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# Leveraging the zebrafish to model organ transplantation

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## Abstract

**Purpose of review—**The availability of organs for transplant fails to meet the demand and this shortage is growing worse every year. As the cost of not getting a suitable donor organ can mean death for patients, new tools and approaches that allows us to make advances in transplantation faster and provide a different vantage point are required. To address this need, we introduce the concept of using the zebrafish (*Danio rerio*) as a new model system in organ transplantation. The zebrafish community offers decades of research experience in disease modeling and a rich toolbox of approaches for interrogating complex pathological states. We provide examples of how already existing zebrafish assays/tools from cancer, regenerative medicine, immunology, and others, could be leveraged to fuel new discoveries in pursuit of solving the organ shortage.

**Recent findings—**Important innovations have enabled several types of transplants to be successfully performed in zebrafish, including stem cells, tumors, parenchymal cells, and even a partial heart transplant. These innovations have been performed against a backdrop of an expansive and impressive list of tools designed to uncover the biology of complex systems that include a wide array of fluorescent transgenic fish that label specific cell types and mutant lines that are transparent, immune-deficient. Allogeneic transplants can also be accomplished using immune suppressed and syngeneic fish. Each of these innovations within the zebrafish community would provide several helpful tools that could be applied to transplant research.

**Summary—**We highlight some examples of existing tools and assays developed in the zebrafish community that could be leveraged to overcome barriers in organ transplantation, including ischemia–reperfusion, short preservation durations, regeneration of marginal grafts, and acute and chronic rejection.

### Keywords

organ transplantation; regenerative medicine; zebrafish

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## INTRODUCTION

Organ transplantation is unquestionably a life-saving treatment for most patients with end-stage organ failure. However, the availability of organs for transplant fails to meet the demand and the number of patients on the waitlist continues to increase. The factors, which contribute to this severe donor shortage are many, ranging from social [1], biological [2], and logistical features to complex ethical considerations [3]. At the same time, diverse research efforts, which address interwoven challenges throughout the transplant process, such as expansion of the donor pool by rendering marginal grafts transplantable, extending preservation duration to enable global matching programs, reducing rejection to increase long-term graft survival, xeno-transplantation and engineered organs to minimize reliance on cadaveric organs, and others, may each play a role in solving the organ shortage [4■,5]. Hence, truly eliminating the organ shortage will require efforts from policy makers and educators, clinicians and surgeons, and breakthrough solutions from scientists from extremely diverse expertise including regenerative medicine, biopreservation/cryobiology, genetic/molecular/tissue engineering, and immunology.

When faced with complex scientific challenges, researchers approach the problem at multiple levels to get a full description and understanding. In some cases, in-vitro cell culture approaches are amenable to high throughput screens and provide invaluable information; however, mimicking the 3D structures of whole organs require sophisticated tissue engineering approaches and even still they may not be adequate. On the other hand, the exploration of physiological functions and systemic interactions of heterogeneous organs and between organs requires whole organs or whole organism studies. In this capacity, animal models have been vital for developing new solutions for organ transplantation. However, experimental transplantation has the worst of both worlds in terms of animal models: small animal studies require personnel with specialized microsurgery training, which is difficult and limiting, large animal studies are extremely laborious and expensive, and preclinical human grafts are difficult to obtain and even harder to perform controlled studies.

A new model that is easy to perform transplants, is amenable to high throughput screening, captures the 3D and complex structures of organs, and has a suite of tools to monitor the underlying biology of engraftment would be a tremendous tool for the field. *Danio rerio*, also known as the zebrafish, has become a favored research animal for studying human disease as it has many advantages as a model organism. Although the zebrafish has been successfully leveraged to understand a broad range of human diseases [6–8], the field of organ transplantation has yet to take full advantage of this useful model. Of course, the zebrafish is unlikely to replace any of the current model systems as each has different advantages. Instead, we suggest cross-organism research can have tremendous supplementary impacts, and zebrafish offers an avenue that bridges the gap between simplistic models, such as cell culture and more complex ones, such as mammalian systems. We also recognize that, as is the case for any animal model, this model has limitations. The most obvious of them being some inherent differences in anatomy between fish and mammals that would not allow to model a lung transplant for example.

Here we begin with a discussion of current barriers in organ transplantation focusing only on three main challenges including the limits on warm ischemia time and resuscitation of marginal organs, short preservation durations, and acute and chronic allograft rejection. Subsequently, we discuss studies in zebrafish in the areas of cancer, regenerative medicine, and immunology, and further focus on studies, which use transplantation of stem cells, tumors, parenchymal cells, and immune cells. Each of these zebrafish studies have been strategically selected and presented in a way to highlight potential tools and approaches, which could be leveraged to address these three key challenges in organ transplantation. Of course, the tools and approaches which can be transposed from the zebrafish community to address the complex challenges of organ transplantation are limitless, and those presented below are in no way an exhaustive list.

