this group of patients (range 0-92% $CD133^+$). Of the 102 samples, 64 patients had a $CD34^+$ expressing AML ($CD34^+$ AML defined as CD34 expression in ≥ 10% of blood mononuclear cells), whereas 38 patients had a $CD34^-$ AML ($CD34^-$ AML defined as CD34 expression in ≤ 10% of blood mononuclear cells). In the 64 $CD34^+$ expressing samples, the mean proportion of $CD34^+CD133^+$ nucleated cells was 27 ± 25%, while 32 ± 24% were $CD34^+CD133^-$, 2.5 ± 6% were $CD34^-CD133^+$ and 39 ± 25% were $CD34^-CD133^-$. In the 38 $CD34^-$ samples the mean proportion of $CD34^+CD133^+$ nucleated cells was 0.7 ± 1.3%, while 1.4 ± 1.7% were $CD34^+CD133^-$, 8 ± 20% were $CD34^-CD133^+$ and the majority, 90 ± 20% were $CD34^-CD133^-$. Interestingly, in 5 of the 38 $CD34^-$ samples a significant CD133 expression (range 13-75% $CD133^+$) was observed, while the other $CD34^-$ samples were $CD133^-$ (0.05%-6.7% $CD133^+$).

Correlation of CD133 on AML blasts with CD34, WBC, FAB type, cytogenetic abnormality, overall survival and NOD/SCID engrafting capacity

A subset of 92 patients was analyzed for the correlation between CD133 expression and other prognostic factors and patient outcome. All patients received induction chemotherapy. In this study no correlation between event-free or overall survival and CD133 expression could be observed with analysis of $CD133^+$ set at ≥ vs < 10%, and ≥ vs < 40% and used as a continuous variable ($p > 0.05$). Event-free and overall survival were significantly associated with WBC < 50 x 10^9 cells/L (p=0.009, p=0.005 respectively) and favorable cytogenetics (p=0.014, p=0.010) but not with CD34 expression or FAB subtype in univariate analysis. The data obtained revealed a highly significant positive correlation between the level of CD133 and CD34 expression ($p<0.001$ with $CD133^+$ and $CD34^+$ ≥ 10%; p=0.003 with $CD133^+$ and $CD34^+$ ≥ 40%). No correlation could be observed between CD133 expression and FAB type, cytogenetic abnormality or WBC.

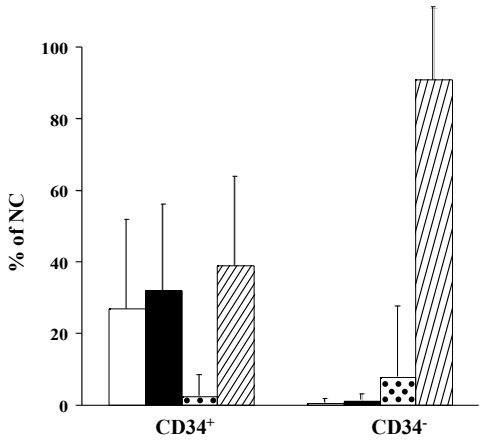

Figure 2: Expression of CD34 and CD133 on AML cells from 102 patients at diagnosis. PB cells from 102 AML patients at diagnosis were stained with CD34-FITC and CD133-PE and analyzed by FACS. The mean % of cells in the $CD34^+CD133^+$ subfraction (open bars), $CD34^+CD133^-$ subfraction (filled bars), $CD34^-CD133^+$ subfraction (dotted bars) and the $CD34^-CD133^-$ subfraction (hatched bars) of 64 $CD34^+$ (CD34 expression ≥ 10%) and 38 $CD34^-$ (CD34 expression ≤ 10%) samples was calculated.
PB = peripheral blood, NC= nucleated cells

In order to determine whether CD34 or CD133 expression was related to NOD/SCID engrafting ability, unsorted PB mononuclear cells (0.5-1 x 10^7 cells) of 72 AML patients were injected into NOD/SCID mice in duplicate. Cells of 36 patients engrafted in these animals. Of the 36 samples capable of engrafting NOD/SCID mice 19 were $CD133^+$ ($CD133^+ \geq 10\%$) and 17 were $CD133^-$ ($CD133^+ \leq 10\%$), while the 36 samples that failed to engraft were $CD133^+$ in 16 cases and $CD133^-$ in 20 cases. No statistical difference ($p=0.62$ for $CD133^+ \geq 10\%$ and $p=0.78$ for $CD133^+ \geq 40\%$) could be found between $CD133^+$ and $CD133^-$ AML regarding their NOD/SCID engrafting ability. Of the 36 engrafting samples, 26 were $CD34^+$ AML ($CD34^+ \geq 10\%$) whereas 10 were $CD34^-$ AML ($CD34^+ \leq 10\%$). Of the 36 samples that failed to engraft 19 were $CD34^+$ and 17 were $CD34^-$. Again, there was no statistical difference ($p=0.23$) between $CD34^+$ and $CD34^-$ AML samples regarding their ability to engraft NOD/SCID mice ($p=0.23$ for $CD34^+ \geq 10\%$ and $p=0.34$ for $CD34^+ \geq 40\%$).

Expression of CD34 and CD133 on AML cells with long term proliferative ability in vitro

In order to determine the phenotype of AML cells capable of producing CFU after long-term culture, PB cells from 12 patients were sorted in four fractions according to their

expression of CD34 and CD133 and subsequently used to establish suspension cultures (SC). Eight patients had high expression of CD34 (40 ± 27% $CD34^+$), while the majority of AML blasts in the other 4 patients were $CD34^-$ (99.6 ± 0.2% $CD34^-$). The $CD34^+CD133^+$ subfraction represented 9 ± 14% of nucleated cells in the 12 patients at sorting, 18 ± 28% were $CD34^+CD133^-$, 3 ± 7% were $CD34^-CD133^+$ and the majority were $CD34^-CD133^-$ (70 ± 35%) (Figure 3). Cells capable of forming primary CFU were present in all four subfractions. Similarly, CFU after 2 weeks in suspension culture (SC) were also present in all four subfractions. However, with increasing culture time, the proportion of total CFU at each timepoint that were from the $CD34^+CD133^+$ subfraction increased, while the proportion of CFU from the other three subfractions decreased. After 8 weeks in SC, 83 ± 28% of CFU were from the $CD34^+CD133^+$ subfraction and 16 ± 28% were from the $CD34^+CD133^-$ subfraction with only 1 ± 4% from the $CD34^-CD133^+$ and 0.2 ± 0.7% from the $CD34^-CD133^-$subfractions (Figure 3).

Cytogenetic analysis of in vitro studies

Primary colonies as well as colonies after SC obtained from all 12 patients appeared morphologically abnormal with often only small cluster-like colonies observed. It was possible to analyse CFU and CFU derived from SC by FISH or standard cytogenetic analysis in some fractions of 6 patients (Table 1). The majority CFU derived from unsorted cells were found to have the respective leukemic change (22/28). CFU derived from SC of unsorted cells at weeks 2-8 were also mainly leukemic (25/41). In 27/72 colonies derived from primary CFU or SC from the $CD34^+CD133^+$ subfraction the leukemic change was observed, 36/69 colonies from $CD34^+CD133^-$ subfractions, 15/28 colonies from the $CD34^-CD133^+$ subfraction and 12/25 of the $CD34^-CD133^-$ subfraction were also leukemic. Overall no selection of leukemic versus normal colonies was seen with CD133 sorting.

In vivo NOD/SCID assay

AML cells from 20 patients were sorted for co-expression of CD34 and CD133 and evaluated for their ability to repopulate sublethally irradiated NOD/SCID mice (Table 2A). Human cells were detected in the BM of mice using unsorted cells from these patients, although the number of human cells in the BM varied considerably (range 0.13-89% $CD45^+$ using 5 x 10^6 cells). With AML cells of 11 patients engraftment was also achieved using cells sorted for CD34 and CD133, however the levels of engraftment were very low in the majority of patients. With 2/11 patient samples, engraftment (0.15% and 1.53%) could only be obtained using cells from the $CD34^+CD133^-$ subfraction, while no engraftment was seen using an equal or higher number of $CD34^+CD133^+$ cells or $CD34^-$ cells. With the other 9 AML samples engraftment was achieved with at least two subfractions and 8 of those samples engrafted using $CD34^-CD133^-$ cells (Table 2A). In summary, from these 11 patient samples, 18 of the 20 mice injected with unsorted cells engrafted, 8/21 mice injected with $CD34^+CD133^+$ cells, 19/34 mice injected with the $CD34^+CD133^-$ cells, 3/8 mice injected with $CD34^-CD133^+$ cells and 10/19 mice injected with $CD34^-CD133^-$ cells also engrafted.

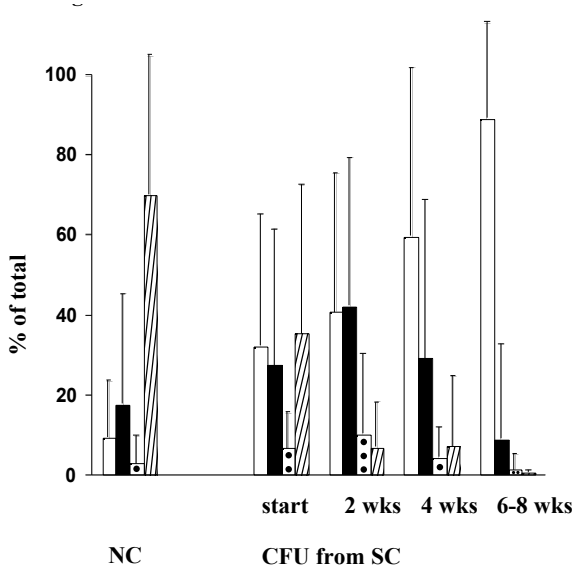

Figure 3: Proportion of AML PB cells and progenitors from 12 AML patients in subfractions sorted for the expression of CD34 and CD133. Percentage of blood cells, primary CFU and CFU after 2-8 weeks in SC in the $CD34^+CD133^+$ subfraction (open bars), $CD34^+CD133^-$ subfraction (filled bars), $CD34^-CD133^+$ subfraction (dotted bars) and the $CD34^-CD133^-$ subfraction (hatched bars). To allow comparison of the proportion of progenitors in each subfraction, CFU frequencies were determined by comparison to a progenitor recovery of 100% (UF) of all four subfractions at each time point.
The mean CFU frequencies of UF of the 12 patient samples at the different time points were 547 ± 965 CFU / 10^5 cells at week 0, 1224 ±1420 CFU / 10^5 cells at week 2, 2146±2168 CFU / 10^5 cells at week4 and 2059 ±3769 CFU / 10^5 cells at week 6-8.
PB = peripheral blood, SC = suspension culture, NC= nucleated cells, UF=unsorted

Histologic and cytogenetic analysis of transplanted cells

In 4 patients, $CD45^+$ cells from all positive NOD/SCID mice injected with unsorted or CD34 and CD133 sorted AML cells were sorted for analysis (Table 2B). Histologic analysis of the $CD45^+$ sorted cells showed > 80% blast cells in every case regardless the subfraction injected. FISH could be performed on $CD45^+$ cells from NOD/SCID mice injected with cells of 2 patients (+13 and inv16). In both cases, cells derived from recipients of unsorted cells had the leukemic karyotype. Furthermore, abnormal cells (+13) could be detected in $CD45^+$ cells from the recipient of the $CD34^+CD133^-$ subfraction of one patient, while in the other patient $CD45^+$ cells showed the abnormal karyotype (inv16) in all mice regardless the subfraction of cells injected.

Table 1: *No. of Colonies detected with the leukemic change by FISH or Cytogenetics following in vitro Assays*

Patient No.	Karyotype	Positive Colonies from CFU blast	Positive Colonies after 2-4 weeks of SC	Positive Colonies after 6-8 weeks of SC
Unsorted				
069	+13	4/10	ND	5/8
201	+8	10/10	3/8	ND
230	-4q	ND	7/11	ND
319	-Y	8/8	3/4	3/4
353	-Y	ND	4/6	ND
$CD34^+CD133^+$				
069	+13	2/8	ND	3/8
201	+8	0/8	0/8	ND
230	-4q	ND	14/14	ND
319	-Y	8/8	ND	ND
353	-Y	ND	0/7	0/7
364	-Y	0/4	ND	ND
$CD34^+CD133^-$				
069	+13	2/7	ND	3/8
201	+8	ND	2/8	ND
230	-4q	ND	9/12	ND
319	-Y	8/8	1/4	3/4
353	-Y	ND	2/7	2/7
364	-Y	4/4	ND	ND
$CD34^-CD133^+$				
069	+13	3/7	2/7	4/8
319	-Y	6/6	ND	ND
$CD34^-CD133^-$				
069	+13	2/8	3/7	5/8
230	-4q	ND	2/2	ND

Abbreviations: SC, suspension culture.
Positive colonies were defined as ≥ 60% cells with the leukemic karyotype in one colony.

Table 2A: *AML cells sorted for co-expression of CD34 and CD133 with multiple subfractions capable of engrafting NOD/SCID mice*

Patient	Unsorted	$CD34^+$ $CD133^+$	$CD34^+CD133^-$	$CD34^-$ $CD133^+$	$CD34^-$ $CD133^-$
069	2.4	0	0.15	0	0
284	38; 16; 45	0; 0.34; 0	1.5; 0; 0; 0	0; 0	0; 1.6; 0.14
212	89	16	24	30	16
319	0.83; 0.54	0; 0	1.53	0	0; 0
351	0.12; 0	0.20; 0.25	0; 0.2; 0; 0.13;0.21	ND	0.12
353	1.3; 0	0; 0	0; 0; 0; 0	ND	0.14; 0
267	0.14; 0.45	0; 0.22	0; 0; 0.15; 0	ND	0
142	0.24; 0.15	0.16	0.13; 0.13	ND	0; 0.16
373	0.19	0; 0; 0	0; 0.15; 0.14; 0.20; 0, 0.21; 0.26	0.19	0.30
272	0.16; 0.18	0; 0.28	0.12; 0.36; 0.18	0	0; 0.30; 0.30
379	1.85; 0.16	0; 0.10	0.18; 0	0.14	0.12; 0
Median	0.45	0.00	0.13	0.00	0.06

ND= not done

Table 2B: *Cytogenetical or morphological analysis of $CD45^+$ cells obtained from bone marrow of NOD/SCID mice*

Patient	Subfraction	Mean % engraftment	Mean % blasts	Positive by FISH
069	Unsorted	2.4	91	Yes
	$CD34^+CD133^-$	0.15	82	Yes
284	Unsorted	33	83	N/A
	$CD34^+CD133^+$	0.3	82	N/A
	$CD34^+CD133^-$	1.5	85	N/A
	$CD34^-CD133^-$	0.6	83	N/A
212	Unsorted	89	81	Yes
	$CD34^+CD133^+$	16	86	Yes
	$CD34^+CD133^-$	24	85	Yes
	$CD34^-CD133^+$	30	86	Yes
	$CD34^-CD133^-$	16	83	Yes
319	Unsorted	0.6	91	ND
	$CD34^+CD133^-$	1.5	90	ND

N/A= not applicable, ND= not done

Discussion

It now seems clear that primitive AML progenitors exist within the AML blast population which are uniquely capable of extensive proliferation.[1-4] It is likely that these cells are responsible for maintaining the disease. CD133 is a newly identified cell surface protein that is expressed both on normal CD34$^+$ stem cells[7,8] and on CD34$^-$ cells with *in vivo* repopulating ability.[13] The aim of this study was to investigate whether CD133 is expressed on primitive AML cells with long term proliferation *in vitro* and NOD/SCID repopulating capacity. Furthermore, we investigated the correlation between CD133 expression on AML blasts and FAB subtype, cytogenetic abnormalities, WBC and event-free and overall patient survival.

The expression of CD133 on blood blast cells of 102 AML patients was heterogeneous, with the majority of CD34$^-$ cases being CD133 negative (87%) and the majority of CD34$^+$ samples being CD133 positive (69%). However, both CD34$^+$ AML cells without CD133 expression and CD133 expression on CD34$^-$ blasts could be observed. This shows that while CD133 and CD34 expression are correlated in AML, CD133 expression is not limited only to CD34$^+$ AML cells. Although some studies have reported a correlation between CD34 expression and survival,[21-23] we did not observe a correlation between CD34 or CD133 and event-free or overall survival. CD133 expression was also not correlated with the risk factors FAB type, cytogenetics or WBC. CD133 expression does not seem to be useful as a prognostic factor in AML. NOD/SCID engrafting capacity has been correlated with a poor prognosis in AML.[24] In this study we also observed no correlation between CD133 or CD34 expression and NOD/SCID engrafting ability.

The long-term *in vitro* proliferative ability of cells sorted for co-expression of CD34 and CD133 was evaluated in 12 AML patients. While the majority of cells at sorting were CD34$^-$, the majority of cells capable of extensive proliferation for up to 8 weeks in SC were derived from the CD34$^+$CD133$^+$ subfraction. Waller et al.[25] demonstrated that the Philadelphia chromosome-positive cells were equally distributed between the CD133$^+$ and CD133$^-$ subfractions of CD34$^+$ PB cells from patients with CML. In this study we did not do cytogenetics on sorted fractions directly but did find cytogenetically abnormal primary CFU and abnormal CFU after SC in both the CD133$^+$ and CD133$^-$ subfractions of CD34$^+$ and CD34$^-$ cells.

With 11 patient samples examined, AML cells which were capable of engrafting NOD/SCID mice were found to be derived from more than one CD34 and CD133 sorted subfraction in most cases. With samples of 7 patients, engraftment with both CD34$^+$CD133$^+$ and CD34$^+$CD133$^-$ cells was seen, with AML cells from 1 patient sample neither fraction engrafted, and using samples of 3 patients the CD34$^+$CD133$^-$ fraction engrafted but not the CD34$^+$CD133$^+$ fraction. The CD34$^-$CD133$^+$ subfraction of 4 patient samples was too small to be assessed in the NOD/SCID mice. However, of the remaining 7 AML samples, both CD34$^-$CD133$^+$ and CD34$^-$CD133$^-$ subfractions engrafted using 3 samples, neither fraction engrafted using 2 patient samples and only the CD34$^-$CD133$^-$ fraction engrafted using the other 2 patient samples.

Although, CD133 expression was thought to be exclusively expressed on $CD34^+$ cells of normal marrow, cord blood and fetal liver, Gallacher et al.[13] identified a very rare population of normal $CD34^-CD38^-Lin^-$ cord blood cells with long-term *in vitro* and *in vivo* proliferative ability that express CD133. It has been suggested that the normal $CD34^-CD133^+CD38^-Lin^-$ progenitor is more primitive than the $CD34^+CD38^-Lin^-$ progenitor. In AML, while the cells with long-term *in vitro* proliferative ability were mainly $CD34^+CD133^+$, CD133 did not seem to be differentially expressed on AML $CD34^+$ *in vivo* engrafting cells. Similarly, unlike the normal situation, CD133 did not seem to be differentially expressed on $CD34^-$ AML engrafting cells. $CD34^-$ cells with AML producing ability are found in a minority of patients overall, but have been previously reported.[4,5,26] The high proportion of patients that had cells in all fractions producing low level engraftment in this study is likely due to chance. It is not known whether these $CD34^-$ cells represent a population that is more primitive or more differentiated than the $CD34^+$ AML progenitors.

It does not appear that CD133 will be useful to purify or purge AML progenitors and other cell surface markers will be necessary. If the phenotype of AML progenitors differs from normal primitive progenitors, new purging strategies could be developed, which might improve the results of autologous stem cell transplantation in AML. The ability to purify AML progenitors and to study their functional characteristics in biological assays would facilitate studies designed to investigate the molecular basis of human leukemogenesis.

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Chapter 3

CD33 Expression is Absent on Primitive Acute Myeloid Leukemia Progenitors from Most Patients

Suzanne M. Vercauteren, Rick Zapf and Heather J. Sutherland

Abstract

Acute myeloid leukemia (AML) populations contain a hierarchy of progenitor cells, a small subset of which can regenerate leukemia in immunocompromised mice or produce clonogenic progeny in long-term cultures. CD33 is a myeloid marker that is expressed on all normal myeloid progenitors and monocytes, but is absent on normal hematopoietic stem cells. CD33 is expressed on 75-90% of the AML samples and therefore appears to be an ideal cell surface marker for bone marrow (BM) purging strategies and antibody targeted therapies. We determined expression levels of CD34 and CD33 on AML progenitors with long-term *in vitro* proliferative ability and NOD/SCID engrafting ability. As in normal hematopoiesis, CD33 is absent on the majority of AML progenitors after suspension culture in serum-free medium for 6-8 weeks. NOD/SCID engrafting cells were found only in the $CD34^{+}CD33^{-}$ fraction in three patients while in the remaining six patient samples engraftment was obtained with more than one fraction. In all 9 patient samples with which engraftment was obtained $CD33^{-}$ cells contributed to the engraftment. These data suggest that leukemic primitive progenitors of most AML patients lack the expression of CD33 and therefore these patients will be likely to relapse after CD33 targeted therapies, while a smaller subset of patients have progenitors that express CD33 and may derive more benefit from this approach.

Introduction

AML is thought to arise as a result of a genetic change in an immature stem cell or a multi-lineage progenitor cell, which results in an uncontrolled expansion of those cells and failure to reach terminal differentiation.[1-3] AML cells are heterogenous in phenotype and functional ability. AML cells capable of initiating the leukemia in the immunocompromised mice and generating clonogenic cells after long-term culture *in vitro* are similar in phenotype and are $CD34^+$, $CD38^-$, HLA-DR^- and $CD71^-$.[2,4-6] This phenotype has some similarities to the phenotype of normal progenitors with these functional characteristics. However, normal progenitors express higher levels of CD117 and CD90.[7-9] Cells with the primitive phenotype represent only a small minority of all AML blasts, but seem uniquely capable of propagating the leukemia, suggesting they are AML stem cells. We have observed a close correlation between the phenotypes of *in vivo* and *in vitro* leukemia maintaining cells.[10] This suggests that long-term growth *in vitro* can be used as an assay for AML stem cells.

Existing therapies to treat AML are largely directed against the bulk population of AML cells. Therefore, even when a complete remission is obtained, enough AML stem cells might be spared to allow reoccurrence of the leukemia. Furthermore, autologous stem cell transplants, a treatment modality used in a substantial number of patients, can be contaminated with AML stem cells.[11] The ability to phenotypically distinguish between AML and normal stem cells could be extremely useful to develop purging therapies aimed specifically against AML stem cells for transplantation and to develop therapies which are directed specifically against AML stem cells to target minimal residual disease.

CD33 is a cell surface marker, which is exclusively expressed on myeloid cells.[12] It is expressed on all myeloid progenitors but is down regulated upon differentiation although it is retained on monocytes. Because CD33 is absent on normal hematopoietic stem cells[13,14], but is expressed on the majority of AML patient samples,[15-18] CD33 appears to be a good candidate for purging strategies and antibody targeted therapy. One of the antibody targeted therapies, gemtuzumab ozogamicin (GO or Mylotarg®) has recently been approved by the United States Food and Drug Administration for the treatment of relapsed $CD33^+$ AML in patients older than 60 years. The long-term effects of GO have not yet been established, but it appears that relapse often occurs.[19,20] This could be due to the absence of CD33 expression on AML stem cells. To address this question we determined whether CD33 is expressed on AML progenitors with long-term proliferative ability *in vitro* and *in vivo* repopulating ability. We demonstrate that, although CD33 is expressed on the majority of AML samples it is absent on primitive AML progenitors with long-term *in vitro* ability and is absent on some but not all repopulating cells of a substantial number of patients.

Materials and Methods

Patient Cells

Peripheral blood (PB) cells from 34 AML patients at diagnosis were obtained after informed consent and with approval of the Clinical Research Ethics Board of the University of British Columbia. Patient characteristics are depicted in Table 1. The mean number of blasts in the samples was 69 ± 22%. Patients with FAB M3 type were excluded because of a different treatment strategy given to M3 patients. Blood cells were Ficoll separated to obtain a mononuclear cell population then frozen in Dulbecco's modified Eagle's medium (DMEM) (StemCell Technologies Inc., Vancouver, B.C.) with 50% fetal calf serum (FCS) (StemCell Technologies Inc.) and 10% dimethylsulphoxide (DMSO) and stored at -135°C.

