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Zebrafish Tumor Assays: The State of Transplantation

Alison M. Taylor¹ and Leonard I. Zon^{1,*}

¹ Stem Cell Program and Division of Hematology/Oncology, Children's Hospital Boston, Harvard Medical School, Boston, Massachusetts

Abstract

Tumor transplant studies are important tools for studying cancer biology in a model organism. Transplantation is especially important for assaying tumor cell malignancy and migration capabilities, and is critical for identifying putative cancer stem cell populations. In this review, we discuss the current state of tumor transplantation studies performed in the zebrafish. We address several zebrafish-specific considerations for development of the transplant assay, including choosing recipient animals, transplant methods, and post-transplant observation. We also examine how the zebrafish is an advantageous model for transplantation, particularly with development of the translucent fish. Transplantation has already been critical for characterizing zebrafish models of leukemia, rhabdomyosarcoma, and melanoma. With further development of imaging techniques and other tools, zebrafish tumor transplantation will continue to contribute to our understanding of tumor cell biology.

The ability of tumors to engraft after transplantation into recipient animals has been known for decades³⁷. A malignant population of tumor cells is not only capable of primary engraftment, but also capable of serial transplantation in subsequent recipient animals. Transplantation has been used to validate animal models of cancer^{21,31} and test malignancy of human tumor samples¹³. Transplantation has also been important for drug development, as chemical treatment of transplanted cells has been used to screen for potential therapeutics that might inhibit tumor malignancy¹⁶.

Identifying transplantation capabilities of tumor cells has addressed several key aspects of tumor biology, particularly malignancy, metastasis, and cancer stem cell biology. Studies over fifty years ago demonstrated that, in many cases, human tumor cells that successfully transplant in rodents are more likely to be malignant in patients⁹. A malignant tumor is expected to engraft and develop into a tumor in the recipient animal. This assay for malignancy may also have implications for metastasis, as it looks for tumor cells to migrate from the site of injection and form a new tumor. Additionally, transplantation of tumors in murine models has been crucial for identifying cancer stem cell populations⁶.

Over the past few years, the zebrafish has emerged as a tumor model that complements studies performed in the murine system. The transplantation assay in the zebrafish has developed to be a robust assay. In many ways, the zebrafish as a model system is particularly advantageous for tumor transplantation assays. Zebrafish fecundity provides high numbers of donor and recipient fish, and generating large numbers of transgenic fish is feasible¹⁴. In addition, the generation of a transparent adult fish is particularly beneficial for post-transplant observation⁴³. Yet, technical improvements can be made for further development to improve zebrafish transplantation methods.

*Corresponding Author: Karp Family Research Laboratories, 7th Floor, Room 7211, One Blackfan Circle, Boston, MA 02115, Tel: (617) 919-2069, Fax: (617) 730-0222, zon@enders.tch.harvard.edu.

Uses of the Transplant Assay in Cancer Biology

Transplantation of tumor cells from one animal to another provides information about the malignancy of the tumor. Cells from a tumor that propagate post-transplant have acquired the ability to self-renew and generate more tumor cells. Transplantation as an assay has been especially important for testing blood neoplasias to differentiate a myeloproliferative or lymphoproliferative disorder from leukemia^{3,21}, which is capable of propagation. In this sense, transplantation as an assay can differentiate between a hyperplastic and malignant growth.

Transplantation can also identify putative cancer stem cells, rare populations of a tumor that can form a new tumor. Testing this requires isolation of a subset of tumor cells from the bulk mass, and then utilization of limiting dilution transplant assays to determine whether the isolated population is more transplantable than the bulk tumor. These assays have been extensively tested in mouse models, primarily in leukemia, to identify a cancer stem cell population²². Despite advances in this field, transplantation as an assay for cancer stem cells has recently been called into question, since modification of the transplant procedure itself can dramatically alter the results of the assay in some tumor models³³. Regardless, transplantability is helpful in understanding tumor cell properties.

