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Role of MKL1 in megakaryocytopoiesis and in t(1;22)-associated Acute Megakaryoblastic Leukemia

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Abstract

ROLE OF MKL1 IN MEGAKARYOCYTOPOIESIS AND IN t(1;22)-ASSOCIATED ACUTE MEGAKARYOBLASTIC LEUKEMIA. James A. Troy (Sponsored by Diane S. Krause). Department of Laboratory Medicine, Yale University, School of Medicine, New Haven, CT.

The t(1;22)(p13;q13) translocation creates a fusion of the genes RBM15 and MKL1 and is exclusively associated with the infantile form of Acute Megakaryoblastic Leukemia, or AML variant M7. Although MKL1 is a known coactivator of the Serum Response Factor (SRF) pathway, it is not known whether MKL1 plays an important role in megakaryocytic commitment or differentiation. This project investigated the role of MKL1 in normal megakaryocytopoiesis by using a human $CD34^+$ hematopoietic stem cell (HSC) model. Human $CD34^+$ HSCs were transduced with lentiviral vectors expressing either MKL1 or dominant negative MKL1 (DN-MKL1) and were cultured in conditions promoting megakaryocytopoiesis. After nine days, cells transduced with MKL1-expressing virus had increased expression of the megakaryocyte-associated markers CD41a ($52\pm10\%$ vs. $28\pm15\%$), CD61 ($56\pm15\%$ vs. $26\pm13\%$) and CD42b ($46\pm17\%$ vs. $14\pm5\%$) when compared to cells transduced with the vector alone (all $p < 0.05$). Transduction with the DN-MKL1 lentivirus was unexpectedly also associated with an increase in these same markers, though this experiment was only performed once and will require further investigation. In separate megakaryocyte progenitor assays, MKL1 was associated with an increased frequency of megakaryocyte colony forming units (CFU-Mk) (263 ± 111 vs. 164 ± 61 CFU-Mk per 10,000 cells), though these data did not reach statistical significance. These findings suggest that MKL1 may be an important regulator of megakaryocytopoiesis, and that abnormal MKL1 function may be involved in megakaryoblastic leukemogenesis.

Acknowledgment

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Introduction

Acute Megakaryoblastic Leukemia

Acute megakaryoblastic leukemia (AMKL), also referred to as acute myeloid leukemia (AML) variant M7, is characterized by infiltration of the bone marrow by 20% or more megakaryoblasts and circulation of these blasts in the peripheral blood. [1] Patients with AMKL typically exhibit the signs and symptoms of pancytopenia (anemia, thrombocytopenia and neutropenia), including fatigue, weakness, petechiae, purpura and impaired immune function. AMKL is also generally associated with extensive myelofibrosis and reticulin fiber deposition in the marrow, which can further exacerbate the symptoms related to bone marrow failure. AMKL most commonly occurs in children with one of two cytogenetic abnormalities: trisomy 21 (Down syndrome) or t(1;22)(p13;q13), a translocation involving chromosomes 1 and 22. In adults, AMKL occurs at a lower frequency and is associated with a wide range of cytogenetic changes and complex karyotypes that are different than those most commonly seen in children.

Down syndrome associated AMKL (DS-AMKL)

DS-AMKL is the most common form of AMKL. Approximately 10% of children with Down syndrome are born with a condition called Transient Myeloproliferative Disorder (TMD), in which immature megakaryoblasts are found in the liver, bone marrow and blood. [2] Although most cases of TMD resolve spontaneously, as many as

30% of these cases progress to AMKL and require medical intervention. Overall, the incidence of AMKL is approximately 500 times greater in children with Down syndrome than in the general pediatric population, and AMKL accounts for approximately 62-86% of all AML in these children. [3, 4]

Both AMKL and TMD in patients with Down syndrome are associated with various mutations of the X-linked GATA-1 gene that lead to the production of a truncated GATA-1 protein referred to as GATA-1 short (GATA-1s). [5] Investigations into the function of GATA-1 using GATA-1$^{-/-}$ embryonic stem (ES) cells and GATA-1$^{-/-}$ mice have demonstrated that the loss of GATA-1 is associated with hyperproliferation and failed differentiation of erythro-megakaryocytic precursors. [6, 7] However, DS-AMKL and non-DS-AMKL have been shown to have very different gene expression profiles, [8, 9] suggesting that these forms of AMKL may lead to megakaryoblastic leukemogenesis by drastically different mechanisms. This is further supported by the vastly improved response to therapy in DS-AMKL versus non-DS-AMKL. Down syndrome patients with AMKL have a three year survival as high as 91%, [10] a characteristic that appears to be related to a greater susceptibility of leukemic clones to conventional chemotherapeutic agents such as cytarabine. [11]

AMKL caused by the t(1;22)(p13;q13) translocation

The second most common cytogenetic abnormality in children with AMKL is the t(1;22)(p13;q13) translocation. [12, 13, 14] This form of AMKL occurs predominantly in

very young children, with a median age at diagnosis of only 6 months. [15] The t(1;22) translocation brings together the RBM15 gene on chromosome 1 and MKL1 on chromosome 22, and results in an in-frame fusion that includes all known functional domains of each constituent. [16, 17] The reciprocal MKL1-RBM15 fusion transcript is also expressed, but is only 17 or 25 amino acids in length, depending on which RBM15 isoform in involved, and is not believed to be associated with megakaryoblastic leukemogenesis.
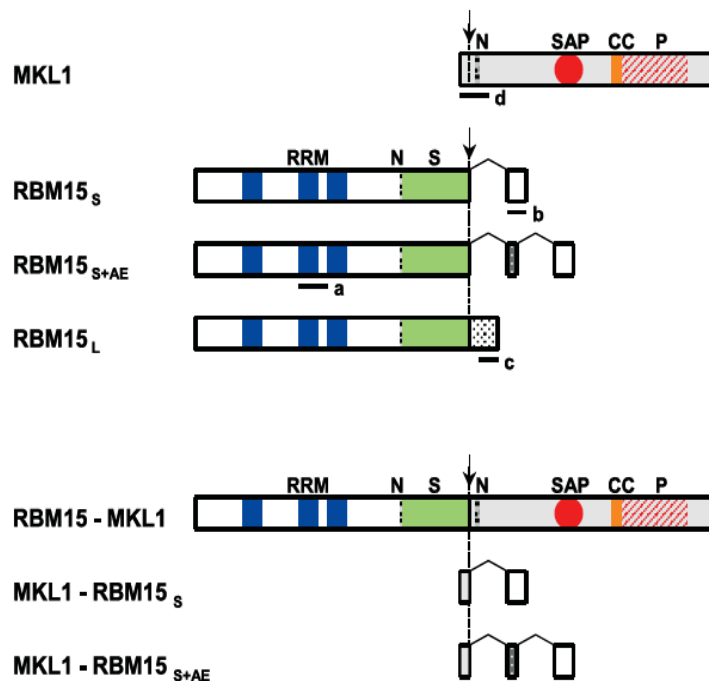
Figure 1: A depiction of RBM15, MKL1, and the corresponding fusion products formed by the t(1;22)(p13;q13) translocation.

Image from Ma, Z., Morris, SW., Valentine, V. et al. (2001). Fusion of two novel genes, rbm15 and mkl1, in the t(1;22)(p13;q13) of acute megakaryoblastic leukemia. Nat Genet 28, 220-221. [17]

RBM15

Though this project was specifically focused on the role of MKL1 in megakaryocytopoiesis, it is also worthwhile to briefly discuss its partner in the t(1;22)(p13;q13) translocation, RBM15. RBM15, also referred to as OTT (for its involvement in the one-twenty two translocation) is a member of the spen (split ends) family that is conserved from Drosophila. In Drosophila, spen is involved in the regulation of EGFR, Ras and Notch-mediated signaling pathways that all can play a role in cell-fate decisions. Notch, in particular, has been shown to have a role in the development of hematopoietic cells. When the Notch receptor is activated, its intracellular domain (NICD) is cleaved, translocates to the nucleus, and interacts with CBF1/RBP-Jκ and an associated corepressor complex. It is thought that NICD activates transcription by causing dissociation of inhibitory cofactors such as HDAC-1 (histone deacetylase) from CBF1/RBP-Jκ and, possibly, by helping to recruit other activators. [18] Notch1, one of four mammalian Notch receptors, activates transcription of HES1, which suppresses the activity of the GATA-1 complex. [19] It has been shown that two members of the spen family, SHARP (SMRT/HDAC1 Associated Repressor Protein) and MINT (which are found in humans and mice, respectively), inhibit Notch signaling by binding to RBP-Jκ. [20, 21] A study of the crystalline structure of SHARP demonstrated that the SPOC domain, which is conserved amongst all spen family genes, mediates this interaction. [22] Based on the homology between RBM15 and SHARP, it is surmised that RBM15 may also be able to inhibit the Notch signaling pathway.

