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Running Title: Leukemia-initiating cells are abundant in zebrafish T-ALL
Abstract

Self-renewal is a feature of cancer and can be assessed by cell transplantation into immune-compromised or immune-matched animals. However, studies in zebrafish have been severely limited by lack of these reagents. Here, Myc-induced T-cell acute lymphoblastic leukemias (T-ALLs) have been made in syngeneic, clonal zebrafish and can be transplanted into sibling animals without the need for immune suppression. These studies show that self-renewing cells are abundant in T-ALL and comprise 0.1% to 15.9% of the T-ALL mass. Large-scale single-cell transplantation experiments established that T-ALLs can be initiated from a single cell and that leukemias exhibit wide differences in tumor-initiating potential. T-ALLs can also be introduced into clonal-outcrossed animals and T-ALLs arising in mixed genetic backgrounds can be transplanted into clonal recipients without the need for MHC matching. Finally, high-throughput imaging methods are described that allow large numbers of fluorescent transgenic animals to be imaged simultaneously, facilitating the rapid screening of engrafted animals. Our experiments highlight the large numbers of zebrafish that can be experimentally assessed by cell transplantation and establish new high-throughput methods to functionally interrogate gene pathways involved in cancer self-renewal.
**Introduction**

T-cell acute lymphoblastic leukemia (T-ALL) is a devastating disease of childhood and is associated with transformation of thymic precursor cells. SCL and LMO1/LMO2 expressing leukemias account for 60% of pediatric cases and are more aggressive and treatment-resistant than the other four subtypes \(^1\), suggesting that distinct molecular pathways associated with transformation affect both tumor aggression and response to treatment. However, shared pathways are also utilized to transform T cells. P16/INK4a deletion is found in a majority of T-ALLs \(^2\), gain-of-function mutations and deletions in NOTCH1 are common \(^3,4\), and MYC is a central regulator of T-ALL formation \(^5\). Taken together, T-ALL comprises a group of diseases that transform T cells by common molecular pathways, but subtypes of disease have different clinical outcomes reflected by the developmental stage at which lymphocyte differentiation is arrested and by the expression of T-cell associated developmental programs.

Rare sub-populations of self-renewing cell types have been identified in mouse and human T-ALL. Cell transplantation experiments utilizing T-ALLs from Pten-deficient mice have identified the c-Kit\(^{mid}\)CD3\(^+\) subpopulation as the leukemia-initiating cell (LIC) \(^6\). This cell population engrafts disease into sublethally-irradiated SCID mice better than either the CD3\(^-\) or c-Kit\(^+\)CD3\(^+\) cells and comprises <0.01% of the total tumor. A rare subpopulation of human T-ALL-initiating cells have also been identified, with the CD34\(^+\)/CD4\(^-\) and CD34\(^+\)/CD7\(^-\) cell types being capable of engraftment when introduced into irradiated NOD/SCID mice \(^7\). In these experiments, 5x10\(^5\)-1x10\(^7\) unsorted leukemia cells were required for engraftment indicating that LICs are also rare in human T-ALL.
Additional xenograft transplantation studies establish that human acute leukemias engraft into NOD/SCID/IL2rγ-null animals but less efficiently when introduced into partially-immune compromised strains of mice. For the two ALLs described in this report, $1 \times 10^6$ unsorted leukemia cells were required to engraft disease ($n = 2$ of 4 or 2 of 5 transplant mice developed disease, respectively), again suggesting that ALL-initiating cells are rare. By contrast, tumor-initiating cells are frequent in syngeneic mouse models of T and B lymphoma and comprise >10% of the tumor mass. In these experiments, one primary Eμ-N-RAS-induced T-lymphoma was assessed for tumor-initiating potential by transplantation into syngeneic recipient animals. All three transplant animals that received ten lymphoma cells went on to develop disease; however, serial transplantation, a hallmark for definitively assessing long-term self-renewal potential, was not reported. Taken together, these opposing reports suggest that there is still quite a controversy in the field governing the numbers of T-ALL cells required to remake tumor and highlight the need for further experiments to accurately determine the cell types and numbers of tumor-initiating cells in human and genetic models of T-ALL.

Transgenic zebrafish models of Myc-induced T-ALL provide a unique platform to study leukemogenesis and self-renewal. Zebrafish T-ALLs develop by 30 days of life and can be induced into various genetic backgrounds by microinjecting the $rag2$-$mMyc$ transgene directly into one-cell-stage zebrafish embryos. Like human T-ALL, zebrafish leukemias are oligo-clonal, and $5 \times 10^8$ cancer cells can be isolated from a single leukemic zebrafish. T-ALL progression can also be visualized in mosaic animals, however, high throughput methods for detecting fluorescent-labeled tumors have not been described. Although the zebrafish model of T-ALL holds immense
promise for defining important pathways in human cancer, the data presented here suggests that tumor-initiating cell number cannot be accurately assessed by current cell transplantation protocols that introduce tumor cells into irradiated non-immune matched recipients\textsuperscript{11,13-21}. If immune barriers can be overcome, the zebrafish model will afford unique opportunities to uncover the mechanisms underlying self-renewal in both normal and malignant cells.

