Introduction

The road from discovery to distribution is a long, dark, and treacherous path for novel cancer therapies, and for good reason. New treatments dubbed as “cures” for certain cancers need to go through rigorous testing to understand their effectiveness in human patients, as well as potential side effects that vary wildly by species and even by patient. For particularly rare or deadly forms of cancer that show themselves in late stages, such as acute myeloid leukemia, finding enough patients with the strength to sustain rigorous treatments and testing is incredibly difficult (1).

Treatment progression could be sped up by creating an alternate pathway for researchers to test novel therapeutics. Genetic engineering may be the key; CRISPR-Cas9 can theoretically be used to induce mutations in healthy bone marrow cells to induce AML and other forms of leukemia to develop and test realistic human tumors without waiting for patients suffering from leukemia willing to participate in a novel study (2). This will also allow patients who undergo new therapies testing using this method to understand the risks and effectiveness more accurately than ever before. This process was first tested in the mice genome to prove its effectiveness, and later on donated human CD34+ Car-T cells (3, 2).

Acute Myeloid Leukemia

Acute myeloid leukemia, or AML, is one of the deadliest types of cancer present in the US. It originates in bone marrow and causes the continuous production of myeloblasts (undifferentiated white blood cells). These cells navigate the body through the circulatory system and spread rapidly to the spinal cord, mouth, and skin. This offers a direct pathway for AML to spread beyond the bone marrow and progress to stages 3 and 4 quickly, one of the reasons AML’s 5-year survival rate is only 28.3% (4). AML treatment typically consists of a combination of chemotherapy, radiation, and stem cell bone marrow transplants. These treatments have made progress over the years, reducing AML’s death-rate from 95.6% in 1975 to 71.7% in 2016. However, this trend has stagnated recently, indicating a roadblock in treatment progression (5).

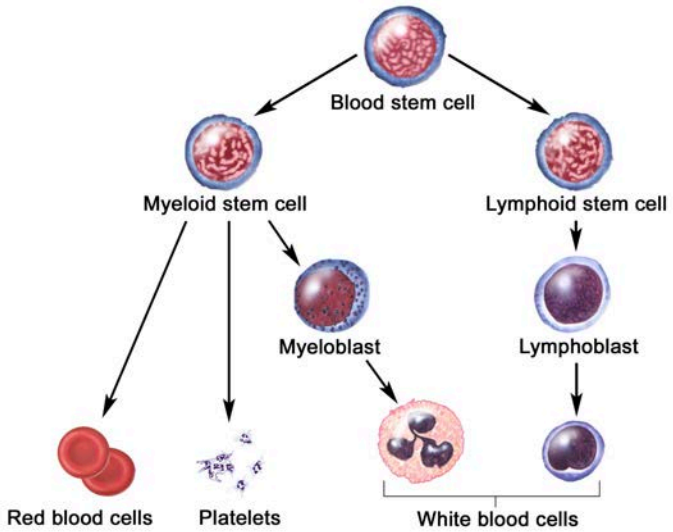

Figure 1: Development of blood stem cells in bone marrow. AML occurs in myeloid stem cells, leading to the over-production of undifferentiated myeloblasts (1).

One way to speed up the approval process for cancer therapies is by testing the therapeutic on "human-like" tumors *before* moving forwards with clinical trials. This allows researchers to screen potential side-effects or flaws more accurately than in mice or other genomes before getting approval to proceed on human patients. Over the last 20 years, this was typically done with patient-derived xenografts (PDX), where a cancer-patient's tumor is removed and injected into a mouse to replicate the tumor's growth while applying novel cancer treatments (5). While more effective than previous techniques, recent research has uncovered that PDX tumors, one transplanted into mice, regularly diverge from their original form and growth patterns. In addition, PDX's cannot be used to test immunotherapy agents due to the inherent differences between mice and human immune systems (3).