Indeed, experiments conducted in the Krause lab have demonstrated a role for RBM15 in myeloid differentiation. A preliminary experiment showed that RBM15-GFP binds to RBP-Jκ and that there is no interaction between RBP-Jκ and GFP alone. Another experiment, using a HES1 driven luciferase assay, showed that 3T3 and HEL (human erythroleukemia) cells transfected with either RBM15 or RBM15-MKL1 expression cassettes exhibited increased Notch-mediated activation of the HES1 promoter. Interestingly, it was found that RBM15-MKL1 increased HES1 activity (and thus, it is presumed, GATA-1 suppression) to a greater degree than RBM15 alone. Finally, shRNA-mediated knockdown of RBM15 caused increased myeloid differentiation of 32DWT18 cell (a myeloid precursor cell line), while expression of RBM15 inhibited myeloid differentiation.[23] Taken together with the finding that GATA-1 is essential for normal megakaryocytic differentiation, this suggests that increased activity of the RBM15-MKL1 product may be involved in megakaryoblastic leukemogenesis.

MKL1

MKL1, also referred to as MAL, BSAC or MRTF-A, is a member of the myocardin family of transcription factors, which also includes myocardin and MKL2. Members of this family have 35% homology, including several highly conserved domains: RPEL repeats involved in actin binding (important for cytoplasmic versus nuclear localization, as discussed below); a basic "B box" and a glutamine-rich domain which bind Serum Response Factor (SRF); SAP (SAF-A, acinus, PIAS), which is thought

to bind matrix attachment regions (MARs) of active chromatin, though deletion has no effect on transcriptional activity in vitro; [24] a leucine zipper-like domain that may facilitate dimerization; and a C-terminal transcriptional activation domain. [25]

Investigations into the functions of the myocardin family members have largely been focused on the role of myocardin in cardiac and smooth muscle differentiation. Myocardin family members can bind Serum Response Factor (SRF) and facilitate the expression of genes that have Serum Response Elements (SRE) in their promoters. The Serum Response Element in the 5' region of these genes binds SRF. The SRE typically consist of one or more CArG (CC-AT-rich-GG) box, though SRE may also be associated with a ternary complex factor (TCF) binding site. It has been shown that p62TCF and MKL1 exhibit mutually exclusive binding to SRF, suggesting that these factors may have complementary roles in the regulation of the SRF pathway.

MKL1, MKL2 and myocardin are all regulated by a RhoA-actin pathway, which controls their cytoplasmic-nuclear translocation. In the presence of G-actin, MKL1 is retained within the cytoplasm via its actin-binding RPEL repeat domain. Activation of the RhoA pathway induces polymerization of actin into stress fibers, and it is thought that the resultant depletion of G-actin allows translocation of MKL1 into the nucleus. [26] There is evidence that phosphorylation or sumoylation of these proteins also plays a role in the regulation of this movement. [27] Studies in skeletal muscle cells have shown that nuclear MKL1 binds to SRF and activates SRE (Serum Response Element) promoters, thereby activating transcription of Immediate-Early genes (IEGs) involved in growth (e.g.

c-fos) and other genes involved in cellular differentiation and regulation of the actin cytoskeleton. [28, 29] In non-hematopoietic cells, transient transfection data show that RBM15-MKL1 product increases activation of SRE responsive genes when compared to MKL1 alone. [24]

A separate study found that BSAC, a murine homolog of MKL1, can inhibit TNF-α mediated cell death in embryonic fibroblasts. [30] This, too, could represent a mechanism by which abnormal activity of MKL1 could contribute to the development of leukemia.

Surprisingly, a study that examined the effects of MKL$^{-/-}$ knockout mice did not identify any significant hematopoietic effects, though several of these mice died in utero due to cardiac abnormalities. Additionally, all surviving MKL$^{-/-}$ female mice exhibited impaired mammary gland function due to defective myoepithelial cell development, resulting in the death of pups born to MKL$^{-/-}$ mothers. [31]

Previous findings suggest that MKL1 may have an important role in megakaryocytopoiesis in both human and murine cells. Levels of MKL1 mRNA increase in E12.5-14.5 murine fetal liver cells that are induced to differentiate along the megakaryocytic lineage, with the highest levels found in mature megakaryocytes. [32] In the human erythroleukemia (HEL) cell line, which can be stimulated to undergo megakaryocytopoiesis with 12-O-tetradecanoylphorbol 13-acetate (TPA), stable transduction of a tet-inducible promoter driving expression of MKL1 cDNA and the addition of doxycycline leads to a significant increase in the number of mature

megakaryocytes (94% vs. 42%) and an increase in the number of cells with ploidy greater than 4N. Gene expression assays using these cells identified those genes that were significantly up- or down-regulated in response to the overexpression of MKL1. These included several genes involved in cytoskeletal structure, as well as cdc2, an important regulator of megakaryocytic endomitosis. Further experimentation showed that siRNA-mediated knockdown of SRF completely abrogated the effects of MKL1. Thus, it has been demonstrated that MKL1 plays an important role in effecting megakaryocytopoiesis, and that these effects are mediated by the SRF pathway.

Hematopoietic stem cells

Hematopoietic stem cells (HSCs) are pluripotent cells that are capable of differentiation into all cell types of the hematopoietic system. In mammalian embryos, the earliest stage of hematopoiesis occurs in the yolk sac and is termed primitive hematopoiesis. At approximately six weeks of gestation, definitive hematopoiesis arises from a region of the dorsal aorta, which is thought to "seed" the liver and spleen, organs that are responsible for fetal hematopoiesis, and the bone marrow, which is the site of hematopoiesis throughout adulthood. Under disease conditions, the liver and spleen can regain some of their fetal hematopoietic functionality. HSCs are quite rare, even in the bone marrow, in which they may represent as few as 1 in 20 thousand nucleated cells. However, they can be isolated by selecting for and against various criteria. For example, HSCs are small in size, are negative for markers of lineage commitment (Lin$^-$), and

positive for CD34. Thus, a sample of bone marrow can be dramatically enriched for a population of hematopoietic precursors. These cells can likewise be isolated from umbilical cord blood or from peripheral blood. A common practice for collection of CD34$^+$ HSCs from peripheral blood is to administer several doses of G-CSF, which stimulates the mobilization of HSCs from within the bone marrow into the bloodstream. These cells are consequently called mobilized peripheral blood stem cells (PBSCs).

HSCs are most commonly isolated for use in stem cell transplants, an important therapeutic option for patients with leukemias, lymphomas, and multiple myeloma. There has also been investigation into transplant as a potentially curative treatment for patients with various non-malignant disorders including metabolic disorders, β-thalassemia, sickle cell disease, or serious autoimmune conditions. Studies have also investigated the potential role of marrow-derived stem cells in repair of ischemic damage, such as following a myocardial infarction, though it is not clear whether HSCs are the cells that are responsible for promoting repair.

HSCs also represent a highly valuable investigational tool for the study of hematopoiesis. In this project, HSCs are an ideal model to study the effects of the gene of interest, MKL1, on the process of megakaryocytopoiesis.

Megakaryocytopoiesis

Megakaryocytopoiesis is the process by which pluripotent hematopoietic cells differentiate and mature into megakaryocytes, culminating in the release of platelets. This

is a complex and tightly regulated process, essential for the maintenance of the thrombopoietic system. In general, megakaryocytopoiesis occurs in the bone marrow, However, as a few CD34^{+} hematopoietic precursors circulate in the peripheral blood, it is possible for some of these cells to engraft in organs such as the spleen and undergo extramedullary megakaryocytopoiesis. This is thought to represent a minor percentage of megakaryocytopoiesis.

One of the earliest markers of megakaryocytic progenitors is the platelet glycoprotein complex IIa/IIIb (GP IIa/IIIb). These markers are also known as CD41a (GPIIb) and CD61 (GPIIIa). CD41 is present on approximately 2% of bone marrow CD34^{+} cells. The earliest identified megakaryocytic progenitor is called the megakaryocyte high-proliferative-potential colony forming cell (Mk-HPP-CFC), which has a remarkably high proliferative capacity, forming large colonies that are visible without a microscope. [33] The Mk-HPP-CFC successively give rise to megakaryocyte burst forming units (BFU-MK) and megakaryocyte colony forming units (CFU-MK), which have progressively decreasing capacity for proliferation.

The process of megakaryocytopoiesis continues with the development of promegakaryoblasts. These cells can not yet be identified by morphologic criteria, but begin expressing additional megakaryocyte-associated markers, such as platelet peroxidase, von Willebrand factor and platelet glycoprotein Ib (also referred to as CD42b).

As the cells continue to mature, there is a progressive increase in the volume of

cytoplasm and increased numbers of platelet specific granules. Mature megakaryocytes are capable of undergoing DNA replication without cell division, a process termed endomitosis. Thus, mature megakaryocytes can acquire DNA content with a ploidy of 64N or greater. These cells are also remarkable for a very high degree of expansion of the plasma membrane, which, due to complex cytoskeletal rearrangements, forms a network called the demarcation membrane system. The cell finally forms long appendages called proplatelets, and the cell undergoes apoptosis, releasing as many as 5,000 platelets per cell. In total, this process is responsible for the production of $2x10^{11}$ platelets per day.

Statement of purpose

Acute Megakaryoblastic Leukemia with the t(1;22)(p13;q13) chromosomal translocation is a form of myeloid leukemia that affects infants and very young children. It is poorly understood how the t(1;22) translocation and its resultant fusion of RBM15-MKL1 promotes megakaryoblastic leukemogenesis. It is likely that the RBM15-MKL1 fusion product has a different activity than wildtype MKL1, possibly acting on pathways affecting megakaryoblastic differentiation and proliferation. The purpose of this project was to investigate the effects of MKL1 on normal megakaryocytopoiesis. Determining whether MKL1 plays a role in megakaryocytopoiesis is a crucial first step toward understanding the fusion protein, which could lead to the design of novel, targeted therapies for infants with AMKL.