AML Cell Phenotyping and Sorting

Prior to sorting, thawed AML cells were suspended in HFN (Hanks medium with 2% FCS and 0.1% sodium azide) at 10^7 cells/ml. Cells were stained for 30 minutes on ice with monoclonal antibodies CD34-FITC (Dr. Peter Lansdorp, Terry Fox Laboratory) used at 4 μg/ml and CD33-PE (Becton Dickinson, San Jose, California) used according to manufacturer's instruction. Separate aliquots were stained with an irrelevant mouse IgG_1-FITC antibody (Becton Dickinson) and mouse IgG_1-PE antibody (Becton Dickinson) as an isotype control. Cells were then washed twice in HFN at 4°C, propridium iodide (PI) at 2 μg/ml was added to the cells prior to the second wash, and the cells were maintained on ice prior to sorting. Cells were analyzed and sorted on a dual laser FACStarplus (Becton Dickinson) on the basis of fluorescence intensity after gating out non-viable (PI^+) cells. Gates were set to exclude 99.9% of isotype control stained cells. Fractions were sorted into DMEM with 50% FCS in microcentrifuge tubes at 4°C. Sorted fractions were washed, resuspended at known cell concentrations and used to initiate colony forming assays (CFU) and suspension culture (SC) assays or for injection into NOD/SCID mice.

Primary CFU Assays

Immediately after FACS sorting, a known number of sorted and control unsorted PB cells were plated in methylcellulose culture medium, (Methocult H4330, StemCell Technologies Inc.), containing 20 ng/ml rhIL-3 (Sandoz, Basel, Switzerland), 20 ng/ml rhIL-6 (Terry Fox Laboratory), 20 ng/ml rhG-CSF (Amgen Canada Inc., Mississauga, Ontario), 20 ng/ml rhGM-CSF (Sandoz) and 50 ng/ml rhSteel Factor (SF) (Terry Fox Laboratory). After 10 days of incubation at 37°C in a 5% CO_2 humidified incubator, AML blast clusters (10-20 cells) or colonies (> 20 cells) were counted and the numbers were pooled to obtain CFU counts.

Table 1: *Characteristics of AML samples*

No.	ID	Age	Sex	FAB Subtype	Cytogenetics	Abnormal metaphases	% of Blasts
1	117-91	36	M	M0	-5, -7	22/25	92
2	122-02	72	F	M0	+11	10/16	75
3	HL034	72	M	M1	Normal		95
4	XO384	21	M	M1	add(6)(p23), t(6,11)(q27,q23)	16/16	80
5	119-16	39	M	M2	+8	14/20	?
6	MO225	68	M	M2	+11	10/10	37
7	105-46	74	F	M2	Normal		?
8	BC212	65	F	M4	Normal		75
9	IX270	62	M	M4	+8, +20	3/25	53
10	106-95	70	F	M4	Normal		16
11	FP126	78	M	M4	Complex	15/15	51
12	YS329	58	M	M4	Complex	9/10	24
13	XZ300	34	M	M4	inv16(p13,q22), t(4,11)(q35,q14)	?	?
14	DW383	52	F	M4	inv(16)	?	64
15	KM069	58	F	M4	+13	6/10	46
16	DK367	53	M	M4	Complex	8/11	61
17	ZU239	56	F	M4	Inv(16), +22	15/15	89
18	MN282	48	M	M4e	Inv(16)	16/16	71
19	XU409	41	M	M4e	Inv(16)	13/20	75
20	FH142	48	M	M5a	Normal		53
21	JH201	46	M	M5a	+8	?	54
22	LW364	68	M	M5a	+Y, +4, +20	?	18
23	NV313	33	F	M5a	Complex	12/12	89
24	115-79	52	F	M5a	+8	10/20	94
25	123-46	17	F	M5a	+8, -5	11/25; 6/25	70
26	117-58	59	M	M5a	Normal		90
27	KE325	36	M	M5a	inv16(p13,q22), 47XY, +22	13/13	37
28	TH407	54	M	M5a	Complex	10/11	97
29	QP353	68	M	M5a	Complex	25/25	84
30	VX284	60	F	M5a	Normal		80
31	VD294	72	F	M5b	+8	11/15	65
32	ND319	55	M		-Y	?	90
33	115-45	44	M	M0/M4	Normal		86
34	116-23	68	M	MDS/ RAEB	+8	14/15	32

Suspension Culture Assays (SC)

Suspension cultures were initiated with a known number of sorted and unsorted cells and maintained generally, as previously described,[2,7] in 1.0 ml of serum free medium containing the growth factor cocktail described. Cultures were maintained at 37°C in a 5% CO_2 humidified incubator and 0.5 ml of fresh medium with growth factors was added weekly. Every second week half the cells and volume of the SC were removed, the cells were washed in DMEM with 10% FCS and cultured in methylcellulose to determine the CFU content of the SC. SC were maintained for 8 weeks then the entire contents of the wells were harvested and assessed for CFU content. To allow comparisons between experiments the proportion of progenitors derived from each sorted fraction at each time point was determined by comparison to a progenitor recovery of 100% from all fractions at that time point. Using PB cells of the AML patients no formation of feeder layers in the SC was observed.

Transplantation of Leukemic Cells into NOD/SCID Mice

Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Jackson Laboratory, Bar Harbour, ME) were bred and maintained in the animal facility of the British Columbia Cancer Research Centre (Vancouver, B.C., Canada) under sterile conditions in sterile micro-isolator cages and were provided exclusively with autoclaved food and water containing 100 mg/l ciprofloxacin and HCl. Twenty-four hours prior to transplantation, mice were irradiated with 3.5 Gy γ-irradiation from a ^{137}Cs source at a dose rate of 1.22 cGy/min. Unsorted AML PB cells and sorted subfractions were suspended in 0.3 ml Alpha minimal essential medium (Alpha MEM)(StemCell Technologies Inc.) with 5% FCS and injected intravenously into the lateral tail vein of 6-8 week old NOD/SCID mice. Whenever possible, various cell concentrations were injected as a semi-quantitative assessment of the number of cells required for engraftment. Normal BM cells were given 15 Gy irradiation using 250kVp X-rays (Philips RT-250, HVL 1.5 mm Cu) at a dose rate of 5.1 Gy/min. In each case, 2 x 10^6 irradiated BM cells were co-injected with the unsorted cells and sorted subfractions to enhance engraftment potential. BM aspirations were performed every 4 weeks on anaesthetised mice as described elsewhere.[21] Eight or 12 weeks post-injection the mice were sacrificed by CO_2 inhalation. BM was removed from the femurs by flushing with Alpha MEM with 5% FCS.

Flow Cytometry Analysis of Murine Tissues

Cell suspensions from femoral BM were lysed in ammonium chloride (StemCell Technologies Inc.) for 20 minutes and then washed in HFN with 5% human serum for blocking of human Fc receptors and prepared for flow cytometric analysis as described.[2,7] Briefly, cells were incubated on ice with an anti-mouse IgG Fc receptor monoclonal antibody (2.4G2) (Dr. P. Lansdorp, Terry Fox Laboratory, Vancouver). Cells were then stained with a human pan leukocyte antibody, 9.4 (anti CD45)-FITC (Dr. P. Lansdorp) for 30 minutes on ice. Separate aliquots were stained with an irrelevant IgG_1-FITC antibody (Becton Dickinson) as an isotype control. Aliquots of normal human PB cells and

BM cells from a non-injected NOD/SCID mouse were also stained with CD45-FITC and the isotype control to serve as positive and negative controls for antibody specificity. Samples were analyzed using a FACScan (Becton Dickinson), gate settings which excluded ≥ 99.9% of the cells in the matched isotype control were used to determine the CD45 expression of each sample. Any positive value (i.e. ≤ 0.1%) for the isotype control was then subtracted from the percentage positive in the CD45 stained samples. We defined human engraftment as expression of ≥ 0.1% $CD45^+$ cells in a sample.

Cytogenetic Analysis

Colonies from primary CFU or from CFU which were derived from SC were evaluated for the leukemia specific cytogenetic change by either standard cytogenetics or by fluorescence in situ hybridisation (FISH) whenever possible. Likewise sorted $CD45^+$ cells from BM removed from NOD/SCID mice were evaluated for the leukemic transformation. For analysis, single colonies were plucked and plated onto slides after colony synchronisation with colcemid.[22] Probes specific for centromeric repeat sequences were used to detect cytogenetic abnormalities in 18 patients. These included D8Z2 (ATCC, Rockville, MD) to detect the +8 abnormality, The Y probe[23] pRY3.4, and a yeast artificial chromosome (YAC) clone 909g3 (Research Genetics) to detect the +13 abnormality. Total plasmid DNAs containing the centromeric specific DNA were labelled with digoxigenin (Boehringer-Mannheim, Mannheim, Germany) by nick translation. The +8 probe was hybridised in Hybrisol VI (Oncor). The signal was amplified by rabbit anti-sheep FITC (Jackson Immuno Research Laboratories, West Grove PA) and detection by sheep anti-digoxigenin-FITC (Boehringer Mannheim, Quebec, Canada). Counterstaining was in PI at 200 ng/ml in Vectashield (Vector Laboratories, Inc. Burlingome, CA). Colonies were defined as positive whenever ≥ 70% of cells contained the respective leukemic karyotype of that patient. Colonies containing only 40-70% of cells with the leukemic change were classified as undetermined and those with < 40% leukemic cells were defined as negative. Whenever both metaphase and interphase cells could be scored approximately 100 cells were counted per colony.

Results

Expression of CD34 and CD33 on AML blasts

Peripheral blood mononuclear cells from 30 AML patients used for either our *in vitro* or *in vivo* experiments were evaluated for co-expression of CD34 and CD33. These patients were FAB M0 (n=2), M1 (n=2), M2 (n=2), M4 (n=9), M4e (n=1), M5a (n=10), M5b (n=1). One patient had myelodysplastic syndrome with RAEB and two patients had an undetermined (UD) subtype. Blasts represented 67 ± 23% of total blood cells. Of the 30 samples, 26 expressed CD33 on ≥ 10% of blood mononuclear cells, and 16 patients expressed CD33 on ≥ 50% of blood mononuclear cells. Of the 30 patients, 19 patients had a $CD34^+$ AML ($CD34^+$ AML defined as CD34 expression in ≥ 10% of blood mononuclear cells), while 11 patients had a $CD34^-$ AML. In the 19 $CD34^+$ expressing samples, the

mean proportion of $CD34^+CD33^+$ nucleated cells was 18 ± 17%, while 43 ± 26% were $CD34^+CD33^-$, 19 ± 21% were $CD34^-CD33^+$ and 19 ± 19% were $CD34^-/CD33^-$ (Figure 1). In the 11 $CD34^-$ samples the mean proportion of $CD34^+CD33^+$ nucleated cells was 1.3 ± 2.6%, while 0.4 ± 0.6% were $CD34^+CD33^-$, 59 ± 23% were $CD34^-CD33^+$ and 39 ± 24% were $CD34^-CD33^-$ (Figure 1). CD33 expression was higher in the $CD34^-$ samples (63 ± 24%) compared to the $CD34^+$ samples (35 ± 27%).

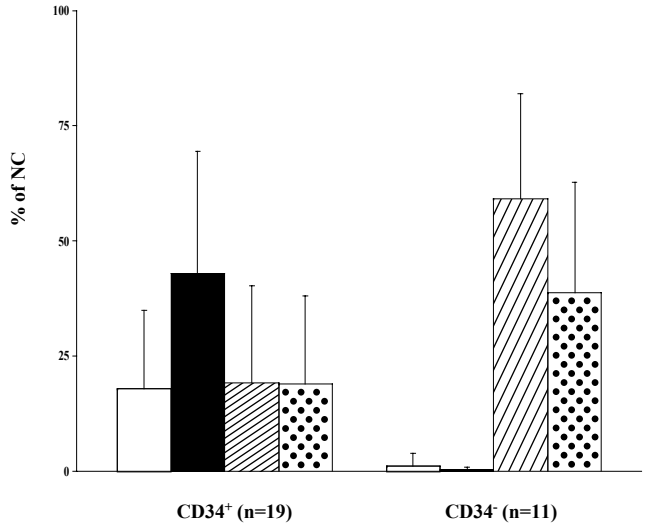

Figure 1: Expression of CD34 and CD33 on AML cells from 30 patients at diagnosis.
PB cells from 30 AML patients at diagnosis were stained with CD34-FITC and CD33-PE and analyzed by FACS. The mean % of cells in the $CD34^+CD33^+$ subfraction (open bars), $CD34^+CD33^-$ subfraction (filled bars), $CD34^-CD33^+$ subfraction (hatched bars) and the $CD34^-CD33^-$ subfraction (dotted bars) of 19 $CD34^+$ (CD34 expression ≥ 10%) and 11 $CD34^-$ (CD34 expression ≤ 10%) samples was calculated.
PB = peripheral blood , NC= nucleated cells

Expression of CD34 and CD33 on AML cells with long term proliferative ability in vitro

In order to determine the phenotype of AML cells capable of producing primary CFU and CFU after long-term culture, PB cells from a subset of 26 patients were sorted in four fractions according to their expression of CD34 and CD33 and subsequently used to establish primary CFU assays and suspension culture (SC) assays. Fifteen patients had high expression of CD34 (66 ± 25% $CD34^+$), while the majority of AML blasts in the other 11 patients were $CD34^-$ (1.7 ± 2.7% $CD34^+$). The $CD34^+CD33^+$ subfraction represented 12 ± 17% of nucleated cells in the 26 patients at sorting, 27 ± 30% were $CD34^+CD33^-$, 33 ± 30% were $CD34^-CD33^+$ and 28 ± 24% were $CD34^-CD33^-$ (Figure 2). Cells capable of forming primary CFU were present in all four subfractions. After 2 weeks in suspension

culture (SC) most CFU were found in the $CD34^+CD33^-$ subfraction (61 ± 39%). Similarly after 4, 6 and 8 weeks in SC the % CFU in the $CD34^+CD33^-$ subfraction was 76 ± 33%, 63 ± 48% and 73 ± 44% respectively (Figure 2). After 6-8 weeks in SC, >90% of CFU were observed in the $CD34^+CD33^-$ subfraction in 11 of 17 patients. While in 2 of 17 patients, >90% of the CFU at week 6-8 were observed in the $CD34^+CD33^+$ subfraction, in 3 of 17 patient samples >90% of CFU were observed in the $CD34^-CD33^+$ and $CD34^-CD33^-$ subfractions and in one patient CFU were distributed in all subfractions. These data suggest that in the majority of patient samples the SC-IC is found in the $CD34^+CD33^-$ subfraction.

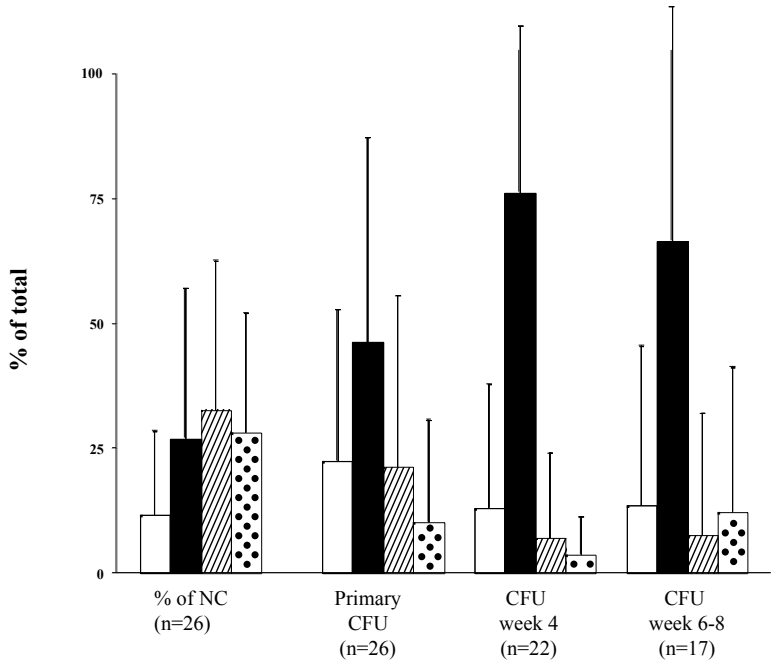

Figure 2: Proportion of AML PB cells and progenitors from 26 AML patients in subfractions sorted for the expression of CD34 and CD33. Percentage of blood cells, primary CFU and CFU after 2-8 weeks in SC in the $CD34^+CD33^+$ subfraction (open bars), $CD34^+CD33^-$ subfraction (filled bars), $CD34^-CD33^+$ subfraction (hatched bars) and the $CD34^-CD33^-$ subfraction (dotted bars). To allow comparison of the proportion of progenitors in each subfraction, CFU frequencies were determined by comparison to a progenitor recovery of 100% (UF) of all four subfractions at each time point.
PB = peripheral blood, SC = suspension culture, NC= nucleated cells, UF=unsorted

Cytogenetic analysis of in vitro studies

It was possible to analyse CFU and CFU derived from SC by FISH or standard cytogenetic analysis in some fractions of 18 patients (Table 2). In Figure 3, an example of abnormal cells in a colony derived from the $CD34^+CD33^-$ subfraction after 4 weeks in SC of a patient with trisomy 8 is depicted. The majority of colonies from primary CFU were abnormal (abnormal defined as at least one abnormal colony of all colonies analyzed) with 11 of 14 patient samples showing an abnormal karyotype in the colonies of the unsorted fraction, 9 of 11 patient samples were abnormal in the $CD34^+CD33^+$ subfraction, 8 of 13 samples in the $CD34^+CD33^-$ subfraction, 6 of 7 in the $CD34^-CD33^+$ subfraction and 6 of 6 in the $CD34^-CD33^-$ subfraction. CFU derived from SC at weeks 2-8 were abnormal in 6 of 10 samples using unsorted cells, 4 of 5 samples using cells from the $CD34^+CD33^+$ subfraction, 5 of 6 samples in the $CD34^+CD33^-$ subfraction, 3 of 3 samples in the $CD34^-CD33^+$ subfraction and 4 of 4 samples in the $CD34^-CD33^-$ subfraction. Of the 11 patients with >90% of CFU in the $CD34^+CD33^-$ subfraction, 5 of 6 samples analyzed were leukemic. In one patient, abnormal colonies could be obtained at any time point using unsorted (12/20), $CD34^+CD33^+$ cells (11/20) or $CD34^-CD33^+$ cells (9/20), while only normal colonies (6/6) were observed at all time points using $CD34^+CD33^-$ cells. No colonies could be obtained with $CD34^-CD33^-$ cells of this patient. Overall these data suggest that the majority of colonies of the patients were leukemic.

Table 2: *Total number of Colonies detected with the leukemic change by FISH or Cytogenetics of 18 patients following in vitro Assays*

Fraction	Positive Colonies From CFU blast	Positive Colonies after 2-4 weeks of SC	Positive after 6-8 weeks of SC
Unsorted	62/105 (59%)	33/87 (38%)	30/52 (58%)
$CD34^+CD33^+$	46/73 (63%)	20/41 (49%)	23/33 (70%)
$CD34^+CD33^-$	50/91 (55%)	15/62 (24%)	27/42 (64%)
$CD34^-CD33^+$	35/51 (69%)	18/21 (86%)	14/20 (70%)
$CD34^-CD33^-$	33/42 (79%)	24/32 (75%)	12/19 (63%)

Abbreviations: SC, suspension culture.
Positive colonies were defined as ≥ 60% cells with the leukemic karyotype in one colony

Table 3: *AML cells sorted for co-expression of CD34 and CD33 with multiple subfractions capable of engrafting NOD/SCID mice.*

Patient	Subfraction	% of NC	Number of cells injected x 105	Engraftment (% $CD45^+$)
069	Unsorted		50	1.0
	$CD34^+CD33^+$	10	8; 8	0; 0
	$CD34^+CD33^-$	7	12	0.1
	$CD34^-CD33^+$	42	10; 10	0.1; 0
	$CD34^-CD33^-$	40	1.3	0
284	Unsorted		100; 10	76; 20
	$CD34^+CD33^+$	15	12	54
	$CD34^+CD33^-$	3	3	0.1
	$CD34^-CD33^+$	61	10;10	0.5; 33
	$CD34^-CD33^-$	21	12	0
212	Unsorted		100; 10	76; 1.7
	$CD34^+CD33^+$	9	5	0.5
	$CD34^+CD33^-$	0.4	1	0.3
	$CD34^-CD33^+$	82	15; 15	0.2; 0
	$CD34^-CD33^-$	8	3.2	0
383	Unsorted		100; 10	3; 0
	$CD34^+CD33^+$	2	9.4; 2.6	0
	$CD34^+CD33^-$	63	2.6	0.1; 0
	$CD34^-CD33^+$	21	0.2	0
	$CD34^-CD33^-$	14	10; 2.6	0
409	Unsorted		100; 10	0.4; 0.1
	$CD34^+CD33^+$	15	10	0.1
	$CD34^+CD33^-$	70	10	0.1
	$CD34^-CD33^+$	8	15	0
	$CD34^-CD33^-$	6	7.7	0
239	Unsorted		100; 10	0; 0.1
	$CD34^+CD33^+$	20	15	0;
	$CD34^+CD33^-$	13	15	0
	$CD34^-CD33^+$	53	10; 10	0.1; 0
	$CD34^-CD33^-$	19	10	0.1

Table 3 (Continued): *AML cells sorted for co-expression of CD34 and CD33 with multiple subfractions capable of engrafting NOD/SCID mice.*

Patient	Subfraction	% of NC	Number of cells injected x 10^5	Engraftment (% $CD45^+$)
115-79	Unsorted		100; 10	0.2; 0
	$CD34^+CD33^+$	1	0.8	0
	$CD34^+CD33^-$	0.05	0.3	5
	$CD34^-CD33^+$	77	20	0
	$CD34^-CD33^-$	21	20	0
115-45	Unsorted		100; 10	0.2; 0.2
	$CD34^+CD33^+$	7	11	0.3
	$CD34^+CD33^-$	32	20	0.5
	$CD34^-CD33^+$	47	10; 10	0.4; 0.1
	$CD34^-CD33^-$	14	7	0.6
123-46	Unsorted		100, 10	0, 0
	$CD34^+CD33^+$	0.6	3.1	0
	$CD34^+CD33^-$	2	1.7	0.7
	$CD34^-CD33^+$	76	ND	
	$CD34^-CD33^-$	21	ND	

ND = not done

In vivo NOD/SCID assay

AML cells from 13 patients were sorted for co-expression of CD34 and CD33 and evaluated for their ability to repopulate sublethally irradiated NOD/SCID mice (Table 3). Human cells were detected in the BM of at least one of the mice injected with unsorted cells from all of these patients, although the number of human cells in the BM varied considerably (range 0.1-76% $CD45^+$ cells). With AML cells of 9 patients engraftment was also achieved using cells sorted for CD34 and CD33, however the levels of engraftment were very low in the majority of patients. With 3 of 9 patient samples, engraftment (range 0.1%-5% $CD45^+$ cells) could only be obtained using cells from the $CD34^+CD33^-$ subfraction, while no engraftment was seen using an equal or higher number of $CD34^+CD33^+$ cells or $CD34^-$ cells. With the other 6 AML samples engraftment was achieved with at least two subfractions and all of those samples engrafted using $CD33^-$ cells (Table 3). In summary, from these 9 patient samples, 13 of 18 mice injected with unsorted cells engrafted, 4 of 13 mice injected with $CD34^+CD33^+$ cells, 8 of 11 mice injected with the $CD34^+CD33^-$ cells, 7 of 13 mice injected with $CD34^-CD33^+$ cells and 2 of 8 mice injected with $CD34^-CD33^-$ cells also engrafted.

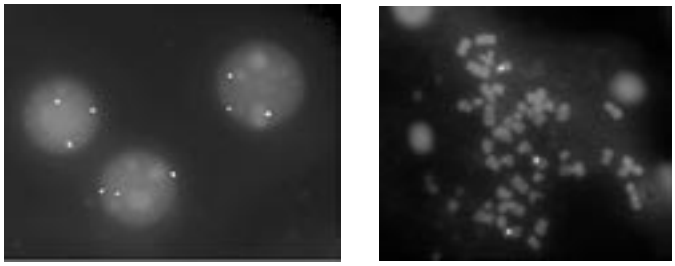

Figure 3: Abnormal cells in the CD34$^+$CD33$^-$ fraction of one AML patient with trisomy 8 after 4 weeks in SC. Colonies from CFU which were derived from 4 weeks SC were evaluated for the leukemia specific cytogenetic change by FISH. Single colonies were plucked and plated onto slides after colony synchronisation with colcemid. The D8Z2 probe specific for the +8 abnormality was labelled with digoxigenin by nick translation. The +8 probe was hybridised in Hybrisol VI. The signal was amplified by rabbit anti-sheep FITC and detection by sheep anti-digoxigenin-FITC. Counterstaining was in PI at 200 ng/ml in Vectashield. Colonies were defined as positive whenever ≥ 70% of cells contained the respective leukemic karyotype of that patient. Colonies containing only 40-70% of cells with the leukemic change were classed as undetermined and those with < 40% leukemic cells were defined as negative. Whenever both metaphase and interphase cells could be scored approximately 100 cells were counted per colony.
CFU=colony forming unit, SC=suspension culture, FISH=fluorescent *in vitro* hybridization, PI=propidium iodide

Discussion

Primitive AML progenitors exist within the AML blast population which are uniquely capable of extensive proliferation and are likely responsible for maintaining the disease.[24] CD33 is a cell surface protein that is expressed on both normal and leukemic myeloid cells.[15-18] Because CD33 is absent on normal hematopoietic stem cells,[13,14] CD33 seems to be a good target for antibody-targeted therapies or purging strategies to treat AML. The aim of this study was to investigate whether CD33 is expressed on primitive AML cells with long term proliferation *in vitro* and NOD/SCID repopulating capacity.