However, with the recent discovery of CRISPR-Cas9 and further research into its functionality, the way scientists test cancer treatments may change forever. Genome sequencing research has shown that most malignancies (cancerous growths) regularly contain mutations in 4 or more "driver" genes, genes that regularly cause cancer (2). Theoretically, CRISPR-Cas9 can be used to deliver these mutations in the mice genome, causing these mice to grow and develop cancer. These tumors will develop more naturally since they are encoded directly into the mouse's genome and can more accurately predict the effects of recently discovered therapies (3). This study paves the way for further research into CRISPR's ability to produce the same tumors in human cells via mutations in the human genome, meaning researchers can take samples of perfectly healthy human cells and mutate them to induce different types of cancer for further research and testing.

Researchers have uncovered one unique feature about bone marrow cells that makes AML and other forms of leukemia especially deadly; they can proliferate autonomously. Since B cells are responsible for producing white blood cells to attack intruders, they need to be able to change their rate of production as quickly as possible during infections and other diseases (3). Further, 60-80% of AML patients were found to have a V617F point mutation; this occurs in the gene responsible for producing JAK2 kinase, a cytokine that induces proliferation through the STAT family of transcriptional activators (5). Additional mutations were found in the myeloid transcription factors that lead to the loss of normal myeloid cell differentiation (6). Myeloid cells typically produce four different forms of white blood cells: leukocytes, megakaryocytes, thrombocytes, and erythrocytes. However, AML patients produces undifferentiated myeloblasts with no real function to the immune system. Together, these mutations allow leukemic myeloblasts to proliferate exponentially and enter the bloodstream under the radar from the rest of the immune system. AML is so difficult to test novel treatments due to its rapid spreading, as patients participating in these clinical trials do not have a lot of time to waste with shot-in-the-dark theories never before tested on true human AML tumors.

Genetic Engineering to Induce Myeloid Malignancies

This is where CRISPR-Cas9 comes into play. By identifying the genes associated with AML, mutations can be added to perfectly healthy human cells to induce the disease in a laboratory setting. From here, the cells can grow into realistic AML tumors to be studied and treated the same way they study the cancer in human patients. Before any testing began, the genes associated with AML were first identified in each genome. For mice, 5 genes were found and characterized as inducing leukemia across samples of mice: EZH2, NF1, TET2, RUNX1, and DNMT3A (2). The genes were identified by chemically inducing mice with AML and sequencing their DNA for mutations; the mice were exposed to carcinogens such as benzene to accurately account for the effects of the environment on the disease (7).

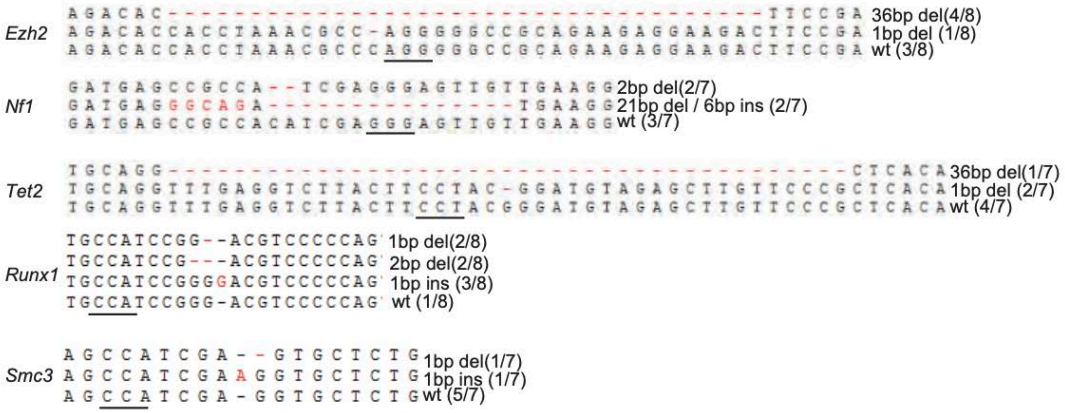

Figure 2: Murine genes targeted as inducing AML

These genes were found to have loss-of-function (LOF) mutations and are recurrently inactivated in mice with myeloid malignancies (2). They result in similar characteristics of AML in humans; no white blood cell differentiation and significantly increased autonomous cell proliferation. Several studies have accounted for the complementary relationship between mice and human AML cells, indicating that success inducing AML in the mouse genome will likely indicate the possibility of the same result on human cells. However, they also noted that the diseased cells react differently to the same treatments and progress differently, so even if it is successful to induce AML in mice cells, they cannot be used to accurately test human therapeutics (8).

