SUPPLEMENTARY APPENDIX

Focused chemical genomics using zebrafish xenotransplantation as a pre-clinical therapeutic platform for T-cell acute lymphoblastic leukemia

Victoria L. Bentley,[1] Chansey J. Veinotte,[2] Dale P. Corkery,[3]Jordan B. Pinder[1], Marissa A. LeBlanc,[1] Karen Bedard,[1] Andrew P. Weng,[4] Jason N. Berman,[2,5*] and Graham Dellaire[1,3*]

[1]Pathology, Dalhousie University, Halifax, NS; [2]IWK Health Centre, Halifax, NS; [3]Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS; [4]Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, BC; and [5]Pediatrics and Microbiology & Immunology Dalhousie University, Halifax, NS, Canada

 doi:10.3324/haematol.2014.110742
Manuscript received on May 15, 2014. Manuscript accepted on September 26, 2014.
**Correspondence: dellaire@dal.ca or jason.berman@iwk.nshealth.ca*

Supplemental Methods:

Cell Culture and Cell Viability Assay

All T-ALL cell lines were obtained from the Weng laboratory and were confirmed by DNA fingerprinting using the PowerPlex 1.2 system (Promega) in January 2013. T-ALL cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco). HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. Mononuclear cells from patient bone marrow aspirate samples performed at the time of diagnosis were obtained by density gradient centrifugation with Lympholyte-H (Cedarlane) and then placed in liquid nitrogen with freezing media until use. Patient lymphocytes were cultured in MarrowMAX bone marrow medium (Gibco) for 24 hours prior to xenotransplantation. Cells were cultured under humidified conditions at 37^{o}C with 5% CO_2. Stock solutions of drugs triciribine (Sigma-Aldrich), rapamycin (Sigma-Aldrich) and Compound E (EMD Millipore) were dissolved in DMSO, and maintained at -20^{o}C.

T-ALL cells were cultured in the presence of vehicle (0.01% DMSO) or each of the inhibitors at varying concentrations for 72 h after which they were subjected to the AlamarBlue (Invitrogen) cell viability assay, and the fluorescence intensity measured using a plate reader (Tecan Infinite M200). Cell viability was calculated as a percentage of vehicle.

Lentiviral Transduction

Lentivirus was obtained by co-transefection of the GIPZshCTRL (Thermo Scientific), pMD2.G, pCMV-8.92, and pCMV-8.93 vectors (described previously (1)) into human HEK-293T cells via calcium-phosphate transfection (Promega), according to manufacturer's directions. After 48 h, media from the transfected cells was filter sterilized using a 0.45μ filter, and the viral media added to Jurkat cells, polybrene was added to a final concentration of 10 μg/mL (Sigma-Aldrich), and cells were centrifuged at 800 x *g* for 30 minutes. Cells were selected using 2 μg/mL puromycin (Sigma-Aldrich).

Generation of Constructs and Luciferase Assay

pMig-Notch1, pMig-Notch1ΔPEST and JH23a luciferase reporter plasmid were available in the Weng laboratory. Site-directed mutagenesis was performed by overlap extension PCR (2). Base substitutions within the Notch1 coding sequence were performed in pMig-Notch1 to create pMig-Notch1-A1696D (GCC to GAC). Plasmids were introduced into HeLa cells with Neon transfection system (Life Technologies). To assay activation of JH23a-dependent transcription, cells were co-transfected with Notch1 expression constructs, a JH23a firefly luciferase reporter plasmid, and an internal control sea pansy *Renilla* luciferase plasmid (Promega). Normalized firefly luciferase activities were measured in whole-cell extracts prepared 48 h after transfection with the Promega dual luciferase kit. Luciferase was measured using a GloMax 20/20 luminometer (Promega).

Sequencing of Patient Exomes

Selected regions were amplified from genomic DNA by PCR. Amplified fragments were purified from an agarose gel, and sequenced using Sanger fluorescent sequencing and

capillary electrophoresis. Sequencing was performed at the DNA Diagnostic Laboratory at the IWK Health Centre in Halifax, Nova Scotia, Canada. Sequence traces were analyzed using MutationSurveyor V.3.97 (Soft Genetics, Inc.).