## CURRENT APPROACHES AND BARRIERS IN ORGAN TRANSPLANTATION

Optimal organ handling for transplantation is critical, beginning with retrieval from the donor, and continuing during storage and transport and finally reperfusion during transplant to the recipient. Each of these stages in organ handling has the potential to cause injury and these compounding injuries subsequently affect long-term graft survival. In general, retrieval of organs from donors after brain death (DBD) is preferable as organs remain perfused with oxygenated blood until the point of organ retrieval. In contrast, donors after circulatory death (DCD) are inevitably exposed to a greater duration of warm ischemia as heart and blood perfusion stops [9,10]. Although approximately 8.9% of total transplants currently use organs from DCD (maximum warm ischemia time for a transplanted DCD liver is 30 min) [11], it is estimated that every year ~6000 livers fall into the category of ‘marginally injured’ warm ischemic livers (between 30 and 60 min warm ischemia). These time constraints are severe barriers and result in large numbers of wasted organs [12]. Strategies that aim to revive organs following warm ischemia include machine perfusion [13]. New information on cellular and molecular events of ischemia–reperfusion injury might provide clues to guide efforts related to the resuscitation of these organs using machine perfusion [14■].

After organ retrieval, the standards for organ preservation use hypothermic storage (+4°C); however, this method only moderately slows down tissue deterioration and organs can only be kept *ex vivo* for a few hours. These suboptimal handling/preservation practices results in severe bottlenecks. Firstly, only the highest quality organs can withstand hypothermic preservation and any cumulative damage, such as warm ischemia can render an organ unusable. Hence, improving organ preservation practices to reduce cold ischemic injury could improve overall organ quality and enable the use of some currently discarded organs thereby increasing the lifetime of the transplanted organ *in vivo*. Moreover, prolonging the storage duration would enable matching over greater distances, therefore, enhancing transplantation outcomes, and decreasing the economic and health-related burden of immunosuppressive therapy [9]. Similarly, expanding the length of ex-vivo organ preservation will allow for more comprehensive quality control analyses, thus alleviating worries about disease transmission between donor and recipient, and turning emergency surgeries into elective ones with improved patient outcomes [10]. Finally, as complimentary fields, such as tissue engineering and regenerative medicine achieve longer term

breakthroughs in the development of synthetic tissues and organs, centralized banking facilities will be required to support this ‘off-the-shelf’ access to life-saving organs.

The next major challenge in organ transplantation is making sure the transplanted organ is not rejected by the recipient, both short-term and long-term. Acute allograft rejection remains a prevalent and serious problem in transplantation, with an incidence of up to 30–40% in liver [15], lung [16], kidney [17], and heart [18] transplantation. Although an acute rejection episode is rarely fatal, these episodes lead to organ injury, compromise the patient’s immune system, and are major determinants of long-term allograft survival [17,19]. In fact, acute rejection events decrease allograft half-life by 34% and increases the risk of chronic rejection [20]. As a result, about one-third of the patients on the kidney transplant waiting list are listed for a retransplant [21], despite the organ shortage.

## ZEBRAFISH IN BIOMEDICAL RESEARCH

The foundation of zebrafish as a vertebrate research model was pioneered by George Streisinger in 1972 at University of Oregon [22] who was the first scientist to produce homozygous zebrafish clones [23]. Zebrafish have many advantages as a model organism as they are inexpensive to breed and maintain. The larvae can be raised in multiwell plates, facilitating the application of high-throughput drug screens. Also, they are particularly suited for biomedical research laboratories as most major organs are formed within 24 h and embryos are translucent facilitating live imaging studies, their genome is sequenced and annotated, and thousands of mutant lines have been generated [24]. According to the zebrafish reference genome, 82% of disease-associated genes have clear zebrafish orthologues [25]. Finally, animals can be mutagenized readily, and robust methods exist for gene editing [25]. As a result, zebrafish have been used in a multitude of subjects ranging from genetics, oncology, immunology, ophthalmology, metabolic and cardiovascular diseases, all the way to regenerative medicine (stem cell transplant, spinal cord regeneration), paving the way to key biomedical advances that allowed us to transform the way we study human diseases. However, there are still many areas where their utility remains to be discovered or explored in more depth and one of them is organ transplantation.

Zebrafish has long been used as a model in cancer research, from understanding tumor biology and immunologic response to screening potential drugs for therapeutics. In fact, discoveries made in zebrafish cancer studies have had immense translational capacity. A stem cell boosting drug FT1050 (a prostaglandin E2 derivative) made it all the way to phase II clinical trials after being discovered in zebrafish [26]. Recently one of those screening studies in zebrafish has led to the proposal of a clinical trial for patients with adenoid cystic carcinoma, which is a rare and fast progressing type of tumor for which there is no standard drug therapy. The screening in zebrafish embryos showed that ATRA (tretinoin) was able to downregulate the expression of the *MYB* gene and slow tumor growth [27]. Another recent study identified that a PARP inhibitor (olaparib) combined with a DNA-damaging agent (temozolomide) was an effective treatment for rhabdomyosarcoma and will soon move into phase II clinical trials [28■■]. Further, many tumor models have been developed in zebrafish for allograft transplantation, which led to the development of immunosuppressed (rag2/c-myb$^{I181N}$) and clonal lines (CG1) [29]. Xenograft transplants into adult fish were facilitated

by creation of PRKDC−/−, IL2rga−/− fish [28■■]. These are significant advances as these lines improve engraftment with-out the need for pretransplant immune ablation. Moreover, this research demonstrates that allograft and xenograft transplantations can be performed with existing tools.