Here, syngeneic zebrafish (CG1-strain) are used to refine cell transplantation of zebrafish cancer and to accurately quantify the numbers of self-renewing cell types in T-ALL. We show that the percentage of leukemia-initiating cell types differs between T-ALLs and comprises between 0.1\% to 15.9\% of the T-ALL mass. Leukemia-initiating potential was also assessed by transplantation of single T-ALL cells, establishing that quantitative differences in leukemia-initiating potential exist between leukemias. By out-crossing CG1-strain fish to different genetic strains, it is also possible to transplant CG1 tumors into mixed genetic backgrounds or to transplant T-ALLs from a mixed genetic strain into CG1 recipient animals. Finally, high-throughput imaging methods are described to score adult transplant zebrafish for T-ALL engraftment. The methods outlined here capitalize on the large numbers of zebrafish that can be transplanted. In total, >1,453 transplant animals were used in these studies and lay the foundation for large scale experiments to define evolutionarily-conserved self-renewal pathways in cancer that are not available in more established models of malignancy.
Material and Methods

Animals

Zebralsh maintenance and developmental staging were conducted as described previously \(^1\). CG1-strain zebrasfish were a gift from Dr. Sergei Revskoy (Feinberg School of Medicine, Northwestern University, Chicago). Albino and *mylz2-GFP* fish were obtained from Dr. Leonard Zon (Children’s Hospital Boston). *rag2-GFP* transgenic fish were created previously \(^1\). Stable transgenic *mylz2-Amcyan, mylz2-mCherry, myogenin-mCherry*, and *creatine-kinase-zsYellow* zebrasfish were generated in the AB-strain background. Specifically, each promoter was amplified from genomic DNA using PCR. The forward primer had a *XhoI* site and the reverse a *BamHI* site. Fragments were PCR purified, digested with *XhoI* and *BamHI* and inserted into the *rag2-GFP* vector. Next, clones were digested with *BamHI* and *HindIII* to release the GFP cassette and either *mCherry, zsYellow*, or *Amcyan* were cloned into the modified vector. Plasmid DNA was digested with *XhoI* to linearize the transgene. DNA was phenol:chloroform extracted, ethanol precipitated, quantified by gel electrophoresis, and diluted to 120 ng/µl in 0.5xTE + 100 mM KCl. Next, AB-strain fish were microinjected at the one-cell stage of life and allowed to grow to adulthood. F0 injected animals were incrossed and the resulting progeny scored to identify stable F1 transgenic progeny. Transgenic F1 animals were outcrossed to AB-strain animals to propagate the line. All animal experiments have been approved by the MGH Subcommittee on Research Animal Care.
Generation of mosaic transgenic animals that develop T-ALL and rhabdomyosarcoma

The rag2-GFP\(^{22}\), rag2-mouse cMyc\(^{11}\), rag2-kRASG12D, and rag2-dsRED\(^{18}\) constructs have been described previously while the rag2-Amcyan and rag2-zsYellow constructs were made essentially as described\(^{11}\). DNA constructs were linearized with either XhoI or NotI, phenol:chloroform extracted, and ethanol precipitated. DNA was diluted in 0.5 X TE + 100mM KCl to 60 ng/microliter, with co-injection of two transgenes having 30ng of each construct/microliter. DNA was microinjected into the one-cell stage animals. Tumor-bearing zebrafish were identified at 30 to 60 days of life based on fluorescent protein expression and subsequently used for cell transplantation experiments when animals were 60- to120-days-old. RNA was also extracted from total tumor and wild-type AB-strain kidney marrow, muscle, or 24 hour embryos. Following Trizol extraction, RNA was treated with DNAse and then made into complementary cDNA. Samples were assessed for expression of lck, immunoglobulin M (IgM), rag2, mouse cMyc, and ef1-alpha as previously described\(^{11}\).

Cell transplantation and fluorescence-activated cell sorting

Primary T-ALLs and RAS-induced rhabdomyosarcomas were transplanted into non-irradiated CG1-, AB-strain, mixed CG1/Albino-strain or CG1/AB-strain recipient fish. T-ALLs were also transplanted into sub-lethally irradiated AB-strain recipients (single dose 23Gy, 2 days prior to transplantation)\(^{11,13}\). Cells were either sorted by fluorescence activated cell sorting (FACS) to isolate fluorescent T-ALL cells (propidium iodide was used to exclude dead cells), or unsorted cells were introduced into recipients.
by intraperitoneal injection (5 microliters injection volume). In some instances, fluorescent T-ALL cells were isolated from transplant recipients and used as donor cells for subsequent serial transplantation. For limiting dilution analysis, red blood cells from CG1-strain zebrafish were used as carrier cells along with T-ALL cells. Transplantation was completed essentially as described\textsuperscript{11,13}.

\textit{Limiting Dilution Analysis}

Limiting dilution analysis was completed using the Bonnefoix linear regression method. Accuracy of this test is determined by correlation coefficient ($R^2$ values). Subsequent analysis using L-calc and limdil software confirmed our limiting dilution calculations and provided 95% confidence intervals and T-test calculations to compare tumor-initiating cell number between samples (Table 1 and Supplementary Table S3 and S5). All three analyses gave similar results.

\textit{Assessing Tumor Engraftment}

Transplant recipient fish were analyzed for fluorescent tumor engraftment using 1) an Olympus SZX16 stereomicroscope outfitted with a Prior Lumen 200 epi-fluorescence illuminator and an Olympus DP-72 color camera or 2) the LED fluorescence macroscope at 10, 20, 30, 35, 45, 60, and 85 days post-transplantation. The anterior portion of leukemic fish was fixed in 4% paraformaldehyde, processed, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin. Additionally, leukemias were also analyzed by cytospin and May-Grunwald Giemsa staining to confirm lymphoblast morphology.
The LED fluorescence macroscope and image analysis

The inverted LED fluorescence macroscope is comprised of digital video cameras capable of still image photography (Logitech QuickCam, model S5500) that are positioned underneath a suspended plexiglass cover (Supplementary Figure S4). High-intensity light-emitting diode (LED) lights illuminate samples from below (405-420 nm blacklight, 420-470 nm blue light, 500 nm-570nm green light, incandescence light for brightfield imaging, PAR20 style bulbs), and a set of filters fit over the camera lenses allowing only emitted light of specific wavelengths to be detected. Two digital cameras interface with a Lenovo computer and images are captured using the AMCap imaging software. Video exposure, gain, intensity, and capture rate are controlled within the AMCap Software package. Brightfield and fluorescent images were imported into Photoshop and merged. For dual spectrum imaging, video was simultaneously captured using two cameras fitted with different filter sets. Digital images from each video frame were merged using the Osirix software.