Studying tumor dissemination post transplantation also aids in the understanding of migration, homing, and vasculature induction properties of tumor cells. Tumor cells injected into the tail vein of a mouse enter the blood stream, at which point migration and metastatic potential of the tumor cells can be studied⁷. Immunocompromised mice can serve as recipients for human tumor cells, allowing similar types of studies on human samples²⁷. Xenotransplantation provides an opportunity to study human tumor cells *in vivo*.

Zebrafish Transplant Methods

Choosing a Recipient Fish

Choosing a recipient animal for the donor tumor cells is a crucial question in both zebrafish and murine systems. Because the major readout from most transplant studies is engraftment, the sensitivity of this readout can dramatically alter the results. Initial tumor transplants in the fish used wild-type recipients, and whether a recipient has engrafted may be subjective if it is based solely on visual identification of a growing mass. For this reason, transplants into wild-type animals may require sacrifice and histological examination of each recipient. The recently developed transparent zebrafish named *casper* enables a much more sensitive technique for visual identification of engrafted tumors. Casper fish, a cross between the nacre and roy lines, lack melanocytes and iridophores, allowing for increased translucency in the adult fish⁴³. As a result, when tumors are transplanted into this line, it is much easier to visualize engraftment, tumor growth, and metastasis. For example, pigmented zebrafish melanoma cells can be scored after transplant into the casper fish, and tumor size can be measured over time without sacrificing the fish (Figure 2a). Additionally, since pigmented cells can interfere with fluorescent signal, transplant into casper fish will allow imaging of fluorescently labeled cells in the adult (Figure 2b). Using confocal microscopy, single cell resolution of transplanted GFP-positive cells is possible.

Transgenic fish with fluorescently labeled tissues are another option for a recipient. To identify the effect of tumor cells on vasculature, cells can be transplanted into *fli1*-EGFP positive embryos or adult fish, and vasculature development and angiogenesis can be monitored post-transplant³⁸. This approach is a powerful method to track fluorescently labeled tissues.

Irradiation/Immune Ablation

An important consideration with the recipient animal is to prevent rejection and allow engraftment. As few isogenic strains have been developed in the zebrafish²⁶, irradiation is often used to suppress the immune system of adult fish and prevent rejection of transplanted cells. Single doses of 20–25 Gy are sublethal, and at least 90% of fish tolerate this dose⁴¹. Tumors engraft at doses as low as 20 Gy given two days prior to irradiation³¹. The immune system of larval fish is also a consideration for assay development. During development, immature T cells arise in the thymus by 3–4 days post fertilization (dpf)¹⁸, and the zebrafish immune system is functional starting at 28 days¹⁷. To ablate T cells, larval fish from 5 days to 1 month of age can be treated in 25 µg/mL to 250 µg/mL of dexamethasone at one to three days prior to transplant^{18,38}. A 15 Gy dose of gamma-irradiation will also ablate T cells in six day old embryos to one-month old larvae³⁸. For transplants into zebrafish embryos, immunosuppression has not been used, as T cells and B cells are not present until 3–4 dpf⁴⁰.

Cell Isolation and Transplant Methods

Cells directly isolated from a zebrafish tumor can be used for transplant into an immunosuppressed recipient fish. In our lab, we generally inject 100,000 cells into each adult recipient, though these numbers can vary from 1000 to 3,000,000 cells^{21,43}. For transplant, one option is to disaggregate the primary tumor and then transplant a defined number of cells directly from the whole tumor population. Because whole tumors contain a mixture of malignant cells, stroma and blood, a more direct assay for malignant cells is to sort cells by flow activated cell sorting (FACS), allowing transplant of specific cell populations. FACS can be used to separate different blood cell populations⁴¹ or fluorescently labeled cell populations¹⁹ prior to transplant. By sorting populations, the malignant capability of each population can be determined. A third option is to remove tumor cells and use an inducible expression system to activate an oncogene *ex vivo* prior to transplantation, providing spatial and temporal control. Alternatively, chemical treatment *ex vivo* prior to transplantation can be used to test potential cancer therapeutics.

