IGF signaling contributes to malignant transformation of hematopoietic progenitors by the MLL-AF9 oncoprotein

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Malignant transformation of normal hematopoietic progenitors is a multistep process that likely requires interaction between collaborating oncogenic signals at critical junctures. For instance, the MLL-AF9 fusion oncogene is thought to contribute to myeloid leukemogenesis by driving a hematopoietic stem cell-like "self-renewal" gene expression signature in committed myeloid progenitors. In addition, insulin-like growth factor (IGF) signaling has been implicated in self-renewal/pluripotency in hematopoietic and embryonic stem cell contexts and supports cell growth/survival by activation of downstream pathways, including phosphatidylinositol 3-kinase/Akt and Ras/Raf/extracellular signal–regulated kinase. We hypothesized that IGF signaling could be an important contributor in the process of cellular transformation and/or clonal propagation. Utilizing an MLL-AF9 mouse bone marrow transplantation model of acute myelogenous leukemia, we discovered that committed myeloid progenitor cells with genetically reduced levels of IGF1R were less susceptible to leukemogenic transformation due, at least in part, to a cell-autonomous defect in clonogenic activity. Rather unexpectedly, genetic deletion of IGF1R by inducible Cre recombinase had no effect on growth/survival of established leukemia cells. These findings suggest that IGF1R signaling contributes to transformation of normal myeloid progenitor cells, but is not required for propagation of the leukemic clone once it has become established. We also show that treatment of mouse MLL-AF9 acute myelogenous leukemia cells with BMS-536924, an IGF1R/insulin receptor–selective tyrosine kinase inhibitor, blocked cell growth, suggesting its efficacy in this model may be due to inhibition of insulin receptor and/or related tyrosine kinases, and raising the possibility that similar IGF1R inhibitors in clinical development may be acting through alternate/related pathways.

Acute myelogenous leukemia (AML) is an aggressive malignancy of immature myeloid progenitors with variable, although generally poor, outcomes. Characteristic chromosomal translocations that lead to expression of novel fusion oncoproteins have been defined and subdivide the disease into clinically relevant prognostic groups. Translocations involving the MLL locus and its various partners are among the most common and best studied [1]. A prototypic example is the t(9;11) chromosomal translocation between MLL and AF9, which leads to the expression of a novel fusion oncoprotein with transcriptional activity and drives an aberrant gene expression program. Enforced expression of the MLL-AF9 fusion protein in bone marrow (BM) progenitor cells leads to rapid development of a lethal AML-like disease in mice [2–4]. The MLL-AF9 fusion oncoprotein enacts a self-renewal program in committed myeloid progenitors in a fashion that is dependent on Wnt/β-catenin signaling, and leukemic granulocyte-monocyte progenitors (GMPs) are thought to represent leukemia stem cells in this disease model [2,5].

Insulin-like growth factor 1 receptor (IGF1R) is a receptor tyrosine kinase sharing high homology to the insulin receptor (InsR), which forms homodimers with itself or

heterodimers with InsR to recognize its ligands, IGF-1 and IGF-2 [6]. IGF signaling is important for normal growth of tissues during development [7] and has been implicated in self-renewal/pluripotency in hematopoietic and embryonic stem cell contexts [8,9]. IGF signaling also plays an important role in supporting growth/survival of cancer cells arising in many different tissue contexts [6]. A number of studies have investigated the role of IGF1R in AML and found that it is highly expressed and that cell growth/viability is sensitive to pharmacologic inhibitors designed to target IGF1R [10–13]. IGF1R is one of the most abundantly phosphorylated receptor tyrosine kinases in AML cells, particularly in cases with wild-type fms-like tyrosine kinase receptor-3 [14]. AML blasts secrete IGF-1/2 and derive growth/survival advantage through autocrine signaling, and secretion of these growth factors is increased dramatically in matched relapse samples [11,12]. Both small molecule tyrosine kinase inhibitor and blocking antibody therapies targeting IGF1R have shown activity against AML cells in preclinical studies [10–13]; however, there has been considerable debate about the mechanism and specificity of these reagents, especially with regard to cross reactivity with InsR and other related signaling molecules [15,16].

In our previous studies examining the role of IGF1R signaling in T-cell acute lymphoblastic leukemia (T-ALL), we observed decreased levels of IGF1R expression to compromise leukemia-initiating cell activity in a retroviral NOTCH1 mouse BM transplantation model [17]. To begin to explore whether this effect was specific to T-ALL, or a more general feature common to other hematological malignancies, we performed a similar analysis to test the effect of reduced IGF1R expression on AML using the well-characterized retroviral MLL-AF9 BM transplantation model [2–4]. We describe here that decreased IGF1R expression significantly impairs malignant transformation of committed GMPs with an associated cell-autonomous decrease in clonogenic activity. In contrast to T-cell lymphoid leukemias, myeloid leukemias with decreased IGF1R are readily transplantable into secondary recipients, and unexpectedly, genetic deletion of IGF1R had no effect on bulk cell proliferation. Treatment of both murine and human AML cells in vitro with small molecule tyrosine kinase inhibitors that preferentially target IGF1R/InsR results in reduced cell growth. To our knowledge, these data represent the first assessment of IGF1R signaling in AML using a genetically defined system, and suggest the efficacy of selective IGF1R/InsR inhibitors in clinical development may be due in part to activity against tyrosine kinases other than IGF1R.

Materials and methods

Mice

The IGF1R^{neo} strain, originally generated on the 129 background [18], was backcrossed onto C57BL/6 for more than 20 generations and kindly provided by M. Holzenberger. Wild-type C57BL/6 mice were used as recipients for all transplantation experiments. All animals were housed in the BC Cancer Agency Animal Resource Centre under pathogen-free conditions and in compliance with protocols approved by the University of British Columbia Committee for Animal Care.

Flow cytometry

For sorting/analysis of hematopoietic progenitor subsets, whole BM cell suspensions were labeled with fluorochrome-conjugated antibodies against CD34 (#11-0341), CD16/32 (#12-0161), and CD117 (#17-1171) from eBioscience (San Diego) and Ly-6A/E (#122513) from Biolegend (San Diego, CA, USA). Cells were also concurrently labeled with a cocktail of biotinylated lineage-specific antibodies against CD3e (#13-0031), CD4 (#13-0041-85), CD8a (#13-0081-82), B220 (#13-0452), CD19 (#13-0193), TER119 (#13-5921), Ly-6G (#13-5931), and CD127 (#13-1271) from eBioscience (San Diego, CA, USA), followed by streptavidin-conjugated peridinin chlorophyll protein complex/Cy5.5 (#405214) from Biolegend (San Diego, CA, USA). For analysis of surface IGF1R expression, human cell lines were incubated with αIR3 (Calbiochem, San Diego, CA, USA), followed by allophycocyanin-conjugated secondary antibody. Intracellular Akt activation was measured by labeling of formaldehyde-fixed, methanol-permeabilized cells with an antibody against phospho-Akt (Ser473; catalog #4075, Cell Signaling Technology, Danvers, MA, USA). Lentiviral CreERT2-transduced cells were discriminated by labeling with phycoerythrin-conjugated anti– nerve growth factor receptor antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). Flow sorting analysis was performed on FACSAriaII, FACSCalibur, and FACSCantoII cytometers. Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Retroviral transduction/BM transplantation

An MLL-AF9 complementary DNA encoding amino acids 1–1461 of MLL fused to the last 90 amino acids of AF9 [2] was subcloned into the MigR1 (MSCV-IRES-GFP) retroviral vector. Replication-defective retrovirus was generated by transient transfection of PlatE [19] or 293T cells as described [20]. For primary transplantation experiments, mouse hematopoietic stem/progenitor subsets were fluorescence-activated cell sorted (FACS) from BM, resuspended in MLL-AF9 retroviral supernatant with supplemental cytokines (10 ng/mL murine interleukin [mIL]-3, 10 ng/mL mIL-6, and 20 ng/mL murine stem cell factor; Peprotech, Rocky Hill, NJ, USA) and 7 μg/mL polybrene (Sigma, St Louis, MO, USA), and centrifuged at 1800 *g* for 1 h. Transduced green fluorescent protein (GFP)$^{+}$ cells were then FACS sorted 42 h later, and injected by tail vein into lethally irradiated (810 rad) recipient mice along with 2×10^5 whole BM cells from C57BL/6 donors to rescue normal hematopoiesis. Mice were monitored daily and those developing clinical signs of disease were sacrificed immediately and tissues were harvested for analysis.