Comparable with previous studies, we found CD33 expression on more than 10% of cells in 88% of the samples.[15-18] Interestingly, CD33 expression was higher on the CD34$^-$ samples compared to the CD34$^+$ samples. We can speculate that the CD34$^+$ AML samples are less differentiated and therefore express lower amounts of CD33. We found leukemic clonogenic progenitors as measured in the primary CFU assay in all four sorted subfractions. This is in contrast to previous reports, that showed that CD33 was expressed on the majority of clonogenic progenitors.[25-27] Of more interest was the expression of CD33 on long-term *in vitro* and *in vivo* repopulating cells, because those cells are thought to be responsible for maintenance of the leukemia *in vivo*. We found that suspension-culture

initiating cells (SC-IC) with proliferative ability in SC for up to 8 weeks from most patients lack the expression of CD33 and thus would not be targetted by CD33 antibody based therapies. Cytogenetic analysis confirmed the leukemic nature of the SC-IC in the majority of cases. However, one of the six patient samples that proliferated up to 8 weeks in culture had normal $CD34^+CD33^-$ SC-IC, while SC-IC in the other fractions had an abnormal karyotype, suggesting that in this case the $CD34^+CD33^-$ subfraction may contain normal cells therefore a subset of patients could be identified that may have a more durable remission with CD33 targeting approaches. Guan et al. found that using the Human Androgen Receptor Assay, cytogenetically normal colonies were polyclonal in 80% of the analyzed cases, suggesting that indeed these cells may be normal.[28] *In vivo* experiments also demonstrated that in a proportion of patients, the majority of primitive AML progenitors express CD34 and lack the expression of CD33, like normal hematopoietic progenitors. However, as we have observed in a previous study,[29] engraftment with $CD34^-$ cells was significant. A more primitive normal $CD34^-$ cell capable of long-term engraftment in immunodeficient mice exists.[30,31] Although a leukemic $CD34^-$ cell capable of engraftment has also been identified,[29,32] it is not clear whether this cell is more primitive or more differentiated than the $CD34^+$ AML progenitors. In general, we have reported a close correlation between the long-term SC-IC and the SL-IC.[10]

The high expression of CD33 on the majority of AML blast cells but not on normal stem cells makes it an obvious candidate for CD33 targeted therapies and CD33 based purging strategies in this disease. However, our study indicates that the cell responsible for maintaining the disease lacks the expression of CD33 in many but not all patients and will therefore be spared by those therapies. Studies using gemtuzumab ozogamicin, a CD33 targeted toxin, indeed showed reduction of the bulk AML blasts and the survival of patients appeared longer, but relapse was frequent even over the short follow-up period.[19,20] In this study we found a subset of patients who would be particularly good candidates for CD33 based therapies as their AML stem cells expressed this antigen. Selecting patients on the basis of CD33 expression on primitive progenitors for therapy could reduce unnecessary toxicity for the patient and is cost effective. The search for cell surface markers specifically expressed on leukemic primitive progenitors should be continued in order to develop disease-specific treatments.

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Chapter 4

Telomerase is Limiting the Growth of Acute Myeloid Leukemia Cells

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The first two authors contributed equally to this work.

Leukemia in press

Abstract

Telomeres play an important role in the proliferation and senescence of normal and malignant cells. To test the role of telomerase in acute myeloid leukemia (AML), we expressed the telomerase reverse transcriptase (hTERT) gene, a dominant-negative DN-hTERT (D868A, D869A) gene, or a gene encoding green fluorescence protein (GFP) in the leukemia cell line K562 and in primary AML cells from different patients using retroviral vectors. Cells transduced with hTERT exhibited elevated levels of telomerase activity compared to GFP-controls, whereas cells expressing DN-hTERT had decreased telomerase activity. K562 populations transduced with DN-hTERT showed reduced clonogenicity, telomere dysfunction and increased numbers of apoptotic cells compared to GFP or hTERT-transduced cells. Two of four clones transduced with DN-hTERT died after 30 and 53 population doublings, respectively. Transduced AML cells were tested in primary colony forming unit (CFU) and suspension culture assays. Relative to hTERT and GFP-transduced controls, AML cells transfected with DN-hTERT produced fewer CFU and showed lower engraftment after transplantation into sublethally irradiated ß$_2$-microglobulin$^{-/-}$ NOD/SCID mice. We conclude that telomerase is limiting the growth of the leukemic cell line K562 and primary AML progenitor cells. Our data warrant further studies of the therapeutic use of telomerase inhibitors in AML.

Introduction

Telomeres in human cells consist of 2-15 kb of non-coding double-stranded T_2AG_3 repeats and 50- to 150-nucleotide overhangs of single-stranded repeats[1] and associated proteins.[2] Telomeres protect the ends of chromosomes from fusion, exonucleolytic degradation, aberrant chromosomal recombination and functional telomeres are required for proper segregation of chromosomes during mitosis.[3-6]

The human telomerase complex, a ribonucleoprotein polymerase, is able to synthesize terminal $(T_2AG_3)_n$ telomeric repeats de novo and can compensate for the loss of telomeric DNA.[7] It consists of a catalytic subunit with reverse transcriptase activity encoded by the hTERT gene, a RNA template encoded by the hTERC gene and associated proteins.[8,9] In general, normal human somatic cells have only low or undetectable telomerase activity with hematopoietic progenitor cells and activated T and B lymphocytes being notable exceptions. Telomerase is required to compensate for the loss of telomeres resulting from the end replication problem, exonuclease processing of 5' ends and lesions induced by radiation or oxidative stress.[10-14] Cells from many tissues show progressive shortening *in vivo* and *in vitro* which could result in their replicative senescence and selection of abnormal cells.[15-18]

Numerous types of cancer, including acute myeloid leukemia (AML), use telomerase activity to maintain telomeres and prevent replicative senescence or apoptosis. Variable levels of telomerase have been detected in up to 85% of all AML's.[19-21] AML is a highly aggressive disease in which an uncontrolled proliferation and maturation of myeloid progenitors is observed.[22] AML is thought to arise from genetic changes in a primitive hematopoietic progenitor or stem cell.[23] As in normal hematopoiesis, heterogeneity in the proliferative and self-renewal abilities of AML cells exists, suggesting that AML cell populations within individual patients are organized in a hierarchy.[24-26] AML progenitors and stem cells can be detected using *in vitro* and *in vivo* assays.[27] While a considerable number of AML blasts is capable of forming colonies in colony forming assays,[25,28,29] only a minority of AML cells have the ability to initiate and sustain long-term proliferation *in vitro* or to engraft non obese diabetic severe combined immune deficient (NOD/SCID) mice.[25,29] Cells with long-term *in vitro* proliferative capacity and cells capable of engrafting immune deficient mice are thought to be uniquely capable of maintaining the disease. These assays are therefore of great interest to determine the efficacy of treatment regimens aimed to eradicate AML stem and progenitor cells.

Telomerase is expressed in most AML cells and telomerase inhibition could potentially lead to eradication of leukemic stem cells. Several point mutants in the reverse transcriptase motifs of the catalytic subunit of hTERT have been identified and shown to markedly reduce telomerase activity upon overexpression in telomerase positive cells.[30,31] Most likely, dominant-negative constructs disrupt telomerase activity by binding to limiting levels of the essential telomerase RNA template resulting in inactive telomerase complexes. In order to study the role of telomerase in AML, we have examined the effect of overexpression and

disruption of telomerase activity in the human leukemia cell line K562 and in primary AML progenitors. Our results show that telomerase is a promising target to inhibit the proliferation of AML cells.

Materials and Methods

Patient cells

Peripheral blood (PB) cells from 12 AML patients at diagnosis were obtained with informed consent and approval of the Clinical Research Ethics Board of the University of British Columbia. Patients with FAB M3 type were excluded because of the unique characteristics of this AML subtype. Blood cells were subjected to density separation using Ficoll Hypaque (Amersham Biosciences, Uppsala, Sweden). Mononuclear cells were frozen in Dulbecco's modified Eagle's medium (DMEM) (StemCell Technologies Inc., Vancouver, Canada) with 50% fetal calf serum (FCS) (StemCell Technologies Inc.) and 10% dimethylsulphoxide (DMSO) (Sigma-Aldrich, Oakville, Ontario) and stored at -135°C. The patient characteristics of the samples used in this study are shown in Table 1.

Table 1: *Characteristics of AML samples used in this study*

No.	ID	Age	Sex	Source Subtype	FAB	Cytogenetics	% of Blasts	TRAP
1	109-14	22	M	PB	M1	Normal	90	++
2	110-36	37	F	PB	M1	Normal	78	+
3	NV253	78	F	PB	M1	n.a.	93	+
4	111-41	25	M	PB	M2	+4; t(8,21)	94	+/-
5	IT369	52	M	PB	M2/MDS	Normal	17	+/-
6	105-46	74	F	PB	M2	Normal	30	++
7	GHN323	50	F	PB	M4 eo	inv 16; +22	55	+/-
8	DK367	53	M	PB	M4	-4; -12; -17; del5(q12,q23) etc.	61	+/-
9	108-24	58	M	PB	M4	Normal	96	++
10	114-04	60	F	PB	M4	Normal	26	+
11	BC212	65	F	PB	M4	Normal	75	+/-
12	VW372	65	F	PB	M4	Normal	89	+/-

Retrovirus production

Gene transfer was achieved using retrovirus-mediated gene transfection. We constructed murine stem cell virus (MSCV)-based retroviral vectors[32] containing the gene for enhanced green fluorescent protein (GFP; Clontech, Palo Alto, CA) under the control of the phosphoglycerate kinase (PGK) promoter with or without the full-length hTERT cDNA (kindly provided by Dr. Robert Weinberg, Massachusetts Institute of Technology, Boston, MA) or DN-hTERT (D868A, D869A) cDNA (kindly provided by Dr. Lea Harrington, University of Toronto, Toronto, Canada). Helper-free retrovirus pseudotyped with the Gibbon Ape leukemia virus envelope for efficient infection of human cells was generated using PG13 packaging cells.[33]

Retrovirus-mediated transfection of K562

K562 cells were cultured in DMEM with 10% FCS and 2 mM L-glutamine (StemCell Technologies Inc.). Cells were harvested, washed once in phosphate-buffered saline (PBS) (StemCell Technologies Inc.) and resuspended at a density of $1x10^6$ cells/ml in retrovirus-containing supernatant along with 4 µg/ml hexadimethrine bromide (Polybrene) (Sigma-Aldrich). K562 cells were cultured overnight, washed once with PBS and further expanded for 5 to 6 days prior to subsequent experiments.

Retrovirus-mediated transfection of AML cells

Primary AML cells were thawed and cultured in Iscove's Modified Dulbecco's Medium (IMDM) (StemCell Technologies Inc.) containing 20% FCS, 50 ng/ml rhSteel Factor (SF) (Terry Fox Laboratory, Vancouver, Canada) and 10 ng/ml rhIL-3 (Novartis, Basel, Switzerland). At day 2 or 3, cells were harvested and counted. $1\text{-}5x10^6$ cells were then plated in this medium onto non-tissue culture 6-well plates (Falcon, Becton Dickinson, Bedford MA) coated with 6-10 µg/cm^2 RetroNectin™ (Takara Shuzo Ltd., Otsu, Japan) preloaded with 4 ml of retrovirus-containing supernatant. Plates were coated either at 4°C overnight or at 37°C for 2 hours (h), subsequently blocked with 2% bovine serum albumin (BSA, Calbiochem-Novabiochem, San Diego, CA) for 30 min at 37°C, and then washed once with PBS prior to use. After 4 h, cells were harvested from the wells by vigorous pipetting and washed once with PBS. Cells were resuspended in IMDM containing 20% FCS, 50 ng/ml SF and 10 ng/ml rhIL-3 and kept in 6-well plates for tissue culture (Falcon) overnight. The transfection procedure was repeated on two consecutive days. After transfection, cells were further expanded for 5 to 6 days and then sorted for GFP expression. The efficiency of transfection was estimated from the percentage of cells expressing GFP at around 0.8% to 34.3%.

Flow cytometry

Viable GFP$^+$ K562 cells or GFP$^+$ primary AML cells (PI$^-$) were isolated at a purity of >99% using a FACStarplus (Becton Dickinson, San Jose, CA) as described previously.[34] Cells were sorted and known cell numbers were used to initiate proliferation assays, colony

forming assays (CFU), suspension culture (SC) assays and transplantation assays. Both single cell clones and bulk populations were obtained from the K562 cells and used in proliferation assays.

Proliferation assays

A known number of sorted K562 clones and bulk cells were plated in media. Once a week, cell counts were performed using trypan blue. The number of population doublings (PD) was calculated from the average cell count, using the following equation: PD = ^{10}log (number of cells counted after expansion) - ^{10}log (number of cells seeded)/^{10}log 2.

Primary CFU assays

Immediately after FACS sorting, a known number of sorted primary AML cells were plated in methylcellulose culture medium (Methocult H4330, StemCell Technologies Inc.), containing 20 ng/ml rhIL-3 (Novartis), 20 ng/ml rhIL-6 (Terry Fox Laboratory), 20 ng/ml rhG-CSF (Amgen Canada Inc., Mississauga, Ontario), 20 ng/ml rhGM-CSF (Novartis) and 50 ng/ml rhSteel Factor (SF) (Terry Fox Laboratory). After 10 days of incubation at 37°C in a 5% CO_2 humidified incubator, AML blast clusters (10-20 cells) or colonies (> 20 cells) were counted and the numbers were pooled to determine the primary CFU frequency per 10^5 cells calculated as follows: the number of colonies/ the number of cells plated x10^5.

Suspension culture assays

Suspension cultures (SC) were initiated with a known number of transduced AML PB cells sorted for their GFP expression and maintained generally, as previously described,[29,35] in 1.0 ml of serum free medium containing 20 ng/ml rhIL-3 (Sandoz, Basel, Switzerland), 20 ng/ml rhIL-6 (Terry Fox Laboratory), 20 ng/ml rhG-CSF (Amgen Canada Inc., Mississauga, Ontario), 20 ng/ml rhGM-CSF (Sandoz) and 50 ng/ml rhSteel Factor (SF) (Terry Fox Laboratory). Cultures were maintained at 37°C in a 5% CO_2 humidified incubator and 0.5 ml of fresh medium with growth factors was added weekly. Every second week, half the cells and volume of the SC were removed, the cells were washed in DMEM with 10% FCS and cultured in methylcellulose to determine the CFU content of the SC. SC were maintained for 8 weeks then the entire contents of the wells were harvested and assessed for CFU content. To allow comparisons between experiments, the frequency of progenitors per 10^5 cells at each time point was calculated from the number of colonies obtained by plating a given number of cells. No feeder layers were formed in SC initiated with PB cells of the AML patients.

Transplantation of transduced AML cells into in ß2-m$^{-/-}$ NOD/SCID mice

$ß_2$-microglobulin deficient nonobese diabetic/severe combined immunodeficient ($ß_2$-m$^{-/-}$ NOD/SCID) mice (Jackson Laboratory, Bar Harbor, ME) were bred and maintained in the animal facility of the British Columbia Cancer Research Centre (Vancouver, Canada)

under sterile conditions in sterile micro-isolator cages. The animals were provided exclusively with autoclaved food and water containing 100 mg/l ciprofloxacin and HCl. Twenty-four hours prior to transplantation, mice were irradiated with 3.5 Gy γ-irradiation from a ^{137}Cs source at a dose rate of 1.22 cGy/min. A known number of sorted AML PB cells transduced with the MSCV vector alone or with the MSCV vector containing hTERT or DN-hTERT were suspended in 0.3 ml Alpha minimal essential medium (Alpha MEM)(StemCell Technologies Inc.) with 5% FCS and injected intravenously into the lateral tail vein of 6-8 week old $ß_2$-$m^{-/-}$ NOD/SCID. BM aspirations were performed every 4 weeks on anaesthetized mice as described elsewhere.[36] Eight or 12 weeks post-injection the mice were sacrificed by CO_2 inhalation. BM was removed from the femurs by flushing with Alpha MEM with 5% FCS.

Flow Cytometry analysis of murine tissues

Cell suspensions from femoral BM were lysed in ammonium chloride (StemCell Technologies Inc.) for 20 min and then washed in Hanks medium (StemCell Technologies Inc.) with 5% human serum for blocking of human Fc receptors and prepared for flow cytometric analysis as described.[29,35] Briefly, cells were then stained with a Cy5-labeled anti-human CD45 antibody for 30 min on ice. Unlabeled cells were used as control. Samples were analyzed using a FACScan (Becton Dickinson), to determine the percentage of cells expressing CD45 using gate settings that excluded ≥ 99.9% of the events in the unlabeled control cells. Human engraftment was defined as expression of ≥ 0.1% $CD45^+$ cells in a sample.

Telomerase repeat amplification protocol assay

Telomerase activity was measured by telomeric repeat amplification protocol (TRAP) assay using an end-labeled telomerase substrate (TS) primer as described.[37] Cell extracts were obtained from a positive control cell line (K562). Extension of the TS primer by telomerase was performed for 30 min. at room temperature, and the products generated were amplified by 30 cycles of polymerase chain reaction (PCR) at 95°C for 60 seconds, 50°C for 45 seconds and 72°C for 60 seconds using the ACX anchored return primer. Half of the amplified products were resolved on a 12% polyacrylamide gel and visualized by a phosphoimaging system (Storm 820, Molecular Dynamics Inc., Sunnyvale, CA). The results of the semi-quantitative TRAP assay were used to rank telomerase activity in primary AML cells relative to the activity measured in K562 cells. High telomerase levels (++) were defined as levels comparable to the telomerase level in K562 cells. Intermediate levels (+) are defined as readily detectable levels but lower compared to K562. The definition of low levels ($^+$/-) of telomerase activity is telomerase activity that is just detectable using this assay.

Telomere length analysis

The telomere length of cells before and after transfection and selection was measured using in situ hybridization and flow cytometry (flow FISH) as described.[38]

Cytospin preparation

Cells were counted and 2-4x10^4 cells were resuspended in 100 µl medium containing 10% BSA (Calbiochem-Novabiochem) and spun at 1250 rpm for 1 min. with high acceleration in a cytocentrifuge (Cytospin-2, Thermo Shandon). The slides were air dried overnight, stained with Wrights Giemsa stain (Hematek 2000, Bayer Diagnostics, Tarrytown, NY) and analyzed.

Calculations and statistical analysis

The Friedman Ranks Test was used to study the correlation between the telomere length of K562 cells transduced with GFP, hTERT and DN-hTERT (n=8). For a correlation between GFP and DN-hTERT-transduced primary AML cells the non-parametric Wilcoxon Signed Ranks Test was used (n=7). For both tests a significant level of 0.05 was chosen.

Results

Disruption of telomerase activity in K562 leads to telomere dysfunction and cell death

To study the role of telomerase in leukemia, we first transduced the leukemia cell line K562 with retroviral vectors encoding hTERT, DN-hTERT (D868A, D869A) and GFP. Transduced cells were sorted on the basis of GFP expression. Cells transduced with DN-hTERT showed a reduced clonogenicity (frequency of proliferating clones from transduced cells immediately after sorting) compared to the GFP-control or hTERT-transduced cells (DN-hTERT 4.2 ± 0.9%; GFP 2.6 ± 28.9%; hTERT 1.9 ± 27.3%; n=2). When DN-hTERT cells were sorted for high and low GFP expression, we observed that clones expressing high levels of GFP showed a reduced proliferation rate. No such effect was seen for clones with low GFP expression. These results suggest that GFP expression in cells transduced with the DN-hTERT gene corresponds to the level of transgene expression and suppression of telomerase activity. Two of four DN-hTERT-transduced clones (clone 2 and 3) died after, respectively, 90 days (53 PDs) and 60 days (30 PDs) in culture. Another clone showed reduced proliferation while still expressing a low level of GFP and another clone lost GFP expression altogether and continued to proliferate like the GFP-control. Overexpression of hTERT in four K562 clones did not alter the proliferation compared to controls (Figure 1).

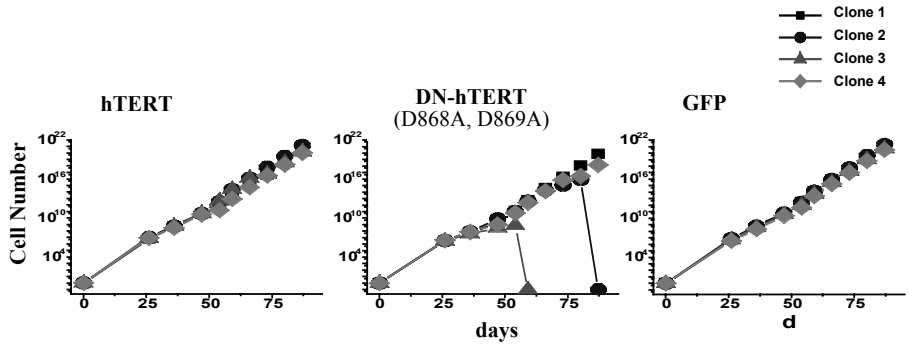


Figure 1: Proliferation of K562 cells transduced with hTERT, DN-hTERT (D868A, D869A) and a GFP-control vector. After transfection cells were sorted and cloned. For each vector four clones were expanded up to 4 months and every week cell counts were performed. Two of the four clones (triangles and circles) transduced with the DN-hTERT died after 60 days (30 PDs) and 90 days (53 PDs). Clone 4 (diamonds) exhibited a reduced proliferation while expressing a low level of GFP. In clone 1 (squares) GFP expression could not be detected after several weeks in culture, suggesting that either the transgene was lost or silenced.

Morphological analysis of the DN-hTERT clones at day 14-20 after cloning revealed multiple anaphase bridges indicative of telomere dysfunction (Figure 2).[31,39] Furthermore, many cells showed morphological features typical of apoptosis such as nuclear chromatin condensation, cell shrinkage and cell fragmentation into apoptotic bodies. GFP-control and hTERT-transduced cells exhibited similar morphology as untransduced K562 cells.

To determine the telomerase activity in the transduced clones after 14 days in culture, we performed telomerase (TRAP) assays. Telomerase activity in DN-hTERT clones that later died in culture was severely reduced. Both the hTERT and the GFP-control clones contained high levels of telomerase activity. Telomere length analysis by flow-FISH revealed shorter telomere length in DN-hTERT clones compared to the GFP-control (Figure 3). Interestingly, although telomerase activity appeared high in both the GFP-control and the hTERT clones, telomere length in the hTERT clones was significantly longer compared to the GFP-control clones (p=0.002).

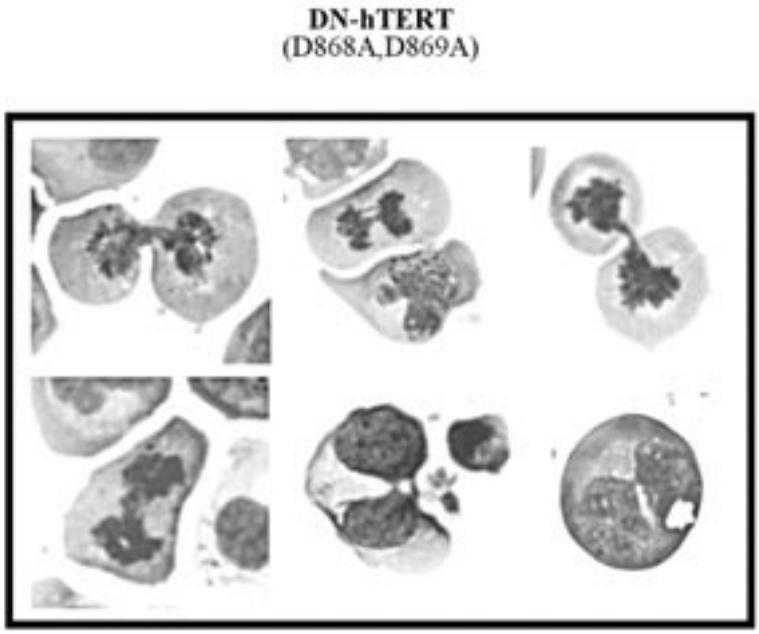


Figure 2: Microscopic appearance of clones 2 and 3 of K562 cells transduced with DN-hTERT (D868A, D869A) after 20 days of expansion. Cytospins of different clones were prepared and stained with Wrights Giemsa stain. As shown DN-hTERT (D868A, D869A)-transduced cells displayed anaphase bridges indicative of telomere dysfunction and apoptotic morphology. No changes relative to untransduced K562 cells were seen for GFP-control or hTERT-transduced cells.