Some mouse and human tumor cells are also capable of engraftment in two dpf zebrafish embryos and blastulas. For transplantation into 2 dpf embryos, between 50 and 2000 cells can be injected into the yolk or near the vasculature plexus, though there is contrasting evidence regarding the upper limit of cells that can be injected without causing toxicity^{10,28}. These cells can also be labeled with CiM-DiI or other fluorescent labels. For transplantation into embryos at the blastula stage, 1 to 100 cells are sufficient for engraftment^{24,39}.

Tumor cells can be injected into either the zebrafish embryo or adult. Previous experiments in the adult zebrafish involve tumor transplantation of cells into the peritoneal space (i.p.) of sublethally irradiated fish^{21,43}. Intracardiac injection has been used for hematopoietic cell transplantation⁴² and may be extended to tumor transplantation. Retroorbital injection techniques are also being developed (R. White, personal communication). Human or mouse cells are generally transplanted into the yolk of the two day old embryo or into the blastula^{24,39}.

Post-transplant Observation

Observation of recipient animals post-transplant will determine if engraftment has occurred. This is the crucial limitation of all transplantation studies, since a tumor transplanted into the peritoneal cavity may not be externally visible yet still can cause the death of the recipient. In general, engraftment in a wild-type adult fish can be seen around 10–14 days post transplant. If the adult casper fish is the recipient and transplanted cells are pigmented or labeled with GFP, then engraftment can be seen at approximately 5 days post transplant. When setting up a new transplantation model, it is important to correlate engraftment by visual scoring with

histological examination of sacrificed fish. If the concordance between visual and histological examination is high, then visual scoring is faster and more amenable to high-throughput approaches.

If engraftment has occurred, continued growth and disease development can also be observed. This can be assayed by visualization of transplant cells if they are fluorescent or pigmented, especially if the casper fish is the recipient. If this is not the case, fish can be monitored for external signs of disease development²³. The timeline for long-term engraftment is often 3–4 weeks, but this may differ for each tumor model. Again, monitoring long-term engraftment is much easier if the casper fish is the recipient so transplanted cells can be observed over time without sacrificing the transplanted fish. If a tumor has developed in the recipient fish, this can be removed and transplanted into a new recipient to test whether the tumor cells can serially transplant, a hallmark of self-renewal ability.

One of the complications of the transplantation assay is that “no engraftment” can result either when the cell population is not capable of engrafting or when the injection itself is not successful. When developing the transplantation assay, it may be useful to have an independent marker of successful injection, especially if the transplanted cells are not labeled or pigmented. Previous studies have used fluorescent microspheres as a readout of successful injection³⁸.

In the case where embryos are the transplant recipient, they can often survive at least one week post transplantation for observation. Engraftment can be observed soon after transplant in the embryo, even immediately in blastula transplants. The effects of transplantation into a 2 dpf embryo on the vasculature can be seen by 3 dpf²⁸. Transplant into the zebrafish embryo can also be used to study developmental signaling pathways, as the zebrafish is an excellent model for studying early development³⁹.

Zebrafish Tumor Transplants to Date

Leukemias and Myeloproliferative Disorders

One of the first fish tumor models developed as a stable transgenic zebrafish line was Myc-induced T cell acute lymphoblastic leukemia (ALL). When transplanted, lymphoblasts from these tumors were able to home to the thymus²¹. Tel-AML1 induces B-ALL, and transplantation of these leukemic kidney marrow cells leads to disease formation 6–9 weeks post transplant³⁴. Notch1 overexpression in Rag2 positive cells induces T-ALL that can propagate in a recipient animal⁵. More recently, T-ALL models have been developed where tumors can be serially transplanted, and engrafted hosts die in a matter of weeks⁸. These studies demonstrate that transplanted leukemia cells can engraft and perpetuate the tumor in zebrafish models of leukemia.

In a KRAS-induced model of myeloproliferative disorder (MPD), the disease could be transplanted one time, but further serial transplants did not result in MPD or leukemia²³. These results established that oncogenic KRAS alone was not enough to confer self-renewal properties and malignancy in blood cells, in agreement with experiments performed in a mouse model of MPD³. Interestingly, *ex vivo* induction of KRAS prior to transplantation also induced MPD after primary transplant. This demonstrates how transplantation provides an additional approach for tissue-specific and temporally-controlled oncogene induction which may be useful in tumor models with particularly potent oncogenes or developmental regulators.