Zebrafish Husbandry, Embryo Collection & Embryo Staging

Zebrafish were maintained according to standard protocol (3). *Casper* (4) embryos were collected and grown in E3 embryo media (5 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$, and 0.16 mM $MgSO_4$, pH 7.5, supplemented with 1 x 10-5% Methylene Blue [v/v]) at 28.5^{o}C. Embryos were developmentally staged according to standard protocol(5).

Xenotransplantation Of Human Leukemia Cells Into Zebrafish Embryos, Dissociation, & Immunofluorescence

Mutant *casper* zebrafish embryos at 48 hours post fertilization (hpf) were anesthetized with 0.090 mg/mL Tricaine (Sigma-Aldrich) and used for cell transplantation using a protocol adapted from Haldi et al. (6,7). Briefly, CM-DiI (Invitrogen)-labeled leukemia cells were loaded into a pulled glass micropipette and approximately 50 cells were delivered, as a single injection, into the yolk sac of each embryo (injection conditions: 0.1 seconds, 2.5 psi) using a PLI-100A Pico-Liter Injector (Warner instruments) while under observation using a Zeiss SteREO DiscoveryV8 microscope. Following injection, embryos were allowed to recover at 28°C for 1 h before transfer to 35°C where they remained for the duration of the experiment.

For T-ALL cell lines: At 48 hours post-injection (hpi), only embryos with a uniform fluorescent cell mass at the site of injection were used for proliferation studies. Embryos xenotransplanted with human cancer cells were then maintained in groups of 15-20 within individual Petri dishes prior to drug treatment. At 48 hpi, drugs were added at concentrations that did not adversely affect the embryos (i.e. at or below 50% of the maximum tolerated dose (MTD50) for 48 hpf embryos treated for 48 h) directly to the fish water, at indicated concentrations, and embryos were incubated for 48 hours until 96 hpi. Embryos treated with 0.1% (v/v) DMSO served as a negative control for drug efficacy. For the fluorescence imaging, a filter with excitation/emission wavelengths of 550/605 nm was used and all embryos were photographed under the same settings. Groups of 15-20 xenografted fish were dissociated into a single cell suspension and leukemia cells were enumerated (7).

For separated bone marrow patient samples: At 96 hpi, drugs were added to the fish water at indicated concentrations, and embryos were incubated for 48 hours until 144 hpi. Fluorescent imaging was performed as indicated above. Groups of fish were dissociated and engraftment fold change enumerated by counting cells positive for PML bodies (expressed in human cells but not zebrafish cells). This was accomplished by performing cytospin (cytospin conditions: 300rpm for 10 minutes, using a Shandon cytospin 3 (Global Medical instrumentation)) of dissociated embryos, and immunohistochemistry for PML bodies (primary antibody: Rabbit Anti-PML (Santa Cruz sc-5621); secondary antibody: DyLight Donkey Anti-Rabbit IgG 649 (Abcam)). Fluorescent images were acquired on a custom built Zeiss Axio Observer Z1 inverted microscope equipped with