Transduction of primary AML cells with hTERT, DN-hTERT and GFP

To determine the role of telomerase in primary AML cells, we determined telomerase activity in samples from 12 patients with various AML subtypes and cytogenetic abnormalities (Table 1). In all 12 samples, telomerase activity could be readily detected although at variable levels (Figure 4). The primary AML cells from all patients were transduced with DN-hTERT and GFP-control and in 8 cases also with the hTERT vector using a modification of our transfection protocol for human T-lymphocytes.[40] Using this strategy, gene transfer was obtained in all cases be it at variable efficiency ranging from 0.8 to 34.3%. Examples of results are shown in Figure 5a. No major differences in transduction efficiency between the three vectors were observed (10.7 ± 9.0% for the DN-hTERT, 13.1 ± 10.9% for the hTERT and 8.4 ± 6.9% the GFP-control). After 5 to 6 days in culture, the transduced AML cells were sorted for GFP expression and a TRAP assay was performed to determine telomerase activity and the efficiency of gene transfer. High telomerase activity was detected in hTERT-transduced cells compared to GFP-controls. In contrast, telomerase activity in DN-hTERT cells appeared to be reduced compared to the GFP-controls (Figure 5b).

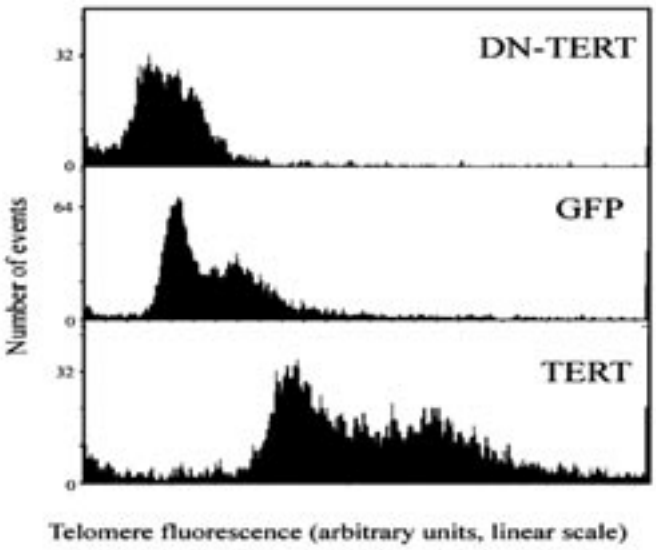


Figure 3: Loss of telomere repeats in K562 cells transduced with DN-hTERT. K562 cells transduced with hTERT, DN-hTERT (D868A, D869A) and a GFP-control vector were sorted and cloned. The telomere fluorescence histograms of representative clones following flow FISH[38] are shown. Note, compared to the GFP-control, that the telomere fluorescence in DN-hTERT-transduced cells was lower whereas the telomere fluorescence in hTERT-transduced cells was higher.

Telomerase activity in AML cells influences mean CFU frequency

To study the function of telomerase in AML progenitor cells, a known number of DN-hTERT or GFP-control cells were plated into primary colony forming unit (CFU) and suspension culture (SC) assays to determine the CFU and SC initiating cell content (SC-IC). In the primary CFU assays, colonies could be observed in all 7 samples and no differences in CFU frequency between DN-hTERT and GFP-control-transduced cells were observed (mean CFU frequency 4.0 $x10^3$ ± 4.3 $x10^3$ vs. 2.8 $x10^3$ ±1.9 $x10^3$ CFU/10^5 cells, respectively). However, after 4 weeks in SC, colonies were observed in only 2/7 samples transduced with DN-hTERT, while growth was observed in 5/7 of GFP-control samples. In the two DN-hTERT samples (BC212 and NV253) in which growth was observed, the frequency of SC-IC was reduced in sample BC212, while in sample NV253 it was comparable to the GFP-control (Figure 6). In two samples (GHN323 and IT369), no growth in either GFP-control or DN-hTERT transduced cells was observed. Overall, the CFU frequency after four weeks of suspension culture was 7.9$x10^2$ ± 1.8$x10^3$ CFU/10^5 cells in DN-hTERT transduced cells compared to 1.1$x10^4$ ± 2.7$x10^4$ CFU/10^5 cells for the GFP-control.

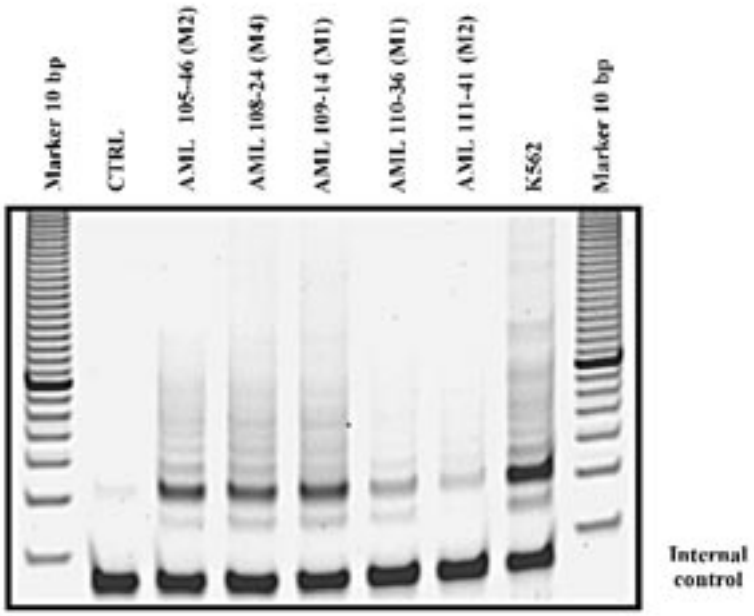


Figure 4: Telomerase activity in primary AML cells. Telomerase activity in 2.0 x 10^3 AML blast after 2 days in culture was analyzed by TRAP assay. Negative controls using DEPC-water and positive control extracts obtained from the cell line K562 were used in each experiment. All primary AML samples showed various degrees of telomerase activity.

One AML sample (110-36) was transduced with hTERT, DN-hTERT and GFP-control (Figure 6). The primary CFU assay again revealed no differences in primary CFU frequency for all 3 populations. After 2 weeks in SC, the CFU content of the DN-hTERT-transduced cells was reduced, while no colonies were observed after 4 weeks in SC. In contrast, colonies could be observed following SC for up to 6 weeks in the hTERT cells and up to 4 weeks in the GFP-control cells.

hTERT-transduced AML cells show high engraftment in ß$_2$-m$^{-/-}$ NOD/SCID mice

Two primary AML samples (105-46 and 108-24) transduced with hTERT, DN-hTERT and GFP-control vectors were sorted for GFP expression and similar cell numbers were transplanted into sublethally irradiated ß$_2$-m$^{-/-}$ NOD/SCID mice in duplicate (Table 2). BM was aspirated after 4 weeks and analyzed for engraftment of GFP-positive and CD45-positive cells. In general, higher engraftment levels were obtained using the hTERT-transduced AML cells compared to GFP-transduced cells, while AML cells transduced with DN-hTERT engrafted at lower levels compared to the GFP-control (Table 2). After 8 weeks, no human cells were observed in any of the mice except in one animal injected with hTERT-transduced cells from sample 105-46 (0.5%).

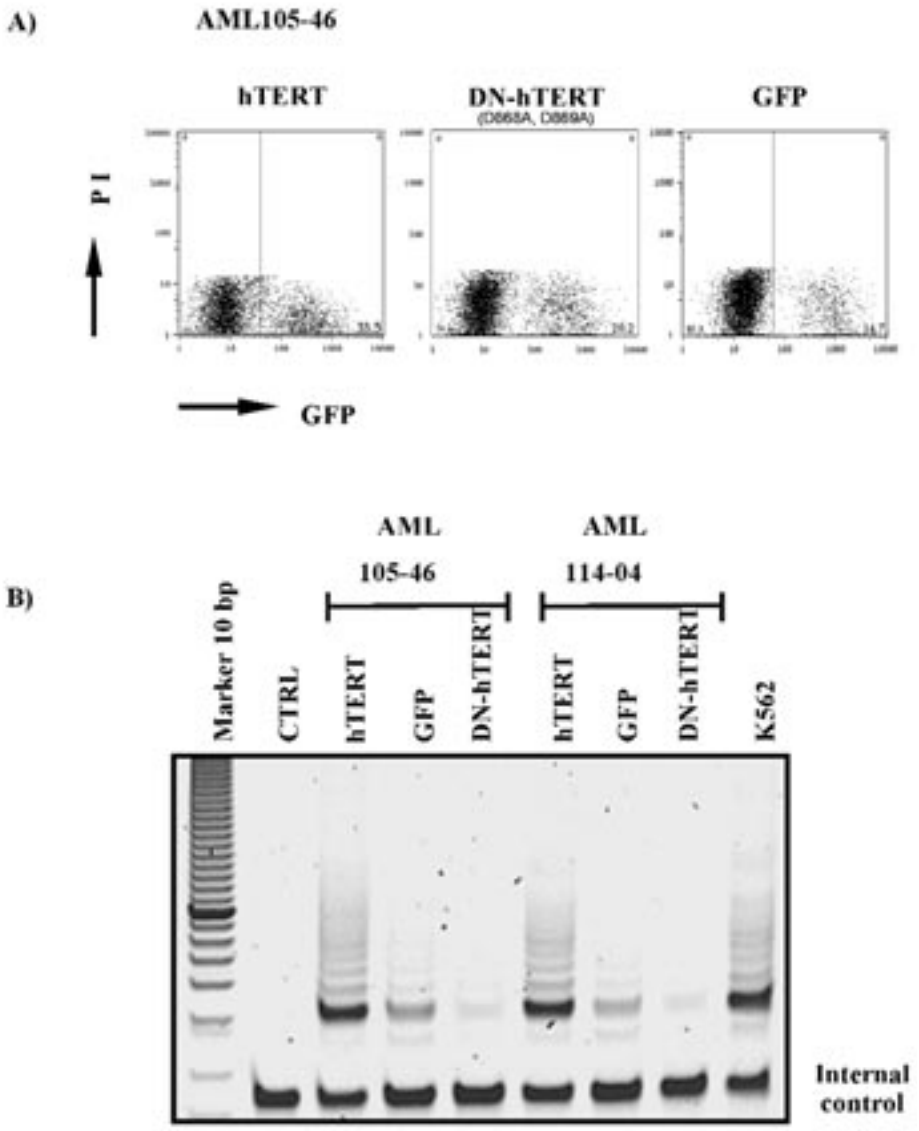
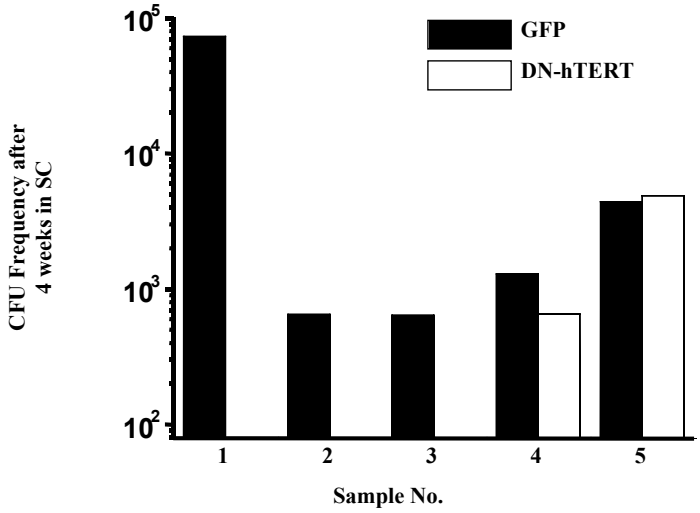


Figure 5: Effective transduction of primary AML cells. After transduction with retroviral vectors, AML cells were expanded for 10 days and analyzed for GFP expression. (a) GFP expression in AML cells from sample 105-46 transduced with hTERT, DN-hTERT and GFP. (b) AML samples 105-46 and 114-04 transduced with hTERT, GFP-control and DN-hTERT (D868A, D869A) were analyzed for telomerase expression by TRAP assay (2.10^3 cells). Negative control using DEPC-water and positive control extracts obtained from the cell line K562 were used.

Table 2: *NOD/SCID assay using two AML samples*

Patient Sample injected ($x10^5$)	Numbers of cells GFP	% of CD45 expression at week 4		
		hTERT	DN-hTERT	
105-46	50	5.6	11.3	2.6
	50		9.6	1.4
	36	5.3		
108-24	1.5	10.5		
	1.25	7.4	12	3.6
	0.7	2.9	2.6	



Sample No.	Patient ID	Week 0			Week 4		
		GFP	DN-hTERT	hTERT	GFP	DN-hTERT	hTERT
1	DK367	$4.8X10^3$	$5.2X10^3$	ND	$7.3X10^4$	0	ND
2	110-36	$8.3X10^2$	$6.7X10^2$	$8.8X10^2$	$6.5X10^2$	0	$2.5X10^3$
3	VW372	$1.9X10^3$	$2.4X10^3$	ND	$6.4X10^2$	0	ND
4	BC212	$4.4X10^3$	$3.8X10^2$	ND	$1.3X10^3$	$6.5X10^2$	ND
5	NV253	$3.0X10^3$	$2.8X10^3$	ND	$4.4X10^3$	$4.9X10^3$	ND
6	GHN323	$1.9X10^2$	$3.0X10^2$	ND	0	0	ND
7	IT369	$4.5X10^3$	$1.3X10^4$	ND	0	0	ND
Mean		$2.8X10^3$	$4.0X10^3$	$8.8X10^2$	$1.1X10^4$	$7.9X10^2$	$2.5X10^3$
SD		$1.9X10^4$	$4.3X10^3$		$2.7X10^4$	$1.8X10^3$	

Figure 6: DN-hTERT-transduced AML cells show reduced CFU frequencies 4 weeks after SC. Cells were sorted for GFP expression and a known number of GFP-positive cells were subjected to primary colony forming unit assays (CFU) (week 0) and suspension culture assays (SC). Every 2 weeks, half of the cells were plated in methylcellulose and colonies were counted at day 10 to calculate the progenitor frequency per 10^5 cells. The CFU frequency of the AML cells from the five patients in which growth was observed are shown. The table shows the progenitor frequencies of the primary CFU assay and the progenitor frequencies after 4 weeks in SC. Cells from one patient (110-36) were transduced with hTERT in addition to GFP and DN-hTERT. The hTERT-transduced showed a higher progenitor frequency compared to the GFP-control.

Discussion

Activation or upregulation of telomerase is believed to play an important step in the progression of most human malignancies.[41] To study the effect and significance of telomerase activity in AML, we disrupted and overexpressed telomerase activity in the human leukemia cell line K562 as well as in various primary leukemia samples from different patients using retroviral vectors encoding hTERT, DN-hTERT (D868A, D869A) or GFP genes.

While telomerase levels in K562 and primary leukemia cells transduced with DN-hTERT were reduced, no direct obvious effect on the proliferation of these cells was observed. However, delayed effects were observed that appeared to be triggered by telomere dysfunction as cells exhibited anaphase bridges, apoptotic cell death and a reduced colony forming ability several weeks after transfection and expansion.[31] The effect was specific to the inhibition of telomerase activity as the overexpression of wild-type hTERT, which differs from the mutant DN-hTERT (D868A, D869A) by two amino acids, did not affect viability or proliferation. In contrast, we observed an increase in the CFU content in hTERT-transduced AML cells as well as a higher engraftment into sublethally irradiated $ß_2$-$m^{-/-}$ NOD/SCID mice. Our results support the idea that telomerase activity is required for telomere maintenance and proliferation in leukemic cells.[42]

Interestingly, two of four K562 clones transduced with the DN-hTERT developed resistance to the effect of the DN-hTERT expression. One clone remained GFP-positive with a very low level of GFP expression, indicative of a very low expression of the transgene. Another clone completely lost GFP expression, possibly through gene silencing or loss of the transgene. These results suggest that inhibition of telomerase acts as strong selective disadvantage for telomerase positive malignant cells. In view of the difficulty to completely inhibit telomerase activity using DN-hTERT and retroviral transduction strategies, our results may have underestimated the effect of telomerase inhibitors. More effective telomerase inhibition most likely will result in increased genomic instability due to telomere dysfunction and may possibly favor selection of cells that can bypass the effect of telomerase inhibition.[43]

The transfection of primary AML progenitor cells with retroviral vectors has been problematic.[44,45] This is the first report of effective transfection of AML progenitor cells with retroviral vectors for hTERT and DN-hTERT (D868A, D869A). The protocol described in this study may find application to genetically mark AML cells and to modify leukemia cells to test novel therapeutic approaches and generate tumor vaccines.[44-49] Further improvements in transfection efficiency could probably be obtained by targeting noncycling cell populations using lentiviral vectors.[50]

Our results support the notion that telomerase activity in AML is associated with disease progression and that high levels of telomerase activity indicate a poor prognosis.[19,20,51-53] As telomerase activity appears largely restricted to malignant cells, telomerase activity measurements may help with the prognosis and the evaluation of antileukemic treatments

and disease progression. The value of telomerase inhibition in leukemia treatment needs to be established in clinical trials in combination with standard antileukemic agents.[42,54,55]

Acknowledgements

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Chapter 5

Constitutively Active Notch4 Promotes Early Human Hematopoietic Progenitor Cell Maintenance While Inhibiting Differentiation and Causes Lymphoid Abnormalities in Vivo

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Abstract

Notch transmembrane receptors are known to play a critical role in cell fate decisions in multiple tissues including the hematopoietic system. Notch*4* is structurally distinct from Notch1-*3* and has not been extensively studied in hematopoiesis. By PCR we find Notch*4* expression in human marrow cells and in CD*34*$^+$ and CD*34*$^-$ populations. When the constitutively active intracellular domain (IC) of Notch1 or *4* was overexpressed in normal human marrow or cord cells, we found a reduced colony forming and short-term proliferative ability. The primitive progenitor content of stromal based myeloid long-term cultures was significantly increased. Notch*4*-IC/green fluorescence protein (GFP) transduced cord cells transplanted into ß$_2$-microglobulin deficient NOD/SCID mice resulted in significantly higher levels of engraftment of both GFP$^+$ and GFP$^-$ populations as compared to controls. GFP$^+$ cells in bone marrow and spleen of transplanted animals gave rise to an immature T cell population, while B cell development was blocked. These results indicate that activation of Notch*4* results in enhanced stem cell maintenance, a block in monocytic and erythroid differentiation and the adoption of a T cell fate by the common lymphoid progenitor.

Introduction

The highly conserved Notch gene family plays an important role in cell fate decisions in a wide range of lineages in invertebrates and vertebrates.[1,2] In mammals four Notch receptors have been identified (Notch1-4) and five ligands Jagged1, Jagged2, and Delta1, 3 or 4. Notch activation in mammals occurs through binding to one of its ligands, which results in the translocation of the intracellular domain (IC) to the nucleus.[3,4] Notch receptors have an extracellular domain, containing tandem epidermal growth factor (EGF) repeats and 3 Notch repeats, a single transmembrane domain and an IC, containing 6 cdc10/ankyrin repeats, putative nuclear localization signals and a C-terminal OPA/EST region.[1] Although the four Notch receptors are similar in structure, Notch4 appears to be evolutionary the most distant from other Notch members with fewer EGF repeats, a significantly shorter IC, no transactivating domain and no cytokine responsive elements.[5] Notch4 also differs from the other Notch receptors by its expression pattern in the mouse, which is strongest in endothelial cells and male germ cells.[6] Notch4 has known effects on embryonic endothelial development[7] and active forms of Notch4 inhibit angiogenesis.[8] Furthermore, the constitutively active IC of Notch4 (Int-3) has oncogenic activity in the mouse mammary gland[9] and is detected in human breast, lung and colon carcinoma cell lines.[10]

The hematopoietic system is a complex and tightly regulated process in which pluripotent stem cells undergo proliferation and differentiation to produce mature blood cells of the various lineages, while maintaining a compartment of uncommitted cells. A role for Notch receptors in hematopoiesis was first proposed after a translocation in human T cell leukemia was identified involving the Notch1 gene.[11] Overexpression of the IC of Notch1 in murine bone marrow resulted in T cell leukemia and thymoma in mice receiving transduced marrow.[12] Furthermore, Notch1-IC overexpression has been shown to induce thymic-independent accumulation of double-positive (DP) T cells at the expense of B cells in the marrow of mice, suggesting that Notch1 signalling could favor the T cell fate in a common lymphoid precursor.[13] Activated forms of Notch2 and Notch3 have been implicated in thymic lymphoma,[14-16] while a role in T cell leukemia has not been reported so far. Notch signalling also plays a role in the maintenance of hematopoietic stem cells[17-21] and in the inhibition of differentiation.[22-24] The role of Notch4 in the hematopoietic system has not been studied. However, Notch4 has GATA recognition sites, transcription factors which play a role in lineage commitment in hematopoiesis,[25,26] in its promoter region suggesting that Notch4 transcription plays a role in hematopoiesis just like Notch1, 2 and 3.[5] Furthermore, Singh et al. detected Notch4 expression in maturing macrophages using single cell PCR.[27]

To study the role of Notch4 in hematopoiesis we determined expression of Notch4 in bone marrow cells and expressed the constitutively active form of Notch4 in human cord and marrow cells and compared it to Notch1-IC overexpression. Our studies indicate that Notch4 is expressed in normal marrow as well as in its $CD34^+$ and $CD34^-$ populations. In addition, Notch1-IC and Notch4–IC overexpression results in an inhibition of short-term

proliferation, with a decrease in monocytic and erythroid differentiation, but an increased long-term proliferative ability. Furthermore, transplantation of Notch4-IC transduced cord cells results in higher levels of human engraftment in the bone marrow. and the development of immature T cells in the bone marrow of immunodeficient mice. These results suggest, that Notch4 activation in primary human hematopoietic cells is similar to Notch1 activation in that it results in a decrease of differentiation into some of the mature blood cells. Furthermore, as is seen with Notch1 activation, the common lymphoid progenitor may adapt a T cell fate instead of a B cell fate when Notch4 is overexpressed in human cord cells.

Materials and Methods

Virus production

The cDNA of the intracellular domain (IC) of Notch1 (kindly provided by S. Artavanis-Tsakonas) and HA-tagged Notch4-IC (kindly provided by L. Li)[5] were cloned into a MSCV-IRES-GFP vector (MIG, from R.K. Humphries) to generate MSCV-Notch1-IC-IRES-GFP and MSCV-Notch4-IC-IRES-GFP vectors. Helper-free virus was obtained by transfecting amphotrophic Phoenix packaging cells[28] cultured in DMEM plus 10% fetal calf serum (FCS, StemCell Technologies, Vancouver, BC) using $CaPO_4$ and harvesting the virus-containing medium (CM) 24 hours later as described.[29] Virus-CM was filtered through a 0.45 μm filter and used freshly each time.

Cells

Bone marrow cells from normal healthy donors were obtained after informed consent and with approval of the Clinical Research Ethics Board of the University of British Columbia. Low-density (<1.077 g/ml) cells were isolated by centrifugation on Ficoll-Hypaque (Amersham Pharmacia) and fractionated directly, or later, after being cryopreserved in 10% DMSO plus 90% FCS. Cord blood cells were obtained with informed consent from the mothers undergoing caesarean delivery of normal full-term infants and low density (<1.077 g/ml) cells isolated using Ficoll-hypaque (Pharmacia, Piscataway, NJ). Primary lineage (CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, glycophorin A) depleted cord cells, which are highly enriched for $CD34^+$ cells (~70%), were obtained using StemSep™ columns (StemCell Technologies).