Results

CG1-strain zebrafish

Eggs were manually extruded from a single Golden strain female, fertilized with UV-inactivated sperm, and subjected to heat shock to produce gynogenetic diploid animals as previously described 23. Gynogenetic females were raised to adulthood and their eggs were used in a second round of heat shock. The resulting F3 progeny were
incrossed to create the CG1-strain (gift from Dr. Sergei Revskoy and Igor Mizgirev, Northwestern University, Chicago). The CG1 clonal fish line has not been described previously and is not related to the CB1 or CW1 lines.

To validate that CG1-strain fish are genetically similar, a genome-wide survey was performed on 281 loci in four CG1 animals and four AB-strain zebrafish. In total, 275 of 281 CA repeat polymorphisms are identical in all four CG1 fish. The primer sets used in this analysis are scattered throughout the genome and are present on all 25 chromosomes (Supplementary Table S1). Of these primer combinations, 63 distinguished allelic differences between CG1 and AB fish, and as expected the AB-strain sibling fish were heterogeneous at many loci (n = 165 of 281). We conclude that CG1 fish are 97.9% identical.

T-ALLs arising in CG1-strain zebrafish can be transplanted into non-irradiated, CG1 recipients

rag2-mouse cMyc (mMyc) and rag2-GFP, rag2-dsREDexpress, rag2-zsYellow, or rag2-Amcyan constructs were co-injected into one-cell stage CG1-strain animals. Because injection of multiple transgenes into one-cell stage embryos leads to co-segregation and co-expression in developing tumors, T-ALLs that develop were fluorescently-labeled. T-ALLs were first detected in the thymus of mosaic animals and disseminated widely as leukemias progressed, similar to previous reports. Leukemias expressed T-cell specific lck, rag1, and mouse cMyc, but failed to express high levels of immunoglobulin M (IgM) or the muscle-specific factor, desmin, confirming that leukemias were T-cell in origin (Supplementary Figure S1). T-ALL cells were
disassociated from 60- to 120-day-old leukemic fish and subjected to FACS with dead cells being excluded by propidium iodide (94.0-97.3% pure, 97.9-98.9% viable). Fluorescent-labeled T-ALL cells were analyzed by cytospin to confirm lymphoblast morphology (n = 12, Supplementary Figure S2) or transplanted into non-irradiated CG1- and AB-strain animals and gamma-irradiated AB-strain fish at limiting dilution (23Gy, 2 days prior to transplantation, 5x10⁴, 1x10⁴, 1x10³, and 1x10² T-ALL cells supplemented up to 5x10⁴ cells with red blood cells (RBCs), n = 3 tumors analyzed total).

Fluorescent-labeled T-ALL cells from CG1-strain animals engrafted robustly into both non-irradiated CG1 recipients and irradiated AB-strain animals (AB+IR) by 10 days post-transplantation (Figure 1A,D and Supplementary Table S2); however, AB+IR recipients had fewer engrafted animals when compared to CG1 recipients at both 10 and 20 days post-transplantation (Compare Figure 1J to 1K) and less tumor burden (Figure 1A,B and 1D,E). Most T-ALLs arising in AB+IR recipient fish had completely regressed by 30 days post-transplantation, while those arising in CG1 fish continued to grow (Figure 1C,F, J-K). Sub-lethal irradiation of AB-strain recipient animals also led to increased death of recipient animals, with 21 of 124 AB+IR recipients dying by 45 days post-transplantation compared to 5 of 114 CG1-recipients (p = 0.003, Fisher exact test). This difference could not be accounted for by death due to tumor burden (Supplementary Table S2). Limiting dilution experiments establish that leukemia-initiating frequency was underestimated by 20 to 33 fold when primary leukemias were introduced into AB-irradiated recipient animals (Supplementary Table S3). As expected, T-ALL cells failed to engraft into non-irradiated AB-strain recipients¹¹ (Figure 1G-I). Taken together, our data show that limiting dilution analysis and cell transplantation into irradiated AB-strain
animals will severely underestimate tumor-initiating cell number due to inefficient ablation of the immune system and irradiation-induced lethality of recipient fish.

*Leukemia-initiating cells comprise 0.1%-1.4% of the primary T-ALL*

To assess the number of self-renewing cells contained within the bulk of the primary T-ALL tumor mass, fluorescent-labeled T-ALL cells were isolated from CG1-strain zebrafish and introduced into CG1-strain recipients at limiting dilution (1x10^4, 1x10^3, 1x10^2, 1x10^1 T-ALL cells supplemented up to 2x10^4 cells with RBC carrier cells, purity 95-99%, viability 94-99.9%, n = 4 T-ALLs analyzed). Engraftment was assessed at 20, 30, 45, and 85 days post-transplantation by epi-fluorescence stereomicroscopy (raw data is presented in Supplementary Table S4) and leukemia-initiating cell number was calculated after 85 days post-transplantation using three methods: the Bonnefoix limiting dilution method \textsuperscript{24}, the L-calc statistical software (Stem Cell Technologies), and the web-based limdil program (http://bioinf.wehi.edu.au/software/elda/index.html). All three analyses gave similar results (Table 1 and Supplementary Table S5). In total, these experiments suggest that leukemia initiating cells are abundant in T-ALL and comprise between 0.1% to 1.4% of the primary tumor mass. Moreover, these results show that leukemia-initiating frequency can differ between T-ALLs (compare #6 to #8, p=0.0001).