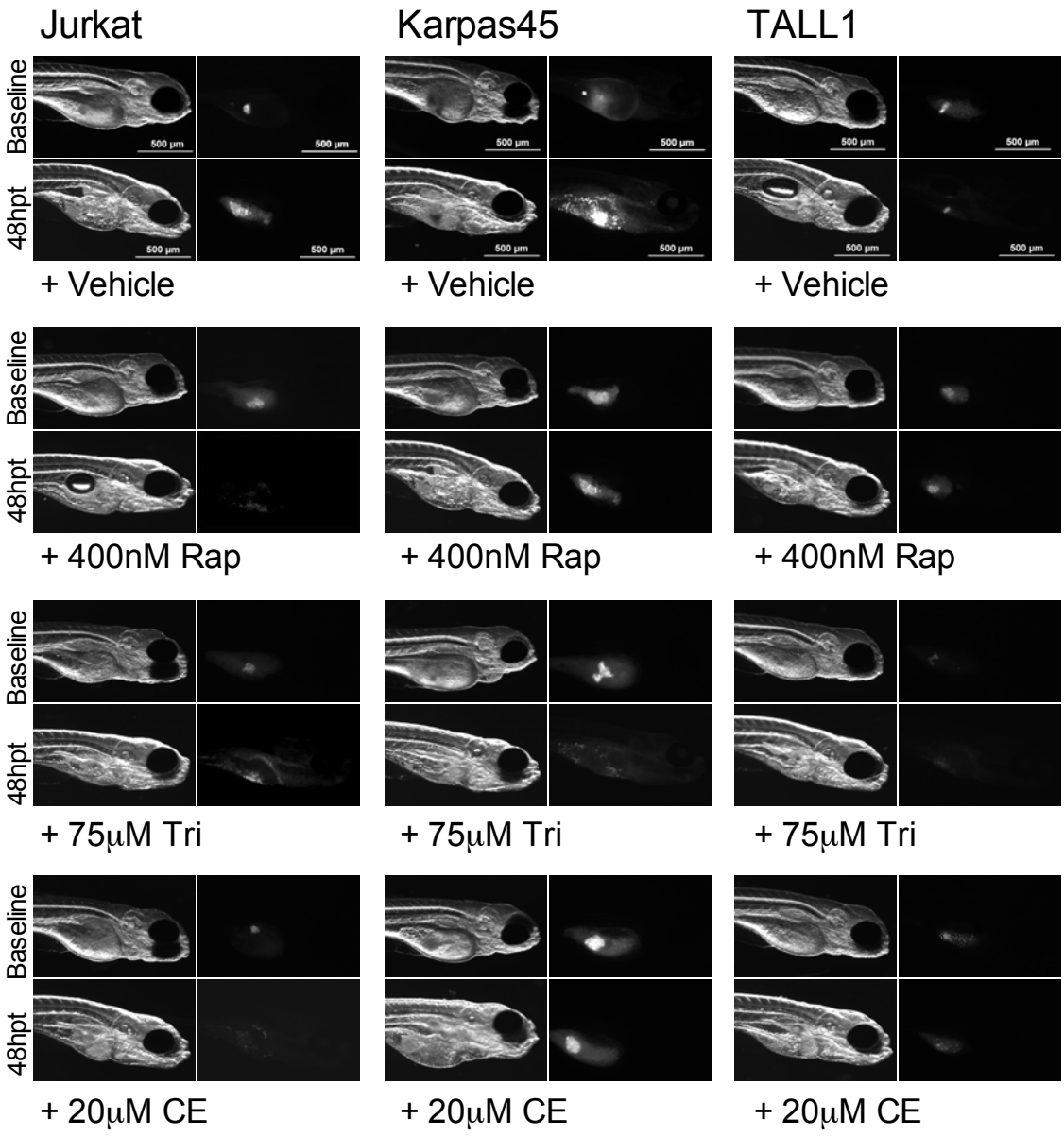
405 nm, 488 nm, 561 nm and 633 nm diode-based lasers (Intelligent Imaging Innovations (3i)) and a confocal spinning-disk unit (CSU-X1)(Yokagowa). Cells were observed using a 10X objective and images were recorded using an Evolve 512 electron-multiplying charge-coupled device (EMCCD) camera (Photometrics) and Slidebook 5.1 Software using the mosaic tool (Intelligent Imaging Innovations (3i)).

Supplemental Figures:

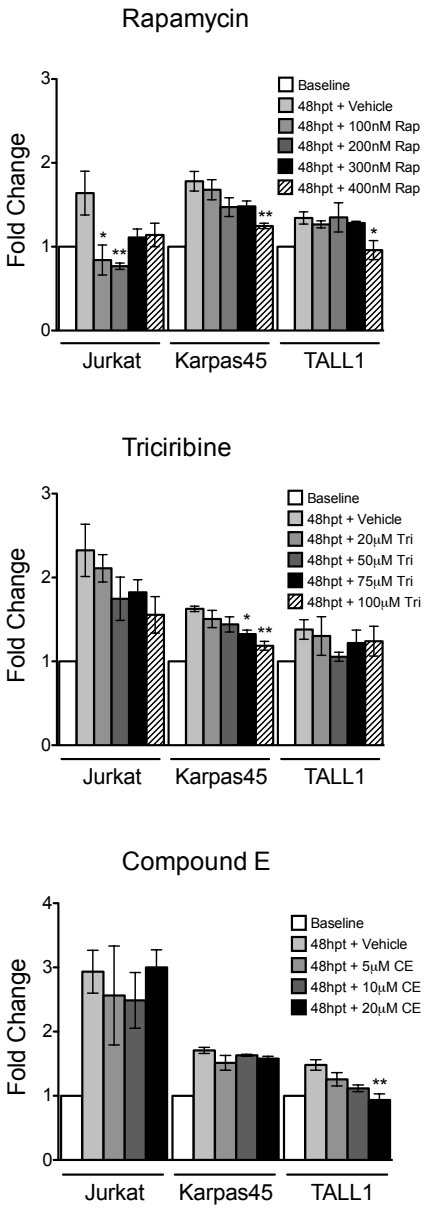
Supplemental Table 1. Maximum tolerated dose of drugs.