Retroviral Transduction

Cells to be transduced were first cultured at $1\text{-}2x10^5$ cells/mL for 48 hours in complete serum free medium consisting of Iscove's medium supplemented with BSA, Insulin and Transferrin (BIT', StemCell Technologies), 40 μg/ml low density lipoproteins and 10^{-4} M 2-mercapto-ethanol (both from Sigma Chemicals, St. Louis, MO) to which the following 5 purified recombinant human growth factors (GFs) were added: Flt-3 ligand (FL, 100

ng/mL, Immunex Corporation, Seatlle, WA), Steel Factor (SF, 100 ng/mL, produced in the Terry Fox Laboratory), interleukin-3 (IL-3, 20 ng/mL, Novartis, Basel, Switserland), interleukin-6 (IL-6, 20 ng/mL, Cangene, Mississauga, ON), and granulocyte colony-stimulating factor (G-CSF, 20 ng/mL, StemCell Technologies Inc.) as previously described.[30] At the end of this 2 day pre-stimulation period, the cells were resuspended in filtered virus-CM supplemented with the same 5 GFs. Protamine sulfate (5 mg/mL, Sigma) was added and the cells were placed in fibronectin coated petri dishes previously loaded twice with virus-CM (30 minutes each). After 12 hours the cells were harvested and resuspended in fresh virus-CM containing the 5 GFs and protamine sulfate. This infection procedure was performed 3 times. After 36 hours the cells were transferred to fresh serum free media. Cells for *in vivo* studies were used at this time-point, while cells for *in vitro* studies were incubated for a further 48 hours prior to harvesting and further analysis. Expression of the Notch4-IC construct was confirmed in 3 cord samples with real-time PCR with primers to the IC of Notch4. Notch4-IC expression was about 1000x higher in the Notch4-IC transduced cord cells than in the MIG transduced cord cells in all 3 samples.

Flow Cytometry

Viable GFP^+ cord or normal marrow cells (PI^-) were isolated at a purity of >99% using a three laser FACStarplus (Becton Dickinson, San Jose, CA) as described previously.[31] Cells were sorted into serum free media in microcentrifuge tubes at 4°C and known cell numbers were used to initiate colony forming assays (CFU), suspension culture (SC) assays and long term cultures. For the phenotype analyses, cells were stained with anti-CD34 (8G12)-Cy5 (Dr. P. Lansdorp), anti-Glycophorin-A(10F7)-PE, anti-CD71 (OKT-9)-PE (both from Dr. P. Lansdorp), anti-CD41a-PE (Pharmingen, Mississauga, ON), anti-CD38-PE, anti-CD13-PE, anti-CD33-PE, anti-CD11b-PE, anti-CD20-PE or anti-CD3-PE (all from Becton Dickinson). Cells were then analyzed on a FACScalibur using Cell Quest software (Becton Dickinson) with gates set to exclude ≥ 99.9% of the cells in the matched isotype control.

RT-PCR Analyses

cDNA from 2 human marrow samples and FACSsorted $CD34^+$ cells or $CD34^-$ subpopulations was kindly provided by Dr. C. Eaves. Aliquots of 1-5 µl of the first round of PCR generated from the initial amplification of total cDNA were subjected to a second round of PCR amplification in 50 µl volumes of 1×P*fx* amplification buffer (GIBCO/BRL), 0.3 mM each dNTP (Amersham Pharmacia), 1mM $MgSO_4$, 1 unit of Platinum P*fx* DNA polymerase, and 10 pmol of specific primers for the extracellular domain of Notch4 (5'-TAGGGCTCCCCAGCTCTC-3' and 5'-GGCAGGTGCCCCCATT-3') to give a DNA fragment of 486 bp. The primers started in different exons, to make sure that genomic DNA contamination did not interfere with the results. As a positive control cDNA of Human Aortic Endothelial Cells (kindly provided by Dr. A. Karsan), known to express human Notch4, was used. Forty cycles (94°C for 30 sec, 60°C for 1 min, 72°C for 30 sec) were then performed.

Real-Time PCR

Primers for Notch1, Notch2, Notch3, Notch4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific amplification were designed using the Primer Express software (PE Applied Biosystems, Fostercity, CA, USA). For Notch1 the sense primer CGGGTCCACCAGTTTGAATG and anti-sense primer GTTGTATTGGTTCGGCACCAT were used, for Notch2 sense primer TTTGGCAACTAACGTAGAAACTCAAC and anti-sense primer TGCCAAGAGCATGAATACAGAGA, for Notch3 the sense primer ATGCAGGATAGCAAGGAGGA and the anti-sense primer AAGTGGTCCAACAGCAGCTT, for Notch4 the sense primer CCCAGGAATCTGAGATGGAA and anti-sense primer CCACAGCAAACTGCTGACAT and for GAPDH the sense primer CGACAGTCAGCCGCATCTT and the anti-sense primer ACCTTCCCCATGGTGTCTCA were used. One step Real-Time PCR was performed using the GeneAmp 5700 Sequence Detection System Mix (PE Applied Biosystems). Reactions were performed in triplet in a 50 µl total volume containing 25 µl SYBR Green Master Mix (PE Applied Biosystems), 1-10 ng cDNA, sense and anti-sense primers and ddH_20. The primer concentrations used were the minimum primer concentrations giving the lowest threshold cycle (C_t) and maximum ΔR_n, while minimizing non specific amplification. The optimal concentration for both the sense and anti-sense primer for Notch1 and Notch2 was 100nM and for Notch3, Notch4 and GAPDH was 300nM in a 50 µl reaction. Dissociation curves were run to detect non-specific amplification, however in none of the experiments non-specific amplification was observed. To relatively quantitate Notch expression the $\Delta\Delta C_t$ method was performed as described in User Bulletin #2: ABI PRISM 7700 Sequence Detection System.

Cultures

A known number of transduced cord cells ($5\text{-}10x10^4$) were cultured in complete serum free media with the same 5 growth factors described above and maintained at 37°C in a 5% CO_2 humidified incubator and cell counts were performed at day 3, 7, 14 and 21 using trypan blue staining. At day 7 and 21 cells were also harvested for FACS analyses. To detect long-term-culture-initiating-cells (LTC-IC) $1x10^5$ transduced cord cells were placed in 2.7 mL Myelocult media (StemCell Technologies) with 10^{-6} M Solucortef and seeded onto a confluent layer of irradiated (80 cGy) mouse embryonic fibroblasts engineered to produce human SF, human IL-3 and human G-CSF).[32] Cultures were incubated at 37°C in a 5% CO_2 humidified incubator and received a weekly half-media change. After 5 weeks both the adherent and the non-adherent cells were harvested, pooled, counted and plated into methylcellulose-based medium (StemCell Technologies Inc.) supplemented with 20 ng/ml rhIL-3 (Sandoz), 20 ng/ml rhIL-6 (Terry Fox Laboratory), 20 ng/ml rhG-CSF (Amgen Canada Inc., Mississauga, Ontario), 20 ng/ml rhGM-CSF (Sandoz), 50 ng/ml rhSteel Factor (SF) (Terry Fox Laboratory) and 3 units/ml erythropoietin (StemCell Technologies Inc.). Colonies were counted after 21 days of culture. To detect primary colony forming cells 500 transduced cord or marrow cells were plated in serum free media-containing methylcellulose cultures (StemCell Technologies) with human SF (50 ng/mL) and 20 ng/mL of each IL-3, IL-6, G-CSF and GM-CSF. After 10 days colonies were counted.

Transplantation of Notch transduced Cord Blood cells into in ß$_2$-microglobulin$^{-/-}$ NOD/SCID mice

ß$_2$-microglobulin deficient nonobese diabetic/severe combined immunodeficient (ß$_2$-microglobulin$^{-/-}$ NOD/SCID) mice (Jackson Laboratory, Bar Harbour, ME) were bred and maintained in the animal facility of the British Columbia Cancer Research Centre (Vancouver, Canada) under sterile conditions in sterile micro-isolator cages. The animals were provided exclusively with autoclaved food and water containing 100 mg/l ciprofloxacin and HCl. Twenty-four hours prior to transplantation, mice were irradiated with 3.5 Gy γ-irradiation from a ^{137}Cs source at a dose rate of 1.22 cGy/min. A known number of unsorted cord cells transduced with MIG or MIG-Notch4-IC were resuspended in 0.3 ml Alpha minimal essential medium (Alpha MEM)(StemCell Technologies Inc.) with 5% FCS and injected intravenously into the lateral tail vein of 6-8 week old ß$_2$-microglobulin$^{-/-}$ NOD/SCID. BM aspirations were performed every 4 weeks on anaesthetized mice. Twelve weeks post-injection the mice were sacrificed by CO_2 inhalation. BM was removed from the femurs by flushing with Alpha MEM with 5% FCS. Spleens and thymus were removed and single cell suspensions were made for FACS analysis.

Flow Cytometry analysis of murine tissues

Cell suspensions from femoral BM and spleens were lysed in ammonium chloride (StemCell Technologies Inc.) for 20 min and then washed in Hanks medium (StemCell Technologies Inc.) with 5% human serum for blocking of human Fc receptors and prepared for flow cytometric analysis. Thymus cells were also incubated with 5% human serum in Hanks medium. Cells were then stained with a Cy5-labeled anti-human CD45 antibody (Dr. P. Lansdorp, Terry Fox Laboratory) in combination with CD71-PE, CD34-PE, CD33-PE, GlycophorinA-PE, CD41a, CD11b-PE, CD20-PE, CD3-PE, CD4-PE or CD8-PE for 30 min on ice. Unstained cells were used as control. Samples were analyzed using a FACScan (Becton Dickinson), gate settings which excluded ≥ 99.9% of the cells in the unstained control, were used to determine the lineage markers on CD45$^+$/GFP$^+$ cells in each sample. We defined human engraftment as expression of ≥ 0.1% CD45$^+$ cells in a sample.

Statistical analysis

Results are shown as the mean ± SD of values obtained in independent experiments. Differences between groups were assessed using the 2-tailed Student's t-test. For the *in vivo* data a paired t-test was performed with the mean values of the marrow aspirations at week 9 and 12. A significant level of 0.05 was chosen.

Results

Notch4 is expressed in human marrow cells with highest levels in primitive progenitors

To investigate the expression of Notch4 in human hematopoietic cells, cDNA from low density human marrow cells and the primitive progenitor population selected by FACSsorting based on the CD34 expression was subjected to RT-PCR. As shown in Figure 1, Notch4 expression was readily detectable in total marrow and in both the $CD34^+$ cells and the $CD34^-$ subpopulations of two marrow samples. In order to quantify expression in subpopulations of the most primitive progenitors ($CD34^+/CD38^-$), more mature progenitors ($CD34^+/CD38^+$) and non-progenitor marrow ($CD34^-$), real-time PCR on cDNA extracted from FACSsorted populations from marrows was performed. Notch1 and 4 expression was significantly higher in the most primitive $CD34^+CD38^-$ populations as compared to unsorted cells (Figure 2A) with the same trend observed for Notch2 and 3. Notch1 expression levels varied between unsorted marrows approximately 4 fold (range 0.34-1.42) as compared to an unsorted bone marrow sample 1. When the expression of Notch2, 3 and 4 were compared to Notch1 expression using the $\Delta\Delta C_t$ analysis in 5 unsorted bone marrow samples, Notch2 was expressed at 1.8±1.0x Notch1, Notch3 was expressed at 0.5±0.9x Notch1 and Notch4 was expressed at 0.3±0.5x Notch1 on average. In the $CD34^+CD38^-$ progenitor population Notch4 expression was 1.4±1.7x Notch1, while Notch3 and Notch4 had a lower expression.

Constitutively active Notch1 or 4 decreases myeloid colony formation and short-term in vitro proliferation

To examine the effect of constitutively active Notch1 and 4 on human myeloid differentiation, human marrow and lineage depleted cord blood (cord) cells were transduced with Notch1-IC or Notch4-IC in the MIG vector or control MIG. In four experiments the mean marrow transduction efficiency was 11.1 ± 8.9% for MIG 0.3 ± 0.3% for Notch1-IC and 5.0 ± 3.3% for Notch4-IC as measured by GFP positivity on FACS two days after transduction. The mean transduction efficiency in cord blood experiments was 27.5 ± 12.5% for MIG (n=10), 7.8 ± 2.1% for Notch1-IC (n=9) and 18.3 ± 9.3% for Notch4-IC (n=10). For both cord and normal marrow cells, GFP^+ cells were sorted and used to initiate primary colony assays. Total myeloid colony forming cells were markedly (3-10 fold) and significantly reduced in the Notch1-IC and Notch4-IC transduced cultures as compared to control (Figure 3). Interestingly, although BFU-E were observed with MIG control transduced marrow, these colonies were not observed using Notch1-IC or Notch4-IC transduced marrow cells (Figure 3a). Using cord cells erythroid colonies were also significantly reduced in the Notch4-IC transduced cultures (p=0.001) (Figure 3b). Using marrow cells a significantly lower number of granulocyte-macrophage colonies were seen with the Notch4-IC as compared to the MIG control (Figure 3b). However this effect was not significant when cord blood cells were assessed.

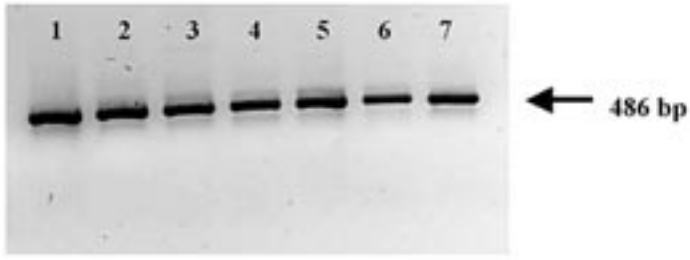

Figure 1: Notch4 expression in human marrow samples as detected by RT-PCR. cDNA of a positive control and two human marrow samples and their $CD34^+$ and $CD34^-$ subpopulations were subjected to RT-PCR. In all 7 samples Notch4 was readily detectable. 1= human aortic endothelial cell line; 2 = normal marrow sample 1; 3 = $CD34^+$ cells of marrow sample 1; 4 = $CD34^-$ cells of marrow sample 1; 5 = normal marrow sample 2; 6 = $CD34^+$ cells of marrow sample 2; 7 = $CD34^-$ cells of marrow sample 2.

Transduced cord blood cells sorted for GFP^+ were also used to initiate suspension cultures (SC) in serum free media with growth factors as described. GFP expression in GFP sorted cultures after 1 and 3 weeks was 47 ± 33% and 19 ± 11% respectively (n=7) for Notch1-IC, 42 ± 30% and 27±18% respectively for Notch4-IC (n=10) and 75 ± 23% and 68 ± 12% respectively for MIG control cultures. GFP expression in the Notch1-IC and Notch4-IC cultures was significantly reduced as compared to the MIG control ($p \leq 0.01$ in all cases). Cell numbers at day 3 were 2-4 fold lower in the Notch1-IC and Notch4-IC transduced cord cultures compared to the control ($p < 0.001$ and $p=0.003$ resp.) and remained reduced at day 7 ($p=0.1$ and $p=0.05$), day 14 ($p=0.03$ and $p=0.02$) and day 21 ($p=0.05$ and $p=0.003$). The phenotype of the cells present was analyzed at week 1 and 3 (Table 1). Total GFP^+ cells from progenitor ($CD34^+$), erythroid ($GlycophorinA^+$), myelomonocytic ($CD33^+$, $CD13^+$ and $CD11b^+$) and megakaryocytic ($CD41a^+$) lineages were all reduced in the Notch1-IC and Notch4-IC cultures as compared to the MIG control with the exception of megakaryocytic lineage cells, which were unchanged by Notch1-IC. Glycophorin A^+ cells were reduced more profoundly than the others (18-37 fold). However, due to heterogeneity of Glycophorin A expression in the MIG control this only reached significance in the Notch4-IC transduced cultures at week 1. These results suggest, that transduction with Notch1-IC and Notch4-IC results in inhibition myeloid differentiation when cultured with myeloid promoting growth factors.

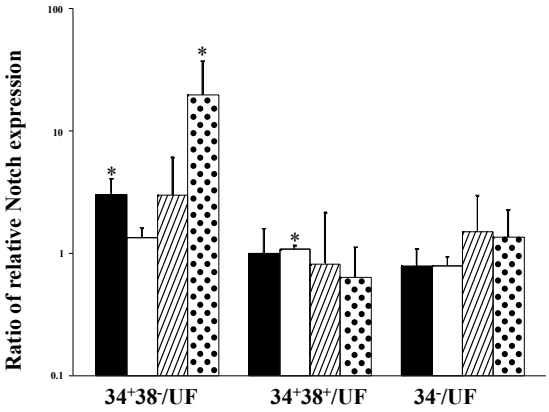

Figure 2: Relative Notch expression in human marrow samples and human hematopoietic progenitors as measured by real-time PCR. cDNA of unsorted marrow and marrow sorted for CD34 and CD38 were used for real-time PCR using Notch primers and SybrGreen. Notch expression in the sorted subpopulations was compared to its unsorted population and relative expression of Notch1-4 in the progenitors expressed. The expression of the sorted population was compared to the unsorted population in a paired t-test. Notch1 black bars; Notch2 white bars; Notch3 shaded bars; Notch4 dotted bars. * $p \leq 0.05$

Notch1 or Notch4 activation results in an increase in long-term culture initiating cells in vitro

To determine whether the long-term proliferative ability of cord cells was also altered by the overexpression of Notch1-IC or Notch4-IC, a known number of GFP^+ cord cells were maintained in stromal based long-term cultures for 5 weeks as described and then subcultured in semisolid medium for colony counts. As shown in Table 2, a significant increase in colonies derived from long term cultures initiated with Notch1-IC ($p=0.04$ and $n=5$) and Notch4-IC ($p=0.04$ and $n=5$) transduced cord cells as compared to the control was observed. There was no difference in phenotype of the colonies between the control and the Notch-IC transduced cells in that all cultures produced similar proportions of mixed granulocytic-erythroid colonies, granulocyte-macrophage colonies and erythroid colonies with similar morphology. This suggests that although Notch1 or 4 overexpression results in inhibition short-term proliferation it increases long-term proliferation of cord blood cells.

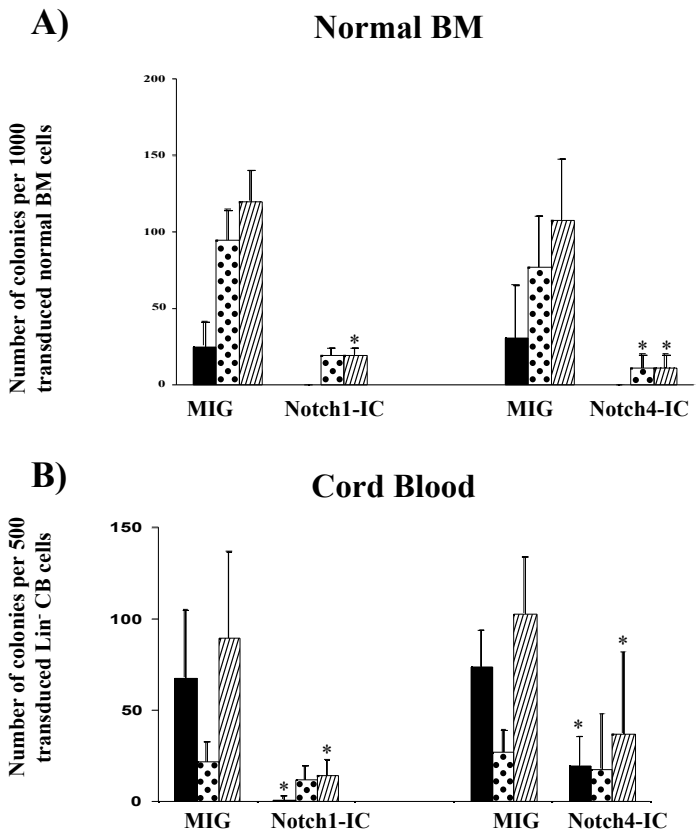

Figure 3: Decreased colony-forming ability of Notch1-IC and Notch4-IC transduced normal marrow and cord cells compared to MIG transduced cord blood cells. Normal marrow cells (A) (Notch1-IC n=3, Notch4-IC n=4) and cord blood (B) (Notch1-IC n=4, Notch4-IC n=5) were retrovirally transduced with MIG, Notch1-IC and Notch4-IC and sorted for GFP. Cultures were initiated with 1000 normal marrow cells (A) or 500 cord blood cells (B) into methylcellulose in the presence of IL-3, IL-6, G-CSF, GM-CSF and Steel Factor (SF) and colonies were counted 10 days after initiation. A paired t-test was performed to determine statistical differences between colony numbers in Notch1-IC, Notch4-IC and MIG. Erythroid colonies black bars; granulocyte-monocytic colonies dotted bars; total colonies shaded bars. * $p \leq 0.05$

Table 1: *Cell surface marker expression on cultured lineage depleted cord blood cells transduced with MIG, Notch1-IC or Notch4-IC.*

A)

Week 1 GFP+ cells	MIG ($x10^4$)	Notch1-IC ($x10^4$)	Fold Δ	MIG ($x10^4$)	Notch4-IC ($x10^4$)	Fold Δ
CD34+	2.6±2.5	0.6±0.3	4	1.6±1.8	0.5±0.6	3
GlycophorinA+	9.4±12.3	0.4±0.6	24	11.2±11.5	0.3±0.4*	37
CD33+	70.9±28.6	38.5±29.6	2	134±152	24.6±18.9	5
CD13+-	81.5±37.1	38.1±29.0	2	154±150	28.1±21.7	5
CD41a+	2.6±2.3	3.0±3.8	1	6.2±10.7	1.8±1.7	3
CD11b+	56.2±29.8	7.1±8.9*	8	72.1±59.2	7.6±9.7*	9
Total	223.2	87.7		379.1	62.9	

B)

Week3 GFP+ cells	MIG ($x10^4$)	Notch1-IC ($x10^4$)	Fold Δ	MIG ($x10^4$)	Notch4-IC ($x10^4$)	Fold Δ
CD34+	13.9±6.6	1.7±1.3*	8	8.9±8.7	2.1±1.1	4
GlycophorinA+	3.8±7.6	0.2±0.2	19	1.8±3.1	0.1±0.1	18
CD33+	513±380	28.5±16.7*	18	503±105	77.2±64.8*	7
CD13+-	484±247	34.9±20.3*	14	486±72.1	83.5±85.0*	6
CD41a+	4.8±5.0	4.1±3.8	1	7.5±12.5	1.6±1.5	5
CD11b+	360±202	21.1±13.9*	17	295±90.1	35.5±15.7*	8
Total	1380	90.5		1302	200.0	

Table 1: Cell surface marker expression on cultured lineage depleted cord blood cells transduced with MIG, Notch1-IC or Notch4-IC. Lineage depleted cord cells were transduced with MIG, Notch1-IC (n=7) or Notch4-IC (n=10). Cells were sorted for GFP+ and placed in suspension culture. After 1(A) and 3(B) weeks in culture cells were stained with monoclonal antibodies against CD34, Glycophorin A, CD33, CD13, CD41a and CD11b and the numbers of GFP positive cells per 105 input cells expressing the markers calculated from cell counts and analysis with FACS.
* $p<0.05$

Table 2: *Colony numbers from week 5 LTC of 1x10^5 of Notch1-IC, Notch4-IC and MIG transduced cord cells*

	MIG	Notch1-IC		MIG	Notch4-IC
Cord 1	250	450	Cord 6	114	245
Cord 2	200	820	Cord 7	430	1140
Cord 3	1730	2930	Cord 8	910	1290
Cord 4	535	600	Cord 4	535	700
Cord 5	863	1730			
Mean ± SD	716±626	1318±1025*	Mean ± SD	522±290	847±408*

Table 2: Colony numbers from week 5 LTC of 1x10^5 of Notch1-IC, Notch4-IC and MIG transduced cord cells. Lineage depleted cord cells were retrovirally transduced with MIG (n=9), Notch1-IC (n=5) and Notch4-IC (n=4) and sorted for GFP. 1x10^5 GFP$^+$ cord cells were put into long-term cultures. Cultures were maintained at 37°C in humidified incubators and had weekly media changes with long-term culture media. After 5 weeks cultures were trypsinized and cells were plated into methylcellulose in the presence of IL-3, IL-6, G-CSF, GM-CSF, Steel Factor and erythropoietin and colonies were counted 20 days after plating. * p≤ 0.05

Cord cells overexpressing the intracellular domain of Notch4 give rise to a CD4$^+$CD8$^+$ population in the bone marrow and spleen of ß$_2$-microglobulin$^{-/-}$ NOD/SCID mice

To assess the effect of Notch4 overexpression in lineage depleted cord cells *in vivo*, cord cells were retrovirally transduced with MIG or Notch4-IC for 36 hours. The average transduction efficiency of the four cord samples used in these experiments was 22.6 ± 13.4% for MIG and 15.3 ± 10.7% for Notch4-IC. Unsorted transduced cord cells (1.5x10^5 - 1x10^6 cells/mouse with 3 mice in each group) were harvested and injected into the tail vein of sub-lethally irradiated ß$_2$-microglobulin$^{-/-}$ NOD/SCID mice and the bone marrow evaluated for engraftment (CD45$^+$ cells) at week 6, 9 and 12 and spleen and thymus evaluated at week 12. The mean level of marrow engraftment in the 4 experiments was significantly higher in the mice injected with Notch4-IC transduced cord blood cells as compared to the control at week 6 and week 9 in both the GFP$^+$ and the GFP$^-$ population (Figure 4). Cells from week 9 and 12 mouse marrow were co-stained with CD45 and the lineage markers CD71, CD34, CD33, GlycophorinA, CD41a, CD11b, CD20, CD3, CD4 and CD8. CD34$^+$ cells were more frequent in the GFP$^+$CD45$^+$ population of the Notch4-IC mice as compared to the control (14 ± 18% versus 1 ± 3% p=0.05 at week 9 and 12). CD33 expression on GFP$^+$CD45$^+$ cells of Notch4-IC mice was lower compared to the control mice (5 ± 13% in Notch4-IC mice versus 44 ± 45% in MIG mice p=0.05). CD11b expression was only analyzed at week 12 and the expression of this marker on GFP$^+$CD45$^+$ cells of Notch4-IC mice was significantly lower compared to the control (10 ± 9% versus 37± 24% resp.

p=0.05). Mice injected with Notch4-IC transduced cord cells had an increased GFP^+CD3^+ population with 50 ± 20% of GFP^+ cells expressing $CD3^+$ in the Notch4-IC mice as compared to 8 ± 21% in the MIG mice ($p<0.01$) at week 9 and 12 combined. Cells were stained for CD4 and CD8 and analysis revealed a $CD4^+CD8^+$ population in the bone marrow of mice injected with Notch4-IC transduced cord cells (27 ± 21% of GFP^+CD45^+ cells), which was not seen in the mice injected with MIG transduced cord cells (0 ± 0% of GFP^+CD45^+ $p<0.01$) (Figure 5). When cells were stained for CD20 a slightly lower percentage of GFP^+CD45^+ cells from the bone marrow Notch4-IC mice (6 ± 11%) were positive for CD20 as compared to the control (22± 19% resp. p=0.13).