In the subsequent study analyzing artificially induced AML in human cells, 7 mutations were previously identified to induce the disease via genetic analysis of myeloid samples from patients. The mutations, also LOF mutations, were located in the following genes: TET2, ASXL1, DNMT3A, RUNX1, TP53, NF1, EZH2 (2). Compared to the mutations identified in the mice genome to induce the same disease, the similarities are uncanny.

Methodology

Mouse Genome

Genetic editing was done via *Streptococcus pyogenes* Cas9 complementary DNA (cDNA) and chimeric small guide RNA (sgRNA). The complementary DNA contained mutations in the 5 genes previously identified as inducing AML in mice: EZH2, NF1, TET2, RUNX1, DNMT3A. Lentiviral vectors were generated based on the pLKO_TRC005 backbone, and reporter vectors were generated by replacing the eGFP fluorescent reporter with plasmodim artificial chromosomes (PACs) and inserting the RFP657 fluorescent reporter gene. The Cas9 target sites were introduced directly after the start codon of RFP657. These vectors were cultured in RPMI medium with 10% FBS and 100 U/mL of penicillin. Erythroleukemia cells were transduced with CRISPR-Cas9 reporter vectors with 4 ug/mL hexadimethrine bromide and selected 48 hours after transduction with 2 ug/mL puromycin for 72 hours and verified via flow cytometry (2).

Bone marrow was isolated from the femurs and tibias of 6-10-week-old female C57Bl/6 mice. The marrow was subject to erythrocyte lysis, followed by magnetic bead selection for CD117-positive cells. These cells were then stained with PacificBlue-labelled GR1 and CD11B

(obtained from BioLegend) and flow sorted. The cells were cultured with 50 ng/mL murine Thpo and 50 ng murine Scf for 48 hours and transduced with concentrated lentiviral supernatant. Blood nucleated cells were then purified peripherally and stained with May-Gruenwald stain, and a bone marrow histology was assessed from paraffin-embedded tissue sections. Cell colony assay were done in cytokine-supplemented methylcellulose and assessed 7 days after seeding. The assays were plated with 25,000 C57B1/6 wild-type bone marrow cells or 25,000 leukemic marrow cells (2).

All experiments and analysis were done with 4-5 mice per group, and no blinding or randomization was performed. Secondary transplant of leukemic mice was performed by transplanting 5 x 10^6 primary bone marrow cells into sublethally (650cGy) irradiated C57B1/6 mice. To assess the genetic editing efficiency, gDNA was isolated using a Qiagen DNA Blood Mini kit according to the manufacturer's instructions. The genetic region next to the CRISPR target site was amplified for each region by PCR to attach Illumina adaptors and add barcodes unique to each mouse. The libraries of each mouse were pooled and purified, and sequencing was performed using MiSeq (2).

Human Genome

Similar to the study using mouse bone marrow cells, genetic editing was done via *Streptococcus pyogenes* Cas9 cDNA and sgRNA. The Cas9 lentiviral transduction was constructed with LOF mutations in 7 genes targeted as driver genes of AML: TET2, ASXL1, DNMT3A, RUNX1, TP53, NF1, EZH2. Mutations in SMC3, U2AF1, and SRSF2 genes were induced as negative controls to introduce LOF mutations distinctly separate from LOF mutation linked to AML. The sgRNAs were tested for RFP657 fluorescent reporter protein expression. Each sgRNA was then tested by transducing the reporter cell line with a lentiviral vector containing Cas9-P2A-GFP driven by EFS promoter. Cells were analyzed for GFP and RFP657 positivity 12-14 days after transduction using flow cytometry, and each sgRNA's editing efficiency was determined using the following formula (3):

$$\%\ \textit{editing efficiency} = (\%\ \textit{RFP + untrasduced cells}) - (\%\ \textit{RFP + of GFP + transduced cells})$$

Genetic editing was done on donated male CD34+ cells; the study used only male donors as they are easier to target *STAG2*, an X-linked gene frequently mutated in male patients with myelodysplastic syndrome (MDS) and secondary AML. Each experiment was conducted with 1-40 million CD34+ cells from each donor, with the same donor cells used for different areas of the experiment (3). The cells were nucleofected with equal amounts CAG-Cas9-2A-GFP plasmid and cloned U6-sgRNA plasmid using Nucleofector II and CD34+ nucleofector kits, both obtained from Lonza. Control CD34+ cells were nucelofected using the same process with non-targeting sgRNA. After nucleofection, the cells were cultured in a cytokine supplemented medium for 16-24 hours prior to *in-vitro* analysis or transplantation *in-vivo* (3).