The ability to induce disease following serial transplantation is a hallmark of self-renewing cancer cells. To determine if leukemia-initiating potential is retained after serial transplantation, fluorescent-labeled leukemias were isolated from primary transplant animals that received 1x10^4 T-ALL cells and introduced at limiting dilution into CG1 secondary recipients (purity 93.9-95.7%, viability 94.3-97.2%). Most T-ALLs
retain similar proportions of LICs when compared to primary leukemias (n=3, Table 1 and Supplementary Table S5). Serial transplantation confirms that leukemia-initiating potential is found in a large portion of T-ALL cells.

*Single cell transplantation confirms that tumor-initiating potential differs between T-ALLs*

Although leukemia-initiating cells are abundant in zebrafish T-ALL, their number differs between leukemias (Table 1). To further validate that T-ALLs can exhibit marked differences in tumor-initiating potential, single cell transplants were completed using either primary leukemias (n = 2), a single passage T-ALL (n = 1), or a serially-passaged T-ALL that had high leukemia-initiating capacity (T-ALL #9, serial passage three). For single cell transplants, cells were sorted into 96-well plates with each well supplemented with 2x10^4 RBCs. Test sorts verified that single cells were found in most wells (n = 45 of 48, Supplementary Figure S3). One GFP-labeled primary leukemia engrafted 2 of 105 transplant animals by 60 days post-transplantation while single cells from the other primary and one-time passaged T-ALL failed to engraft disease by 120 days post-transplantation (n=0 of 210), indicating that tumor-initiating cell number was low in these T-ALLs. Tumor purity and viability were very high in all three samples following FACS (>95% viability and >97% purity), and 1x10^4 cells from these same leukemias lead to engraftment in all recipient animals (n = 6 per tumor). One CG1-strain T-ALL was serially transplanted three times (T-ALL #9). This leukemia had high leukemia initiating frequency and engrafted robustly into recipient animals (n = 7 of 44, viability 97.9% and purity 94.1%, Figure 2). These results highlight the large scale single cell transplantation
experiments that are now possible in zebrafish (n=359 animals) and confirm that leukemias can exhibit wide variation in leukemia-initiating potential with ≤ 0.1% to 15.9% of all leukemia cells having the capacity to remake tumor (p<0.003, Fisher Exact test).

T-ALLs can be transplanted into CG1-outcrossed animals

Non-tumorigenic somatic cells can alter the tumor microenvironment and can affect recruitment of tumor vasculature, invasion, and metastasis. These processes can be assessed experimentally by transplanting tumor cells into syngeneic recipients that harbor specific genetic lesions. Moreover, introduction of tumors into various fluorescent transgenic zebrafish lines will allow these processes to be visualized in vivo. To determine if CG1-strain zebrafish are amenable to such analysis, T-ALLs from CG1 fish were transplanted into the progeny of CG1-strain zebrafish crossed to Albino homozygous animals. Remarkably, T-ALLs engrafted robustly into CG1/Albino recipients (n = 24 of 31, Figure 3A-C and Table 2), however, less efficiently than pure CG1 recipients (n = 27 of 27, p = 0.01, Fisher Exact Test). T-ALLs from CG1-strain zebrafish were introduced into the progeny of CG1 fish crossed with 1) wild-type AB-strain, 2) rag2-GFP (AB-strain, ^22^), or 3) mylz2-mCherry (AB-strain) transgenic zebrafish. In contrast to experiments using Albino animals, T-ALLs did not engraft efficiently into F1 AB-strain outcrossed recipients (n = 1 of 23 CG1/AB recipient animals developed T-ALL, p < 0.001, Fisher Exact Test). However, T-ALLs displayed increased engraftment potential when introduced into progeny resulting from two rounds of outcrossing to CG1 fish - a mating of CG1 fish to CG1/AB fish (p = 0.001, Fisher Exact
Test). In total, 8 of 19 CG1 x CG1/AB animals engrafted T-ALL (Figure 3D-I, and Table 2), and by the third out-cross to CG1-strain fish, all animals were able to engraft T-ALLs arising from CG1-strain fish (n=18 of 18). Taken together, our experiments show that it will now be possible to outcross CG1-strain zebrafish to specific lines of interest, allowing T-ALLs from CG1-strain fish to be efficiently transplanted into mixed mutant and/or transgenic backgrounds without the need for MHC matching or immune ablation by gamma-irradiation. Our experiments also indicate that Albino strain fish are more closely related to CG1 fish and that strain differences will have major impacts on the number of outcrosses required to create syngeneic recipient animals.

_T-ALLs from a mixed CG1 genetic background can be transplanted into CG1 recipients_

Generating zebrafish tumors that have heritable genetic lesions will be important for identifying genes that modulate tumor engraftment, metastatic potential, and self-renewal. As such, T-ALLs were generated in zebrafish of mixed genetic backgrounds by microinjecting \textit{rag2-mMyc} and \textit{rag2-GFP} or \textit{rag2-dsREDexpress} into one-cell-stage 1) CG1 embryos, 2) the offspring of a CG1 by Albino homozygous cross (CG1/Alb), or 3) the progeny of a CG1/Alb by CG1 cross (CG1/Alb x CG1). T-ALLs arising in mosaic transgenic animals were transplanted into non-irradiated CG1-strain recipient animals (Table 2). As expected, T-ALLs from CG1-strain zebrafish engrafted robustly into CG1 recipients (n = 3 T-ALLs, 10 fish per tumor, data not shown), while T-ALLs from CG1/Albino fish failed to engraft into CG1 recipients (n = 3 tumors assayed, Table 2). By contrast, a subset of T-ALLs arising from CG1/Alb x CG1 fish engrafted into non-irradiated CG1-strain recipients (n = 2 of 6 T-ALLs assayed), and in cases where
transplantation was possible, the percent of animals with tumor engraftment was remarkably high (n = 22 of 23 transplanted zebrafish engrafted T-ALLs). Similar results were also seen using a kRASG12D-induced transgenic model of embryonal rhabdomyosarcomas (Supplementary Table S6). Specifically, injection of $\text{rag2-}k\text{RASG12D}$ into AB-strain $\text{mylz2-mCherry}$ transgenic animals that had been outcrossed to CG1 fish three times produced transplantable rhabdomyosarcomas. All four rhabdomyosarcomas assayed could engraft with 100% efficiency when introduced into CG1 recipient animals (n=25 of 25, Supplementary Table S6). Taken together, these experiments provide new methodologies to rapidly introduce existing transgenic reporter lines and genetic mutations into syngeneic zebrafish.