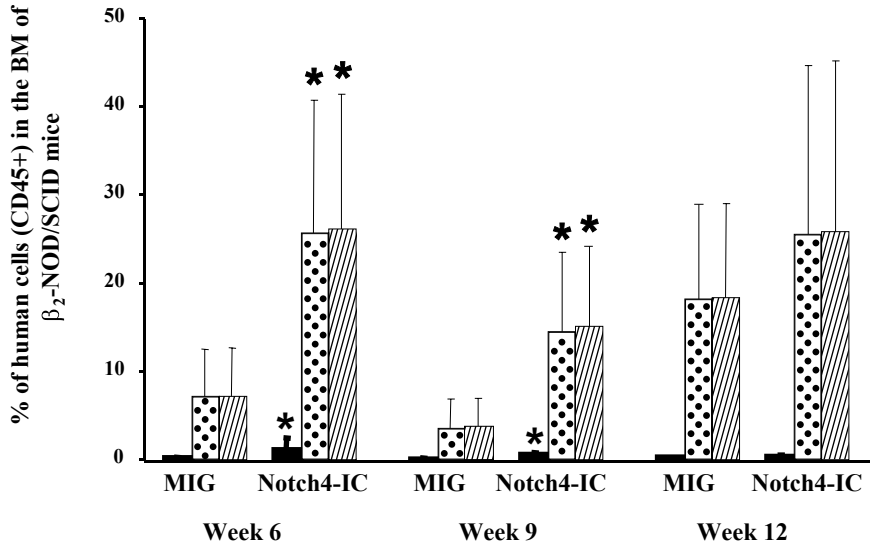

Figure 4: Increased engraftment of Notch4-IC transduced cord cells compared to MIG transduced cord cells in ß$_2$-microglobulin$^{-/-}$ NOD/SCID mice. Lineage depleted cord cells were retrovirally transduced with MIG and Notch4-IC (n=4) and injected in sublethally irradiated ß$_2$-microglobulin$^{-/-}$ NOD/SCID mice. Every 3 weeks bone marrow aspirations were performed and cells were stained with CD45-Cy5. Cells were analyzed by FACS to determine human engraftment. $CD45^+GFP^+$ cells black bars; $CD45^+GFP^-$ cells dotted bars; Total $CD45^+$ cells shaded bars. * $p \leq 0.05$

In the spleens the average engraftment in the Notch4-IC mice was 10.2 ± 18.2% compared to 3.1 ± 5% in the MIG mice. GFP^+ cells were observed in the spleens of the mice in only 2 of the 4 experiments. In those 2 experiments GFP^+ cells in the MIG mice contributed to the $CD20^+$ B cell population (63 ± 24% of GFP^+CD45^+ cells), while GFP^+ cells in the Notch4-IC mice did not (0.1±0.1% cells of GFP^+CD45^+). Moreover, in 4/6 Notch4-IC mice GFP^+ cells gave rise to a GFP^+CD3^+ population (1.1±1.3% of $CD45^+$ cells), which was

primarily $CD4^+CD8^+$ (0.8±0.3% of $CD45^+$ cells). No GFP^+CD3^+ population in the spleens of the MIG mice was observed. In the four experiments no human engraftment was observed in the thymus of the sacrificed animals. However, in an earlier individual experiment prior to deciding on our techniques, in which we cultured the transduced cord cells for three days before injecting them in the mice, engraftment was seen. High levels of engraftment ($CD45^+$ cells) were observed in the thymus of all three Notch4-IC mice (94 ± 3%), whereas in the three MIG mice engraftment in the thymus was lower (23 ± 37%). Interestingly, although the majority of $CD45^+$ cells in the thymus of the Notch4-IC mice were GFP^- (98 ± 1%), in the MIG mice a substantial number of $CD45^+$ cells were GFP^+ (43 ± 32%). The mean percentage of total $CD45^+$ cells expressing CD3 was 69 ± 6% in the Notch4-IC mice and 41 ± 36% in the MIG mice. The percentage of $CD4^+CD8^+CD45^+$ cells in the thymus of the mice was 92 ± 4% in the Notch4-IC mice and 52 ± 24% in the MIG mice. We are currently further investigating this finding.

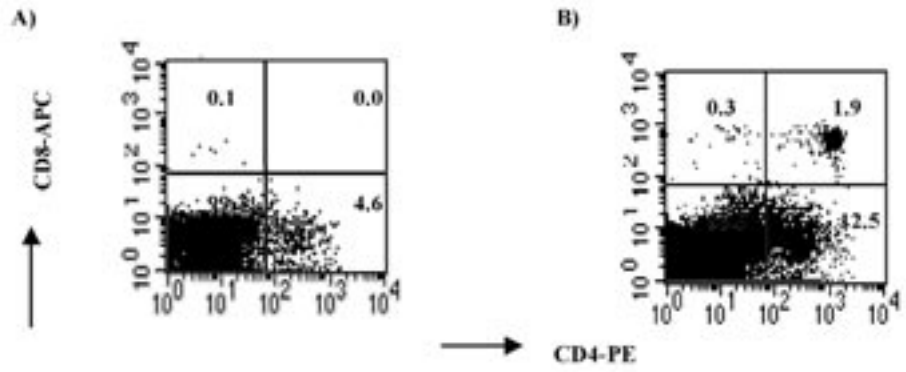

Figure 5: Notch4-IC transduced cord cells give rise to a $CD4^+CD8^+$ population in the bone marrow of ß$_2$-microglobulin$^{-/-}$ NOD/SCID mice. Marrow from week 9 and 12 ß$_2$-microglobulin$^{-/-}$ NOD/SCID mice transplanted with MIG and Notch4-IC (n=4) transduced cord blood cells was analyzed for phenotype by FACS. Murine marrow cells were stained with CD4-PE and CD8-APC along with PI. A) marrow from MIG mouse within viable cells B) marrow from Notch4-IC mouse within viable cells.

Discussion

Emerging evidence suggests an important role for Notch genes in influencing cell fate decisions during early hematopoiesis. It is also known that Notch is important during development, and dysregulated Notch is associated with malignancy. However, with the availability in mammals of four Notch receptors, five ligands, and numerous co-regulatory molecules, Notch signalling is complex, and the extent of redundancy which might exist in Notch functions is unknown. It is known that both Notch1 null mice and Jagged1 null mice are embryonic lethal,[33,34] suggesting non-overlapping functions for Notch genes and ligands, at least during development. We have evaluated the expression and function of Notch4 in human hematopoiesis, a molecule previously poorly studied in this setting.

Here we demonstrate that Notch4, like Notch1, 2 and 3 is expressed in human marrow cells and like Notch1 has the highest expression in the more primitive $CD34^+CD38^-$ sorted cells. Using a quantitative PCR method we find the expression Notch1 and 2 somewhat higher as compared to Notch3 and 4 in unsorted human marrow cells. In order to evaluate the role that Notch4 could play in the differentiation and maintenance of primitive progenitor cells we overexpressed constitutively active forms of Notch4 in human lineage depleted cord cells or bone marrow and functionally and phenotypically analyzed these cells.

Constitutively active Notch4 produced a marked reduction in primary colony formation and inhibited short-term *in vitro* proliferation affecting both the erythroid and the myelomonocytic lineages. This is consistent with some previous reports, in which an inhibition of short-term proliferation and colony-forming ability was also observed after Notch activation through its ligand Jagged1.[35,36] Other groups reported an increase in colony-forming ability upon Jagged stimulation[17-19] or overexpression of Notch1-IC. [24] However, in most cases cells were cultured for up to 14 days with Jagged1 before colony assays were performed. When we cultured Notch1-IC or Notch4-IC transfected cells on growth factor producing stromal cells in long-term cultures for 5 weeks, before plating them into semisolid media we also observed an increase in colonies, suggesting that Notch4 or Notch1 signalling results in an increase in more primitive myeloid progenitors. This is consistent with the higher expression levels of these genes in the $CD34^+CD38^-$ populations. In this study the Notch1 and Notch4 induced inhibition of differentiation was most dramatic in the erythroid lineage cells. Hadland et all. reported an increase in erythroid colony formation by $Notch1^{-/-}$ embryonic stem cells, suggesting the erythroid lineage may be differentially more influenced by Notch activity.[37] Erythroid differentiation requires the erythroid cell- and megakaryocyte-specific transcription factor GATA-1, but this factor does not appear to be regulated by Notch1 nor is the hematopoietic-specific transcription factor TAL-1.[38] One of the downstream effectors could be GATA-2, which is necessary to keep primitive progenitors in an undifferentiated state. It has been shown that Notch1 signalling can sustain GATA-2 expression and thereby prevents differentiation.[23] Whether Notch4 can also sustain GATA-2 expression has not been studied thus far. However, other transcription factors could play a role in Notch mediated inhibition of

erythropoiesis. For example, erythroid Kruppel-like factor (EKLF), which plays a role in erythroid differentiation, interacts with the corepressors mSin3A and histone deacetylase HDAC1, proteins which also interact with downstream targets of Notch signalling. [39]

When Notch4-IC cord cells were evaluated for their engraftment potential in ß$_2$-microglobulin$^{-/-}$ NOD/SCID mice, engraftment levels were higher in bone marrow and spleen as compared to the control cord cells. Increased repopulating ability has been observed upon overexpression of Notch1-IC in murine cells[40,41] as well as upon stimulation of human cells with Delta1 and Jagged1 ligand,[17,20,42] but increased engraftment of human hematopoietic cells upon overexpression of Notch-IC has not been previously demonstrated. Engraftment was increased in both the GFP$^+$ and the GFP$^-$ population, suggesting the possibility that Notch signalling might influence the expression levels of Notch receptor and or ligands on both GFP$^+$ and GFP$^-$ cells. A non-cell autonomous effect of Notch1 has been proposed by Kawamata.[43] However, it also may be that the expression of the Notch4-IC or Notch1-IC gene is differentially downregulated after homing and/or engraftment. This is suggested by the much lower expression of GFP in Notch1-IC and Notch4-IC GFP$^+$ sorted cells after 3 weeks in culture (19 ± 11% and 27 ± 18% respectively) as compared to the GFP expression in MIG control GFP$^+$ sorted cells in culture (68 ± 12%). Consistent with the higher levels of engraftment we also observed a higher percentage of CD34$^+$GFP$^+$ cells and a decrease in the percentage of myeloid and monocytic cells in the marrow of the animals.

The function of Notch on the myeloid compartment *in vivo* is still controversial. Both promotion and inhibition of myeloid differentiation upon Notch signalling has been suggested.[41,43-45] Human cord cells overexpressing the intracellular domain of Notch1, have limited potential to develop into monocytes in the marrow of NOD/SCID mice.[44] This is consistent with our *in vivo* data in which we saw a marked reduced CD11b expression on Notch4-IC transduced cord cells both *in vivo* and *in vitro*. This system is not suitable to analyze the effect of Notch overexpression on erythroid differentiation because erythroid development does not occur in the mice upon transplantation of human cells.

Suppression of B cell proliferation and abnormal maturation of T cells have been observed in mice transplanted with Notch1-IC transduced murine marrow and human cord cells.[43] Notch4-IC cord cells like Notch1-IC transduced cord cells can become immature CD4$^+$CD8$^+$ T cells in marrow and spleen and are unable to differentiate into B cells. We only found abnormal T cells and inhibited B cell differentiation in the GFP$^+$ population consistent with results from De Smedt et al. who reported abnormal B and T cell development in Notch1-IC transduced human cord cells but not in untransduced cells.[44] This suggests that the common lymphoid progenitor may adapt a T cell fate instead of a B cell when Notch4-IC is overexpressed. It is interesting that overexpression of the downstream effectors of Notch, HES1 and HES5, causes a partial block in B cell development but no T cell abnormalities were seen, suggesting that other downstream effectors play a role.[45] The thymic engraftment we observed in one experiment after culturing Notch4-IC cord cells for 3 days before injection into the mice could have been due to the culture, because Ohishi et al showed that human cord cells cultured with soluble

Delta1 resulted in high levels of thymic engraftment.[42] It is possible that cells overexpressing Notch4-IC in culture up-regulate other Notch genes or ligands e.g. Delta1. However, further experiments are needed to elucidate the role of Notch4-IC in thymic engraftment and the effect of culturing of Notch4-IC transduced cord cells.

Altogether, our data show that Notch4-IC, like Notch1-IC, plays a role in hematopoiesis in that it enhances engraftment, maintains stem cells, suppresses B cell development while inducing abnormal double positive T cells and suppresses myeloid differentiation. This suggests that Notch4 dysregulation could be involved in lymphohematopoietic malignancies.

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Chapter 6

Acute Myeloid Leukemia Progenitors Express Abnormal Levels of Notch Receptors and Respond Differently to Jagged1 Stimulation Compared To Normal Progenitors

Suzanne M. Vercauteren, Rick Zapf and Heather J. Sutherland

Abstract

Members of the Notch gene family are found on primitive hematopoietic progenitors and play a role in the differentiation and proliferation of hematopoietic cells. This study examines the expression of the four Notch receptors in AML cell lines and primitive AML progenitors using quantitative real-time PCR and the proliferative response of AML cells to stimulation of Jagged1. The expression of the four Notch receptors in AML cell lines and primary AML cells was heterogeneous compared to normal bone marrow (BM). In normal BM cells the ratio of Notch1-4 expression in the $CD34^+CD38^-$ fraction/unsorted cells was higher compared to the ratio's of $CD34^+CD38^+$ fraction/unsorted cells and the $CD34^-$ fraction/unsorted cells. In AML cells the ratio's of Notch1 and Noth2 expression in the three sorted fraction were very similar to each other, suggesting that the usual upregulation of Notch1 and 2 seen in normal primitive progenitors, with subsequent lower expression in $CD34^-$ cells, is dysregulated during differentiation in AML. The ratio's of Notch3 and Notch4 expression in the $CD34^+CD38^-$ fraction/unsorted cells, $CD34^+CD38^+$ fraction/ unsorted cells and $CD34^-$ fraction/unsorted cells in AML cells was heterogeneous with both extremely high and low expression levels observed. When normal BM cells and AML cells were cultured for 3 days on S17 or S17+Jagged1 cells and subsequently analyzed phenotypically and in *in vitro* assays, we observed an increase in total $CD34^+CD38^-$ cells in normal BM but not in AML samples. Furthermore, a significant increase in primary CFU was observed with normal BM cells pre-stimulated with Jagged1 compared to control, while this was not observed with AML cells. Interestingly, long-term culture's (LTC) on S17 or S17+Jagged1 cells resulted in a significant decrease in LTC-initiating cell (LTC-IC) content when normal BM cells were cultured on S17+Jagged1 cells compared to S17 cells, while a significant increase in AML LTC-IC content was observed on S17+Jagged1 stroma.

Introduction

In normal hematopoiesis a hierarchical organisation of progenitor cell types is observed. The phenotype of those different subsets of progenitors has been identified using *in vitro* and *in vivo* functional assays. The long-term culture-initiating cell (LTC-IC) is preferentially detected in the $CD34^+CD38^-$ subpopulation,[1] whereas the more differentiated colony-forming cells (CFU) are frequently detected in the $CD34^+CD38^+$ subpopulation.[2] Normal progenitors capable of repopulating non obese diabetic/severe combined immunodeficienct (NOD/SCID) mice have been detected in the $CD34^+CD38^-$ subpopulation and with a low frequency in the $CD34^-$ populations.[3-6]

Although in acute myeloid leukemia (AML) a deregulation of proliferation and a block in differentiation is observed, the same hierarchical organisation as in normal hematopoiesis persists.[7-9] Largely, the same phenotype of subpopulations has been observed in AML with the NOD/SCID leukemia initiating cell and the suspension-culture-initiating cell (SC-IC) being $CD34^+CD38^-$[9,10] while the phenotype of CFU's is more heterogenous.

So far, the mechanisms controlling proliferation and differentiation in normal hematopoiesis have been poorly understood. However, more and more evidence arises for a role of the Notch receptor family in controlling cell fate decisions as well as proliferation in a wide variety of tissues including the hematopoietic system.[11] A role for Notch in hematopoiesis was first proposed after the human homologue of Notch1 was cloned from T cell acute lymphatic leukemia cells containing a translocation involving the Notch1 gene.[12] In mammals, four Notch receptors have been identified (Notch1-4), which are highly conserved transmembrane glycoprotein receptors.[13] The Notch receptors consist of an extracellular domain, containing a variable number of epidermal growth factor (EGF)-like repeats and lin-12/Notch repeats, which are involved in ligand binding and Notch activation, a single transmembrane domain and a intracellular domain.[14] The intracellular domain contains a RAM domain, which binds CSL proteins (CBF1, Su(H), LAG-1), cdc10/ankyrin repeats, a nuclear localisation signal and a C-terminal OPA/EST region. Physiological activation of Notch occurs through the binding of Notch to one of its ligands (Jagged and Delta in mammals). These ligands are members of the DSL (Delta, Serrate, LAG-2 in non-vertebrates) family. Following activation, Notch is cleaved within the transmembrane domain, resulting in the translocation of the Notch intracellular domain to the nucleus where it activates transcription of HES genes via association with the CSL proteins.[15,16] HES genes encode for basic Helix-Loop-Helix (bHLH) proteins, which function as transcriptional repressors. Notch signalling can also occur via a CBF-1 independent pathway.[17,18]

Notch1 is expressed on both $CD34^+Lin^-$ and $CD34^+Lin^+$ cells in human BM as well as on $CD34^-$ cells although in reduced levels.[19] Furthermore, single cell RT-PCR has revealed a wide expression of Notch receptors, its ligands and modulators of Notch signalling within the hematopoietic system.[20] We have compared the expression of the four Notch receptors in human normal BM and its progenitors and found that all four Notch receptors are

expressed in the hematopoietic system and the expression of the four Notch receptors is higher in the progenitor cell population compared to more mature populations (Chapter 5).

Functional studies of Notch1 in normal hematopoiesis have shown that, in general, Notch1 activation by ligand binding or overexpression of a constitutively active form of Notch results in maintenance of primitive progenitor cells and in some studies even an expansion of the animal repopulating cells.[21-28] Although the role of Notch receptors in lymphoid development has been well established, [29-31 32,33] the role of Notch in myeloid differentiation remains controversial. Initial reports suggested that Notch signaling inhibits the differentiation of the murine myeloblast cell line 32D upon stimulation with G-CSF or GM-CSF.[34,35] In contrast, more recent reports suggest the promotion of differentiation of the 32D cell line in response to growth factor stimulation upon activation of Notch.[36,37] Notch signalling through its ligands also plays a role in monocyte apoptosis and differentiation.[38,39] Overexpression of the intracellular domain of Notch1 in the erythroleukemia cell line K562 resulted in suppression of erythroid but not of megakaryocytic differentiation.[40] We found that the overexpression of the intracellular domain of Notch1 or Notch4 resulted in the maintenance of primitive progenitors, with an inhibition of erythroid, myeloid and monocytic differentiation both *in vitro* and *in vivo* (Chapter 5). It is likely that the Notch family is important in the orderly progression of normal events in hematopoietic differentiation. Thus, it seemed relevant to investigate how expression of the four Notch receptors in the development of AML compared with their normal counterparts. Therefore, we sorted normal and AML cells in the $CD34^+CD38^-$, $CD34^+CD38^+$ and the $CD34^-$ populations and determined the Notch expression in these defined populations and in AML cell lines using Real-Time PCR. We observed a relatively low level of Notch1 and Notch2 expression in the $CD34^+CD38^-$ population compared to the $CD34^-$ population in the majority of AML patients, while expression of Notch3 and 4 was more heterogeneous in this population. To investigate whether AML and normal BM cells function differently after ligation with the Jagged1 ligand, AML and normal BM cells were cultured for 3 days on S17 or S17+Jagged1 stroma cell lines and analyzed for phenotypic changes using FACS and functional differences by determining the number of primary CFU and CFU after suspension culture (SC) and long-term culture (LTC). The total number of $CD34^+CD38^-$ normal BM cells was expanded at a significant higher level when cultured on S17+Jagged1 stroma cells compared to S17 cells, while in AML cells the total number of this phenotypic population decreased with or without Jagged1 stimulation. Primary CFU frequency of normal BM was significantly higher on S17+Jagged1 stroma compared to S17 while CFU frequency after SC or LTC was lower compared to S17. In contrast, LTC-IC frequency of AML cells was significantly higher on S17+Jagged1 cells compared to S17 cells. These results demonstrate a deregulated expression of the Notch genes in the majority of AML samples and abnormal responses to Jagged1 stimulation, suggesting a possible role for the Notch regulatory pathway in the etiology of this disorder.

Materials and Methods

Patient Cells and Cell lines

Bone marrow (BM) cells from normal healthy donors and peripheral blood (PB) cells from patients at diagnosis of AML were obtained after informed consent and with approval of the Clinical Research Ethics Board of the University of British Columbia. The mean number of blasts in the samples was 74 ± 23%. Patients with FAB M3 type were excluded because of specific characteristics of this subtype of AML. Blood cells were Ficoll separated to obtain a mononuclear cell population then frozen in Dulbecco's modified Eagle's medium (DMEM) (StemCell Technologies Inc., Vancouver, B.C.) with 50% fetal calf serum (FCS) (StemCell Technologies Inc.) and 10% dimethylsulphoxide (DMSO) and stored at -135°C. The cell lines K562 (ATCC Rockville, MD, USA), NB4 and Kasumi-1 (Quadra Logic Technologies) were cultured in RPMI (StemCell Technologies Inc.) + 10% FCS. KG-1 (ATCC) was cultured in DMEM + 20% FCS, AML193 was maintained in Iscove's Modified Eagle's Medium (StemCell Technologies Inc.) supplemented with 5 µg/ml transferrin + 5 µg/ml insulin + 2 ng/ml GM-CSF and the cell line Mo7e was cultured in DMEM + 10% FCS + 10% 5637 conditioned medium (Terry Fox Laboratory) + 5 x 10^{-5} M ß-mercapto-ethanol (Terry Fox Laboratory) + 5ng/ml rIL-3 (Sandoz, Basel, Switzerland). The stromal cell lines S17 and S17 expressing human Jagged1 (Dr. Tariq Enver, Institute of Cancer Research, London, UK) were cultured in α-Minimal Essential Medium (StemCell Technologies Inc.) + 20% FCS. All cultures were maintained at 37°C in a 5% CO_2 humidified incubator.

AML Cell Phenotyping and Sorting

Prior to sorting, thawed AML cells were suspended in HFN (Hanks medium with 2% FCS and 0.1% sodium azide) at 10^7 cells/ml. Cells were stained for 30 minutes on ice with monoclonal antibodies CD34-FITC (Dr. Peter Lansdorp, Terry Fox Laboratory) used at 4 µg/ml and CD38-PE (Becton Dickinson, San Jose, California) used according to manufacturers instruction. Separate aliquots were stained with an irrelevant mouse IgG_1-FITC antibody (Becton Dickinson) and mouse IgG_1-PE antibody (Becton Dickinson) as an isotype control. Cells were then washed twice in HFN at 4°C, propridium iodide (PI) at 2 µg/ml was added to the cells prior to the second wash, and the cells were maintained on ice prior to sorting. Cells were analyzed and sorted on a dual laser FACStar^{plus} (Becton Dickinson) on the basis of fluorescence intensity after gating out non-viable (PI^+) cells. Fractions were sorted into DMEM with 50% FCS in microcentrifuge tubes at 4°C. Sorted fractions were washed, resuspended and used to extract RNA.