Prior to analysis, a fraction of GFP+ cells were cultured for an additional 5 days to maximize Cas9 cutting. 200,000-500,000 cells were transplanted per mouse, and transfection efficiency was estimated by GFP expression and assessed using flow cytometry to maintain efficiency between 60-80% for each group. Transfected colonies were cultured on a fully supplemented methylcellulose medium for 14 days (3).

8-10-week-old male and female NSGS mice were retro-orbitally injected with 200,000-500,000 cells per mouse, with 3-5 recipients per analysis. The mice were monitored daily for

presence of disease and sacrificed at designated times after transplantation. Peripheral blood was collected from the retro-orbital cavity using heparinized glass capillary and automated differential blood cell country were determined via hematology. Mice were examined for the presence of tumors, enlarged lymph nodes, or other abnormalities, and organs were collected for further cell and histopathologic analysis. Peripheral blood and bone marrow from mice were analyzed for the contribution of human CD45+ hematopoiesis by flow cytometry (3).

Results

Mouse Genome

Transduction of genetically edited cells into irradiated recipient mice caused significant myeloid skewing of hematopoiesis (production of blood cells, P = 0.007) and severe reduction of B cells (P = 0.001) compared to the control group transduced with control vectors containing non-targeting sgRNA. Peripheral blood from leukemic mice revealed increased granulocytes and circulating blasts, indicative of early AML patients (Figure 3A). Bone marrow histology revealed suppression of normal myeloid differentiation (Figure 3B). Bone marrow pathology confirmed AML in secondary transplant recipient mice (2).

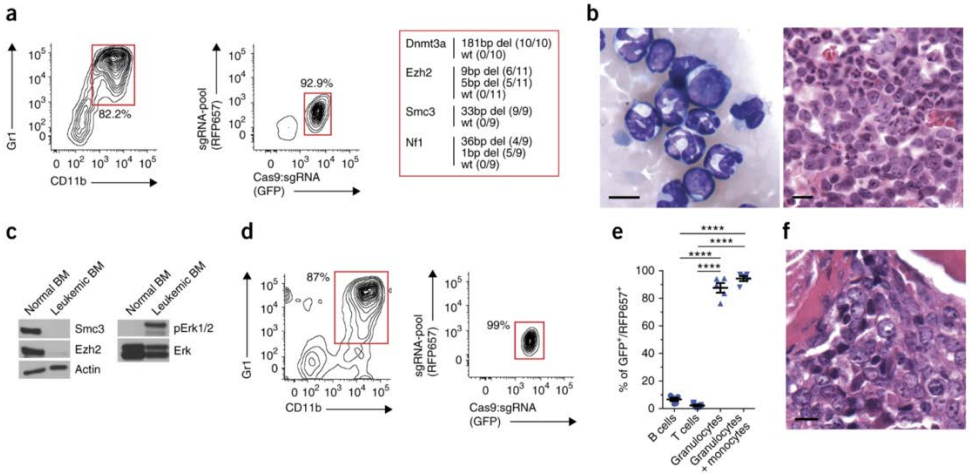

Figure 3: (a) flow cytometry analysis of diseased C57B1/6 mouse for expression of introduced Cas9-sgRNA vectors, with detected mutations located in the right panel. (b) Peripheral blood smear (left) and bone marrow (right) showing granulocytic cells (2).

Protein expression in the EZH2 and SMC3 genes were significantly reduced, consistent with the mutations detected in both genes. Clonal mutations were identified in TET2, DNMT3A, RUNX1, NF1, and EZH2 genes. None of the mice containing control nontargeting sg-RNA developed disease during the course of the experiment (2).