Drug	MTD	MTD50	Treatment concentration
Rapamycin	2.5 μM	1.25 μM	400 nM
Triciribine	200 μM	100 μM	75 μM
Compound E	ND	ND	20 μM

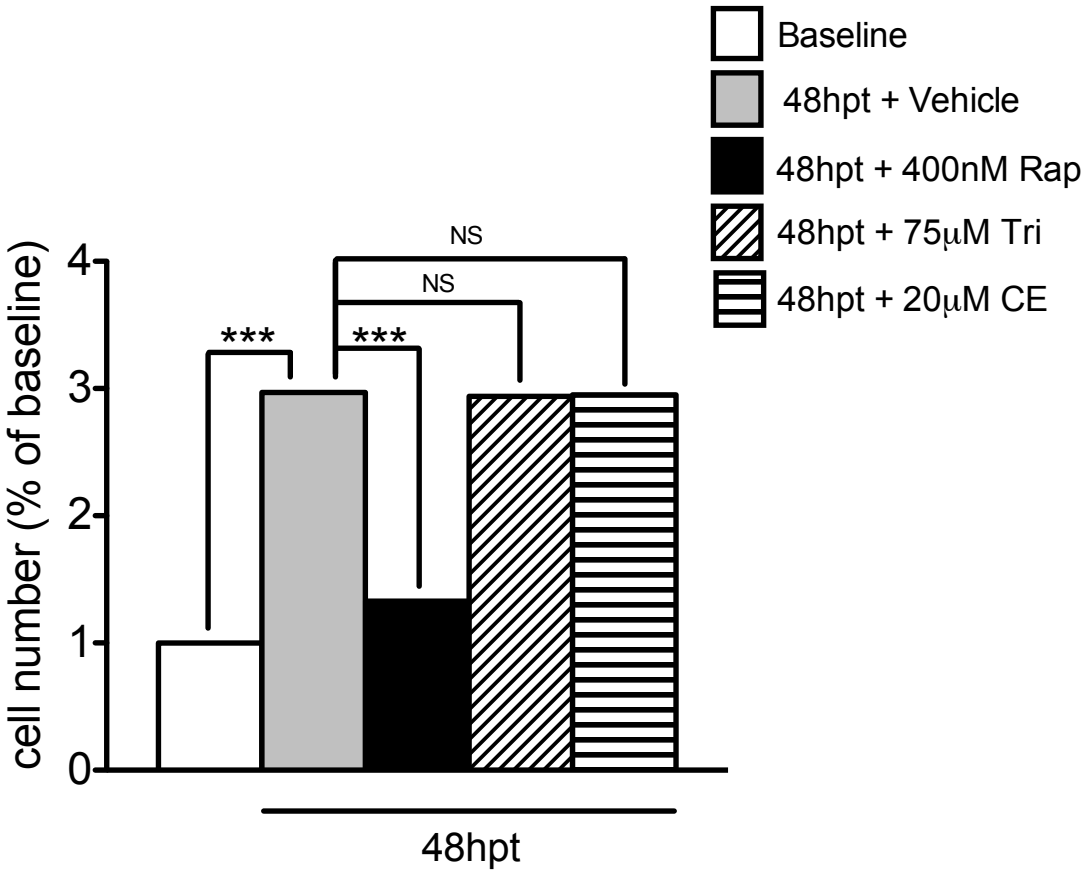
Determination of the maximum tolerated dose (MTD) for compound E, triciribine, and rapamycin was conducted in non-XT embryos. Embryos were placed in 96-well plates and increasing concentrations of each drug was added to E3 embryo media, and following 48 h of treatment, embryos were examined for viability. The MTD was defined as the lowest drug concentration to kill embryos after 48 h of treatment. Due to poor solubility above 40 μM, the MTD for compound E was not determined (ND). No drug treatment exceeded half the MTD (termed MTD50).