RT-PCR and Real-Time PCR

Total cellular RNA from sorted and unsorted BM cells, sorted and unsorted PB of AML patients and the AML cell lines was prepared using Trizol reagent (Life Technologies,

Burlington, Canada). RNA was DNAse I treated to remove any contaminating DNA following the manufacturer's instructions (Life Technologies). Synthesis of first-strand cDNA was carried out as according to manufacturer's instructions using random primers. Primers for Notch1, Notch2, Notch3, Notch4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific amplification were designed using the Primer Express software (PE Applied Biosystems, Fostercity, CA, USA). For Notch1 the sense primer CGGGTCCACCAGTTTGAATG and anti-sense primer GTTGTATTGGTTCGGCACCAT were used, for Notch2 sense primer TTTGGCAACTAACGTAGAAACTCAAC and anti-sense primer TGCCAAGAGCATGAATACAGAGA, for Notch3 the sense primer ATGCAGGATAGCAAGGAGGA and the anti-sense primer AAGTGGTCCAACAGCAGCTT, for Notch4 the sense primer CCCAGGAATCTGAGATGGAA and anti-sense primer CCACAGCAAACTGCTGACAT and for GAPDH the sense primer CGACAGTCAGCCGCATCTT and the anti-sense primer ACCTTCCCCATGGTGTCTCA were used. One step Real-Time PCR was performed using the GeneAmp 5700 Sequence Detection System Mix (PE Applied Biosystems). Reactions were performed in triplet in a 50 µl total volume containing 25 µl SYBR Green Master Mix (PE Applied Biosystems), 1-10 ng cDNA, sense and anti-sense primers and ddH_20. The primer concentrations used were the minimum primer concentrations giving the lowest threshold cycle (C_t) and maximum ΔR_n, while minimizing non specific amplification. The optimal concentration for both the sense and anti-sense primer for Notch1 and Notch2 was 100nM and for Notch3, Notch4 and GAPDH was 300nM in a 50 µl reaction. Dissociation curves were run to detect non-specific amplification, however in none of the experiments published here non-specific amplification was observed. To relatively quantitate Notch expression the $\Delta\Delta C_t$ method was performed as described in User Bulletin #2: ABI PRISM 7700 Sequence Detection System and relative Notch expression levels of all samples compared to unsorted cells of normal BM1 were calculated.

Functional studies of primary AML cells cultured on Jagged1 expressing stromal layers.

S17 cells and S17 cells + Jagged1 were irradiated and plated at $1.5x10^5$ cells in 24 well plates and maintained overnight. After 16-24 hours, the media was removed and AML cells or normal BM cells resuspended in serum-free media containing 20 ng/ml rhIL-3 (Sandoz, Basel, Switzerland), 20 ng/ml rhIL-6 (Terry Fox Laboratory), 20 ng/ml rhG-CSF (Amgen Canada Inc., Mississauga, Ontario), 20 ng/ml rhGM-CSF (Sandoz), 50 ng/ml rhSteel Factor (Terry Fox Laboratory) and 3 units/ml erythropoietin (StemCell Technologies Inc.) were added to the stroma layers. After 3 days in culture the wells were trypsinized (StemCell Technologies Inc.) resuspended in serum free media, counted and aliquots used in primary CFU assays, suspension culture (SC) assays and used for FACS analysis.

Primary CFU Assays

A known number of cultured normal BM and AML cells were plated in methylcellulose culture medium, (Methocult H4330, StemCell Technologies Inc.), containing 20 ng/ml rhIL-3, 20 ng/ml rhIL-6, 20 ng/ml rhG-CSF, 20 ng/ml rhGM-CSF, 50 ng/ml rhSteel Factor

and 3 units/ml erythropoietin. After 10 days of incubation at 37°C in a 5% CO_2 humidified incubator, AML blast clusters (10-20 cells) or colonies (> 20 cells) were counted and the numbers were pooled to obtain CFU frequencies.

Suspension Culture Assays

A known number of cultured normal BM and AML cells were plated in SC. SC were maintained at 37°C in a 5% CO_2 humidified incubator and 0.5 ml of fresh medium with growth factors was added weekly as previously described.[10,41] Every second week half the cells and volume of the SC were removed, the cells were washed in DMEM with 10% FCS and cultured in methylcellulose to determine the CFU frequency of the SC-IC. SC were maintained for 8 weeks then the entire contents of the wells were harvested and assessed for SC-IC frequency.

Long-term Culture Assays

To detect long-term-culture-initiating-cells (LTC-IC) a known number of AML or normal BM cells (5-10x10^5 cells) were placed in 2.7 mL Myelocult media (StemCell Technologies Inc.) with 10^{-6} M Solucortef and seeded onto a confluent layer of irradiated S17 or S17+ Jagged1 stroma cells. Cultures were incubated at 37°C in a 5% CO_2 humidified incubator and received a weekly half-media change. After 5 weeks both the adherent and the non-adherent cells were harvested, pooled, counted and plated into methylcellulose-based medium supplemented with 20 ng/ml rhIL-3, 20 ng/ml rhIL-6, 20 ng/ml rhG-CSF, 20 ng/ml rhGM-CSF, 50 ng/ml rhSteel Factor and 3 units/ml erythropoietin. Colonies were counted after 21 days of culture.

Statistical Analysis

Comparison of relative Notch expression levels between AML populations and normal BM populations was performed by Student's t-test. To study differences between AML cells cultured on S17 cells and S17+ Jagged1 expressing cells the Student t-test was also performed. Statistical significance was assigned when the probability that there was no difference between two variables was ≤ 0.05.

Results

Expression of the four Notch receptors in AML cell lines, primary AML cells and AML progenitors

In order to determine whether Notch receptors are abnormally expressed in AML, we first investigated the relative Notch1-4 levels in normal BM of 9 healthy volunteers and 6 AML cell lines using real-time PCR. Normal BM1 was set as the reference at 1.00. Table 1 enlists the relative Notch expression levels in normal BM and the AML cell lines K562, Kasumi-1, KG-1, AML193, Mo7e and NB4. In normal BM, Notch1 was expressed at 0.6-8.8x the

level of normal BM1. In the AML cell lines, Notch1 was barely detectable in AML 193 (0.04x) and low in NB4 (0.3x), whereas in K562 (3.4x), Kasumi-1 (1.1x), KG-1 (1.8x), and Mo7e (1.9x) expression levels were similar to normal BM. Notch2 expression in normal BM was 0.1-2.1x normal BM1. Notch2 expression in the AML cell line Mo7e was barely detectable, whereas in the other 5 cell lines the Notch2 expression levels ranged from 0.2-0.6x. Notch3 expression in normal BM was very heterogeneous (range 2.4-49x normal BM1) and the same pattern was observed in the AML cell lines. In the Kasumi-1 cell line (376x normal BM1) and the AML 193 cell line (249x normal BM1) Notch3 expression was very high. Notch4 expression in normal BM ranged from 0.25-35x NBM1, while in the AML cell lines Notch4 was barely detectable in the cell lines KG-1 (0.01x normal BM1) and NB4 (0.01x normal BM1).

Table 1: *Relative Notch expression of normal BM and AML cell lines.*

	Notch1 (x normal BM1)	Notch2 (x normal BM1)	Nocth3 (x normal BM1)	Notch4 (x normal BM1)
Normal BM	3.8±3.4	1.0±0.7	18±23	14±15
K562	3.4	0.2	37	15
Kasumi-1	1.1	0.2	249	0.3
KG-1	1.8	0.6	5.4	0.01
AML193	0.04	0.6	376	1.3
Mo7e	1.9	0.01	45	0.2
NB4	0.3	0.5	11	0.01

Table 1: Relative Notch expression of normal BM and AML cell lines. RNA was extracted from 9 normal BM samples and 6 AML cell lines. RNA was used in Real-time PCR experiments using the ΔΔCt method. Notch expression levels were calculated as a relative increase or decrease compared to normal BM1.
BM bone marrow

To investigate whether Notch is abnormally expressed in primary AML cells and AML progenitors, PB cells from 11 AML patients at diagnosis and normal BM cells of 7 healthy donors were sorted into $CD34^+CD38^-$, $CD34^+CD38^+$ and the $CD34^-$ fractions. In unsorted primary AML cells Notch expression was similar to normal BM with Notch1 expressed at 3.3±4.6x normal BM1 (range 0.16-15.5), Notch2 expressed at 1.5±1.3x normal BM1 (range 0.04-4.1), Notch3 expressed at 1.3±1.6x normal BM1 (range 0.01-4.5) and Notch4 expressed at 0.8±2.2x normal BM1 (range 0-6.1). Interestingly, similar to the generally very low expression of Notch4 in the cell lines, Notch4 expression was ≤0.02x normal BM1 in 5 of 8 AML patients.

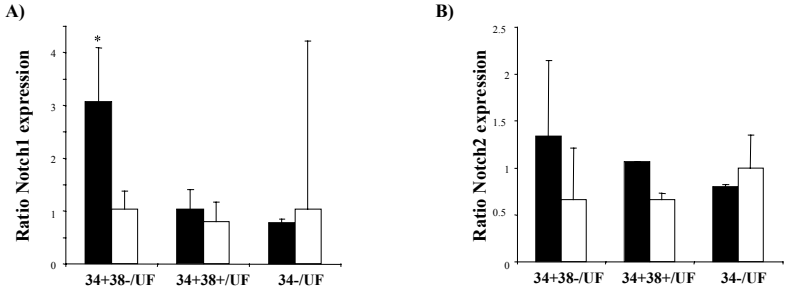

Figure 1: Ratio's of Notch expression in normal BM and AML progenitors. RNA was extracted from sorted CD34$^+$CD38$^-$ cells, CD34$^+$CD38$^+$ cells and CD34$^-$ cells and from unsorted cells from 5 normal BM samples (closed bars) and from peripheral blood of 13 AML patients (open bars). Relative Notch expression was determined by the amount increase/decrease compared to normal BM1. Ratio's were calculated by dividing the relative Notch expression in the subpopulation by the relative expression in the unsorted cells. A) Ratio's of relative Notch1 expression in normal BM and AML subpopulations. B) Ratio's of relative Notch2 expression in normal BM and AML subpopulations.
BM bone marrow * $p<0.05$

To allow comparison of Notch expression levels in the different populations per sample, we calculated the ratio of Notch expression in the sorted populations divided by the Notch expression in unsorted cells of that sample. In normal BM, Notch1 and Notch4 expression is significantly higher (p=0.05 and p=0.04 respectively) in the primitive progenitor fraction (CD34$^+$CD38$^-$ fraction) compared to unsorted cells, with the same trend observed for Notch2 and 3 (Chapter 5). When the results from normal cells were compared to AML there was a significant difference between the ratio of Notch1 expression in the CD34$^+$CD38$^-$ fraction/unsorted cells in normal BM (mean 3.1 ± 1.0) as compared to the ratio in AML cells (mean 1.0 ± 0.6) ($p<0.01$)(Figure 1). The same trend was observed with the ratio of Notch2 expression in the CD34$^+$CD38$^-$ fraction/ unsorted cells (normal BM mean 1.3 ± 0.3; AML cells mean 0.7 ± 0.7; p=0.07). Furthermore, a significant lower ratio of Notch2 expression was observed in the CD34$^+$CD38$^+$ fraction/unsorted cells in AML samples (mean 0.7±0.3) compared to normal BM (mean 1.0±0.1; p=0.01). Overall, in AML samples, the ratio of Notch1 expression in the 3 sorted populations was very similar, while the ratio of Notch2 expression increased slightly upon maturation. We have determined Notch3 and 4 expression in 5 patient samples and both Notch3 and 4 expression were very heterogeneous in these samples. In two patient samples Notch3 expression was similar to normal BM in all three fractions, in two patients, Notch3 was barely detectable in all fractions and in one patient a very high (137x normal BM1) level of Notch3 expression was observed in the CD34$^+$CD38$^-$ fraction, while in other fractions a normal expression level was observed. Notch4 was barely detectable in 3 of 5 AML patient samples. In one sample a relatively high (5x normal BM1) Notch4 expression level was observed in the CD34$^-$ fraction with barely detectable Notch4 expression in the CD34$^+$CD38$^-$ fraction or the CD34$^+$CD38$^+$ fraction. In the same patient with high Notch3 expression in the

$CD34^+CD38^-$ fraction cells a very high level (526x normal BM1) of Notch4 expression was observed in the same fraction with very low levels of Notch4 in the other 2 fractions. Interestingly, the ratio of Notch1 (0.2x normal BM1) and Notch2 (0.7x normal BM1) expression in the $CD34^+CD38^-$ fraction/unsorted cells of this patient seemed relatively low. These results suggest that the usual upregulation of Notch1 and 2 in the $CD34^+$ subpopulation with subsequent downregulation in the $CD34^-$ subpopulation is dysregulated in AML progenitor cells. Notch3 and 4 expression in AML progenitors is more heterogeneous.

Table 2: *Total number and number of CD34+CD38- cells of normal BM and AML cells pre-cultured on S17/S17+Jagged1 stroma cell lines.*

Per 10^5 input cells	Cell number ($x10^4$)		$CD34^+CD38^-$ cells($x10^4$)			Cell number ($x10^4$)		$CD34^+CD38^-$ cells ($x10^4$)	
	S17	S17J	S17	S17J		S17	S17J	S17	S17J
NBM 1	3.8	5.3	ND	ND	AML 1	2.4	1.6		
NBM 2	6.7	12	ND	ND	AML 2	6.8	6.5	15	15
NBM 3	9.4	10	ND	ND	AML 3	5.7	5.2	9.1	8.9
NBM 4	7.7	8.0	ND	ND	AML 4	5.4	5.7	3.5	5.2
NBM 5	4.4	3.4	19	17	AML 5	8.7	7.8	0.8	2.5
NBM 6	4.9	6.7	72	78	AML 6	2.5	4.2	0.8	1.2
NBM 7	4.4	3.4	8.7	18	AML 7	1.0	1.1	0.1	0.3
NBM 8	4.4	6.0	14	29	AML 8	4.1	4.1	0.3	0.2
NBM 9	5.0	7.2	17	56	AML 9	3.6	4.5	0.5	0.9
NBM 10	3.0	4.0	9.4	31	AML 10	0.7	1.0	0.4	0.4
NBM 11	1.9	2.6	3.2	8.5	AML 11	1.7	2.5	0.8	1.0
NBM 12	1.8	3.6	3.1	11	AML 12	4.2	4.9	0.1	0.9
NBM 13	14	13	18	21	AML 13	4.0	2.1	0.1	1.8
NBM 14	18	21	24	48					
NBM 15	16	19	137	182					
Mean ± SE	7.0 ± 5.0	8.4 ± 5.7*	30 ± 40	45 ± 50*	Mean ± SE	3.9 ± 2.3	0.8 ± 0.1	2.7 ± 4.6	3.2± 4.6*

Table 2: Total number and number of $CD34^+CD38^-$ cells of normal BM and AML cells pre-cultured on S17/S17+Jagged1 stroma cell lines. 15 normal BM samples and 13 AML samples were cultured for 3 days on irradiated S17 or S17+Jagged1 cells. Cells were counted and analyzed for CD34 and CD38 expression by flow cytometry. The number of total or $CD34^+CD38^-$ cells at day 3 on S17 or S17+Jagged1 cells per 10^5 input cells was calculated. BM bone marrow NBM = normal bone marrow * $p<0.05$

Stromal Jagged1 expression influences the number of normal BM and AML progenitors

To investigate whether Notch signalling alters the functional abilities of AML progenitors as compared to normal cells, we used cultured AML and normal cells with the stromal cell line S17 which expresses the Notch ligand Jagged1. We first evaluated whether the phenotype of bulk normal BM and AML cells is altered when stimulated with a Notch ligand. 1.2-2.5 x 10^6 AML cells (n=13) or 0.4-2.5 x 10^6 normal BM cells (n=15) were placed for 3 days on S17 or S17+Jagged1 cells in duplicate. After 3 days cells were counted, placed in MC and SC or analyzed by FACS for CD34 and CD38 expression (Table 2). After 3 days of culture the mean number of total viable normal BM cells was significantly higher ($p<0.01$) on S17+Jagged1 stroma cells compared to S17 cells. There was no difference between the mean number of total AML cells on S17 and S17+Jagged1 cells at day 3. A significant higher number of $CD34^+CD38^-$ normal BM cells ($p<0.01$) and $CD34^+CD38^-$ AML cells ($p=0.02$) was observed after culture on S17+Jagged1 cells compared to S17 cells. However, although the total number of $CD34^+CD38^-$ normal BM cells increased after 3 days on S17 and S17+Jagged1 cells, the total number of $CD34^+CD38^-$ AML cells decreased when cultured for 3 days on S17 or S17+ Jagged1 cells. This suggests that Notch ligation can increase slightly both normal and AML primitive progenitors as compared to controls.

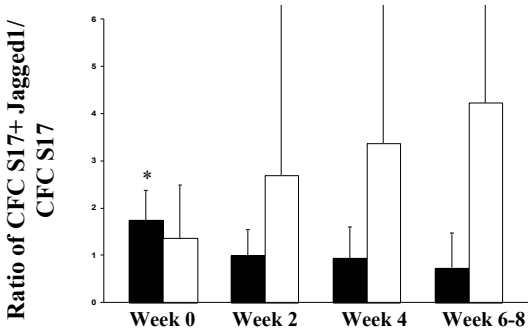

Figure 2: Ratio of the CFU frequency of normal BM cells and AML cells maintained in SC on S17+Jagged1 divided by the frequency of cells maintained on S17 cells. A known number of cultured normal BM (closed bars) and AML cells (open bars) were plated in SC. Every second week half the cells and volume of the SC were removed and cultured in methylcellulose to determine the CFU content of the SC. SC were maintained for 8 weeks then the entire contents of the wells were harvested and assessed for CFU content.
CFU colony forming cell, BM bone marrow, SC suspension culture * $p<0.05$

Stromal Jagged1 expression influences the functional ability of normal BM and AML progenitors

To investigate whether Jagged1 influences the maintenance of functional AML progenitors *in vitro*, a known number of normal BM and AML cells cultured for 3 days on S17 or S17+Jagged1 stroma cells were plated into MC. In addition, cultured normal and AML cells were placed in SC for up to 8 weeks. There was a significant increase ($p<0.01$) in mean CFU frequency of normal primary CFU when cells were cultured on S17+Jagged1 cells compared to S17 cells (Figure 2). This effect was not observed when AML cells were used. When normal BM cells were cultured in SC with S17 or S17+Jagged1, a lower CFU frequency was observed when BM cells were cultured on S17+Jagged1 cells compared to S17. In contrast, AML CFU after SC showed a higher CFU frequency on S17+Jagged1 cells compared to culture on S17 cells with time. To further investigate this phenomenon we cultured normal BM (n=5) and AML (n=12) cells in LTC with S17 and S17+Jagged1 as stroma feeders. After 5 weeks in LTC, the LTC-IC frequency of normal BM cells on S17+Jagged1 cells was significantly lower ($p=0.04$) compared to the LTC-IC frequency on S17 cells (Table 3). In contrast, the LTC-IC frequency of AML cells cultured on S17+Jagged1 cells was significantly higher ($p=0.02$) compared to the LTC-IC frequency on S17 cells. These data suggest that Jagged1 stimulation of normal BM cells results in a decrease of primitive progenitors or a decrease in their functional ability. In contrast, stimulation of AML cells with Jagged1 increases the number or functional ability of primitive progenitors.

Table 3: *CFU frequency of 5 normal BM samples and 12 AML samples after 5 weeks in LTC on S17 or S17+Jagged1 stroma cells.*

	CFU/10^5 after LTC			CFU/10^5 after LTC	
	S17	S17J		S17	S17J
NBM 1	21	2.1	AML 1	0.2	0.6
NBM 2	13	0.4	AML 2	9.1	37
NBM 3	52	45	AML 3	8	16
NBM 4	24	6.1	AML 4	0.4	1.0
NBM 5	21	22	AML 5	0.5	0.5
			AML 6	0.1	0.3
			AML 7	0.1	3.3
			AML 8	0.0	3.1
			AML 9	74	93
			AML 10	57	79
			AML 11	1.7	14
			AML 12	4.0	4.1
Mean ± SE	26 ± 15	15 ± 19*	Mean ± SE	13 ± 25	21 ± 32*

Table 3: CFU frequency of 5 normal BM samples and 12 AML samples after 5 weeks in LTC on S17 or S17+Jagged1 stroma cells. 5 normal BM samples and 12 AML samples were cultured in LTC on irradiated S17 or S17+Jagged1 cells. After 5 weeks both the adherent and the non-adherent cells were harvested, pooled, counted and plated into MC assays. Colonies were counted after 21 days of culture.
NBM normal bone marrow, CFU colony forming cell, LTC long-term culture, MC methyl cellulose * $p<0.05$

Discussion

It seems clear that the Notch gene family plays an important role in cell fate decisions within the hematopoietic system. We and others have shown that constitutively active Notch genes or Notch ligation promotes *in vivo* repopulating cell proliferation and maintenance while blocking terminal erythroid and myelomonocytic differentiation (Chapter 5).[28,34,40,42] These findings suggest that Notch is an important regulator of the fate of stem cells and myeloid committed progenitors. Notch genes have also been implicated in a wide variety of malignancies including lymphoid leukemias and lymphomas, and downstream pathways of Notch interact with other known oncogenic pathways such as Hox. As a key feature of AML is the failure of terminal myeloid differentiation, we were interested to see whether Notch genes are abnormally expressed, either as a primary or secondary event, in bulk AML cells and in the phenotypically defined primitive AML progenitors thought to be responsible for maintaining the disease. [7,9,10] Furthermore, in order to evaluate the functional response of AML progenitors to Notch we co-cultured AML cells and normal BM cells with a stromal cell line expressing the Notch ligand Jagged1.

We have evaluated Notch gene expression as a potential marker for altered Notch signalling. While a direct correlation may not exist, some evidence for a correlation is suggested in that Notch receptors are expressed at the highest levels in the primitive $CD34^+CD38^-$ subpopulation where it has been shown to be active in cell fate determinations (Chapter 5).[19] A marked overexpression of Notch, which might be predicted if Notch were important in driving AML cell behavior, was only seen in the AML cell lines Kasumi-1 (FAB type M2, myeloblastic leukemia) and AML193 (FAB type M5, monocytic leukemia) in which Notch3 expression was extremely high. We also observed very high levels of Notch3 and Notch4 in the primitive AML progenitor population of one patient with FAB M5. In the AML cell lines NB4 (FAB type M3, promyelocytic leukemia) and KG-1 (FAB type M6, erythroleukemia) all four Notch receptors were expressed at a very low level compared to normal BM. Constitutively active forms of Notch1 and Notch4 inhibit erythroid differentiation. It is possible that the Notch receptors are downregulated to try to overcome this block in differentiation possibly caused by other genetic changes. While normal cells have higher Notch expression in the $CD34^+CD38^-$ population, in AML cells, Notch1 and 2 are expressed in the $CD34^+CD38^-$, the $CD34^+CD38^+$ and the $CD34^-$ subpopulation at comparable levels in the majority of the samples, suggesting that the usual downregulation of Notch1 and 2 is perturbed in AML samples.