Solid Tumors

Transplantation assays have also been used to study zebrafish solid tumors. Cells from zebrafish BRAF^{V600E};p53^{-/-} melanomas regenerate highly invasive tumors after serial transplantation³². Transplantation of these tumors into transparent adult zebrafish

demonstrated metastatic capability, as cells were able to disseminate far from the transplantation site⁴³. Transplantation of a zebrafish RAS-induced embryonal rhabdomyosarcoma (RMS) was used to identify a cancer stem cell population. In this model, populations of cells were labeled with α -actin GFP (a late marker of muscle cell differentiation) or Rag2-dsRed (an early marker of differentiation). Transplantation of sorted populations showed that Rag2⁺ (red) cells were more capable of engrafting in primary and serial transplants, so enrichment for Rag2 expression enriches for a cancer stem cell population. Additionally, limiting dilution assays were used to determine that only ten Rag2⁺ cells are required for transplantation whereas other cell populations required much higher number of cells²³.

Xenotransplantation

Human tumor cell lines can be transplanted into zebrafish embryos for study of tumor cell migration, metastasis, and effect on vasculogenesis. Several groups have shown that transplantation of human tumor cell lines into zebrafish embryos or larvae induces increased vasculature formation and endothelial cell gene expression^{10,28-38}, providing a model by which to study tumor-induced vascularization. Transplant into embryos at the blastula stage shows whether tumor cells are capable of engraftment, invasion, and developmental effects²⁴⁻⁴². More recently, fluorescently labeled pancreatic tumor cells and TGF β -stimulated transformed mammary epithelial human cells (but not pancreatic tumor cells), were shown to be capable of invading and metastasizing into other tissues when injected into 48 hpf embryos²⁵. Primary human pancreatic cancer cells also demonstrated invasiveness post-transplant that could be modulated by protease inhibitor treatment. These types of experiments demonstrate how the zebrafish can be used to study and compare pro-angiogenic and metastatic behavior of human tumor cells. It is still important to note that a limitation of xenotransplants to date is the inability to successfully engraft in adult fish. Addressing some of the immunological issues around adult transplantation (see below) will address this issue in the future.

Advantages of Using the Zebrafish System

Numbers

One advantage of the zebrafish as a model is the ability to easily generate and maintain large numbers of fish, and this greatly increases the throughput of the transplant assay. It is reasonable to generate large numbers of fish with tumors, and even more feasible to generate hundreds of recipient fish. For adult transplantation assays, 100–200 or more transplants can be completed in a single day, which is generally more than what is standard for a mouse model of transplantation, where often only 2–3 animals are injected per experimental group¹⁶. In embryo transplant experiments, hundreds to thousands of recipient embryos can be generated each day, although practically only about 300–700 one cell stage embryos can be reasonably injected per day.

Generation of Transgenics

The ease of making transgenics in the zebrafish, particularly with fluorescent markers, is advantageous for study of tumor transplantability. Although targeted knockdown technology is still under development in the zebrafish¹, creating transgenic lines is a powerful tool in zebrafish biology. With the Tol2 transposase-based system of transgenesis, injection of a few hundred embryos is enough to create a stable transgenic line¹⁴. This allows generation of a variety of tumor models, and, if needed, a large number of mosaic fish. It is also feasible to generate inducible models, where an oncogene can be expressed under control of a responsive promoter. Using a heat-shock or alternative promoter, an oncogene can be induced *ex vivo* prior to transplantation²³. An oncogene can also be activated at a specific stage of development, or tissue-specific promoters can be used to limit oncogene expression to the tissue of interest.

These methods provide spatial and temporal control over oncogene expression, decreasing toxicity and potentially creating a more accurate model of human disease.

In addition to facilitating tumor model generation, fluorescence can also be used to label cells expressing the transgene, providing a simple way to observe or sort tumor cells. Fluorescent transgenics can also label populations of interest within a tumor by co-injection of constructs with an oncogene of interest and a fluorescent marker driven by the same promoter²⁰. This may be useful to label cells expressing a specific transcription factor or in a particular differentiation state.