**High-Throughput imaging using the LED fluorescence macroscope**

Having accurately determined the leukemia-initiating cell number in zebrafish T-ALL and developed methods to introduce tumor cells into a variety of genetic and transgenic backgrounds, we wanted to streamline the methods for detecting tumor engraftment in recipient animals. For example, screening transplant fish for engraftment using a conventional fluorescence-dissecting microscope is laborious and time-consuming because screening is limited to visually inspecting one fish at a time (Figure 4Q-T). To expedite screening of both normal and tumor-bearing fluorescent transgenic zebrafish, a low-cost, high-throughput machine has been developed that can score a whole Petri dish of adult transgenic zebrafish - the LED fluorescence macroscope (Supplementary Figure S4). In total, the LED fluorescence macroscope is capable of discriminating five colors (Figure 4) including: Amcyan (blacklight, 480/30 filter), GFP
(blacklight, 520/30 filter), zsYellow (blue light, 575/52 filter), dsREDexpress (blue light, 610/40), and mCherry (green light, 610/40 filter or 640/35). The emission/excitation spectra for each fluorescent protein are shown in Supplementary Figure 5.

To validate the sensitivity of the macroscope in detecting fluorescent transgenic zebrafish, six stable transgenic lines that express Amcyan, GFP, zsYellow, or mCherry were scored using the LED fluorescence macroscope (Supplementary Table S7). 140 of 141 fluorescent transgenic animals could be discriminated using the LED fluorescence macroscope (filter and light combinations shown in Supplementary Table S8) whereas non-transgenic zebrafish were also negative by macroscope analysis (n = 53 of 53). Unanesthetized animals can be imaged in real-time using either video or still image capture, further facilitating the rapid screening of transgenic zebrafish (Supplementary Figure S6, Supplementary Movies 1-4). Collectively, these results indicate that the LED fluorescence macroscope is exceedingly accurate at identifying stable transgenic lines, can image live fluorescent zebrafish in real-time without the need for anesthesia, and can effectively discriminate multiple fluorescent proteins.

In order to assess if the LED fluorescence macroscope could be used to improve the detection of transplant engraftment, Myc-induced T-ALLs were generated that express Amcyan, GFP, zsYellow, or dsREDexpress and were transplanted into non-irradiated, syngeneic recipient animals (1.5x10^4 leukemia cells). LED fluorescence macroscope imaging reliably identified engrafted animals by 30 and 45 days post-transplantation for all four fluorophores (n = 140 of 140, Figure 4I-P, Supplementary Table S7) when compared to conventional epi-fluorescence microscopic analysis (Figure 4Q-T, Supplementary Figure S7). The LED fluorescence macroscope could also image
fluorescent-labeled rhabdomyosarcomas that had been transplanted into CG1-strain recipients, suggesting that imaging methods are not limited to T-ALL (Supplementary Figure S8). Finally, the LED fluorescence macroscope also identified animals that had engrafted T-ALL from single cells. 105 animals transplanted with single primary GFP-labeled T-ALL cells were scored by both conventional epi-fluorescence microscopy and macroscopic analysis. LED fluorescence macroscope imaging identified two engrafted animals that had developed prominent GFP-labeled T-ALL by 60 days post-transplantation. Similar results were also obtained with a dsRED-labeled T-ALL that had been serially passaged and contained large numbers of cells with tumor-initiating potential (Figure 5A-C). Specifically, sixty-four single cell T-ALL transplants were completed, and subsequently, animals were analyzed for engraftment at 30 and 45 days post transplantation. All nine fish that engrafted T-ALL could be scored by either an epi-fluorescence stereomicroscope or the LED fluorescence macroscope (Figure 5A-C).

Together, the results indicate that it is now possible to perform high throughput imaging of single cell transplants, allowing the unprecedented study of self-renewal and clonal evolution within fluorescent leukemias. Moreover, a whole Petri dish of up to 30 animals can be scored simultaneously, compared to single inspection using an epi-fluorescence stereomicroscope.

The LED fluorescence microscope also allows for simultaneous imaging of multiple fluorescent proteins using two cameras fitted with different filter sets. In total, 5 of 6 fluorescent combinations could be resolved by multi-spectral imaging (Supplementary Table S9) and can easily delineate Amcyan from mCherry (Figure 5D), GFP from mCherry (Figure 5E), and zsYellow from mCherry (Figure 5F). Moreover, still
images and movies of live fish could be captured in un-anesthetized, swimming animals, indicating that multi-spectral analysis can also be completed in real-time (Figure 5 and Supplementary Movies 5-7). Multi-spectral imaging can also be used to image T-ALL transplant recipient animals. In these experiments, $5 \times 10^5$ Amcyan-labeled T-ALL cells were introduced into twice outcrossed mylz2-mCherry transgenic zebrafish and imaged for engraftment at 30 days post-transplantation using a epi-fluorescence microscope (Figure 5G) or the LED fluorescence macroscope (Still image in Figure 5H or single frame of Supplementary Movie 8 is shown in Figure 5I). Together, our work describes a novel methodology to simultaneously capture real-time, multi-channel images and video of live dual fluorescent transgenic fish, a technique not available by traditional epi-fluorescence stereomicroscopy.