Colony-forming cell assay

Cells were plated in 20% Iscove's modified Dulbecco's medium, 20% fetal bovine serum (FBS), 20% BIT9500, and 40% methylcellulose base medium (Stem Cell Technologies, Vancouver, BC, Canada) with supplemental cytokines (10 ng/mL mIL-3, 10 ng/mL mIL-6, 10 ng/mL murine granulocyte-macrophage colony-stimulating factor, and 20 ng/mL murine stem cell factor; Peprotech). For serial colony-forming cell (CFC) assay, primary

colonies from individual plates were pooled, washed in phosphate-buffered saline, and replated under identical conditions as primary cultures.

Cell culture

Human AML cell lines were cultured in RPMI-1640 with 10% FBS, 2 mM GlutaMAX, 1 mM sodium pyruvate, and antibiotics (Invitrogen, Carlsbad, CA, USA). Primary mouse MLL-AF9 leukemia cells were cultured in the same media, but with 20% FBS, 55 μM 2-mercaptoethanol, and supplemental cytokines (10 ng/mL mIL-3, 10 ng/mL mIL-6, 10 ng/mL murine granulocyte-macrophage colony-stimulating factor, and 20 ng/mL murine stem cell factor; Peprotech, Rocky Hill, NJ, USA). Cell growth assays were performed by direct addition of the fluorometric indicator dye resazurin (CellTiterBlue; Promega, Madison, WI, USA) to cells in culture media, incubation for 30 to 120 min at 37°C, and reduced dye measured by fluorometry. Cell viability was measured by flow cytometry for propidium iodide exclusion. Cell proliferation was measured by bromodeoxyuridine incorporation (BrdU Flow Kit; BD, New Jersey, NJ, USA).

Drugs/antibody reagents

Small molecule dual IGF1R/InsR inhibitors BMS-536924 and BMS-754807 were obtained under material transfer agreement from the manufacturer. These were resuspended in dimethyl sulfoxide at 10 mM, then serially diluted in Hank's Balanced Salt Solution (Invitrogen) before addition to culture media. Antibodies used for Western blotting were against IGF1Rα (sc-712; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and extracellular signal–regulated kinase 2 (sc-154; Santa Cruz Biotechnologies).

Ligand stimulation assay

Cells were serum starved overnight, pretreated for 1 h with tyrosine kinase inhibitor, and then stimulated for 10 min with either FBS or recombinant insulin. Cells were then fixed immediately by addition of formaldehyde to 1.5%, permeabilized with 100% ice-cold methanol, labeled with anti-pAkt antibody, and analyzed by flow cytometry.

Results

Reduced IGF1R expression impedes leukemogenic transformation of myeloid progenitors

To study the role of IGF1R signaling in myeloid leukemogenesis, we employed a hypomorphic allele of IGF1R, termed *IGF1R^{neo}*, in which an integrated neo cassette in the 2nd intron results in altered/inefficient messenger RNA splicing and thus reduced levels of protein expression. Full-length IGF1R protein is expressed at ∼60% of wild-type levels in normal tissues of IGF1R$^{neo/neo}$ mice [18], and at ∼30% of wild-type levels in T-ALL leukemias generated by transduction of IGF1R$^{neo/neo}$ BM with activated NOTCH1 retrovirus [17]. To generate AML efficiently on the IGF1R^{neo} background, we transduced BM-derived hematopoietic progenitors with retrovirus encoding the MLL-AF9 fusion oncogene, followed by transplantation into histocompatible recipients. This retroviral MLL-AF9 transduction/transplantation model is well characterized and has proven useful in defining hematopoietic stem cell (HSC)–like self-renewal programs that are induced by MLL-AF9 in committed myeloid progenitors [2,3]. Accordingly, leukemic GMPs are thought to be the self-renewing leukemia initiating-cells, or LICs, in this model.

Bone marrow from age and sex-matched wild-type vs IGF1R$^{neo/neo}$ mice was harvested and Lin$^-$ c-kit$^+$ Sca-1$^+$ (LSK) and GMP subsets sorted by FACS (Supplementary Figure E1; online only, available at www.exphem.org). These purified populations of cells were then transduced with MLL-AF9/GFP retrovirus, GFP$^+$ cells sorted by FACS, and similar numbers of transduced cells injected intravenously into lethally irradiated C57BL/6 recipient mice, along with a 2×10^5 dose of wild-type whole BM to rescue normal hematopoiesis (Supplementary Table E1; online only, available at www.exphem.org). Mice were subsequently monitored daily for clinical signs of leukemia (e.g., hunched position, decreased activity/grooming); animals attaining a standardized morbidity score were euthanized and tissues analyzed at necropsy.

MLL-AF9–transduced LSK cells from both wild-type and IGF1R$^{neo/neo}$ donors produced aggressive immature myeloid leukemias in recipient mice with similar penetrance and latency (Fig. 1A, solid lines), and there was no significant difference in disease burden/distribution or immunophenotype (Supplementary Figure E2; online only, available at www.exphem.org). Importantly, reduced IGF1R expression in IGF1R^{neo} leukemias was confirmed by Western blot (9% ± 3% of wild-type levels; Fig. 1B). In contrast to the situation with LSK cells, there was a significant effect of reduced IGF1R on the ability of MLL-AF9 to transform GMP cells. Although 36% of mice receiving MLL-AF9–transduced wild-type GMPs developed AML, none of the mice receiving MLL-AF9–transduced IGF1R^{neo} GMPs developed disease within the 180-day observation period (Fig. 1A, dashed lines).

To address whether the relative resistance of IGF1R^{neo} GMPs to transformation by MLL-AF9 could be due to altered hematopoietic differentiation on the IGF1R^{neo} background, we examined BM cellularity and abundance of hematopoietic stem/progenitor subsets in IGF1R$^{neo/neo}$ mice as compared to age- and sex-matched wild-type controls. There were no appreciable differences in either total BM cellularity (Supplementary Figure E3A; online only, available at www.exphem.org) or relative proportions of hematopoietic stem/progenitor populations, including lineage marker–negative (Lin$^-$) progenitors, HSC-containing LSK cells, common myeloid progenitors (Lin$^-$ c-kit$^+$ Sca-1$^-$ CD34$^+$ FcγRII/III^{lo}), megakaryocyte-erythroid progenitors (Lin$^-$ c-kit$^+$ Sca-1$^-$ CD34$^-$ FcγRII/III$^-$), or GMPs (Lin$^-$ c-kit$^+$ Sca-1$^-$ CD34$^+$ FcγRII/III^{hi}) [21] (Supplementary Figure E3B; online only, available at www.exphem.org). Additionally, analysis of peripheral blood counts revealed no significant differences between IGF1R$^{neo/neo}$ and wild-type mice (Supplementary Figure E4; online only, available at www.exphem.org). These data suggest that hematopoietic differentiation is not