Human $CD34^+$ hematopoietic progenitor cells were used as a model to study the effects of MKL1 on megakaryocytopoiesis. These cells were transduced with viral vectors expressing MKL1 or dominant negative MKL1 and were cultured in conditions that favor megakaryocytopoiesis. Cells were assessed for morphologic evidence of differentiation, expression of megakaryocyte-associated antigens and the presence of committed megakaryocytic progenitors.

**Transduce cells to overexpress
or block activity of MKL1
(days 1 and 2)**

MKL1 or
dominant
negative MKL1

9 days

**CD34+ hematopoietic
progenitor cells**

**Culture with thrombopoietin
(Tpo) +/- other cytokines**

**Assess effects on megakaryocytic
proliferation and differentiation
(day 9)**
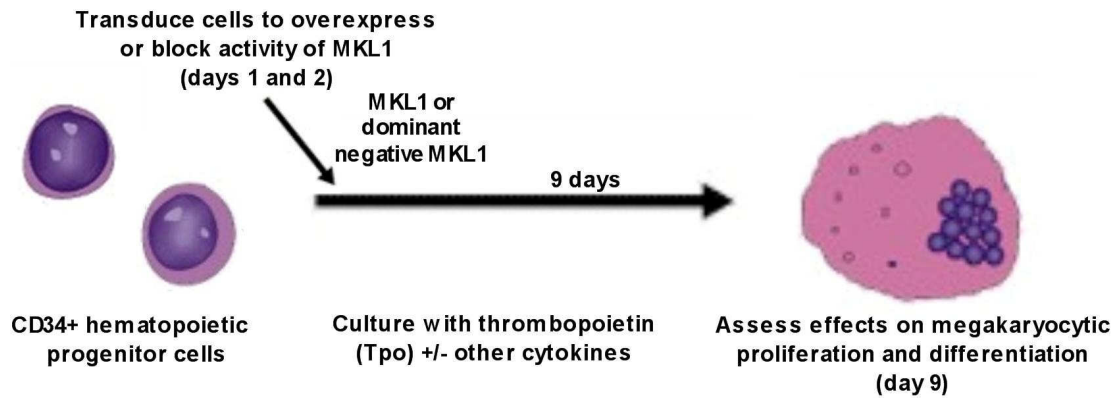
Figure 2: This schematic illustrates the overall experimental plan, in which CD34-selected human mobilized peripheral blood hematopoietic stem cells are transduced with lentiviral vectors expressing MKL1 or dominant negative MKL1, and then cultured in conditions that favor megakaryocytopoiesis. The cells are then assessed for morphologic and phenotypic evidence of megakaryocytic differentiation and maturation.

Materials and methods

Construction of retroviral and lentiviral plasmids (James Troy, Matthew Renda and Lin Wang)

Human and murine pcDNA3-MKL1 plasmids were generously provided by Stephan W. Morris, St. Jude's Children's Research Hospital.

A human dominant negative MKL1 plasmid, pCMVx3-FLAG7.1-C630, was a generous gift from Dr. Ron Prywes, Columbia University. This plasmid includes the first 630 amino acids of the hMKL1 coding sequence, and acts as a dominant negative MKL1 by suppressing MKL1 activity on SRE-containing reporters. [24] Dr. Prywes also donated pm18, a Luciferase reporter with a modified c-fos promoter containing a CArG box (a SRF binding site) but without a binding site for ternary complex factor (TCF). pm18 is one of the reporters that has been used to test dominant negative MKL1 function, and was similarly used in this work.

The lentiviral plasmid, pCCL-C-MND, was generously provided by Dr. Carolyn Lutzco, Children's Hospital of Los Angeles. [34] pCCL-C-MND includes a phosphoglycerate kinase (PGK) promoter driving expression of GFP, and a second promoter, MND, which can be used to express an inserted coding sequence. This MND-driven site was used to create MKL1 and dominant negative MKL1 expressing lentiviral vectors.

A retroviral MKL1 vector, MigR1-human C630, was made by digesting

pCMVx3-FLAG7.1-C630 with Ssp I and Hinc III, digesting MigR1 (MSCV-IRES-GFP, a vector derived from murine stem cell virus) with Hpa I, and ligating the appropriate fragments with T4 DNA ligase. Subsequent sequencing identified a known 150 bp deletion within the C630 coding region, present on the parent vector.

pCCL-human C630 was prepared by digesting both pCCL-C-MND and MigR1-human C630 with EcoRI and ligating the appropriate fragments.

A murine dominant negative MKL1 variant was designed by performing two successive mutagenesis reactions on MigR1-murine MKL1, which includes an in-frame C-terminal His tag. First, a Bsi WI site was created at approximately position 630. A second Bsi WI site was added at the end of the MKL1 coding sequence, but prior to the His tag coding sequence. This plasmid was digested with Bsi WI to excise the region between the two Bsi Wi restriction sites and religated with T4 DNA ligase. This brought about a truncated MKL1 coding sequence but kept the His tag sequence in frame.

MigR1-human MKL1 was initially difficult to clone directly, so it was made by a complicated strategy that started with MigR1-FLAG-C630 plasmid, a dominant negative construct that includes a truncated MKL1 sequence. MigR1-FLAG-C630 and pcDNA-FLAG-human MKL1 were digested with Sfi I and Bam HI in order to append the latter portion of the MKL1 coding sequence onto the dominant negative construct. However, this has a 150 bp deletion within the MKL1 sequence, as noted above. Thus, a multiple cloning site (MCS) was inserted upstream of MKL1, and Mlu I (within the MCS) and Sfi I were used to excise the first portion of the MKL1 coding sequence, which included the

deletion, and this was replaced using a different fragment from pcDNA-FLAG-human MKL1.

To prepare the MKL1-expressing lentivirus, pcDNA-FLAG-human MKL1 was digested with Bam HI, and Klenow fragments were used to create blunt ends. These blunt ends were ligated with an Eco RI linker. The resulting plasmid was digested with Eco RI and ligated to an Eco RI digested fragment from the pCCL-C-MND lentiviral vector.

A murine MKL1 lentivirus was prepared in a similar manner as the human version described above. The pCCL-C-MND lentiviral vector was digested with Eco RI and Klenow fragments were used to create blunt ends. The lentiviral vector backbone was ligated to a Pme I digested insert from the pcDNA-murine MKL1 plasmid.

Luciferase assays

Several dominant negative MKL1 plasmids were obtained or produced in order to assess the effects associated with blocking MKL1 activity. The pcDNA-hMKL1 C630 plasmid was generously provided by Dr. Ron Prywes, Columbia University. C630 includes the first 630 bases of the human MKL1 coding sequence. As described above, the MKL1 C630 coding sequence was cloned into MigR1 to create a retroviral vector. A murine dominant negative vector was also designed by truncating the MigR1-mMKL1 coding sequence at the equivalent position in the murine MKL1 coding sequence.

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS), 2 mM L-glutamine (Gibco) and penicillin/streptomycin

(Gibco). When the cells reached greater than 90% confluence, the media was changed to antibiotic-free DMEM plus 10% FBS and 2mM L-glutamine. Cells were transfected with the pm18 Luciferase reporter, the PRL-TK Renilla control, and varying combinations of the pcDNA-hMKL1 and dominant negative MKL1 constructs described above. The pcDNA backbone was added to normalize the total amount of DNA in each sample. The transfection was performed in the presence of Lipofectamine 2000 and Opti-MEM for a total incubation of 5 hours. After transfection, the cells were allowed to recover overnight in DMEM plus FBS and L-glutamine. This was followed by a period of serum starvation, in which the cells were incubated in DMEM plus 0.2% FBS and L-glutamine for 24 hours, to block early serum-stimulation of the RhoA actin pathway. Finally, cells were placed in DMEM plus 20% FBS for three hours.

To prepare the samples for the Luciferase assay, cells were lysed with the Luciferase assay lysis buffer and briefly frozen at -80º C. The assay was performed on an automated luminometer, which adds 100 mcL successively of each of the Firefly Luciferase and Renilla substrates (Biotium) and measures the light output at the appropriate intervals. These experiments were performed in triplicate and p values were calculated by performing unpaired t tests on the relative Luciferase/Renilla values.

Production and purification of retroviral and lentiviral vectors

Retroviruses and lentiviruses were prepared as vectors for the delivery of MKL1 and dominant negative MKL1 expressing cDNA. To make Mig-MKL1, Mig-DN-MKL1,

and the Mig R1 control, these plasmids were cotransfected with the PCL Ampho packaging vector into 293FT cells in Opti-MEM media supplemented with Lipofectamine 2000. The cells were incubated for 5 hours, after which the media was changed, and the cells were subsequently incubated in DMEM plus 10% FBS, L-glutamine and penicillin/streptomycin. Starting on the day after transfection, the virus-containing supernatant was collected for 3 days. The supernatant was filtered through a 0.45 um syringe filter (to remove cellular debris) and concentrated in a centrifugal filter unit (Amicon Ultra-15) at 4000 rpm in a Sorvall RT7 centrifuge for approximately 20 minutes at 4º C. This concentrated viral supernatant was frozen at -80º C. A sample of the virus was titrated by a titered transduction of HeLa cells, assessed for GFP expression by FACS.