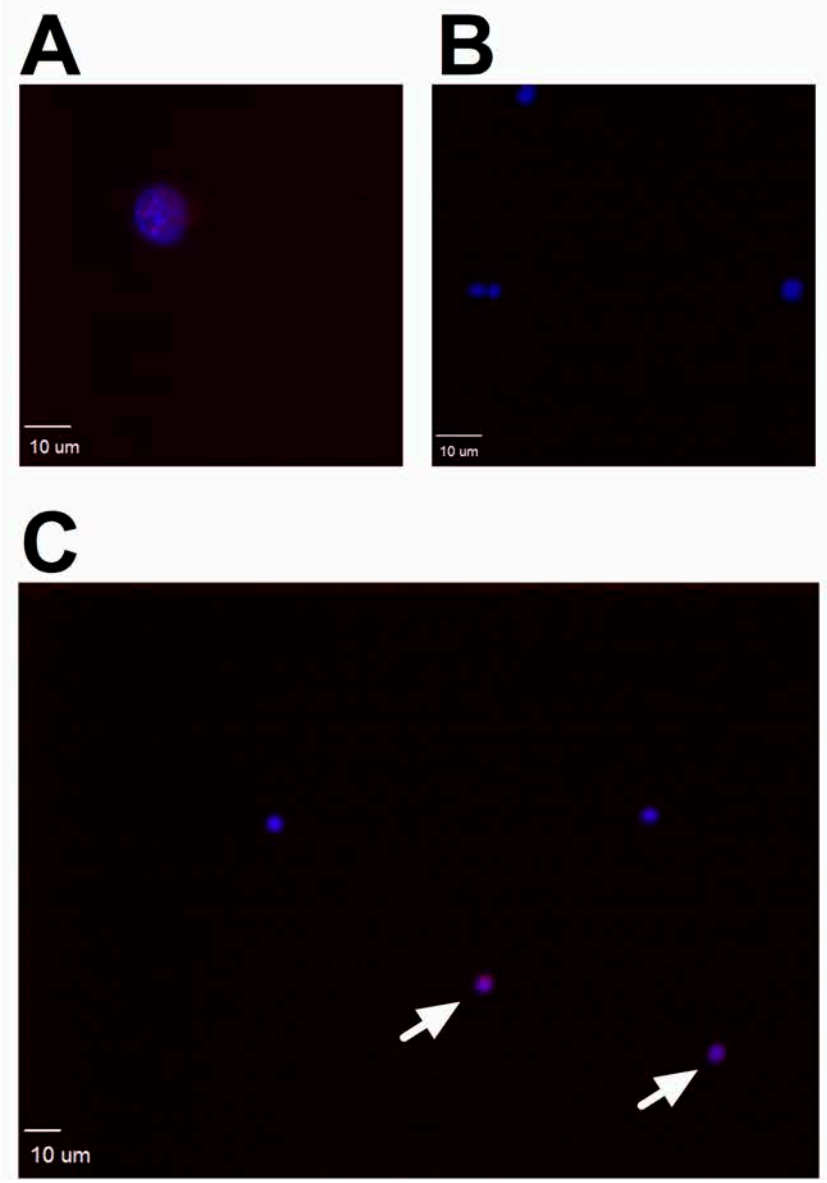
Supplemental Figure 1. Representative brightfield and fluorescent images of zebrafish embryos transplanted with T-ALL cell lines at Baseline (0 hpt, 48 hpi) and 48 hpt (96 hpi) with or without drug. Zebrafish embryos were xenotransplanted with CmDiI-labelled T-ALL cell lines at 48 hpf. Cells were given 48 h to engraft in zebrafish embryos, embryos were screened for fluorescent masses (48 hpi, 0 hpt is referred to as 'baseline'), and then were treated with indicated drug for 48 h. Embryos were imaged at baseline and 48 hpt, and groups of embryos were dissociated at fluorescent cells enumerated at each time point. Drug doses: compound E 20 µM; triciribine 75 µM; rapamycin 200 nM. hpf = h post-fertilization; hpt = h post-treatment; hpi = h post-injection.