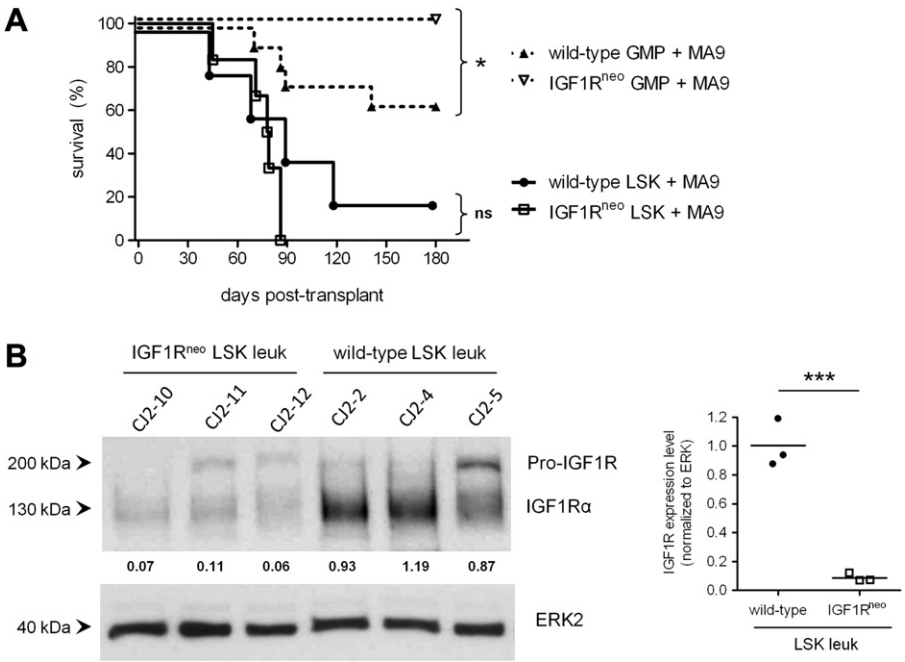

Figure 1. Reduced IGF1R impedes leukemogenic transformation of committed myeloid progenitors by MLL-AF9. (**A**) Survival of C57BL/6 mice transplanted with MLL-AF9–transduced hematopoietic stem (LSK) or GMP cells from wild-type or IGF1R^{neo} donors. Bone marrow from age- and sex-matched wild-type and IGF1R$^{neo/neo}$ mice was harvested and LSK/GMP subsets were FACS sorted, transduced with MLL-AF9 retrovirus, and sorted again for successfully transduced (GFP$^+$) cells. Similar numbers of transduced cells were then transplanted into lethally irradiated syngeneic/congenic C57BL/6 recipients by tail vein injection along with a radioprotective "rescue" dose of whole BM. Mice were monitored for clinical signs of morbidity and euthanized after attaining a predefined end point based on standardized scoring of clinical symptoms. Pathologic involvement by acute myeloid leukemia was confirmed at necropsy. NS = nonsignificant; $^*p < 0.05$ (log-rank test). (**B**) Western blot analysis of IGF1R protein expression level in primary acute myeloid leukemias derived from MLL-AF9–transduced LSK cells as in (**A**). Cell lysates were prepared from cell populations containing >95% GFP$^+$ tumor cells. Numbers below the IGF1R panel indicate relative IGF1R expression level after normalization to extracellular signal–regulated kinase 2 loading control. Statistical analysis of the normalized IGF1R expression values is presented at the right with means indicated by horizontal bars. $^{***}p < 0.001$ (Student's *t* test).

substantially distorted in IGF1R$^{neo/neo}$ mice, and support the conclusion that GMPs with reduced levels of IGF1R are qualitatively less susceptible to transformation by MLL-AF9. Although we did not observe a similar defect in transformation of IGF1R^{neo} LSK cells, this cannot be concluded definitively because recipient mice were not injected with identical numbers of MLL-AF9–transduced IGF1R^{neo} vs wild-type LSK cells (Supplementary Table E1; online only, available at www.exphem.org).

Reduced levels of IGF1R do not markedly compromise leukemia-initiating cell activity

We have observed previously that acute T-cell leukemias generated on the IGF1R^{neo} background by transduction with activated NOTCH1 retrovirus are markedly compromised in leukemia-initiating activity as assayed by serial transplantation. In fact, the calculated leukemia-initiating cell frequency in IGF1R^{neo} T-ALL is 1 in 14,000,000 cells (1 in 0.6–3.5 $\times$ 10^7 cells; 95% confidence interval), or approximately 2000-fold lower than wild-type ([17] and data not shown). To address whether this might also be the case for MLL-AF9 acute myeloid leukemias, we transplanted primary leukemias into secondary recipient mice and scored for disease propagation. In contrast to T-ALL, we observed IGF1R^{neo} AMLs to produce disease readily in secondary recipients similar to wild-type leukemias (Fig. 2). These data suggest reduced IGF1R expression does not markedly compromise leukemia-initiating cell activity in MLL-AF9 AMLs as it does in NOTCH1 T-ALLs. Further studies will be needed to explore the basis for this difference in IGF1R dependence between T-ALL and AML leukemia-initiating cells.

Reduced levels of IGF1R compromise clonogenic activity of MLL-AF9–transduced GMPs

Given that transduction of GMPs from IGF1R^{neo} mice with MLL-AF9 failed to generate AML disease, we sought to explore whether reduced IGF1R signaling might directly affect proliferation/survival in a cell autonomous fashion. To address this point, we employed CFC assays, a standard in vitro culture method for assessing the ability of committed myeloid progenitors to form colonies in semi-solid media. GMP cells were sorted from wild-type vs IGF1R^{neo} BM, transduced with MLL-AF9 or empty (GFP only) retrovirus, and sorted again to separate transduced (GFP$^+$) from non-transduced (GFP$^-$) cells. Both GFP$^+$ and GFP$^-$ subsets

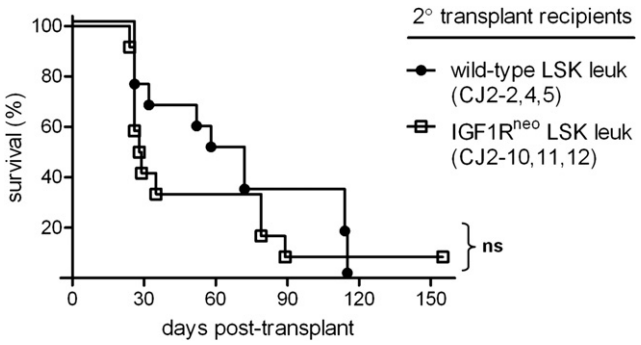

Figure 2. Reduced IGF1R expression does not markedly impair leukemia-initiating cell activity of MLL-AF9 AMLs. Survival of nonirradiated, secondary transplant recipient mice injected by tail-vein with 500,000 primary MLL-AF9 leukemia cells as generated in Figure 1. Mice were monitored for clinical signs of morbidity and euthanized at the same clinical end points as used for evaluation of primary transplant recipients. Pathologic involvement by acute myeloid leukemia was confirmed at necropsy. Data are summarized for three independent primary leukemias of each genotype. NS = nonsignificant (log-rank test).

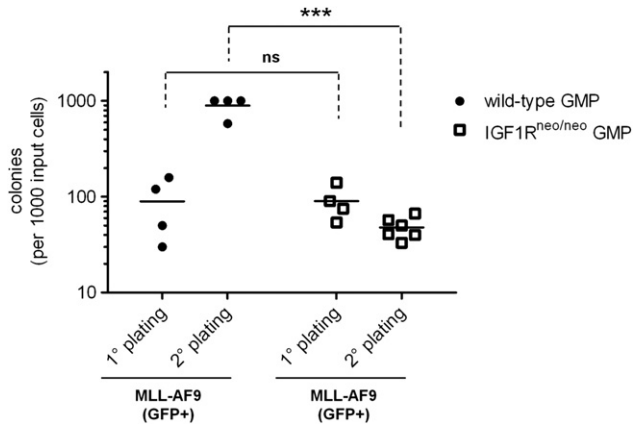

Figure 3. Reduced IGF1R expression impairs clonogenic activity of MLL-AF9–transduced GMPs. Clonogenic activity as measured by CFC assay. GMPs were FACS sorted from the BM of age- and sex-matched wild-type and IGF1R$^{neo/neo}$ donor mice and transduced with MLL-AF9 retrovirus. Transduced (GFP$^+$) cells were then FACS sorted and plated in methylcellulose-containing complete media with supplemental cytokines. Colonies were counted manually after 20 days incubation and visually confirmed for appropriate expression of GFP using an inverted fluorescent microscope. For secondary platings, colonies arising in the first plating were pooled and an equivalent number of live GFP$^+$ cells as seeded in the first plating were replated into secondary cultures. Means are indicated by horizontal bars. NS = nonsignificant; ***$p < 0.001$ (Student's t test).

were then plated in methylcellulose-containing media under standard myeloid CFC conditions [2], and colony formation was assessed 20 days later.

Empty virus (GFP$^+$) and nontransduced (GFP$^-$) cells produced only 0 to 1 colony per 1000 input cells from either background (Supplementary Figure E5; online only, available at www.exphem.org), and no colonies formed on secondary replating. Consistent with oncogenic MLL-AF9 function, MLL-AF9 (GFP$^+$) transduced cells yielded ~90 colonies per 1000 input cells, but with no significant difference between wild-type and IGF1R^{neo} background (Fig. 3). There was also no apparent difference in the size distribution of resulting colonies (data not shown). Upon serial replating, however, essentially every cell from primary wild-type CFCs yielded a secondary colony (~1000 colonies per 1000 input cells), whereas primary IGF1R^{neo} CFCs yielded only ~50 secondary colonies per 1000 input cells. These data support that reduced levels of IGF1R negatively affect in vitro clonogenic activity of MLL-AF9–transduced GMPs, and suggest the inability of these cells to produce leukemia in vivo may be due at least in part to cell autonomous signaling defects. Further studies would be needed to address potential effects of reduced IGF1R signaling on other leukemogenic phenotypes, such as self-renewal, homing/engraftment, interactions with microenvironmental niches, and immune evasion.