Lentiviral concentrates were formed in a similar manner to make pCCL-MKL1, pCCL-DN-MKL1, and the pCCL vector control. These lentiviral plasmids were cotransfected into 293FT cells with the packaging, rev, and vsv-g plasmids in Opti-MEM and Lipofectamine 2000, as described above. Supernatant was likewise collected each day for the following three days. However, the lentiviral supernatant was filtered through a large-volume 0.45 um bottle-top flask (Sigma-Aldrich) and concentrated by ultracentrifugation in a Beckman SW41 rotor at 25,000 rpm for 2 hours at 4º C. After forming a viral pellet, the supernatant was removed, the virus was resuspended and frozen as described above. The lentiviral preparations were also titered in HeLa cells.

CD34$^+$ HSC collection (Wendy Haskell)

Healthy human donors were injected with G-CSF to mobilize CD34$^+$ HSCs. Peripheral blood mononuclear cells were collected by apheresis for the primary purpose of HSC transplantation. CD34$^+$ cells were isolated by immunomagnetic selection, using the Baxter Isolex Cell Separation Device (Miltenyi Biotec). These cells were cryopreserved in 10% DMSO. The use of human subjects was approved by the Yale Institutional Review Board (IRB). Surplus cells from these collections were used in the following experiments.

Thawing CD34$^+$ HSCs (adapted from Stem Cell Technologies protocol)

Frozen vials of CD34$^+$ HSCs were thawed quickly in a 37º C water bath. Cells were transferred to a 50 mL conical tube (Falcon). 15 mL of Iscove's modified Dulbecco's medium (IMDM) plus 2% FBS and penicillin/streptomycin (Gibco) were added dropwise. The tube was subsequently filled to 50 mL with the same media and gently mixed. The cells were pelleted by centrifugation at 1000 rpm for 10 minutes. Supernatant was removed and the cells were resuspended at a concentration of 100,000 cells/mL in serum free expansion media (SFEM, Stem Cell Technologies) plus penicillin/streptomycin and various cytokines.

Determination of optimal cytokines to induce human mobilized peripheral blood CD34^{+} cells to undergo megakaryocytopoiesis

CD34^{+} cells, as described above, were cultured in various cytokine combinations in order to determine the ideal conditions for megakaryocytopoiesis. The following cytokine combinations were tested: 100 ng/mL thrombopoietin alone; 100 ng/mL thrombopoietin plus 50 ng/mL stem cell factor; and 100 ng/mL thrombopoietin, 50 ng/mL stem cell factor (SCF) and 0.01 ng/mL IL-3 (all cytokines purchased from Peprotech).

Proliferation of these cells was assessed every 2-3 days by staining non-viable cells with Trypan Blue and counting the remaining cells on a hematocytometer. Cytospins were performed to assess the degree of megakaryocytic differentiation in response to culture with various cytokines. CD34^{+} cells were adjusted to a concentration of 10,000-20,000 cells / 100-200 mcL in SFEM media. These samples were placed onto glass microscopic slide by centrifugation in the Cytospin apparatus at 500 rpm for 7 minutes. Slides were air dried, then stained with Wright-Giemsa. The cytospins were examined using an Olympus BX51 microscope, and images were collected with a Cooke Sensicam QE camera. Uniform segments of each slide were counted for the total number of cells and the total number of megakaryocytes under each culture condition.

FACS experiments were also performed to assess the expression of megakaryocyte-associated markers. Samples composed of 100,000-200,000 cells were washed with FACS buffer plus 10% FBS, and distributed into a V-well plate. Samples

were incubated with anti-CD32 to block non-specific binding, washed again, and then incubated with various combinations of differentiation-specific antibodies, including anti-CD41a, anti-CD42b, anti-CD61, anti-CD13 and anti-CD33. The corresponding immunoglobulin isotypes were used as controls. Cells were analyzed by FACS using a FACSCalibur and the FlowJo flow cytometry software.

Comparison of retroviral and lentiviral transduction efficiency in human CD34^{+} cells

Human mobilized peripheral blood CD34^{+} cells were thawed as previously described and cultured in SFEM (StemCell Technologies) plus 100 ng/mL TPO (Peprotech), 50 ng/mL SCF (Peprotech), 2 mM L-glutamine (Gibco) and penicillin/streptomycin (Gibco). On days 1 and 2, cells were transduced with the GFP-expressing MigR1 virus, GFP-expressing pCCL lentivirus, or underwent a mock transduction, by spinfection in a Sorvall RT centrifuge at 1000 rpm for 2 hours at 32º C in the presence of 8 ng/mL polybrene. After spinfection, the media was changed and the cells were allowed to recover overnight. Cells were subsequently analyzed by FACS for the expression of GFP, to determine whether the retroviral or lentiviral vectors were optimal for transduction of these cells.

Comparison of polybrene and Lipofectamine 2000 as transduction enhancing agents

There is evidence in the literature that Lipofectamine 2000 may serve as a better transduction-enhancing agent than polybrene for human CD34^{+} cells. [35] Thus, we

performed a direct comparison of polybrene and Lipofectamine 2000, using the pCCL-dominant negative MKL1 virus. Two-day differentiated human mobilized peripheral blood CD34^{+} cells were distributed evenly (approximately 230,000 cells/well) into four separate wells of a 12-well plate. Three wells received aliquots of pCCL-DN-MKL1 virus. The final well received an equivalent volume of DMEM plus 10% FBS, to serve as a mock transduction control. All wells were adjusted to include 100 ng/mL TPO, 50 ng/mL SCF, 2 mM L-glutamine and penicillin/streptomycin. Separate wells received either polybrene (at a final concentration of 8 ng/mL) or Lipofectamine 2000 (either 1 mcg or 10 mcg in a total volume of 1 mL). Samples were centrifuged at 2000 rpm for 2 hours at 37º C. After spinfection, the media was replaced with fresh SFEM plus TPO, SCF, L-glutamine, and penicillin/streptomycin at the concentrations listed above. After 2 days, cells were assessed by FACS for the expression of GFP, and data was analyzed with the FlowJo flow cytometry software.

FACS Analysis of MKL1 and dominant negative MKL1 transduced cells (James Troy and Matthew Renda)

Human mobilized peripheral blood CD34^{+} cells were thawed as previously described and cultured in SFEM (StemCell Technologies) plus 100 ng/mL TPO (Peprotech), 50 ng/mL SCF (Peprotech), 2 mM L-glutamine (Gibco) and penicillin/streptomycin (Gibco). On day 2, the cells were pelleted, resuspended in a total volume of 1.5 mL, and distributed into 3 wells of a 12-well plate. Each well received

either pCCL, pCCL-MKL1, or pCCL-DN-MKL1 viral supernatant, or an equivalent volume of DMEM. Transductions were performed at a multiplicity of infection (MOI) of approximately 50-100, except for pCCL-DN-MKL1, which had MOI of only approximately 5 due to lower viral yields. Cytokines, L-glutamine and penicillin/streptomycin were present in the same concentrations as listed above. Polybrene was added at a concentration of 8 ng/mL, and plates were "spinfected" in a tabletop centrifuge at 1000 RPM, 32° C, for 2 hours. An additional 2 mL of complete medium was added and the cells were incubated for at least 10 hours to recover. A second spinfection was then performed as described above, after which the cells were placed in fresh medium at a concentration of approximately 100,000 cells/mL.

On day 9, the cells were prepared for FACS analysis, to determine the expression of megakaryocyte-associated markers and the expression of other lineage defining markers. Cells were incubated with anti-CD32 to block non-specific binding, and then incubated with anti-CD41a, anti-CD42b, anti-CD61, anti-CD13 and anti-CD33. Cells were counted on a BD FACSCalibur and analysis was performed using the FlowJo flow cytometry software.

Cells from the same donor were used for all repeats of this experiment, to avoid potential differences associated with different individuals.

Assessment of the effect of Retronectin on the efficiency of lentiviral transduction into CD34$^+$ cells

In one experiment, a direct comparison was made to determine the potential benefit of using Retronectin-coated plates for lentiviral transduction into CD34$^+$ cells. A 50 mcg/mL solution of Retronectin was prepared and added to one well of a non-tissue culture treated plate. After incubation for 2 hours at 25º C, the Retronectin solution was replaced with a 2% BSA for another 30 minutes. The plate was then washed with PBS and stored at 4º C until use. The transduction protocol was otherwise performed as described above.

Megakaryocyte progenitor assays (James Troy and Matthew Renda)

CD34$^+$ cells were thawed, cultured and "spinfected" as described above with pCCL and pCCL-MKL1, except that on day 4, cells were underwent FACS sorting to isolate GFP$^+$ expressing cells.

Cells were pelleted and resuspended in 5 mL PBS plus 0.5% FBS, filtered through a coarse filter to remove cell clumps, pelleted in a FACS tube, and resuspended in approximately 500 mcL PBS plus 0.5% FBS. The samples were subsequently kept on ice, and sorted for GFP$^+$ cells by the flow cytometry technician using a Dako MoFlo cell sorter. The cells were resuspended in the complete medium described above.