Supplemental Figure 2. *In vivo* proliferation of T-ALL cell lines in the zebrafish xenograft (XT) model. Quantification of cell proliferation was performed by dissociating groups of xenografted fish at baseline (0 hpt) and 48 hpt and enumerating fluorescently labeled cells as described in Corkery et al. *Brit J Haematol*, 2011. All drug treatments are shown as a fold change of the number of leukemia cells when treatments were initiated (baseline). Means +/- SEM; N=3 (N = the number of independent experiments, with 15-20 embryos per group per experiment). Baseline = Untreated fish at 0hpt; all other treatments are at 48 hpt; hpt = h post-treatment. Significant decrease in cell number was determined using 1-way ANOVA followed by Dunnett's multiple comparison test.



Supplemental Figure 3. Proliferation response of GFP-expressing Jurkat cells in the zebrafish xenograft (XT) model is comparable to CM-DiI-labeled Jurkat cells following treatment with targeted agents. Jurkat cells were transduced with GIPZshCTRL, and GFP-positive cells were selected. GFP-expressing Jurkat cells were xenotransplanted into 48 hpf embryos and following 48 h of treatment with indicated drug groups of 15-20 embryos were sacrificed and the number of GFP-positive cells enumerated. N=1 (N = the number of independent experiments, with 15-20 embryos per group per experiment). Baseline = Untreated fish at 0hpt; all other treatments are at 48 hpt; hpt = h post-treatment; hpf = h post-fertilization. P*<0.05, P**<0.01, P***<0.001 for significant decrease in number of cells determined using unpaired 2-tailed student's *t* test.



Supplemental Figure 4. Human cancer cells can be differentiated from zebrafish cells by immunohistochemistry for promyelocytic leukemia (PML) bodies. Cytospins showing that A. Human Jurkat cell are positive for PML bodies (shown in red). B. Zebrafish do not contain PML bodies. C. Dissociated zebrafish engrafted with human Jurkat cells. White arrows indicate human cells (positive for PML bodies). Fluorescent images were acquired on a custom built Zeiss Axio Observer Z1 inverted microscope equipped with 405 nm, 488 nm, 561 nm and 633 nm diode-based lasers (Intelligent Imaging Innovations (3i)) and a confocal spinning-disk unit (CSU-X1)(Yokagowa). Panel A was taken using a 60X objective and panels B and C were taken using a 40X objective. Images were recorded using an Evolve 512 electron-multiplying charge-coupled device (EMCCD) camera (Photometrics) and Slidebook 5.1 Software.

References:

1. Bedard K, Attar H, Bonnefont J, Jaquet V, Borel C, Plastre O, et al. Three common polymorphisms in the CYBA gene form a haplotype associated with decreased ROS generation. Hum Mutat [Internet]. 2009 Jul [cited 2014 Aug 17];30(7):1123–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19388116

2. Higuchi R, Krummel B, Saiki RK. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res [Internet]. 1988 Aug 11 [cited 2014 Jan 26];16(15):7351–67. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=338413&tool=pmcentrez&rendertype=abstract

3. Westerfield M. The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio). Eugene: University of Oregon Press; 1995.

4. White RM, Sessa A, Burke C, Bowman T, LeBlanc J, Ceol C, et al. Transparent adult zebrafish as a tool for in vivo transplantation analysis. Cell Stem Cell [Internet]. 2008 Feb 7 [cited 2014 Jan 18];2(2):183–9. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2292119&tool=pmcentrez&rendertype=abstract

5. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn [Internet]. 1995 Jul [cited 2014 Jan 22];203(3):253–310. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8589427

6. Haldi M, Ton C, Seng WL, McGrath P. Human melanoma cells transplanted into zebrafish proliferate, migrate, produce melanin, form masses and stimulate angiogenesis in zebrafish. Angiogenesis [Internet]. 2006 Jan [cited 2014 Jan 26];9(3):139–51. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17051341

7. Corkery DP, Dellaire G, Berman JN. Leukaemia xenotransplantation in zebrafish--chemotherapy response assay in vivo. Br J Haematol [Internet]. 2011 Jun [cited 2014 Jan 9];153(6):786–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21517816