Sensitivity of Post-Transplant Observation

The translucent zebrafish embryo has been critical for studying development, and this is also beneficial for the recipient in the transplant assay. Fluorescently tagged transplant cells can be scored in the zebrafish embryo during early development²⁴. With the development of the casper line, fluorescently tagged or pigmented cells can easily be imaged in the adult fish as well. The single cell resolution possible in the zebrafish casper line exceeds the resolution currently possible in mouse transplant models⁴³. Since the fish does not need to be sacrificed for analysis, observation over extended periods of time is feasible. This makes the transparent zebrafish particularly advantageous for post-transplant monitoring.

Chemical Treatment

Unique to the zebrafish system, whole organism treatment is possible by direct administration to the water. In both embryos and adult zebrafish, this method of chemical treatment can be used to modulate regenerative angiogenesis^{4,2}. This treatment provides an approach to test the importance of angiogenesis in tumor formation post-transplantation, and other processes can be assayed in a similar matter. As chemical treatment of zebrafish embryos has proven to be a useful high-throughput screening tool^{32,29}, treatment of embryos prior to or after transplant may also prove effective.

Areas for Future Development in Zebrafish Transplantation

Preventing Death Post-Transplant

Even at sublethal doses of irradiation, the death rate of zebrafish post-transplant is often substantially higher than 10%. Optimization of irradiation dose, including split dose treatments, may decrease the death rate (T. Bowman and J. DeJong, unpublished observations). Additionally, post-transplant care may need to be optimized as irradiated and immunosuppressed fish are susceptible to infection. Maintaining transplant recipients in a clean facility with fewer microorganisms may significantly decrease infection-related death.

It is also possible that alternative and less toxic forms of immunosuppression can be used. These include testing drugs similar to dexamethasone prior to transplant into adult fish. Another alternative is to generate fish lacking T-cell receptors, lacking specific cytokines like IL-2, or having an otherwise impaired immune system. Similar to NOD/SCID mice, these fish would be less likely to reject a transplant, particularly a xenograft of mouse or human cells. Alternatively, typing of MHC genes in the zebrafish may allow for matching of donor and recipients, helping to circumvent graft rejection³⁵. It is also possible that development of these methods will allow engraftment of xenotransplants in the adult.

Variability between Strains

Transplants in mouse are often between isogenic mice, decreasing the possibility of transplant rejection by the recipient mouse. Without isogenic zebrafish strains, transplant rejection and graft versus host disease (GVHD) can cause increased death and decreased engraftment. Unlike

in mouse models where numerous isogenic lines exist, isogenic clonal zebrafish lines have just recently been developed. Serial transplant of chemically-induced tumors is feasible using the clonal CG1 line²⁶. If transgenic tumor models can be generated in these fish lines, they will be increasingly useful for transplant studies. Until that becomes more widespread, it is crucial to determine the ideal recipient line for each study (i.e. AB, Tu, WIK, or others). In mice, there is evidence that the background genotype of the animal can strongly affect tumor behavior³⁰, and it is likely to be similar in the zebrafish as well.

Injection Site

The location of the injection site in zebrafish also substantially differs from mouse models. While most zebrafish tumors are transplanted via i.p. injection, mouse transplants often occur at or near the tissue of interest. Development of methods to transplant in specific tissues will likely improve overall engraftment rates. Orthotopic injection methods should be developed, as this method of injection is better than subcutaneous injection to assess engraftment and metastatic potential in mouse models¹⁵. Although testing migration ability from the peritoneal cavity may be a model of metastasis in some ways, transplant closer to the tissue of interest will likely better represent human tumor cell biology.

Temporal and spatial control

Although there are currently several inducible expression systems available for zebrafish modeling, there are still systems used in the mouse that have not been fully developed for zebrafish tumor models. Tamoxifen-inducible expression may provide higher efficiency and better temporal control¹². The Gal4/UAS inducible system widely used in yeast and drosophila has more recently been developed in the zebrafish system¹¹. Use of these systems for inducible tumor models will provide an additional level of control in the transplant fish assay.