On day 5, sorted cells were used to perform a MegaCult-C assay. Cell concentrations were adjusted to give either 5,000 or 20,000 cells per chamber. The

medium was IMDM plus 50 ng/mL rhTPO, 10 ng/mL rhIL-6 and 10 ng/mL rhIL-3.
Slides were incubated at 37° C for 12 days.

Results

Assessment of dominant negative MKL1 suppression of SRE activation

Three different dominant negative MKL1 constructs, pcDNA-hMKL1 C630, MigR1-hMKL1 C630, and MigR1-mMKL1 C630 were assessed for ability to suppress activity of MKL1. pm18, a modified c-fos promoter was used as the reporter in Luciferase assays because the c-fos promoter contains a Serum Response Factor binding site. As shown in Figure 3, transient transfections of three dominant negative constructs had the ability to significantly suppress the activity of both exogenous and endogenous MKL1. In particular, the pcDNA-hMKL1 C630 construct reduced the activity of exogenously added MKL1 by 96% ($p < 0.0001$). The human and murine MigR1 dominant negative constructs reduced activity by 20% ($p < 0.05$) and 36% ($p < 0.005$), respectively. In the absence of exogenous MKL1, I also assessed the effect of dominant negative MKL1 on endogenous MKL1 activity. pcDNA-hMKL1 C630 reduced the activity of endogenous MKL1 by 87% ($p < 0.005$), compared to 76% ($p < 0.005$) and 86% ($p < 0.005$) seen with the human and murine MigR1 samples.

A similar experiment was conducted to test the effects of these three dominant negative constructs on the activity of murine MKL1. As shown in Figure 4, pcDNA mMKL C630 was highly effective at suppressing the activity of exogenous mMKL1 on the pm18 reporter. However, in this setting, the MigR1 dominant negative constructs (including the murine MKL1 C630) did not significantly block the activity of mMKL1.
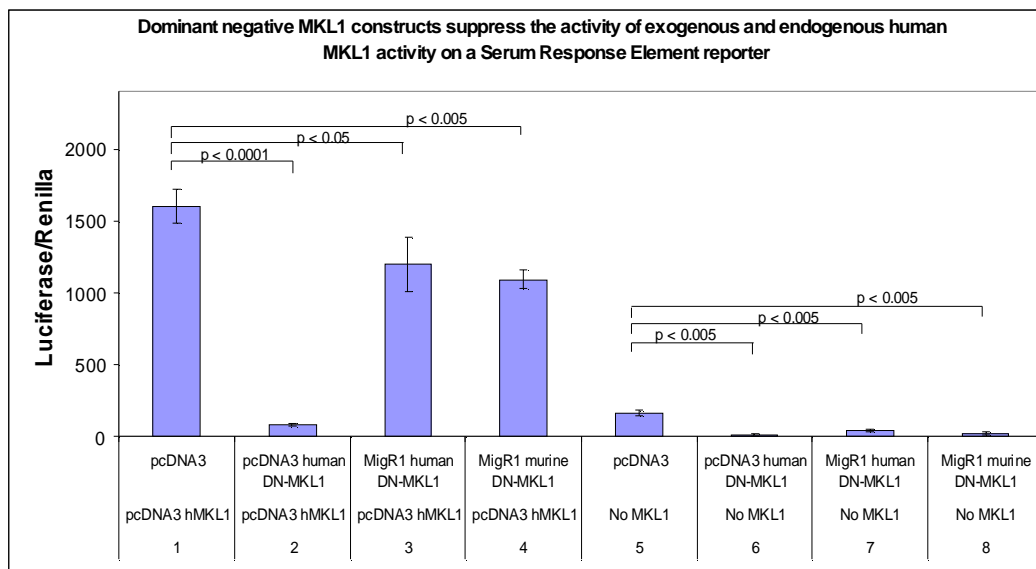
Figure 3: Comparison of the ability of three human dominant negative constructs to suppress the activity of exogenous (columns 1-4) and endogenous (columns 5-8) human MKL1 on a Serum Response Element reporter. The Firefly luciferase readings were normalized against a Renilla control.
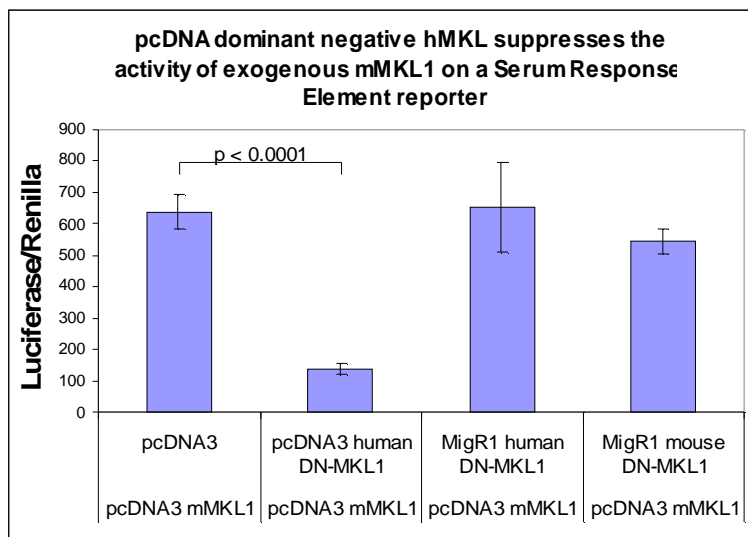

Figure 4: Luciferase assays were also performed to compare the ability of the three dominant negative MKL1 constructs to suppress the activity of exogenous murine MKL1. The Firefly luciferase readings were normalized against a Renilla control.

HSC culture and differentiation

Human CD34$^+$ hematopoietic progenitor cells were cultured in media containing various combinations of thrombopoietin (TPO), stem cell factor (SCF) and interleukin-3 (IL-3) in order to determine the ideal conditions in which to induce these cells to undergo megakaryocytopoiesis. The use of TPO alone yielded the highest proportion of megakaryocytes after 9 days, as measured by the expression of megakaryocyte-associated markers (such as CD41/61, found on immature and mature megakaryocytes) and by morphology, which assesses only mature cells. For example, in one experiment, 50% of cells cultured in thrombopoietin alone were CD41$^+$, compared to only 28% of cells cultured with TPO plus SCF, or TPO, SCF and IL-3. However, the cells cultured in TPO alone had the lowest degree of expansion. The addition of SCF yielded a lower percentage of megakaryocytes but increased numbers of cells, due to either increased proliferation or survival. Cells cultured with TPO plus both SCF and IL-3 had the greatest degree of expansion and the lowest proportion of megakaryocytic differentiation. FACS results, cell counts, and representative images from day 9 cytospins are shown in Figure 5. Results of these studies are further summarized in Table 1.
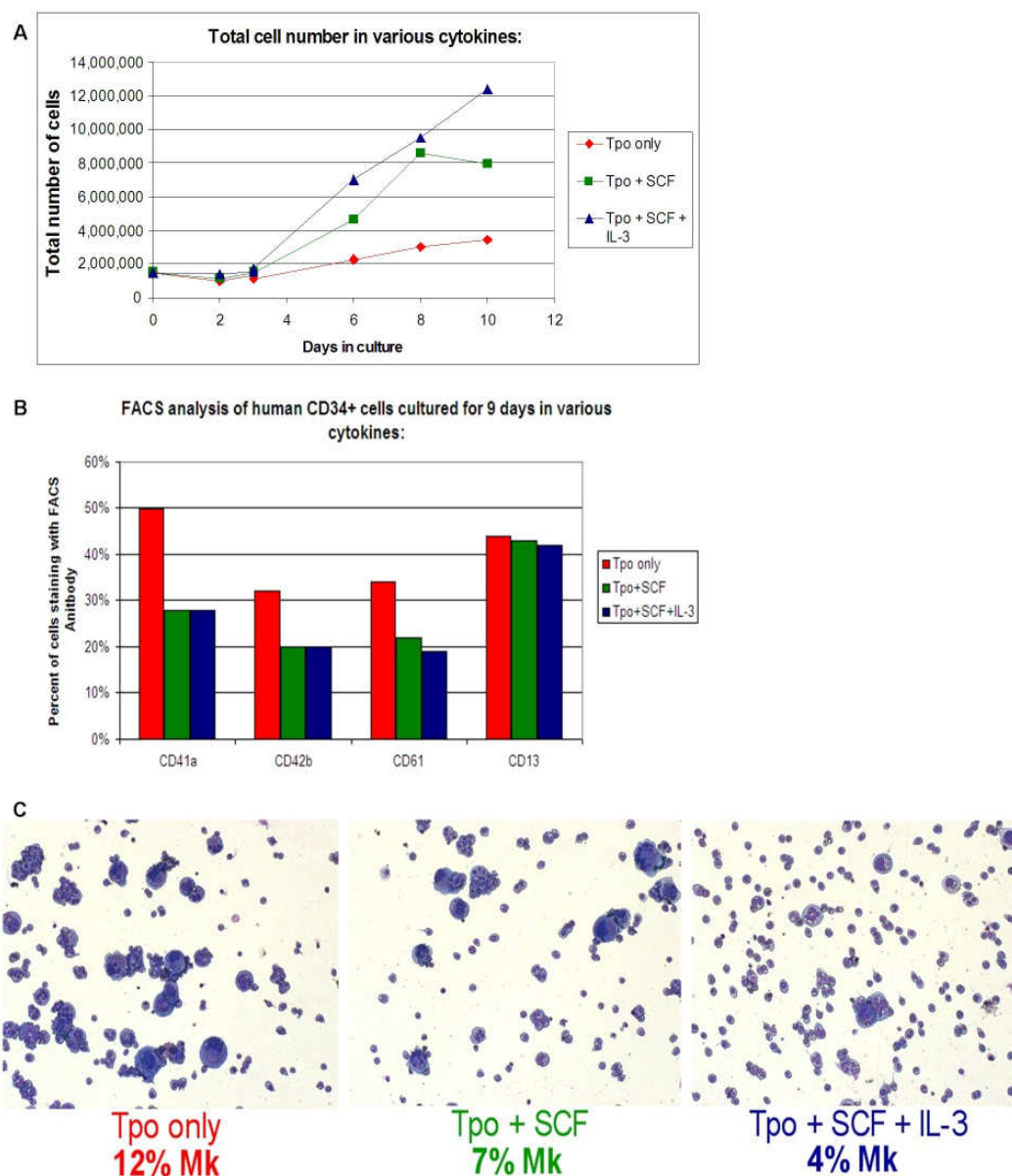
Figure 5: Proliferation and megakaryocytic differentiation of human CD34$^+$ cells in various cytokines. A. Cells were counted at regular intervals to assess the degree of proliferation. B. Flow cytometry was performed to determine the expression of megakaryocyte-associated markers CD41a, CD61 and CD42. CD13 was used to assess myeloid cell differentiation. C. Cytospins were prepared and examined microscopically to assess the degree of megakaryocytic maturation. Tpo = thrombopoietin, SCF = stem cell factor, IL3 = interleukin-3.