Discussion

Zebrafish have become a powerful tool for dissecting molecular pathways in cancer $^{25}$, but assessment of cancer cell self-renewal has been limited by lack of immune compromised zebrafish and genetically-identical animals. For example, rag1-deficient zebrafish have been generated $^{26}$, but have yet to be used in cell transplantation assays. Additionally, immune compromised NOD/SCID zebrafish have not been reported in the literature. Instead, investigators have used sub-lethal gamma-irradiation to ablate the immune system and subsequently introduce tumor cells into adult recipient animals $^{11,13}$-$^{21}$. Pioneering work from Misgireuv and Revskoy have shown that chemically-induced liver cancers arising in clonally-derived, isogenic zebrafish can be successfully transplanted into non-irradiated, sibling zebrafish $^{23}$. However, liver tumors were not
assessed for tumor-initiating cell number, nor were fluorescent reporters used to visualize tumor formation and engraftment. Moreover, transplantation of tumors from mixed genetic backgrounds into clonal zebrafish was not fully described. No additional reports have used clonal fish lines to assess tumor engraftment or to identify self-renewing cell types in cancer.

The data presented here shows that transplantation experiments that use irradiation to ablate the immune system will fail to accurately determine leukemia-initiating cell number. Our previous work suggested that T-ALL-initiating cell number was approximately 1 in 1x10^3 to 1 in 2x10^4 cells 20. Similar leukemia-initiating frequency was also recently reported for genetic models of zebrafish T-cell leukemia that used similar methodologies for introducing T-ALLs into non-immune matched, irradiated recipient animals 21. The detailed studies described here show that cell transplantation into non-immune matched gamma-irradiated animals severely underestimates true cancer-initiating cell number due to inefficient ablation of the immune system, subsequent recovery of immune responses by 20 days post-irradiation, and death of animals due to gamma-irradiation directly. Transplantation experiments using clonal CG1-strain zebrafish reveal that leukemia-initiating cell number is much higher than previously reported with 0.1%-15.9% of T-ALL cells capable of engraftment into syngeneic animals.

Leukemia-initiating cell number is quite high in primary zebrafish T-ALL, signifying that self-renewal may also be a much more common attribute in malignant T-ALL cells than previously suggested 6-8,27. There are several possible reasons that could account for why leukemia-initiating cell number differs so greatly between zebrafish and
human T-ALL. Human T-ALL-initiating cell number has been assessed using xenograft transplantation into partially-immune compromised SCID or NOD/SCID recipient mice. Recent work from Quintana et al. established that melanoma-initiating cell number is vastly underestimated using NOD/SCID recipient animals and reported a 100-fold increase in self-renewing cells following transplantation into NOD/SCID/IL2 receptor-gamma-deficient mice. Moreover, in the one report where human ALLs were introduced into NOD/SCID/IL2 receptor-gamma-deficient animals, it is uncertain if T- or B-ALLs were analyzed and which molecular subtype of leukemia was assessed. Our results in zebrafish raise the interesting possibility that human T-ALLs contain many more leukemia-initiating cells than previously reported, however, additional experiments will be required to determine if self-renewal potential differs across molecular subtypes and if common self-renewal mechanisms are utilized among all T-ALLs.

Having refined the methods for cell transplantation in zebrafish, there is a major need for high throughput imaging modalities to identify animals engrafted with fluorescent-labeled tumors. The LED fluorescence macroscope is inexpensive, can score many fish at one time, and can detect multiple fluorescent channels simultaneously. Use of the LED fluorescence macroscope will not be limited to cancer biology. For example, the LED fluorescence macroscope will aid in the identification of fluorescent transgenic founder fish, can be used in conjunction with transplantation of quantum dots into the peritoneum or musculature to track individual fish within a tank, and can be used for real-time imaging of complex behaviors of adult fish. In our experiments, the LED fluorescence macroscope has sped the screening of stable transgenic zebrafish and transplant engraftment, paving the way for high throughput cell transplantation.
experiments to assess the kinetics of tumor engraftment, cancer stem cell self-renewal, and clonal evolution.

The functional transplantation assays and new high-throughput detection methods outlined here afford unique opportunities to assess molecular pathways involved in leukemia self-renewal. For example, it is possible to deliver cancer modifying transgenes to developing T-ALLs by merely co-injecting \( \text{rag2-mMyc} + \text{rag2-dsREDexpress} + \text{rag2-gene of interest} \) into one-cell-stage CG1 fish. Previous experiments suggest that co-segregation of three transgenes into microinjected zebrafish embryos results in high frequency of co-expression within developing T-ALLs (\(n = 22\) of 23) \(^{20}\). When coupled with FACS and limiting dilution cell transplantation, such approaches will allow direct assessment of transgene effects on self-renewal without the need for establishing stable transgenic animals or for complex breeding strategies to generate compound transgenic zebrafish. These methods are not only amenable to Myc-induced T-ALL, but it will also be possible in any transgene-induced malignancy in zebrafish. It also is now feasible to introduce T-ALL into various genetic backgrounds to assess modifying gene effects on leukemia by breeding a mutant or transgenic line to CG1-strain animals and backcrossing these progeny to CG1 fish. Subsequent generations of backcrossed animals can be injected with cancer causing transgenes and functionally assessed for metastasis, recruitment of vasculature, or self-renewal by engraftment of tumors into CG1 recipient animals. Importantly, these processes can be visualized by transplanting tumors into syngeneic fluorescent reporter lines produced by multiple out-crossings to CG1-strain fish. Such experiments capitalize on functionally assessing immune competency without the need for complex immune matching. Although we have focused on using our high-
throughput methods of cell transplantation and imaging to assess self-renewal in T-ALL, these methods will be broadly applicable to studies involving cell transplantation of hematopoietic stem cells, muscle stem cells, and organ-specific stem cells. Together our results highlight the emergence of the zebrafish as a new model of cancer cell self-renewal and provide new high-throughput methods for functionally assessing tumor-initiating cell number in cancer that do not exist in more established vertebrate models of disease.