Human Genome

Sequencing of CD34+ single cell-derived colonies cultured on methylcellulose was done to analyze the presence of multiplex targeting. 37 of the 88 colonies analyzed (42%) had targeted editing of at least one gene, and 26 (70%) of those colonies had multiple targeted editing within a single cell clone. Analysis of cell-derived colonies show mutated undifferentiated myeloblasts

compared to the nontargeting sgRNA control group, which contained primarily leukocytes. Pools of sgRNA in a total of 50 mice were found to have targeting efficiencies of between 59-100% (3).

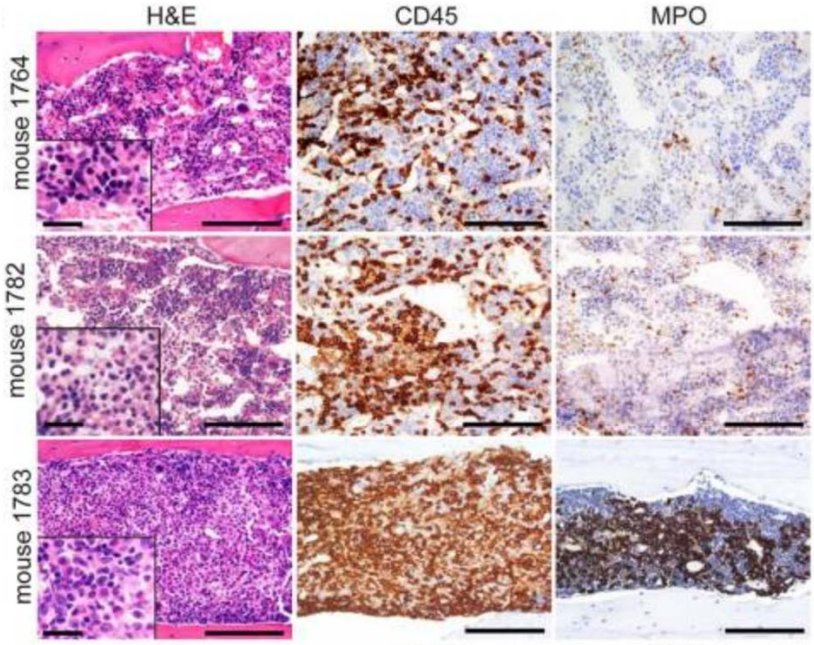

Figure 4: Functional *in vivo* models generated by targeting CD34+ cells. Notable expansion of immature CD45+ myeloid cells in bone marrow of mouse 1783 compared to control mouse 1764 and negative control 1782.

Discussion

Initial results show clear success in CRISPR/Cas9's ability to mutate mouse and human genomes within myeloid cells to induce AML. However, none of the models generated using human CD34+ cells developed leukemia as described by morphologic criteria. The study points to a number of reasons why: murine cytokines do not bind to human cytokine receptors, lack of contribution from human bone marrow stroma and a functional immune system as a result of inherent disadvantages of xenografts, and a focus on acquiring somatic lesions in clonal hematopoiesis over strictly inducing AML. The study mentions that future research using the same genetic editing over a human stroma-based xenograft system could produce different results and develop morphological leukemia (2, 3).

Both studies show the editing efficiency and effectiveness of CRISPR-Cas9, especially when mutating multiple genes in a single cell. They also point to how complex cancer genomes are, and how difficult it is to induce the same progression of AML in a lab setting. Beyond creating cancerous cells via targeted genetic mutations, the environment must also satisfy the cancer's ability to develop; without the system in place for cancerous cells to progress there is no way to test therapeutics designed for later stages (3, 4, 8).

However, both studies have led to the development of targeted therapy drugs for AML patients (3, 10). These drugs are designed to inhibit genetic changes in leukemic cells, designed to affect the body in a fundamentally different way than chemotherapy drugs. Midostaurin and gilteritinib (Rydapt and Xospate, respectively) have been approved as FLT3 inhibitors for people with AML. These drugs are designed to act against the mutations present in AML cells and block

unchecked myeloid cell proliferation, and don't come with the same side effects as traditional chemotherapy (10). Further research is needed to develop more complex models of AML for rigorous testing, but the effects of CRISPR-Cas9 genetic editing on cancer research can already be seen on the market today.

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