Cytokines	Percentage megakaryocytes, as assessed by:				Total number of cells	Absolute number megakaryocytes, as assessed by:	
	CD41a	CD61	CD42b	Morphology		FACS markers (range)	Morphology
TPO	50%	32%	32%	12%	3.4×10^6	$1.1\text{-}1.7 \times 10^6$	0.41×10^6
TPO+SCF	28%	22%	20%	7%	8.0×10^6	$1.6\text{-}2.2 \times 10^6$	0.56×10^6
TPO+SCF+IL3	28%	19%	20%	4%	12×10^6	$2.4\text{-}3.5 \times 10^6$	0.50×10^6

Table 1: Summary of megakaryocytic differentiation and proliferation of human CD34+ cells cultured in various cytokine combinations. CD41a, CD61 and CD42b were used to assess both immature and mature megakaryocytes, while cells were examined by microscope to count the frequency of mature megakaryocytes. TPO = thrombopoietin, SCF = stem cell factor, IL3 = interleukin-3.

Lentivirus is more effective than retrovirus for the transduction of human CD34$^+$ HSC

Human CD34$^+$ peripheral blood stem cells were transduced with GFP-expressing lentivirus (pCCL) or retrovirus (MigR1) to determine which vector is more effective in these cells. Cells were assessed by FACS to determine the percentage of GFP$^+$ cells, using cells which underwent a mock transduction as the control. Transduction with pCCL was associated with GFP expression in 27% of cells, while MigR1 only transduced 6% of cells.
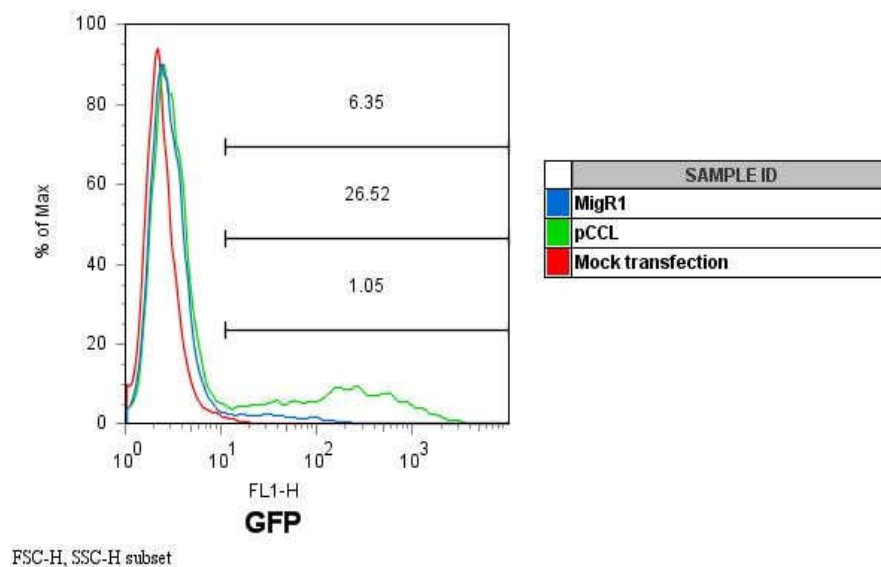
Figure 6: Comparison of the transduction efficiencies of MigR1 and pCCL viral vectors into human CD34$^+$ hematopoietic stem cells. Cells were analyzed by flow cytometry for the expression of GFP, illustrating a superior transduction efficiency of the pCCL lentivirus.

Retronectin does not increase the lentiviral transduction efficiency of CD34$^+$ cells

Retronectin is thought to improve viral transduction efficiency by binding both viruses and cells, and bringing these into close proximity. [36] A direct comparison was performed to determine if Retronectin is beneficial for the transduction of VSV-G pseudotyped lentivirus into peripheral blood CD34$^+$ cells. CD34$^+$ cells were transduced with pCCL-MKL1 using identical protocols, except some cells were placed in a Retronectin-coated well. As shown in Figure 7, the transduction efficiency in the presence of a Retronectin-coated well was approximately 12%, compared to greater than 15% without the addition of Retronectin.
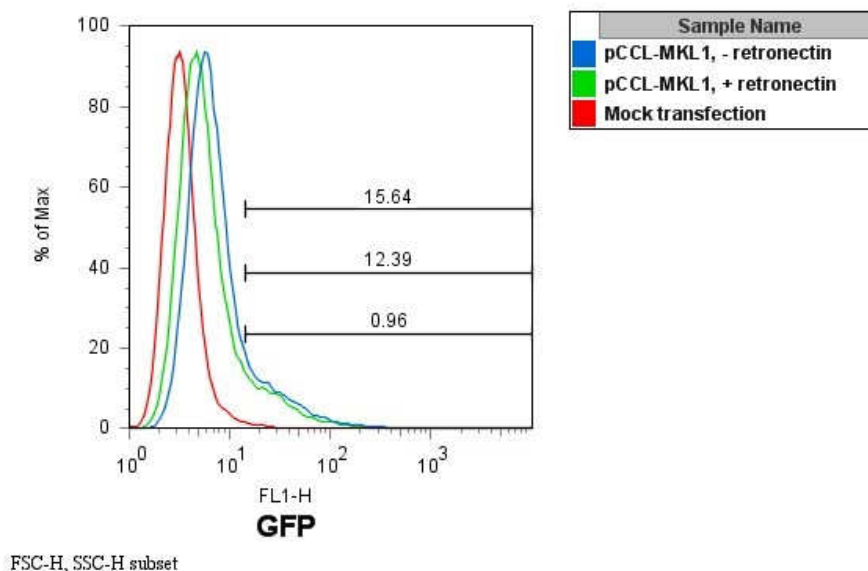
Figure 7: Human CD34$^+$ cells were transduced with lentivirus, with and without the presence of Retronectin. Flow cytometry was performed to assess the expression of GFP, and to determine whether Retronectin was beneficial in enhancing the transduction efficiency in these experiments. Retronectin was not found to be beneficial for lentiviral transduction of human PBSCs.

Polybrene enhances lentiviral transduction of human CD34^{+} cells

Human CD34^{+} cells were transduced with the pCCL-dominant negative MKL1 virus using either polybrene or Lipofectamine 2000 as a transduction-enhancing agent. Additionally, Lipofectamine 2000 was tested at two different concentrations. In Figure 8, it is shown that the highest transduction efficiency, 12%, was obtained with the use of polybrene. The Lipofectamine 2000 samples had 9% and 7% efficiencies at the low and high doses, respectively.
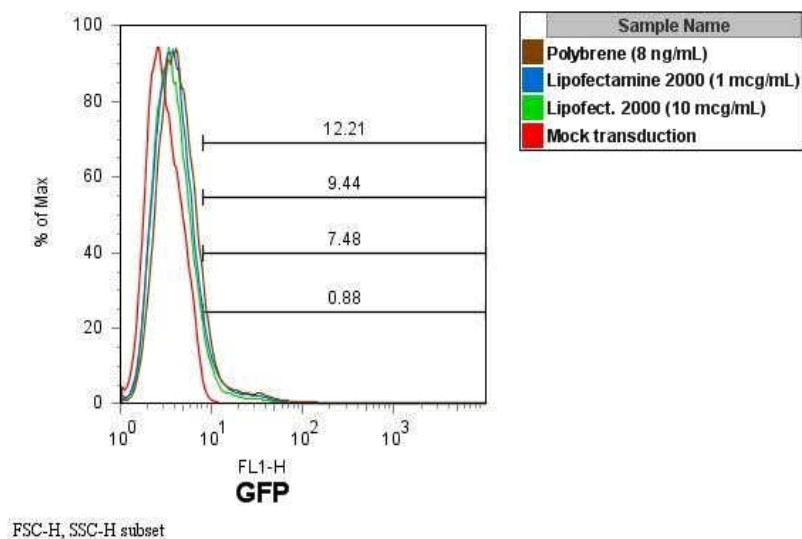

Figure 8: Two polycations, polybrene and Lipofectamine 2000, were compared as "transduction-enhancing agents" for the lentiviral transduction of human CD34^{+} cells. Polybrene was associated with a higher transduction efficiency than Lipofectamine 2000. Note that higher concentrations of Lipofectamine 2000 were also inversely correlated with lower rates of transduction.