Acknowledgments

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Authorship Contributions and Disclosure of Conflicts of Interest

A.C.H.S., A.R.R. designed and performed experiments, wrote portions of the manuscript. C.D.S., M.S.I, J.S.B., N.Y.S, J.L.O.dJ., A.T.C., Y.Z. performed experiments and edited the manuscript. I.V.M. and S.R. produced the CG1 strain zebrafish and edited the manuscript. D.M.L. designed and performed experiments, wrote the manuscript.

L.I.Z. is a founder and stock holder of Fate, Inc. and a scientific advisor for Stemgent.
References


Table 1. Leukemia-initiating cells are abundant in zebrafish T-ALL. Leukemia initiating cell frequency (LIC Frequency) and 95% confidence intervals were calculated by the L-calc program and correlation coefficient $R^2$ values show how well the data fit using the Bonnefoix limiting dilution method. Asterisk denotes that sample #8-1 T-ALL is significantly different from all other samples ($p<0.05$), except #5-1 T-ALL. The raw data used to calculate LIC frequency are shown in Supplementary Table S4.

Table 2. T-ALLs from mixed CG1-backgrounds can be introduced into non-irradiated CG1-recipients. The $rag2-mMyc$ and $rag2-GFP$, $rag2-dsREDexpress$, or $rag2-zsYellow$ transgenes were co-injected into various genetic backgrounds at the one-cell stage of life and T-ALLs that developed were introduced into recipient animals by intra-peritoneal injection. Transplanted animals were scored for T-ALL engraftment at 10, 20, and 30 days. Table is a compilation of the total number of animals that had engrafted disease by 30 days. Albino (Alb) and AB-strain (AB). Mixed* is a combination of three independent experiments where T-ALLs arising in CG1 animals were introduced into AB-strain fish that were once outcrossed to CG1-strain animals (AB/CG1), twice outcrossed (AB/CG1xCG1), or three times outcrossed ([AB/CG1xCG1] x CG1). Not assayed (NA).
Table 1.

<table>
<thead>
<tr>
<th>T-ALL Donor</th>
<th>Recipients per Experiment</th>
<th>LIC Frequency</th>
<th>95% Confidence</th>
<th>Correlation R² value</th>
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</thead>
<tbody>
<tr>
<td>#5 - 1⁰ T-ALL</td>
<td>n = 44</td>
<td>1/250</td>
<td>1/94 to 1/663</td>
<td>0.9802</td>
</tr>
<tr>
<td>#5 - 1⁰ Transplant</td>
<td>n = 42</td>
<td>1/57</td>
<td>1/30 to 1/110</td>
<td>0.9982</td>
</tr>
<tr>
<td>#6 1⁰ T-ALL</td>
<td>n = 46</td>
<td>1/71</td>
<td>1/37 to 1/139</td>
<td>0.9917</td>
</tr>
<tr>
<td>#6 - 1⁰ Transplant</td>
<td>n = 40</td>
<td>1/60</td>
<td>1/32 to 1/114</td>
<td>0.9112</td>
</tr>
<tr>
<td>#7 - 1⁰ T-ALL</td>
<td>n = 40</td>
<td>1/77</td>
<td>1/39 to 1/151</td>
<td>0.999</td>
</tr>
<tr>
<td>#7 - 1⁰ Transplant</td>
<td>n = 41</td>
<td>1/135</td>
<td>1/59 to 1/306</td>
<td>0.9859</td>
</tr>
<tr>
<td>#8 - 1⁰ T-ALL</td>
<td>n = 35</td>
<td>1/845*</td>
<td>1/277 to 1/1212</td>
<td>0.9852</td>
</tr>
</tbody>
</table>

Table 2.

<table>
<thead>
<tr>
<th>Tumor ID</th>
<th>Donor Background</th>
<th>Transplant Cell #</th>
<th>Recipient Background</th>
<th>[AB/CG1 x CG1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>CG1</td>
<td>5x10⁶</td>
<td>5 of 6</td>
<td>NA</td>
</tr>
<tr>
<td>#3</td>
<td>CG1</td>
<td>5x10⁶</td>
<td>3 of 4</td>
<td>NA</td>
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<tr>
<td>#4</td>
<td>CG1</td>
<td>5x10⁶</td>
<td>2 of 6</td>
<td>14 of 14</td>
</tr>
<tr>
<td>Mixed</td>
<td>CG1</td>
<td>5x10⁶</td>
<td>1 of 1</td>
<td>NA</td>
</tr>
<tr>
<td>A</td>
<td>CG1/Alb</td>
<td>5x10⁶</td>
<td>0 of 7</td>
<td>1 of 23</td>
</tr>
<tr>
<td>B</td>
<td>CG1/Alb</td>
<td>5x10⁶</td>
<td>0 of 6</td>
<td>8 of 19</td>
</tr>
<tr>
<td>C</td>
<td>CG1/Alb</td>
<td>5x10⁶</td>
<td>0 of 5</td>
<td>18 of 18</td>
</tr>
<tr>
<td>D.1</td>
<td>CG1/Alb x CG1</td>
<td>5x10⁶</td>
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<td>NA</td>
</tr>
<tr>
<td>D.2</td>
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<td>5x10⁶</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>D.3</td>
<td>CG1/Alb x CG1</td>
<td>5x10⁶</td>
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<tr>
<td>D.4</td>
<td>CG1/Alb x CG1</td>
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<tr>
<td>D.5</td>
<td>CG1/Alb x CG1</td>
<td>5x10⁶</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>D.6</td>
<td>CG1/Alb x CG1</td>
<td>5x10⁶</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
Figure 1. T-ALLs from CG1-strain zebrafish engraft into non-irradiated CG1-strain recipients. GFP-labeled T-ALLs were isolated from primary leukemic fish and 1x10^3 FACS sorted GFP-labeled leukemia cells were transplanted into non-irradiated CG1- and AB-strain animals (A-C and G-I, respectively) or irradiated AB-strain fish (D-F). Transplant fish were scored for engraftment at 10, 20, and 30 days post-transplantation. Panels are merged images of fluorescent and brightfield photographs. The engraftment kinetics differ greatly when T-ALLs are introduced into CG1 (J) or irradiated AB-strain zebrafish (AB+IR, K). Percent engraftment is the percent of recipient animals that have visibly engrafted T-ALL at each time point. Panels J-K are combined data from three independent experiments (n = 238 transplants total). The raw data for this experiment are available in Supplementary Table S2.