Deletion of IGF1R in established leukemia cells has no effect on cell growth/viability

Both IGF1R blocking antibodies and selective IGF1R tyrosine kinase inhibitors have been reported to antagonize growth/survival of human AML cells [10,13], but there has been ongoing debate as to the relative contributions of IGF1R and InsR signaling pathways in this context [15,16]. We took advantage of the defined genetics afforded by our MLL-AF9/IGF1R^{neo} mouse model to address the role of IGF1R in growth/survival of established AML cells. To this end, we cultured three wild-type and three IGF1R^{neo} LSK leukemias in vitro and assayed cell growth by resazurin reduction. Despite some clone-to-clone variation, there was no evidence that reduced IGF1R expression led to a reduced net rate of cell growth (Fig. 4A). This observation is corroborated by the fact IGF1R^{neo} leukemias did not show prolonged latency in secondary transplant assay as compared to wild-type leukemias (Fig. 2). This finding was rather unexpected, given the general importance of IGF signaling in growth regulation, and that IGF1R is expressed at 10-fold lower levels in IGF1R^{neo} leukemias as compared to wild-type controls (Fig. 1B). To explore this point further, we took advantage of the fact that the IGF1R^{neo} allele, which contains flanking loxP sites around the neo cassette in intron 2 as well as around exon 3 [18], can be excised by Cre-mediated recombination to ask whether genetic deletion of IGF1R would compromise AML cell growth. To this end, freshly explanted leukemia cells were transduced with lentivirus encoding tamoxifen-inducible Cre (CreERT2) and a truncated nerve growth factor receptor marker. Cultures were then treated with 4-hydroxytamoxifen for 11 days to induce Cre-mediated deletion of IGF1R in transduced cells. Deletion of IGF1R, which was confirmed by polymerase chain reaction

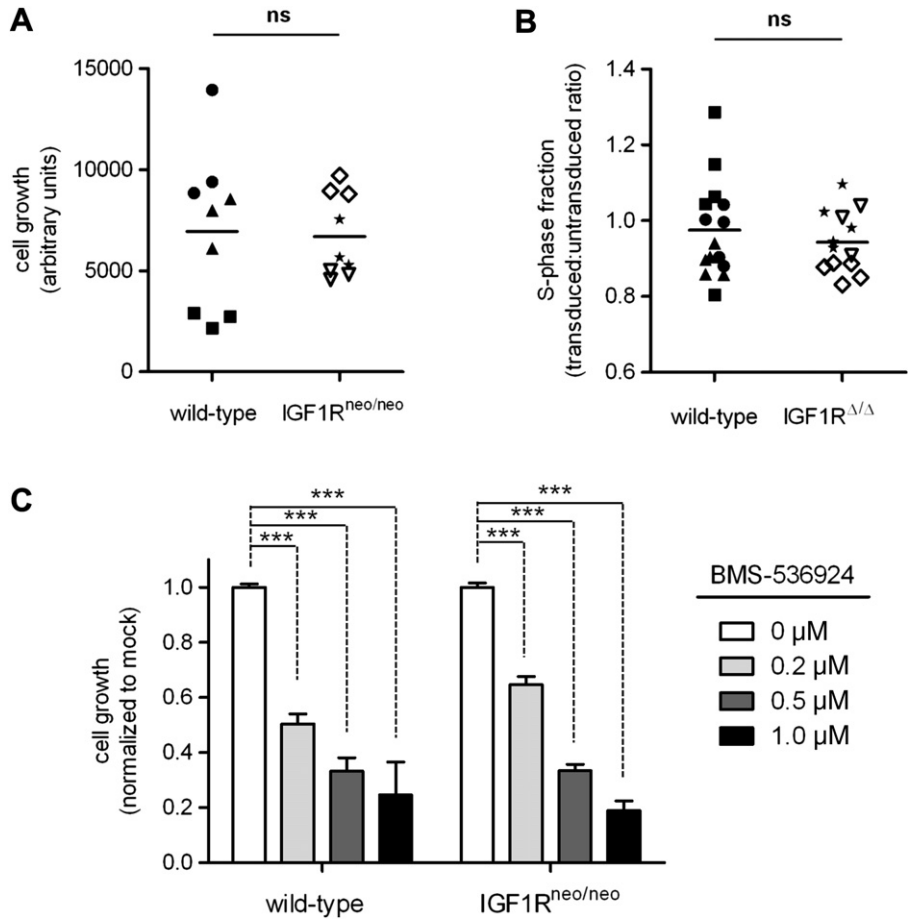

Figure 4. Established MLL-AF9 leukemias do not require IGF1R signaling. (**A**) Growth of MLL-AF9 leukemias with reduced IGF1R expression. Freshly explanted primary MLL-AF9 (LSK) leukemias were cultured briefly on MS5 BM stromal feeders, then equal numbers of cells were plated on bare plastic. Cell growth was measured by resazurin reduction assay 48 hours later. Three independent leukemias (indicated by different shaped data points) of each genotype were assayed in triplicate. Means are indicated by horizontal bars. NS = nonsignificant (Student's *t* test). (**B**) Proliferation of MLL-AF9 leukemias after deletion of IGF1R. Primary MLL-AF9 (LSK) leukemias were transduced with lentiviral CreERT2 (with nerve growth factor receptor [NGFR] marker), then treated with 5 to 10 nM 4-hydroxytamoxifen for 11 days to induce deletion of the IGF1R^{neo} allele. Cell proliferation was then assessed by bromodeoxyuridine incorporation. Transduced cells were discriminated from nontransduced cells in the same culture by labeling for NGFR. Three independent leukemias (indicated by different shaped data points) of each genotype were assayed three to five times each. Means are indicated by horizontal bars. NS = nonsignificant (Student's *t* test). (**C**) Effect of IGF1R/InsR-selective tyrosine kinase inhibitor BMS-536924 on MLL-AF9 leukemias. Primary MLL-AF9 leukemias were seeded at 2.5×10^5 cells/mL and BMS-536924 was added at the indicated doses vs dimethyl sulfoxide vehicle control in complete media. Cell growth was measured 48 hours later by resazurin reduction assay. One independent leukemia of each genotype was assayed in triplicate. Error bars indicate standard deviation. $^{***}p < 0.001$ (two-way analysis of variance with Bonferroni post-test analyses).

assay (Supplementary Figure E6; online only, available at www.exphem.org), had no demonstrable effect on cell proliferation as measured by bromodeoxyuridine incorporation compared to nontransduced cells in the same culture (Fig. 4B). Additionally, we noted the nerve growth factor receptor$^+$ transduced cell fraction to remain stable over extended passage of both 4-hydroxytamoxifen–treated and control cultures (data not shown). Overall, these findings support the conclusion that signaling through IGF1R is neither required for growth/survival of established AML cells, nor is there any incremental growth advantage conferred by increased levels of IGF1R expression in this context.

Tyrosine kinase inhibitors that preferentially target IGF1R/InsR impede AML cell growth and synergize weakly with cytotoxic chemotherapy

In parallel with IGF1R deletion studies described here, we also explored effects of pharmacologic tyrosine kinase inhibitors on AML cell growth. Most small molecule IGF1R inhibitors also affect InsR due to their close homology and, at higher doses, can be expected to cross react with more distantly related receptor tyrosine kinases. One such inhibitor, BMS-536924 [22], significantly impeded growth of both wild-type and IGF1R^{neo} leukemias (Fig. 4C). Because genetic deletion of IGF1R had no effect on cell proliferation (Fig. 4B), we conclude that the efficacy

of BMS-536924 under these assay conditions must be due to inhibition of InsR and/or related receptor tyrosine kinases. In support of this, BMS-536924 blocked insulin-induced signaling as measured by downstream activation of Akt (Supplementary Figure E7; online only, available at www.exphem.org).