FACS Assays

Human peripheral blood CD34$^+$ cells were transduced with either pCCL, pCCL-MKL1, pCCL-DN-MKL1 or underwent a mock transduction. On day 9, FACS was performed to assess the expression of megakaryocyte-associated antigens such as CD41a, CD42b and CD61. As shown in Figure 9, transduction with the MKL1-expressing virus was associated with an increase in expression of each of these megakaryocytic markers when compared to the pCCL control. For example, the pCCL-MKL1 transduced cells were, when averaged across four experiments, 52±10% CD41a$^+$, 46±17% CD42b$^+$ and 56±15% CD61$^+$. In contrast, the pCCL cells were 28±15% CD41a$^+$, 26±13% CD61$^+$ and 14±5% CD42b$^+$. P values for each of these markers were < 0.05, using paired t tests for the four pairs of parallel samples. In one experiment, dual staining with both CD41a and CD61 also demonstrated that these cells were almost entirely dually positive, consistent with the heterodimeric fibrinogen receptor that these factors are known to form.

Surprisingly, cells transduced with pCCL-DN-MKL1 had a similar, if not greater, increase in megakaryocyte antigen expression, though due to poor transduction efficiencies, this experiment was only performed once. In this single experiment, the GFP$^+$ fraction was also 65% CD41a$^+$, 68% CD42b$^+$, and 92% CD61$^+$ (data not shown), greater than pCCL or pCCL-MKL1 transduced cells that were incubated and prepared in parallel. As this experiment was only performed once, it will require further investigation.
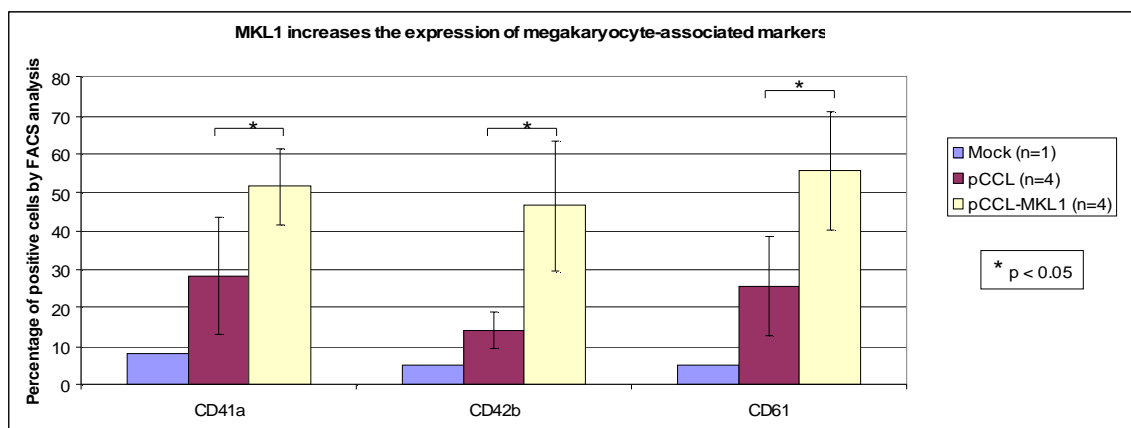
Figure 9: Several experiments were performed to determine the effects of MKL1 and dominant negative MKL1 on megakaryocytopoiesis in human CD34$^+$ hematopoietic stem cells. Cells transduced with either pCCL-MKL1 lentivirus were compared to both a "mock" control and to cells transduced with the lentiviral vector alone. The pCCL-MKL1 transduced cells consistently had greater expression of megakaryocyte-associated markers than the pCCL control.

Megakaryocyte progenitor assays

We assessed the effect of MKL1 on differentiation of megakaryocytic progenitors from human CD34$^+$ peripheral blood stem cells. CD34$^+$ cells were transduced with lentiviral vectors expressing either GFP or both GFP and MKL1. In four different experiments, transduction with pCCL-MKL1 was associated with an increase in the number of megakaryocytic colony forming units (CFU-Mk), compared to cells transduced with pCCL alone, though this finding did not reach statistical significance. pCCL-MKL1 was also associated with a trend decrease in the number of non-

megakaryocytic CFU. Surprisingly, both pCCL and pCCL-MKL1 were associated with greater numbers of CFU-Mk in comparison to cells which underwent a mock transduction. As an additional control in some experiments, the GFP⁻ fraction from the sorts were also used in some of the CFU-Mk assays. Although transduction with pCCL-MKL1 was associated with significant increase in CFU-Mk versus the GFP⁻ cells, the same effect was seen with the cells transduced with pCCL only. Some unsorted ("pre-sort") cells were also tested, to determine the effects of sorting on the survival and/or differentiation of megakaryocytic progenitors. In general, the process of sorting was associated with a decrease in the total numbers of CFU-Mk.
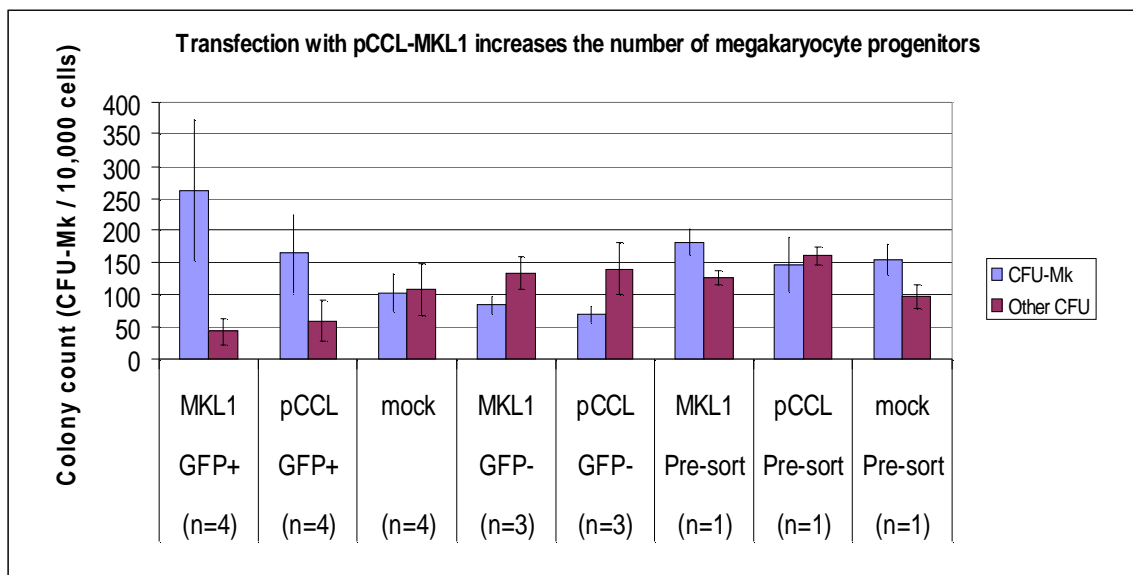
Figure 10: Human CD34⁺ hematopoietic stem cells were transduced with pCCL-MKL1 or pCCL, and sorted for GFP-expressing cells as indicated. These cells were placed into megakaryocyte progenitor assays and cultured for 12 days. Slides were then stained and read for the presence of megakaryocyte colony forming units (CFU-Mk) and other colonies. In four different experiments, the MKL1-expressing cells yielded greater numbers of megakaryocytic progenitors compared to cells transduced with pCCL alone.

Discussion

The goal of this project was to determine the role of MKL1 in the process of megakaryocytic differentiation, which is an important step toward understanding the mechanism by which RBM15-MKL1 is involved in megakaryocytic leukemogenesis in t(1;22)(p13;q13)-associated AMKL. To do so is essential for the development of novel targeted therapies for infants with this particularly aggressive form of leukemia. A human CD34$^+$ hematopoietic progenitor cell model was developed and used to demonstrate that MKL1 indeed promotes megakaryocytopoiesis.