Imaging Techniques

Fluorescence based imaging provides single-cell resolution in the zebrafish system, but other imaging methods are not well developed, particularly bioluminescence and luciferase based assays. These types of approaches are useful in mouse models for measuring tumor growth over time³⁶. Application of these tools to the fish system will provide increased alternatives for post-transplant analysis without sacrificing the recipient fish.

Summary

Transplantation of tumors has already taught us a significant amount of zebrafish tumor cell biology. Tumor malignancy has been demonstrated in multiple zebrafish tumor models by tumor propagation in a transplant recipient. Using fluorescently labeled transgenics, cancer stem cell populations enriched for tumor regeneration capability have been identified in rhabdomyosarcoma. A zebrafish model of melanoma is shown to have metastatic capability when transplanted into translucent fish. Human tumor cells are able to induce vasculogenesis in the zebrafish embryo. These experiments were successful in large part due to the advantages of the zebrafish as a model system. Beyond the ability to maintain large numbers of fish and make a variety of transgenic lines, the generation of the transparent adult fish has greatly enhanced transplantation in the fish. As this tumor assay is further developed, tumor transplantation in the zebrafish will be increasingly important in our understanding of tumor biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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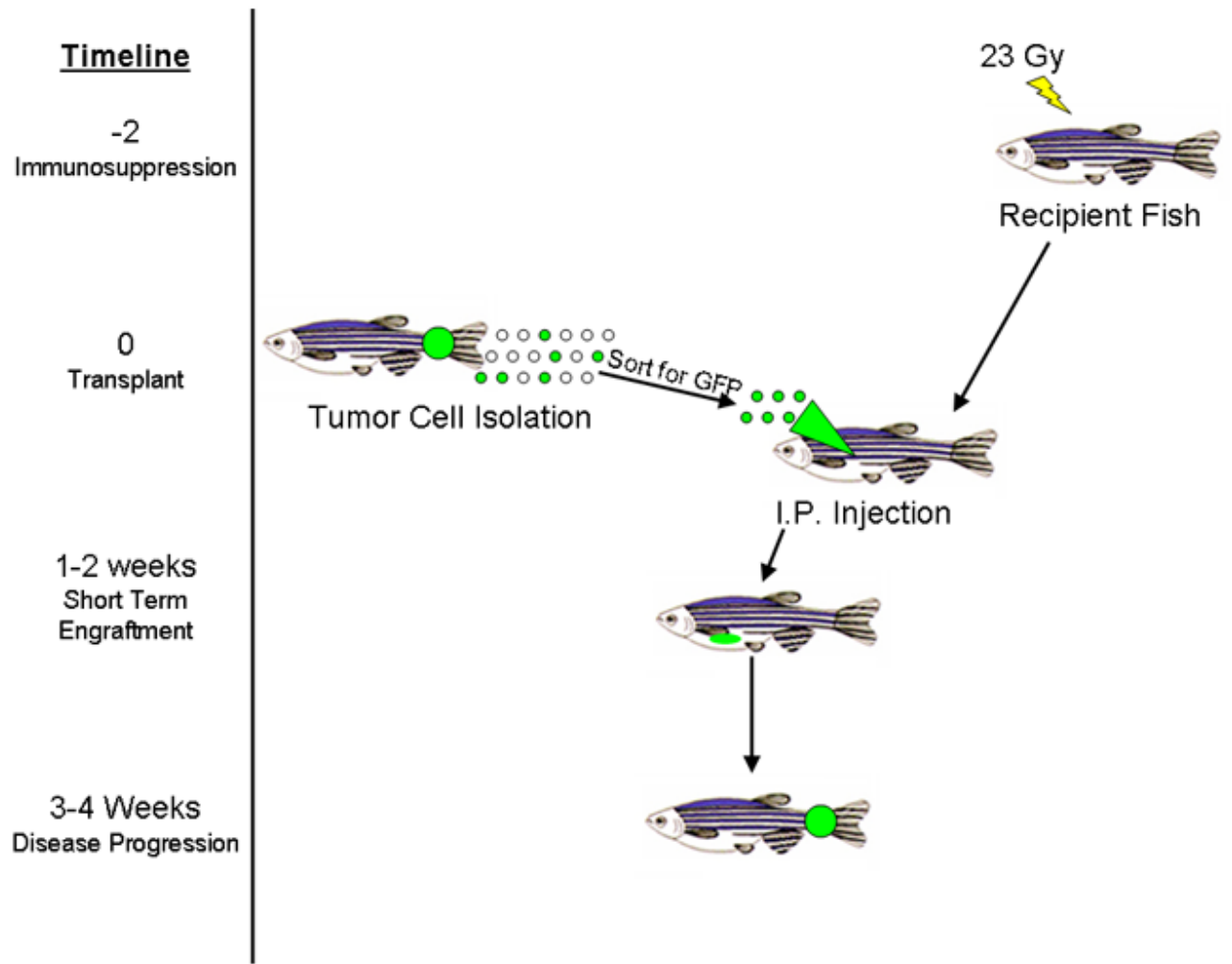