The effect of Jagged1 and other Notch ligands on hematopoietic progenitors in *in vitro* assays is still unclear. While some studies report an increase in CFU frequency after stimulation with Jagged1,[21,26,27] other groups have reported a decrease in CFU frequency.[43,44] Differences may be related to the length of exposure to Jagged1 before plating the cells in CFU assays or to other factors. In addition, some groups have used Jagged1 expressed by stroma cell lines,[43] whereas others have used a soluble form of Jagged1.[21,26,44] It is not clear whether soluble forms of Notch ligands are activating the

Notch pathway or might actually block Notch signalling. Recently, two papers have demonstrated that Jagged1 and Delta1 are capable of translocating to the nucleus themselves suggesting that they may signal as well.[45,46] In this study, we found a modest but significant increase in $CD34^{+}CD38^{-}$ cells in both normal and AML cells after 3 day exposure to the Jagged1 expressing S17. Primary CFU from normal BM cells also increased significantly after a 3-day exposure to Jagged1 expressing S17 cells, while AML CFU were slightly but not significantly increased as compared to the S17 control. After 2, 4, and 6 weeks in SC, derived CFU from normal BM was slightly lower in the cultures previously exposed to 3 days of Jagged1, while CFU from the S17+Jagged1 AML cultures were increased 2–4 fold as compared to the control, however neither change was significant. Similar findings were observed when normal BM or AML cells were co-cultured with the S17 or S17+Jagged1 for 5 weeks and then analyzed for CFU. In normal BM, CFU frequencies from LTC, co-cultures were significantly lower when cells were cultured on S17+Jagged1 cells as compared to S17 cells, while in AML the Jagged1 exposure increased CFU. It seems clear that constitutively active forms of Notch promote the maintenance of primitive progenitors (Chapter 5).[23,28,47] However, the signal that Notch receptors receive through Jagged1 ligation may be different than signalling through constitutively active forms of Notch or other Notch ligands.[48] In AML, we found similar effects of Notch-Jagged1 ligation as compared to normal BM cells when we analyzed phenotype or primary CFU, however using assays that measure more primitive progenitors, opposite effects were observed with significant increases in the LTC assays. This suggests, that either the frequency or the functional ability of primitive AML progenitors increases upon Jagged1 stimulation.

Abnormal Notch expression in AML progenitor cells and an abnormal proliferative response to Jagged1 ligation suggest that the Notch gene family may play a role in AML. Whether abnormal Notch expression has a causative role in this disease or whether abnormal Notch expression is a consequence of other dysregulated genes remains to be elucidated.

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Chapter 7

Summary and General Discussion

Acute Myeloid Leukemia (AML) is characterized by an uncontrolled proliferation and accumulation of myeloid blast cells in the bone marrow, blood and other organs of patients. It is thought that the disease is sustained by a small subset of cells capable of extensive self-renewal and proliferation, which gives rise to the more mature myeloid blast cells.[1-3] These cells, uniquely capable of sustaining the disease, are called AML stem cells. Despite remarkable progress in the treatment of AML over the last decades, the disease is still often fatal mainly due to relapse of the disease.[4,5] Relapse is often the result of minimal residual disease or, in case of an autologous stem cell transplant, contamination of the transplant with leukemic stem cells.[6] Eradication of these AML stem cells is essential to cure the disease. Characterization of the AML stem cells both functionally and phenotypically is important not only to develop new treatment strategies that are disease specific and curative, but also because it will give us new insights into the etiology of this disease.

7.1 Normal and AML Stem Cells: the Phenotypic Characteristics

AML occurs as a result of genetic changes in normal hematopoietic cells.[1,7] Typically, a normal hematopoietic stem cell instead of progenitor cell is the target for transformation for two reasons. First, stem cells have the machinery for self-renewal, while more differentiated cells have to turn that machinery on de novo. Therefore, fewer mutations may be required to maintain self-renewal than to re-activate this potential in progenitor cells. Secondly, because of the relative longevity of stem cells compared to more mature progenitors, there is also a higher change of an accumulation of genetic changes that may lead to the transformation to occur.

To date normal hematopoietic stem cells can only be defined functionally: they self renew and they differentiate into all the lineages of mature blood cells. Intensive efforts have been made to identify the phenotype of the hematopoietic stem cell. The majority of hematopoietic stem cells lack the expression of CD38, CD71, HLA-DR[8-10] and common lineage specific markers such as CD33, CD13, CD20, CD11b, CD3, CD4 and CD8[11] and express CD34 and CD90.[12] The estimated frequency of the competitive repopulating units (CRU) in immuno-deficient mice, a surrogate marker for hematopoietic stem cells, is about 30 times higher in the $CD34^+$ population of the BM (1 per 10^5 $CD34^+$ cells)[13] compared to total BM cells (1 per $3x10^6$ total BM cells).[14] Although CD34 is used extensively in the clinic for the purification of normal hematopoietic stem cells, recent studies have demonstrated the existence of $CD34^-$ stem cells, which appear to be more primitive than the $CD34^+$ cells.[15,16]

AML stem cells share many phenotypic similarities with normal stem cells adding to the body of evidence that the malignant transformation occurs at the stem cell level. AML progenitors can be detected in the same *in vitro* and *in vivo* assays as their normal counterparts.[1,2,17] We have observed a close correlation between the malignant progenitors with long-term *in vitro* proliferative ability and the cell capable of engrafting in the NOD/SCID mouse.[18] The frequency of the SCID-Leukemia Initiating Cell (SL-IC) varies in different reports from as high as 20-1000 SL-IC per 10^6 AML cells[1] to as low as 0.7-45 SL-IC per 10^7 AML cells.[19] A 3 log purification in leukemic stem cells was obtained when

AML cells were sorted for their CD34$^+$CD38$^-$HLA-DR$^-$ phenotype.[3] However, engraftment with CD34$^-$ cells has also been reported.[20] Whether the CD34$^-$ AML cell capable of engraftment is a more primitive progenitor than the CD34$^+$ cell, as has been suggested in normal hematopoiesis, is unclear. One of the few differences between normal and leukemic stem cells is that normal stem cells express CD90, while most leukemic stem cells do not.[21] We could speculate that an initial genetic change in a CD90$^+$ hematopoietic stem cell is followed by additional mutations in a downstream CD90$^-$ progenitor cell that is still capable or regained the ability of engrafting. Another possibility is that mutations in the hematopoietic stem cell result in the loss of CD90 expression.

CD133 is a cell surface antigen that is expressed on a wide variety of stem cells and is rapidly downregulated upon differentiation. In the hematopoietic system, CD133 is expressed on both the CD34$^+$ and CD34$^-$ cells capable of engrafting in immuno-deficient mice, suggesting that this marker is more useful to detect normal hematopoietic stem cells.[16,22] Our data demonstrate that CD34 and CD133 expression is not unique to normal hematopoietic stem cells, because CD34 and CD133 expression could be seen on the majority of AML cells capable of engrafting in immuno-deficient mice. Autologous transplants based on CD34 or CD133 selection could therefore be a source of leukemic stem cells that ultimately could contribute to relapse of the disease.

CD33, a myeloid specific cell surface antigen, is absent on normal hematopoietic stem cells,[11,23,24] but is expressed on the majority of AML cells.[25-27] Hence, this antigen appears to be an ideal candidate for AML specific treatment strategies. Over the last decade, several therapeutic strategies have been developed that use a CD33 antibody for the specific delivery of the anti-leukemic agent.[28-30] Although most patients initially respond to these treatments, relapse often occurs. This is not so surprising given the fact that we showed that the majority of AML stem cells do not express CD33 and will be spared by any of these treatments. However, in our study a few AML patient samples could be selected with CD33 expression on their leukemic stem cells and these patients might benefit by CD33 based therapies. It would be too time consuming to set up functional assays for each individual patient to determine their CD33 expression on primitive AML progenitors. An alternative would be to check for abnormal karyotype in the CD34$^+$CD33$^-$ population, which according to our study contains the majority of primitive AML progenitors.

Immunophenotypic instability of AML cells has been reported and could limit the potential use of the phenotype of AML cells as a possible target for therapeutic interventions. A recent study has reported immunophenotypic changes in 91% of the AML samples upon relapse.[25] Given the instability of the immunophenotype of AML cells, loss of expression of cell surface antigens might occur as a mechanism of resistance to antibody based therapies. Therefore, patients receiving these therapies should be monitored for the development of this mechanism of resistance.

The identification of the immunophenotype of both normal and AML stem cells is not only useful for the development of new treatment or positive selection strategies, it is also a

powerful tool to determine the gene expression profile in leukemic stem cells. In the last decade various methods have been developed to quantitatively determine gene expression in a particular population. For example, gene expression in a certain population can be detected using Real-Time PCR,[31] which can only detect known genes or Serial Analysis of Gene Expression (SAGE), which also detects unknown genes.[32] In addition, methods have been developed to directly compare two different populations (e.g. normal versus leukemic stem cells). Recently, DNA micro-arrays, which can only detect known genes, have been used to compare gene expression profiles of different types of stem cells and of hematopoietic stem cells of different species.[33,34] These methods of gene expression profiling could provide useful information about gene expression or silencing in AML stem cells versus normal stem cells. This information may give us new insights into the fine line between normal and leukemic stem cell behaviour. It may also provide us with new targets for the treatment of AML.

One of the challenges for the applicability of these methods is to obtain a relatively pure stem cell population. In addition, normal and leukemic stem cells only represent a small subset of total hematopoietic cells and because expansion of these cells *in vitro* does not seem to maintain the stem cell properties, it is still a challenge to obtain enough RNA necessary for these procedures. However, considerable progress is being made to amplify RNA from extremely low amounts of RNA[35] and combined with an increasing sensitivity of the methods detection of gene expression in a small number of cells will soon be achievable.

7.2 Normal and AML Stem Cells: Telomerase and Telomeres

The enzyme telomerase is capable of maintaining telomere length, thereby preventing replicative senescence of cells.[36-38] Not surprisingly, many malignant cells, including the majority of AML cells, have found mechanisms to upregulate telomerase.[39] The marked reduction in long-term growth in our *in vivo* and *in vitro* assays indicates that telomerase inhibition decreases the proliferative potential of primitive AML progenitors. Therefore, telomerase is a potential target to eradicate AML stem cells. Although telomerase itself is not an oncogene, it can extend the replicative lifespan of cells, one of the necessities for malignant growth. Interestingly, the telomere length in malignant cells is often shorter than in normal cells upon senescence.[40] This suggests that malignant cells can escape apoptosis for a longer period of time than normal cells. However, if malignant cells can not maintain telomere length, in other words if they do not have telomerase, apoptosis occurs. Four major concerns exist with the use of telomerase inhibitors for the treatment of cancer. First, selection of resistant clones may emerge during anti-telomerase therapy and these clones may be harder to eradicate. We have observed some K562 clones transduced with the dominant negative form of hTERT (DN-hTERT), which returned to proliferate after a period of quiescence. This return of proliferation coincided with the loss of the transgene. However, we also observed one DN-hTERT transduced K562 clone, which dramatically upregulated its endogeneous telomerase activity, while the DN-hTERT transgene was still expressed. Second, an alternative telomerase independent pathway (alternative

lengthening of telomeres, ALT) to maintain telomere length has been described, suggesting that telomerase inhibition may activate telomerase independent pathways. Bryan et al. demonstrated that of the 113 tumors or tumor cell lines studied, 8 cell lines (7%) had ALT.[41] In addition, some tumors used both telomerase and ALT to maintain their telomere length. This suggests that a combination of telomerase and ALT inhibitors may be necessary for the eradication of the malignancy. Third, inhibition of telomerase can lead to telomere shortening, which in turn can cause genetic instability and chromosome fusions. Genetic instability or chromosomal abnormalities may result in the development of malignancies. Indeed, patients with the inherited syndrome dyskeratosis congenita (DKC) have a mutation in one of the components of telomerase, resulting in a deficiency of telomerase activity.[42,43] These patients have a high incidence of certain tumor types, suggesting that the absence of telomerase can cause malignancies.[44] Lastly, normal stem cells, including hematopoietic stem cells, express low levels of telomerase.[45-48] Inhibition of telomerase in stem cells may cause a lack of mature cells of certain types. For example, telomerase inhibition in hematopoietic stem cells may cause bone marrow failure. In patients with DKC, pancytopenia occurs after a median onset of 10 years, indicating that continuous administration of telomerase inhibitors may be relatively safe for several years.[49,50] However, further studies are necessary to determine the effects of telomerase inhibition on normal stem cells.

7.3 Normal and AML Stem Cells: Regulatory Pathways

One of the most important issues in stem cell biology is understanding the mechanisms that regulate the relative balance between self-renewal and differentiation. Cancer can be considered to be a disease of unregulated self-renewal. Many pathways that regulate stem cell self-renewal and differentiation are also associated with cancer. For example, Wnt, Hox, Sonic Hedgehog and Notch signalling all regulate stem cell self-renewal and lead to tumorigenesis when dysregulated. The only exception to date is the HoxB4 gene. Overexpression of HoxB4 results in a 40 fold expansion of murine hematopoietic stem cells *in vitro* and mice transplanted with these cells retain their full lympho-myeloid repopulating potential without malignant transformation.[51]

As Notch receptors are known to play a role in maintaining proliferative cell populations in the undifferentiated state, their putative role in cancer would be to prevent malignant cells from responding to differentiation signals in their immediate environment. Notch could also give malignant stem cells the ability of continuous self-renewal. Interestingly, Notch1 can also act as a tumor suppressor,[52] suggesting that there is a fine balance in Notch expression and that disturbance of this balance causes malignant transformation of the cells. Clearly, constitutive activation of Notch1 causes T cell malignancies in humans.[53,54] Notch activation can inhibit the differentiation of myeloid cells especially erythroid and monocytic differentiation and it maintains normal hematopoietic progenitors.[55-57] In search of differences between normal and AML progenitors we found that Notch receptors are abnormally expressed on AML progenitors. Primitive AML progenitors failed to downregulate Notch1 and 2, while Notch3 and Notch4 expression

appeared very high in certain patients. Further investigations in the expression of Notch in AML would be greatly facilitated by the availability of Notch receptor antibodies.

To date it is not clear whether constitutively active forms of Notch and activation of Notch by the various Notch ligands result in the same downstream effects. It appears that not all ligands have the same effect on the hematopoietic stem cells.[58] In addition, it is unknown whether the use of soluble forms of Notch ligands mediates the same downstream effects as ligands expressed on cells. It has been speculated that soluble forms may actually act as antagonists of this pathway. This knowledge would be helpful to create new ways to selectively expand normal versus leukemic stem cells.

Overall, we can conclude that normal and AML stem cells are very similar in phenotype, hampering strategies to separate them from each other. However, insights in the mechanisms that immortalize AML stem cells and give them the ability to escape regulatory differentiation and self renewal signals will give us new insights in the etiology of AML and may create new treatment strategies.

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Nederlandse Samenvatting

Acute Myeloïde Leukemie (AML) wordt gekenmerkt door een ongecontroleerde celdeling en een blokkade in de differentiatie van myeloïde cellen. Hierdoor ontstaat in het beenmerg van de patient een tekort aan de overige rijpe bloedcellen zoals rode bloedcellen, bloedplaatjes en leukocyten. De meeste patiënten met AML worden behandeld met chemotherapie al dan niet gevolgd door een allogene (van een donor) of autologe (van de patient zelf) stamcel transplantatie. Ofschoon het grootste deel van de patiënten in complete remissie raakt, keert bij de meeste patiënten de ziekte terug.

In het beenmerg vindt de aanmaak van bloedcellen, hematopoïese, plaats. Dit proces wordt in stand gehouden door een kleine populatie stamcellen. Een stamcel moet aan twee eisen voldoen. Ten eerste moet het in staat zijn zichzelf te vervangen (zelf-replicatie). Ten tweede moet een stamcel via tussenstadia van gecommitteerde voorlopercellen kunnen differentieren tot alle rijpe cellen in het bloed. Verschillende functionele testmethoden zijn ontwikkeld om hematopoïetische stamcellen en gecommitteerde voorlopercellen te bestuderen. Hematopoïetische stamcellen kunnen voor reconstitutie van hematopoïetische zorgen in (sub) letaal bestraalde immunodeficiënte dieren, zoals NOD/SCID muizen. Het is tot nu toe onduidelijk of de echte hematopoïetische stamcellen gedetecteerd kunnen worden in beschikbare *in vitro* testen. Er zijn echter kweekcondities bekend waarin primitieve cellen langdurig kunnen worden gekweekt. Gecommitteerde voorlopercellen kunnen gekweekt worden in een kolonie vormende assay. Deze functionele testen in combinatie met flowcytometrische analyse hebben aangetoond dat hematopoïetsiche stam- en voorlopercellen bepaalde antigenen op het cel oppervlak tot expressie brengen. Over het algemeen wordt aangenomen dat normale stamcellen CD34 en CD90 tot expressie brengen maar dat CD38, CD71 en andere met differentiatie geassocïeerde antigenen ontbreken. Gecommitteerde voorlopercellen zijn vaak nog positief voor CD34 maar brengen ook CD38 tot expressie. Op dit moment wordt CD34 in de kliniek gebruikt voor de purificatie van stamcellen. Er blijft echer onduidelijkheid bestaan over het fenotype van stamcellen, want onlangs zijn er studies verschenen die hebben aangetoond dat $CD34^-$ cellen primitiever zijn dan de $CD34^+$ cellen. Aangezien het gebruik van de combinatie van de bovenstaande antigenen slechts een verrijking van het percentage stamcellen geeft maar geen pure stamcel populatie oplevert, kan een hematopoïetische stamcel tot nu toe alleen functioneel gedefinïeerd worden.

AML ontstaat door een genetische mutatie in een hematopoïetische stamcel, die de leukemie kloon voortbrengt. Ondanks de afwezigheid van rijpe leukocyten is er toch een zekere mate van differentiatie van de leukemie stamcellen. Dit wijst op de aanwezigheid van een hierarchie in AML, waarin alleen de AML stamcellen verantwoordelijk zijn voor het in stand houden van de ziekte. Door het fenotype van AML stamcellen te bepalen kunnen we eventuele verschillen in het fenotype van normale en AML stamcellen gebruiken voor de ontwikkeling van methoden om autologe stamcel transplantaties te zuiveren van AML stamcellen. Als er verschillen zijn kunnen we ook nieuwe therapiëen ontwikkelen, die specifiek gericht zijn op leukemie stamcellen. Dezelfde functionele testen en flowcytometrische analyses, die gebruikt zijn om het fenotype van normale hematopoïetische stam- en voorlopercellen te bepalen, kunnen gebruikt worden om AML stam- en voorlopercellen te detecteren. Over het algemeen zijn er weinig verschillen tussen

het fenotype van normale stamcellen en leukemie stamcellen met uitzondering van het antigeen CD90 dat tot expressie gebracht wordt op normale stamcellen maar vaak niet aanwezig is op leukemie stamcellen.

Onlangs is er een nieuw antigeen ontdekt, CD133, dat aanwezig is op normale $CD34^+$ en $CD34^-$ hematopoïetische stamcellen en daarom beter zou zijn dan CD34 voor de isolatie voor normale stamcellen. Wij hebben echter aangetoond dat ook de meeste leukemie stam- en voorlopercellen CD133 tot expressie brengen, waardoor autologe stamcel transplantaties gebaseerd op CD133 selectie ook AML stamcellen kunnen bevatten.

Het CD33 antigeen wordt niet tot expressie gebracht op normale hematopoïetische stamcellen, maar is aanwezig op het oppervlak van alle myeloïde voorlopercellen en op AML cellen van 75-90% van de patiënten. Daarom wordt CD33 gebruikt als antigeen in de ontwikkeling van AML specifieke therapiëen. Wij hebben aangetoond dat CD33 meestal afwezig is op AML stamcellen. Bepaalde patiënten kunnen echter geselecteerd worden die $CD33^+$ leukemie stamcellen hebben. De meeste patiënten zullen dus weinig baat hebben bij het gebruik van CD33 specifieke therapiëen, maar voor een specifieke groep patiënten kunnen deze therapiëen erg nuttig zijn.

Telomeren bevinden zich aan het eind van chromosomen en worden gekarakteriseerd door specifieke DNA sequenties en bijbehorende eiwitten. Telomeren voorkomen genetische instabiliteit en zorgen ervoor dat chromosomen niet met elkaar fuseren. Met iedere celdeling worden telomeren korter tot een kritiek punt bereikt wordt en de cel apoptose ondergaat. Dit mechanisme zorgt ervoor dat cellen niet onbeperkt kunnen delen. Telomerase is een enzym dat in staat is om de lengte van telomeren in stand te houden. Dit is noodzakelijk in sommige cellen zoals stamcellen, die een leven lang moeten kunnen delen. Verschillende types kankercellen hebben mechanismen ontwikkeld waardoor ze telomerase tot expressie kunnen brengen. Dit heeft tot gevolg dat kankercellen schijnbaar onbeperkt kunnen delen. AML cellen van de meeste patiënten brengen ook telomerase tot expressie. Wij hebben AML cellen getransfecteerd met een dominant negatief telomerase gen met tot gevolg dat het telomerase enzym niet meer werkt. Functionele testen met deze getransfecteerde cellen hebben aangetoond dat deze AML stamcellen niet meer goed kunnen groeien en waarschijnlijk apoptose ondergaan. De remming van telomerase kan dus een goede methode zijn om AML te behandelen. Echter het effect van telomerase op normale stamcellen moet eerst goed onderzocht worden.

Ook hebben we gekeken naar de Notch receptoren in normale hematopoïese en in AML. Het is aangetoond dat activatie van de Notch receptoren leidt tot het behoud van normale stamcellen. Verder veroorzaakt constante activatie van Notch, abnormaliteiten in lymfocyten. Het is echter ook aangetoond dat activatie van de Notch receptoren kan leiden tot een blokkade in de differentiatie van myeloïde cellen. Om dit fenomeen te onderzoeken hebben we eerst een geactiveerde vorm van Notch1 en Notch4 tot expressie gebracht in $CD34^+$ geselecteerde navelstreng bloedcellen. Dit had tot gevolg dat deze navelstreng bloedcellen een hoger percentage aan primitieve cellen hadden en minder goed in staat waren om tot myeloïde cellen te differentieren in *in vitro* testen. Verder waren deze cellen

beter in staat om het beenmerg en bloed van immunodeficiënte muizen te reconstitueren. Echter alle muizen hadden afwijkingen aan de T en B lymfocyten en er waren ook minder myeloïde cellen. Het effect dat Notch activatie op normale bloedcellen heeft, bracht ons tot de hypothese dat Notch een rol zou kunnen spelen in het ontstaan van AML. Wij hebben bepaald dat Notch expressie in primitieve AML cellen anders is dan in normale cellen. Activatie van Notch door zijn ligand Jagged1 zorgt voor een hogere proliferatie van AML cellen, terwijl normal primitieve cellen juist minder prolifereren na ligatie met Jagged1. Blokkering van de Notch-Jagged1 route zou daardoor kunnen leiden tot specifieke remming van de proliferatie van primitieve AML cellen.

Wij kunnen concluderen, dat het fenotype van AML stamcellen veel gelijkenis vertoont met normale stamcellen, waardoor het moeilijk is om deze stamcellen van elkaar te onderscheiden en specifieke therapiëen tegen AML stamcellen te ontwikkelen. Het enzym telomerase is wel een goed doelwit om AML te behandelen. Nieuw routes, die zelfvernieuwing en differentiatie van normale en leukemie stamcellen reguleren, zoals de Notch route, kunnen ook leiden tot nieuwe inzichten in het ontstaan en het behandelen van AML.

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CURRICULUM VITAE

Suzanne Vercauteren is geboren op 25 oktober 1970 te Waalwijk.
In 1990 behaalde zij het VWO diploma aan het Onze Lieve Vrouwe Lyceum te Breda.
In dat jaar begon zij haar studie Medische Biologie aan de Faculteit Geneeskunde van de Universiteit Utrecht (UU).
In augustus 1990 behaalde zij haar propedeutisch examen en in 1997 haar doctoraal examen Medische Biologie.
Tijdens deze studie volbracht zij de volgende twee wetenschappelijke stages: gedurende negen maanden een onderzoek onder leiding van Prof. Dr.G.C. De Gast (UU, faculteit Geneeskunde vakgroep Hematologie) naar het effect van de co-stimulatoire antigenen B7-1 en B7-2 op T cel activatie;
daarna onder supervisie van Prof. Dr. R.K.Humphries (Terry Fox Laboratory, Vancouver, Canada), een onderzoek naar de rol van Heat Stable Antigen op B cel activatie en proliferatie.
In 1992 begon zij aan dezelfde universiteit, naast de studie Medische Biologie, ook haar studie Geneeskunde.
Het propedeutisch examen legde zij af in 1993 en in 1996 behaalde zij haar doctoraal examen Geneeskunde.

Haar co-schappen liep zij via de Universiteit Utrecht met uitzondering van Keel- Neus en Oorkunde. Dit co-schap deed Suzanne via het Massachusetts Hospital (Boston, U.S.A.).
Na het behalen van haar licentie als arts in 1999 begon zij tot mei 2003 haar promotieonderzoek naar het karakteriseren van Normale en Acute Myeloide Leukemie stamcellen onder begeleiding van Prof. Dr. A.Hagenbeek en Prof. Dr. H.J.Sutherland aan het Terry Fox Laboratory (Vancouver, Canada).
Het resultaat van dit onderzoek ligt thans voor u.
Van mei tot oktober 2003 werkte Suzanne aan de Terry Fox Laboratory als Post Doctoral Fellow onder begeleiding van Prof. C. Eaves op het gebied van humane embryonale stamcellen.
Sinds oktober 2003 is Suzanne in opleiding tot Hemato-Patholoog aan de University of British Columbia (Vancouver, Canada).

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