We next sought to explore whether dual IGF1R/InsR inhibition was effective on human AML cells. Four human AML cell lines were selected for study and were confirmed to express surface IGF1R at readily detectable levels by flow cytometry (Fig. 5A). We found two of the four cell lines to be sensitive to another dual IGF1R/InsR inhibitor, BMS-754807 [23], which has replaced BMS-536924 in the clinical development pipeline (Fig. 5B). In parallel, cell viability was assessed by propidium iodide dye exclusion, and revealed dead cells in these cultures never to exceed 5%, suggesting that BMS-754807 primarily affects cell proliferation rather than survival (data not shown). These results are concordant with earlier studies utilizing BMS-536924 [13], but formally demonstrate efficacy of the current generation inhibitor, BMS-754807. Although we did not observe BMS-754807 to have appreciable effects on cell survival, it should be noted that our maximum dose assayed was 1 μM. In contrast, BMS-536924 was shown to induce substantial apoptosis of these same cell lines, but only at doses in the 5 to 10 μM range [13].

It has been suggested in the literature that IGF1R may be one of several genes whose expression correlates with chemotherapy resistance in AML [24,25]. We therefore tested whether IGF1R/InsR inhibition might enhance sensitivity to cytotoxic chemotherapy. The two human AML cell lines sensitive to BMS-754807 were treated with various dosing combinations of BMS-754807 and a commonly used anthracycline in AML treatment, daunorubicin, and cell growth was measured by resazurin reduction assay (Fig. 5C). We observed very mild synergy between BMS-754807 and daunorubicin, where the interaction accounted for 8% to 10% of the total variance (interaction p value <0.0001, two-way analysis of variance). Individually, BMS-754807 and daunorubicin were responsible for 25% to 32% and 56% to 61% of the total variance, respectively. These data suggest IGF1R/InsR inhibition may prove useful in combination with standard cytotoxic chemotherapy in human AML and can be expected to show mostly additive, but perhaps slight synergistic, effects. Further studies are needed for validate these observations in primary patient material and also to demonstrate efficacy in vivo.

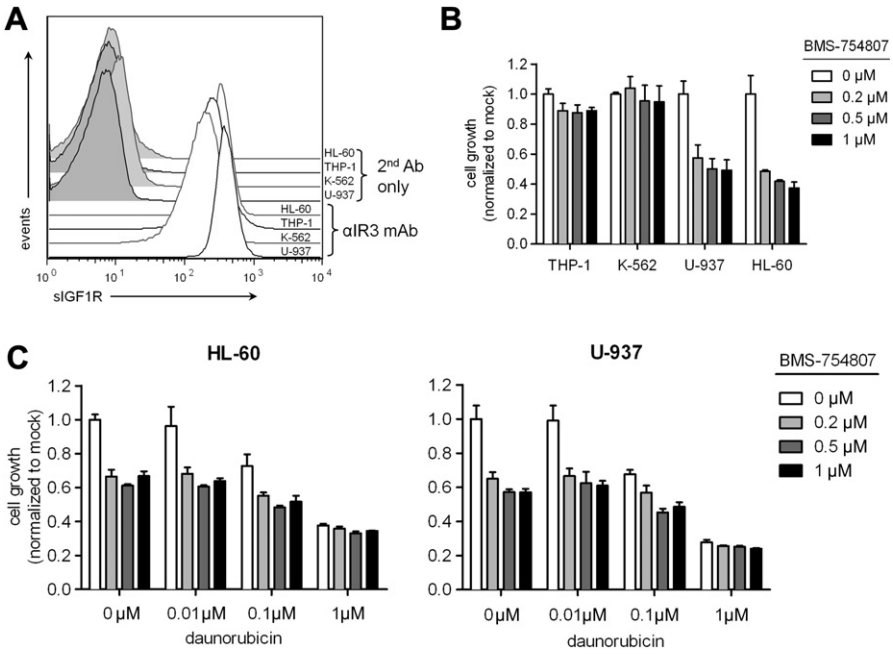

Figure 5. BMS-754807, an IGF1R/InsR-selective tyrosine kinase inhibitor, blocks growth of human AML cells and is weakly synergistic with daunorubicin. (**A**) Flow cytometric analysis of surface IGF1R expression in human AML cell lines. Cells were labeled with primary αIR3 antibody, followed by allophycocyanin-conjugated goat anti-mouse secondary antibody. (**B**) Effect of BMS-754807 on cell growth. Cells were plated at 2.5×10^5 cells/mL and BMS-754807 was added at the indicated doses vs dimethyl sulfoxide (DMSO) vehicle control in complete media. Cell growth was measured 48 hours later by resazurin reduction assay. All assays were performed in triplicate. Error bars indicate standard deviation. (**C**) Effects of BMS-754807 and daunorubicin on cell growth. Cells were plated at 5×10^5 cells/mL and BMS-754807 and/or daunorubicin were added at the indicated doses vs DMSO/water vehicle controls, respectively, in complete media. Cell growth measured 24 hours later by resazurin reduction assay. All assays were performed in triplicate. Error bars indicate standard deviation.

Discussion

Using genetically engineered mice that express decreased levels of IGF1R, we have demonstrated that IGF1R signaling contributes to oncogenic transformation of GMP cells by the MLL-AF9 fusion oncoprotein. As earlier work supports the idea that MLL-AF9 is capable of re-engaging a self-renewal program in committed GMPs [2], our data suggest that IGF signaling can collaborate with MLL-AF9–induced genes to support the leukemic self-renewal program. Of note, IGF signaling has been shown to play an important role in self-renewal of embryonic stem cells [8], and IGF1R is expressed at higher levels in HSCs as compared to more differentiated progenitor subsets [9]. Additionally, IGF1R is differentially overexpressed in $CD34^{+}CD38^{-}$ AML stem cells as compared to their $CD34^{+}CD38^{+}$ differentiated progeny [26]. Further studies will be needed to determine which signaling pathways downstream of IGF1R are most relevant for leukemia initiation/self-renewal, and to what extent dependence on these signals varies with cell/developmental context.

It is interesting to note that the transforming potential of MLL-AF9 is also dependent on its level of expression. Although retroviral transduction drives high-level expression of MLL-AF9 in BM progenitors and is relatively efficient at generating AML disease, knock-in of AF9 into the endogenous MLL locus achieves much lower levels of expression and is comparatively impotent in generating disease [27]. Furthermore, in striking similarity to our observations with reduced IGF1R, MLL-AF9 expression at lower, knock-in levels efficiently transformed LSK cells, but GMPs were resistant to transformation under these conditions. Given this similarity, we considered the possibility that MLL-AF9 might induce expression of IGF1R. Of note, in B-cell acute lymphoblastic leukemia cells, HOXA9, a key mediator of MLL-induced gene programs, positively regulates IGF1R expression [28] and a related MLL fusion protein, MLL-AF4, binds to the IGF1R locus [29]. We were unable, however, to detect changes in IGF1R expression in human AML cells following transduction with MLL-AF9 retrovirus (Supplementary Figure E8A; online only, available at www.exphem.org), and published expression profile data from mouse GMPs transduced with MLL-AF9 retrovirus showed consistent upregulation of known targets Meis1 and Hoxa9 [2], but not Igf1r (Supplementary Figure E8B; online only, available at www.exphem.org). Additional studies will be needed to determine if the dosage-dependent effects of MLL-AF9 and IGF1R on transformation of GMPs are mechanistically related.

It remains unclear what underlying mechanism is responsible for the difference in transformation susceptibility between GMPs and LSKs as revealed either at physiologic MLL-AF9 dosage in the Kersey laboratory's knock-in study [27], or via reduced IGF1R expression in the current study. One possibility could be that the threshold for transformation may be higher in GMPs, where the self-renewal program presumably must be reinitiated, as opposed to HSCs, where it needs only to be maintained. This difference may hold clinical significance in that self-renewal activity of leukemia stem cells that originated by transformation of committed progenitors may be dependent on high levels of MLL-AF9 expression and/or IGF1R signaling, whereas those arising by transformation of HSCs may not. Accordingly, novel therapies targeting these pathways may potentially be more effective against progenitor-derived than stem cell-derived leukemias.