Much of the initial effort in this project was focused on the design and construction of various viral vectors. Several of the plasmids used in this work, such as human and murine pcDNA3-MKL1, pCMVx3-FLAG7.1-C630 (dominant negative MKL1), and the pCCL-C-MND lentiviral vector, were donated from other laboratories. These plasmids were used to construct a complete set of vectors including all permutations of human and murine, retroviral and lentiviral, and wild-type and dominant negative negative MKL1. Luciferase assays demonstrated that the pcDNA3 and MigR1 DN-MKL1 constructs suppress activity of exogenous and endogenous MKL1 on the pm18 reporter. One surprising finding was that the pcDNA3-DN-MKL1 was much more effective at suppressing exogenously-added pcDNA3-hMKL1 than either the human or murine MigR1 DN-MKL1 constructs. This difference may have to do with different levels of cDNA expression between the pcDNA3 or MigR1 plasmids.

Another major component of this work was development of the human CD34$^+$ hematopoietic progenitor cell model. There are several published studies which have sought to determine the optimal conditions for inducing CD34$^+$ cells to undergo megakaryocytopoiesis, adjusting factors such as cytokines and temperature. For example, some researchers have attempted to use combinatorial techniques to evaluate the roles of numerous cytokines, such as thrombopoietin, stem cell factor, Flt-3 ligand, and many others. [37] However, these studies have not always agreed on the ideal combination of cytokines. Furthermore, different investigators have used different sources of hematopoietic stem cells, which may have different responses to equivalent cytokines. For example, there are several very significant differences between HSCs derived from bone marrow versus peripheral blood or umbilical cord blood, and these differences may impact the cells' ability to differentiate along the megakaryocytic lineage. Though some reports indicate the ability to create a highly pure population of megakaryocytes using thrombopoietin alone, [38] this sort of result has not been published using mobilized peripheral blood HSCs. Our findings were that thrombopoietin alone yielded the highest proportion of megakaryocytes, but even so, the total percentage of cells which resembled megakaryocytes by morphology or expressed megakaryocyte-specific markers was significantly below some published results using other sources of hematopoietic progenitor cells. Moreover, the majority of cells under our culture conditions were positive for CD13 and CD33, which are markers of myeloid cells, generally not seen on mature megakaryocytes. This is consistent with the finding that CD34$^+$ cells from

peripheral blood are much more often CD13^{+} and CD33^{+} than CD34^{+} cells obtained from bone marrow. [39] This may be related to the method of mobilization in which donors are given several doses of G-CSF.

One of the challenges with the CD34^{+} cells was achieving a satisfactory transduction efficiency. A major decision was whether to use a Moloney Murine Leukemia Virus (MMLV), such as MigR1, or lentivirus as the vector. MMLV, also referred to as oncoretroviruses, were the first form of viruses used for transduction of hematopoietic cells. However, these viruses have a major limitation: they require that a cell be undergoing cell division for nuclear import and integration of the transgene. This can be overcome by stimulating cell division with various cytokines, but this may have an unwanted effect on the pluripotency of experimental cells. For this reason, lentiviral vectors may be preferred for transgene delivery into HSCs, because they have been shown to be able to transduce quiescent HSCs. [40] In a direct comparison, we also found that the pCCL lentivirus had a greater transduction efficiency than the MigR1 oncoretrovirus in the transduction of human mobilized peripheral blood CD34^{+} cells.

There are many other factors that can affect the transduction efficiency of viral vectors into hematopoietic cells. For example, the presence of cytokines can play an important role. A survey done by Zielske et al. looked at the evidence favoring cytokines in the lentiviral transduction of HSCs. The majority of studies included in this survey found that prestimulation with various combinations of cytokines resulted in higher transduction rates than without prestimulation. [41]

Under some conditions, the use of Retronectin (a recombinant fibronectin fragment) can improve transduction of hematopoietic cells. The rationale behind this is that the Retronectin fragment has domains which are independently capable of binding both the viral envelope as well as the membrane of the target cell, thus bringing these two elements into close proximity. A benefit has been shown with CD34^{+} cells and some types of lentiviral vectors, [36] but this benefit was not seen in the above experiments when using a VSV-G pseudotyped lentivirus.

Other factors that have been shown to improve transduction include the use of cationic polymers, such as polybrene or Lipofectamine 2000, [35] and the use of a "spinfection" or "spinoculation" protocol. [42] Our experiments demonstrated that polybrene was associated with superior transduction efficiency compared to Lipofectamine 2000. Thus, polybrene was used in all subsequent transductions, though transduction efficiencies were still somewhat disappointing. There are few other options for increasing the level of transduction for future experiments. One possibility would be to attempt to generate a much higher lentiviral titer by dramatically scaling up the virus production process. However, this was already being performed at the upper limits of our capabilities, with regard to the equipment and materials involved.

After optimization of the above factors, CD34^{+} cells were transduced with the lentiviral vectors expressing GFP alone, GFP and MKL1, or GFP and dominant negative MKL1. The transduction efficiencies for pCCL-MKL1 and pCCL-DN-MKL1 were quite low in comparison to the pCCL virus alone. It is possible that the smaller size of the

empty viral vector accounts for this difference. In future studies, it may be wise to create a control vector of equal size as the pCCL-MKL1 or pCCL-DN-MKL1 plasmids, perhaps by inverting the insert coding sequence or by inserting stop codons that prevent expression of a full length transcript. Despite these low transduction efficiencies, it is possible to analyze such experimental populations by selecting for GFP$^+$ cells in a FACS analysis or a FACS sort. When examining only the GFP$^+$ cells, transduction with pCCL-MKL1 was associated with a clear and consistent increase in the expression of megakaryocyte-specific markers such as CD41a, CD42b and CD61 in four different experiments compared to cells transduced with pCCL alone. However, there was also a large percentage of CD13$^+$ and CD33$^+$ cells in this experiment, suggesting some degree of granulocytic differentiation. Moreover, the percentage of cells positive for megakaryocytic markers plus the percentage of cells positive for granulocytic markers exceeded one hundred percent, which means there was some overlap between these two populations.

Perhaps the most surprising result found during this experiment was that cells transduced with dominant negative MKL1 lentivirus had a similar trend as those transduced with normal MKL1. That is, both MKL1 and dominant negative MKL1 were associated with an increase in cells positive for CD41a, CD42b and CD61. However, the pCCL-DN-MKL1 experiment was performed only once. Given that previous experiments using HEL cells have shown that MKL1 induces megakaryocytopoiesis via the Serum Response Factor pathway, it was expected that the C630 dominant negative MKL1 would

inhibit megakaryocytopoiesis by blocking this activity. The mechanism for the increase that we observed is completely unclear, though it is possible that dominant negative MKL1 can exert some activity via pathways unrelated to SRF, or that it has different effects on the SRF pathway at earlier versus later stages of megakaryocytopoiesis. It should also be noted that pCCL-DN-MKL1 was not tested for dominant negative activity, even though the other DN-MKL1 constructs were shown to suppress MKL1 function. However, it is difficult to imagine a mechanism by which a different vector could reverse the function of the dominant negative expression product. These interesting DN-MKL1 results will require further experimentation.

The megakaryocyte colony forming unit assays performed in this project provide preliminary evidence that transduction with the MKL1 expressing lentivirus increases the formation of megakaryocyte progenitors. In four experiments, cells transduced with pCCL-MKL1 generated a higher proportion of CFU-Mk than transduction with pCCL alone. These experiments were limited by low transduction efficiencies and thus having very few positively transduced cells, though, fortunately, these assays require a very low number of cells. It is somewhat concerning that transduction with pCCL appeared to promote megakaryocyte colony formation versus cells put through a "mock" transduction. The mechanism for how the empty virus causes such a trend is unclear and is worthy of further investigation.

Implications of this work for the understanding and treatment of Acute Megakaryoblastic Leukemia

There are several forms of leukemia that are known to be associated with various chromosomal translocations. In the past, chemotherapeutic regimens consisted entirely of drugs which non-specifically impair the cell cycle or are toxic to rapidly dividing cells. Though these regimens have proven to be effective in some populations, treatments are generally associated with many chemotherapy-related side effects due to toxic effects on other cells types in the body. However, the recent decade has been notable for greater understanding of the mechanisms by which several chromosomal translocations are involved in leukemogenesis, and the development of novel, targeted treatment options for some of these diseases. For example, imatinib mesylate (Gleevec) was found to be a potent inhibitor of the bcr-abl fusion product that is formed by the Philadelphia chromosome translocation, t(9;22), in chronic myelogenous leukemia (CML), some cases of acute lymphoblastic leukemia (ALL), and rare cases of AML. [43] Gleevec has consequently become an important part of therapy for these types of leukemia.

However, it is not yet clear how the RBM15-MKL1 fusion product is involved in the pathogenesis of t(1;22)(p13;q13)-associated Acute Megakaryoblastic Leukemia, or what roles its component genes play in normal megakaryocytopoiesis. In this project, we have used a human hematopoietic stem cell model to show that MKL1 is an important factor in the process of megakaryocytic differentiation. CD34$^+$ HSCs transduced with MKL1 had both increased expression of megakaryocyte-associated markers CD41a,

CD42b and CD61, and a trend suggestive of increased numbers of committed megakaryocyte progenitors. Thus, it appears probable that one of the mechanisms by which RBM15-MKL1 causes enhanced commitment to the megakaryocytic lineage is by increased activity of the pathways which MKL1 acts upon in normal megakaryocytopoiesis. It is possible that targeted therapies directed at MKL1 and, perhaps, other factors in the Serum Response Factor pathway could one day play an important role in the treatment of infants with t(1;22)(p13;q13)-associated AMKL.

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