Figure 2. Single T-ALL cells can be efficiently transplanted into non-irradiated CG1 recipient. Transplant recipient fish receiving one FACS sorted dsRED+ T-ALL cell at 20 (A,D), 30 (B, E), and 45 days post-transplantation (C, F). One representative animal engrafted T-ALL by 20 days (A-C) while one never developed leukemia (D-F). Images photographed at 0.7X. All panels are merged images of fluorescent and brightfield photographs.

Figure 3. T-ALLs from CG1 fish are capable of engraftment into CG1 outcrossed animals. A GFP-labeled T-ALL engrafted into the progeny of CG1 by Albino fish (CG1/Alb, A-C). Engraftment of T-ALLs into AB-strain zebrafish required two rounds of outcrossing to CG1 fish (D-I). A dsRED-labeled T-ALL engrafted into the progeny of
a CG1 by CG1/AB *rag2-GFP* transgenic animal (D-F) or an Amcyan-labeled T-ALL engrafted into the progeny of a CG1 by CG1/AB *mylz2-mCherry* (G-I). Images photographed at 0.7X. All panels are merged images of fluorescent and brightfield photographs. GFP-labeled thymus is marked by T.

**Figure 4.** The LED fluorescence macroscope can image five fluorescent fluorophores in normal muscle and T-cell acute lymphoblastic leukemia (T-ALL). Whole animal imaging of wild-type (Negative), *mylz2-Amcyan*, *mylz2-GFP*, *creatine kinase-zsYellow*, *mylz2-mCherry* transgenic zebrafish (A-D). Amcyan was imaged using a blacklight and 480/30 filter (A,J,N), GFP (blacklight, 520/30 filter, B,K,O), zsYellow (blue light, 575/52 filter, C), dsREDexpress (green light, 610/40 filter, L,P), and mCherry (green light, 610/40 filter, D). Transgenic zebrafish imaged using an epi-fluorescence stereomicroscope at the lowest magnification (0.7X; E-H). The LED fluorescence macroscope is also capable of imaging engraftment of T-cell acute lymphoblastic leukemia in recipient animals. Whole animal imaging of recipient fish transplanted with $1.5 \times 10^4$ Amcyan-, GFP-, or dsREDexpress-labeled leukemia cells at 30 days post-transplantation (I-L). Animals from panels I-L were mixed to demonstrate that fluorescent-labeled animals can be easily delineated (M-P). Transplant recipients imaged using an epi-fluorescence stereomicroscope at low magnification (0.7X, Q-T). Scale bars are 2cm in panels A-D, I-L and 5mm in panels E-H and Q-T.

**Figure 5.** The LED fluorescence macroscope can detect tumor engraftment of single T-ALL cells and is capable of multi-spectral, real-time imaging. Animals engrafted with a
single dsRED-labeled T-ALL cell can be easily distinguished from non-engrafted animals at 45 days post-transplantation (n = 1 of 10 fish, A; n = 2 of 20 fish, B; n = 3 of 29 fish panel C) by LED fluorescence macroscopy. Multi-spectral imaging using the LED fluorescence macroscope (D-I). Still image capture utilizing dual-spectrum imaging of un-anesthetized mylz2-Amcyan and mylz2-mCherry transgenic animals (D, blue and black light with 520/40 filter and 640/35 filter), mylz2-GFP and mylz2-mCherry (E, blue light with 520/40 filter and 640/35 filter) and creatine kinase-zsYellow and mylz2-mCherry (F, blue light with 575/52 filter and 640/35 filter). Animals were imaged from below at 30 frames per second and a single frame is shown. Multi-spectral imaging can also be used to visualize fluorescent-labeled T-ALLs engrafted into fluorescent recipient animals. A mylz2-mCherry animal engrafted with an Amcyan-labeled T-ALL at 30 days post-transplantation imaged by epi-fluorescence stereomicroscopy (G, 0.7X magnification) or by using the LED fluorescence macroscope (blue light with 480/30 and 640/35 filters, H-I). Panel H shows a control animal (top) along with a dual transgenic leukemic fish (bottom). Three leukemic fish imaged from below at 30 frames per second and a single frame is shown (I). Scale bars are 1cm in panels A-F, H-I; 2mm in G.
Figure 4

A: Negative
B: Amcyan
C: GFP
D: zsYellow
E: mCherry

E: Negative
F: Amcyan
G: GFP
H: orange

I: Negative
J: Amcyan
K: GFP
dsREDex

M: Negative
N: Amcyan
O: GFP
dsREDex

Q: Negative
R: Amcyan
S: GFP
T: red

Bars indicate scale.