Figure 1.

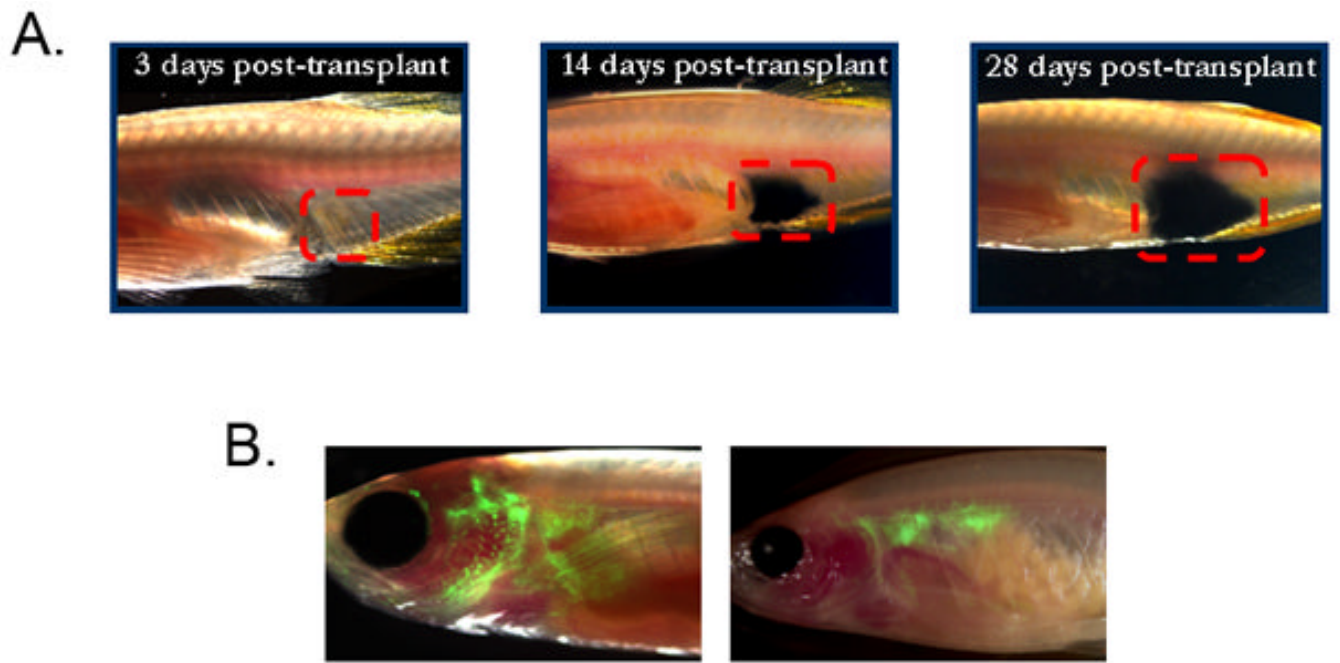


Figure 2.