Despite our observation that genetic deletion of IGF1R has no apparent effects on growth of established murine MLL-AF9 leukemias, it is notable that these mouse cells are sensitive to pharmacologic inhibitors designed to target IGF1R/InsR. This suggests that there may be significant functional redundancy between IGF1R, InsR, and related tyrosine kinases in AML cells, and implies that the relative nonspecificity of small molecule kinase inhibitors may actually be an advantage over IGF1R-specific neutralizing antibodies. Going one step further, our data also raise the possibility that the observed efficacy of anti-IGF1R antibodies against AML cells in vitro [10] may not, in fact, be acting solely by blocking IGF1R signaling, but perhaps also through direct/indirect cytotoxic effects, such as can potentially result from high-density antibody binding to the surface of the cell. Regardless of the actual mechanism, the efficacy of any IGF1R inhibitor strategy will have to be validated in human patients and balanced with associated side effects [15,16].

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Author contributions: C.R.J., V.G., and A.P.W. designed experiments. C.R.J., O.O.S., and S.H.L. performed experiments. J.M.C. and M.M.G. developed and provided the BMS inhibitor compounds. M.H., M.P., and R.K.H. provided reagents, resources and discussed results; C.R.J. and A.P.W. wrote the article.

Conflict of interest disclosure

J. Carboni and M. Gottardis are employees of Bristol-Myers Squibb Company. No financial interest/relationships with financial interest relating to the topic of this article have been declared by the remaining authors.

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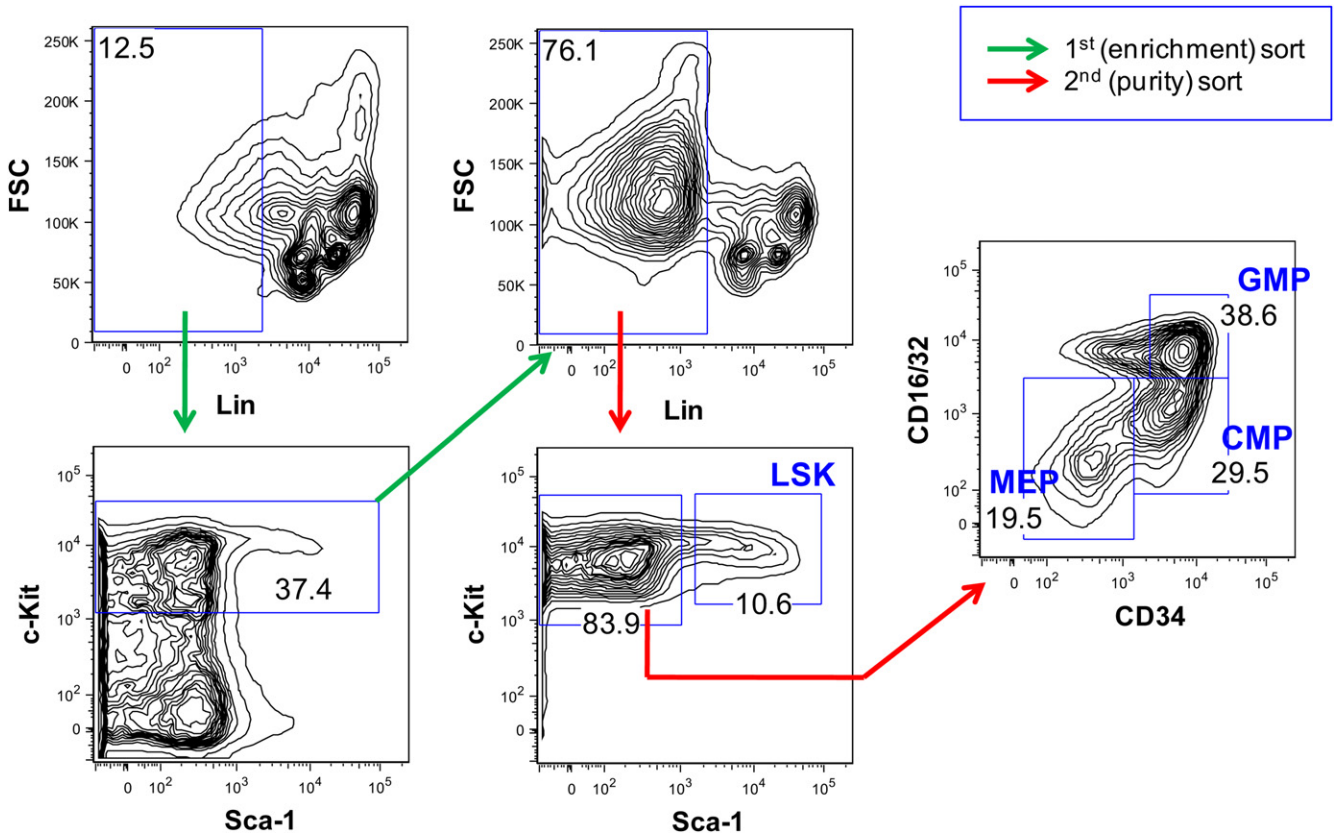
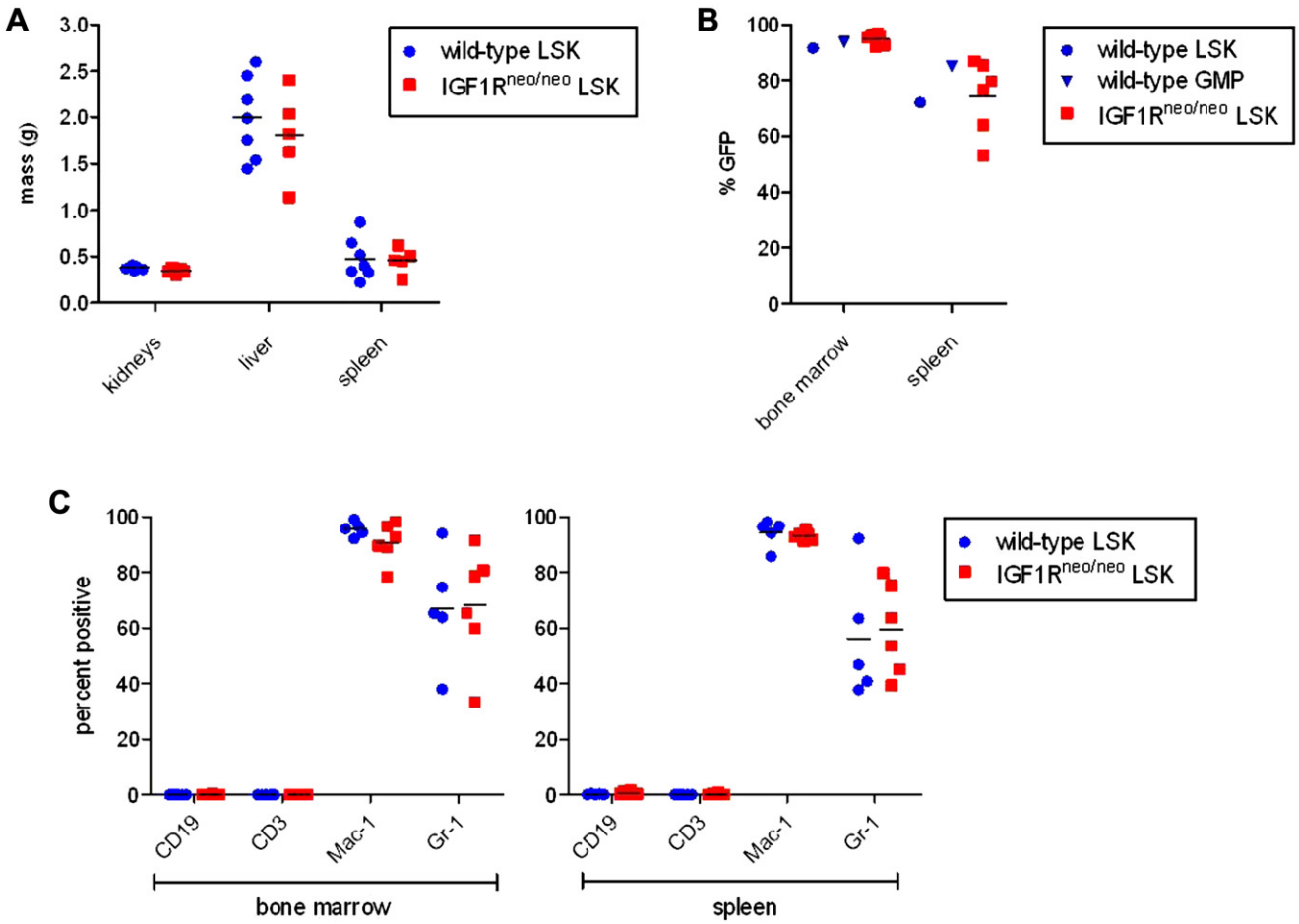
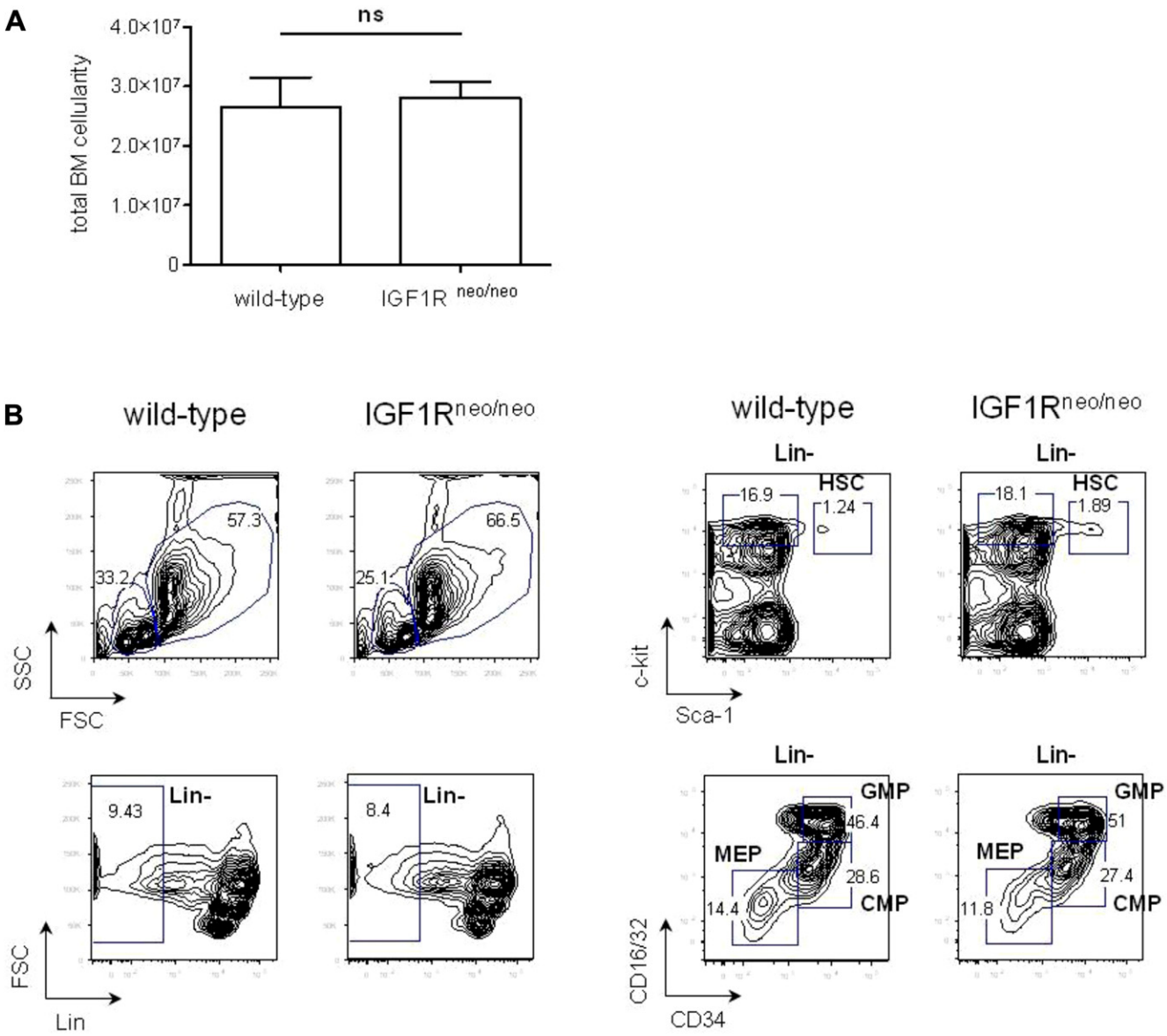


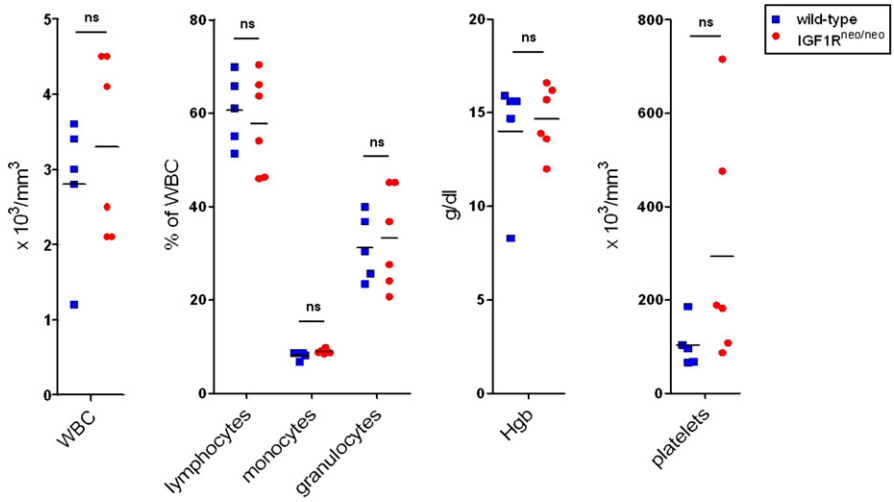
Supplementary Figure E1. FACS sorting strategy for purification of LSK and GMP subsets. Whole BM was harvested, labeled with antibodies against lineage markers (CD3e, CD4, CD8a, B220, CD19, TER119, Ly-6G, and CD127), c-kit, Sca-1, CD34, and CD16/32 (FcγRIII/II), and sorted according to the depicted strategy. Two sequential sorts were utilized, first to enrich for the desired populations, and then repeated a second time to obtain high purity populations. CMP = common myeloid progenitors; FSC = forward light scatter; Lin = lineage; MEP = megakaryocyte-erythroid progenitors.



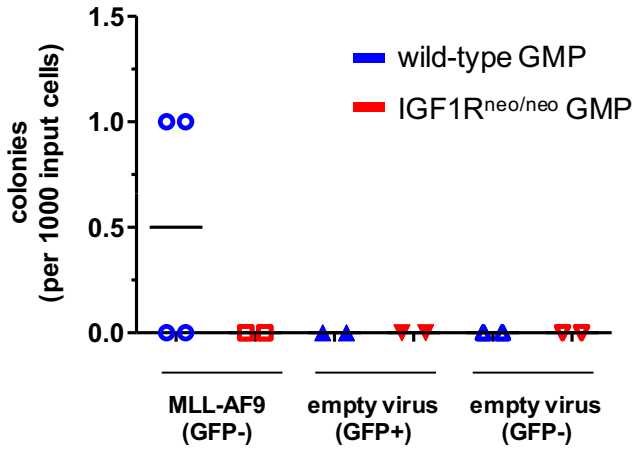
Supplementary Figure E2. Reduced IGF1R does not affect AML disease parameters. (**A**, **B**) Tissue involvement by MLL-AF9 leukemia cells in primary transplant recipient animals. (**A**) Organ weights at necropsy. (**B**) GFP^+ fraction as assessed by flow cytometry of single-cell suspensions derived from involved tissues. Each data point represents a different primary recipient animal. (**C**) Flow cytometric analysis for B-lymphoid (CD19), T-lymphoid (CD3), and myeloid (Mac-1, Gr-1) marker expression by MLL-AF9 leukemic cells from tissues of primary recipient mice. Fraction of cells positive for each marker is indicated within the GFP^+ leukemia cell gate. Each data point represents a different primary recipient animal.