Table 1

Summary of Zebrafish Tumor Transplants to Date

Many groups have previously tested transplantation in both the embryo and adult zebrafish. This table compares the methods used for each type of tumor cell transplanted, including numbers of cells transplanted and post transplant phenotype. Irradiation, when used for immunosuppression, was performed two days prior to transplant.

Model	Publication	Recipient Fish	Immuno-suppression	Transplanted Cells	Engraftment	Disease Development
Myc-induced T-ALL	Langenau et al., 2003	(1) 2 dpf embryos (2) AB adults	(1) n/a (2) 25 Gy	WKM i.p. (1) 10^3 to 10^4 cells (2) 10^6 cells	GFP positive by 7 days	Homing to the thymus and tumor development starting at 14 days
BRAF induced melanoma	(1) Patton et al., 2005 (2) White et al., 2008	(1) Tu adults (2) Casper	(1) 20 Gy (2) 23 Gy	(1) $3*10^6$ cells i.p. (2) $2*10^5$ cells i.p. or i.c.	By two weeks	(1) Melanoma by three weeks (2) Tumor dissemination by 5 to 28 days
Melanoma Xenotransplant	Lee et al., 2005	WT blastula-stage embryos	n/a	1–100 cells	Immediately	n/a
Human melanoma cells	Topczewska et al., 2006	WT blastula-stage embryos	n/a	50–100 cells	Immediately	Masses form by 3 days
Human melanoma cell line	Haldi et al., 2006	Wildtype 2 dpf embryos	n/a	50–100 cells labeled with CM-Dil	By 5 dpf	Melanoma masses by 10 days
Tel-AML1 induced B-ALL	Sabaawy et al., 2006	Wildtype adults	25 Gy	$5*10^5$ WKM cells i.p.		Signs of disease 6–9 weeks post transplant
Notch1-induced T- ALL	Chen et al., 2007	AB fish	23 Gy	10^6 WKM cells i.p.	GFP cells at 7 days	Lymphoid cell infiltration by 21 days
Adenocarcinoma cell line xenotransplant	Nicoli et al., 2007	Wildtype 2 dpf embryos	n/a	1000–2000 cells labeled with CM-Dil or Dio		Increased vasculature by 3 dpf
KRAS-induced rhabdomyosarcoma	Langenau et al., 2007	AB fish	23 Gy	$10\text{-}2*10^4$ FACS sorted tumor cells i.p.	By 7 days	Tumor formation by 14 days
KRAS-induced MPD	Le et al., 2007	AB fish	23 Gy	$3*10^5$ WKM cells i.p.		Disease by 2 months, but serial transplant did not induce disease
Human cancer cell lines	Stoletov et al., 2007	AB fish, flil-EGFP fish	Dexa- methasone	50–300 fluorescently labeled cells i.p.	Immediately post transplant	Microscopic tumors
NRAS induced melanoma	White et al., 2008	Casper fish	23 Gy	$2*10^5$ tumor cells i.p. or i.c.	GFP positive cells at 5 days	Tumors begin to form at 14 days
Ras transformed murine epithelial cells	Marques et al., 2009	Tu, flil-eGFP	n/a	Cells injected into yolk	Immediately post transplant	If TGFB β stimulated, tumors begin to form at 3 days

Model	Publication	Recipient Fish	Immuno-suppression	Transplanted Cells	Engraftment	Disease Development
Primary human pancreatic tumor cells	Marques et al., 2009	Tu, fl1-eGFP	n/a	Cells injected into yolk or via orthotopic injection	Immediately post transplant	Invasion and micrometastasis by 24 hours
Heritable T-ALL	Frazer et al., 2009	WT fish	25 Gy	2.5×10^3 to 1×10^6 GFP ⁺ tumor cells i.p.	Engrafted hosts began to die at 11–13 days	Disease capable of serial transplantation

Dexamethasone was given at 10 µg/mL for 2 days prior to and 1 day post transplant. i.p. = intraperitoneal injection, i.c. = intracardiac injection, WKM = whole kidney marrow, dpf = days post fertilization