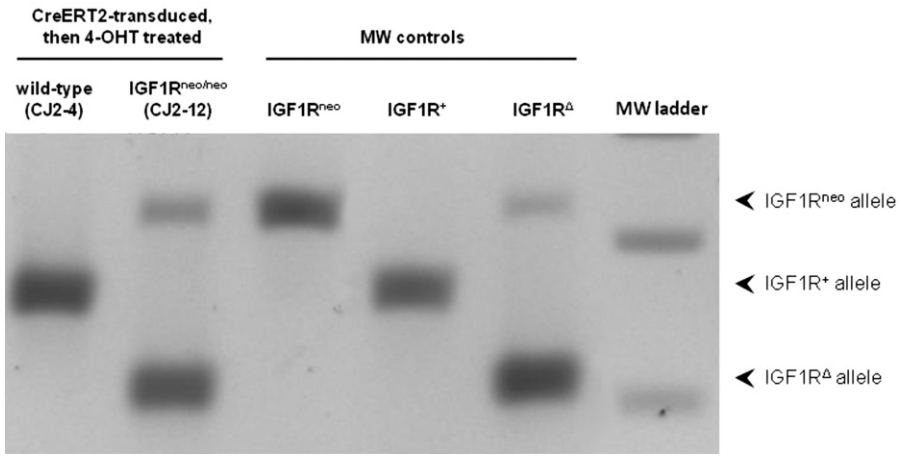
Supplementary Figure E3. BM cellularity/composition is not perturbed in IGF1Rneo mice. (**A**) Total BM cellularity from wild-type vs IGF1Rneo/neo mice. Cells were harvested from bilateral femurs and tibias, pooled, and counted manually following erythrocyte lysis. Data represent means from four mice of each genotype. Error bars indicate standard deviation. NS = nonsignificant (Student's *t* test). (**B**) Flow cytometric analysis of normal BM cell subsets in wild-type vs IGF1R$^{neo/neo}$ mice. Cells were harvested as in (**A**), then stained for the indicated markers and analyzed by flow cytometry. Data depicted are representative of three replicates. CMP = common myeloid progenitor; FSC= forward light scatter; Lin = CD3e, CD4, CD8a, B220, CD19, Ter119, Ly-6G (Gr-1), and CD127 (IL-7Ra); MEP = megakaryocyte-erythroid progenitor; SSC = side light scatter.



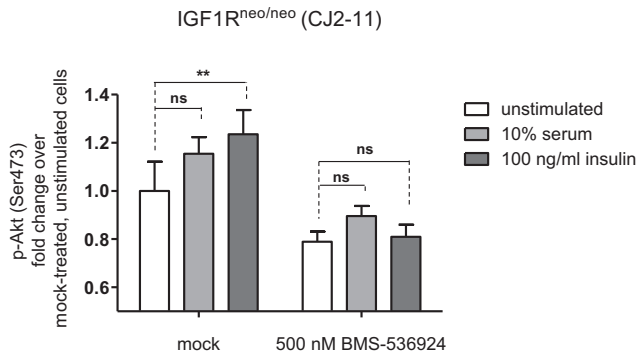
Supplementary Figure E4. Peripheral blood counts are not perturbed in IGF1R$^{neo\ mice}$. Complete blood count analysis of peripheral blood from age and sex-matched IGF1R$^{neo/neo}$ and wild-type mice. Means are indicated by horizontal bars. Hgb = hemoglobin; ns = nonsignificant (Student's *t* test); WBC= white blood cells.



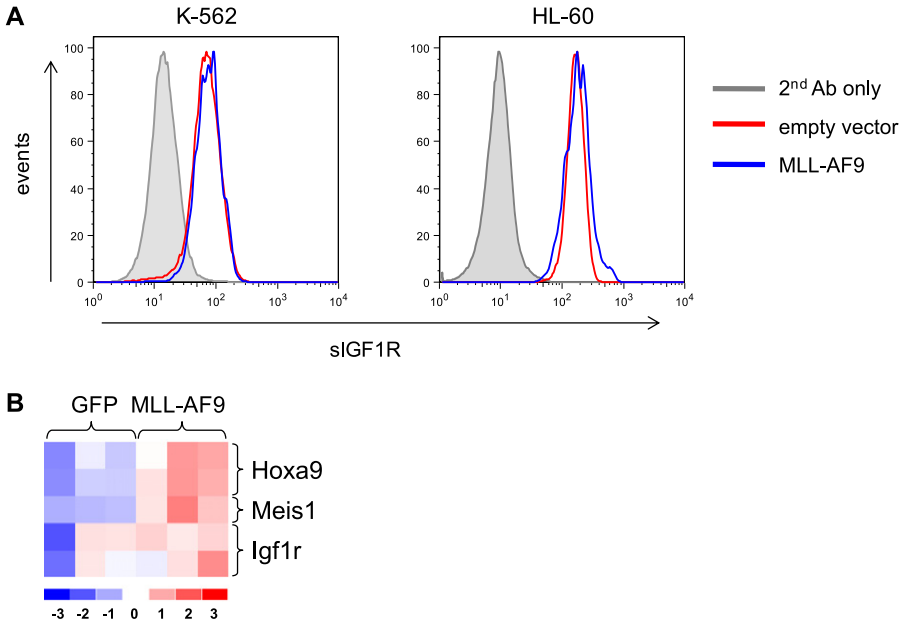
Supplementary Figure E5. Clonogenic activity of untransduced and empty virus-transduced GMPs. Clonogenic activity as measured by CFC assay. GMPs were FACS sorted from the BM of age and sex matched wild-type and IGF1R$^{neo/neo}$ donor mice and transduced with MLL-AF9 retrovirus vs empty retrovirus control. Transduced (GFP$^+$) and nontransduced (GFP$^-$) cells were then FACS sorted and plated in methylcellulose-containing complete media with supplemental cytokines. Colonies were counted manually after 20 days incubation and visually confirmed for appropriate expression of GFP using an inverted fluorescent microscope. Colony yields are depicted from the first plating of nontransduced (GFP$^-$) and empty virus-transduced (GFP$^+$) GMP cells, which served as controls for Figure 3. Means are indicated by horizontal bars.



Supplementary Figure E6. Polymerase chain reaction (PCR) confirmation of IGF1R deletion by lentiviral CreERT2. PCR analysis for IGF1R deletion. Lentiviral CreERT2-transduced. 4-hydroxytamoxifen (4-OHT)–treated MLL-AF9 leukemia cells from Figure 5B were FACS sorted by virtue of the lentiviral nerve growth factor receptor marker and genomic DNA was prepared. Multiplex PCR analysis was performed using primers to discriminate between wild-type (+), neo, and deleted (Δ) alleles of IGF1R. MW = molecular weight.



Supplementary Figure E7. BMS-536924 blocks insulin signaling in MLL-AF9 primary leukemia cells. Flow cytometric analysis for Akt activation by intracellular phospho-Akt (Ser473) level. Primary IGF1R$^{neo/neo}$ leukemia cells from mice transplanted with MLL-AF9–transduced BM LSK cells were cultured briefly in vitro and then serum starved overnight. Cells were then treated with BMS-536924 inhibitor for 1 h before stimulation with either FBS or recombinant insulin for 10 min. Cells were then fixed immediately and permeabilized before flow cytometric analysis. All data are normalized to the mock-treated, unstimulated sample. Error bars indicate standard deviation. ns = not significant; $^{**}p < 0.01$ (two-way analysis of variance with Bonferroni post-test analyses).



Supplementary Figure E8. MLL-AF9 does not regulate IGF1R expression level. (**A**) Flow cytometric analysis of surface IGF1R expression in human AML cell lines transduced with MLL-AF9 retrovirus or empty vector control. sIGF1R = surface IGF1R. (**B**) Microarray expression profile analysis of messenger RNA levels from triplicate samples of mouse GMP cells transduced with MLL-AF9 retrovirus or empty GFP vector control. Normalized data were downloaded from Gene Expression Omnibus (accession #GSE3721) and analyzed with dChip software. All available probesets for Igf1r and known MLL-AF9 targets Hoxa9 and Meis1 are depicted. Scale is expression level normalized across all samples with mean = 0 and standard deviation = 1 for each probeset.

Supplementary Table E1. Cell populations used for transplant analyses

Background	Cell type	MLL-AF9^{+} cells injected/mouse	Normal bone marrow cells co-injected/mouse	No. of mice injected
Wild-type	LSK	350	200,000	5
Wild-type	GMP	5000	200,000	11
IGF1R$^{neo/neo}$	LSK	570	200,000	6
IGF1R$^{neo/neo}$	GMP	5000	200,000	11