One characteristic that made zebrafish a popular model in regenerative medicine is the innate ability of adult fish to regenerate tissues after injury that include fin, spinal cord, retina and even heart [30■]. In 2002, Poss *et al.* [31] were the first ones to demonstrate and characterize the cardiac regenerative response in adult zebrafish after ventricular resection. They reported the complete regeneration of the heart, confirmed by histology and contractile function (visual inspection) within 60 days of surgically removing 20% of the ventricle [31]. Although heart regeneration studies were never performed with the intent of developing new tools/techniques for organ transplantation, they opened the door to what could be considered the first solid organ transplant in zebrafish [32]. Although a partial heart transplant, it highlights the possibility to design studies that focus on solid organ transplants using transgenic lines in a cost effective and relatively higher throughput way as compared with mammalian models and other solid organ transplants could be performed in addition to the heart.

Another field that largely explored the use of zebrafish models is immunology. Zebrafish have been used to study host–pathogen interactions after exposure to a variety of pathogens including bacteria [33], viruses [34] and fungi [35]. Underlying mechanisms in immunology, such as development and maturation of the immune system [36,37], cell migration [38], inflammation [39], innate [40] and adaptive immune responses, using transplantation [41] and immunization [42] models, have been reported. In addition, the immunological response to injury [43] and regeneration [44] and immunological reactions to foreign cells/tissues that ultimately led to the use of immunosuppressive techniques to ensure engraftment have been characterized. MHC class I genes identified on chromosome 19 have also been shown to play a role in immune matching of zebrafish for stem cell transplant studies [45,46]. Transgenic lines with fluorescent immune cells have also been developed. Taken together, these learnings and tools may be leveraged to better understand and observe solid organ rejection in real time.

Each of the tools mentioned above will be discussed in further detail in the following subsections as examples of zebrafish models that have been used for different purposes but could be applied to transplantation studies.

## Tumors

Embryonic and larval stages are usually chosen for transplants as their immune system has not been fully developed, therefore, allowing for short-term survival of transplanted cells. Adult fish are also used as allogeneic transplant models as long as immunosuppression is induced either by drugs or radiation. Other options for using adult fish include the use of clonal syngeneic strains or more recently the use of a newly generated immune-compromised zebrafish mutants [47]. Zebrafish tumor transplant models have been used with a variety of allogeneic tumours including endocrine [48], T-cell acute lymphoblastic leukaemia [49], rhabdomyosarcoma [49], melanoma [49], glioblastoma [48] and xenogeneic

brain [50], breast [51], endocrine [52], and gastric [53] tumors to name a few. These models provide a clear visualization platform to better understand cancer biology *in vivo* and the mechanisms behind progression, metastasis, and efficacy of treatment. A great advance in tumor transplantation was the generation of an optically clear immune-compromised rag2(E450fs) (casper) zebrafish that allows for optimized cell transplantation and engraftment as well as direct visualization of fluorescently labelled allogenic cancer cells at single-cell resolution [49]. Recent advances have now led to engraftment of human tissues using the PRKDC−/−, IL2rga−/− fish [28■■].

Another promising use of zebrafish transplant models in cancer research involves generating 'human avatars' by transplanting patient tumors into zebrafish to screen for drugs that will be more effective to treat each patient's specific tumor, therefore, allowing for the development of precision therapies in oncology [54]. The fact that adult zebrafish can reject allogeneic cells and tissues along with the generation of immune suppressed lines, show that they might be a useful model to better understand the mechanisms involved in organ rejection. The existence of an optically clear mutant (casper) in addition to a variety of transgenic lines expressing fluorescent markers in specific cells and tissues allow for microscopy studies and imaging of cellular processes as they happen in real time *in vivo*.

## Stem cells

Zebrafish models have been highly used in the past decades as a tool to observe and understand the underlying mechanisms of hematopoiesis *in vivo*. Numerous novel cellular interactions as well as possible chemical and genetic targets that regulate hematopoiesis have been revealed using this model [55]. In addition, novel genes required for hematopoietic stem cells (HSCs) induction and regulation have also been discovered as part of efforts to produce bioengineered patient-specific HSCs, and therefore, increase stem cell engraftment [56,57]. Stem cells of different origins have been used for transplantation studies in zebrafish models that include both allogeneic and xenogeneic cell transplantation.

Human HSCs have also been transplanted both at the larval and adult stages [41,58]. The larval stage is used to track cellular trafficking using fluorescently labelled cells that can be observed in real time in the translucent larvae. Staal *et al.* [58] showed that human HSCs injected intravenously into 48 h postfertilization (hpf) larvae survive up to 6 days after transplantation. They also showed by using conditioned medium and cell migration assays that human HSCs are chemoattracted to zebrafish stromal and endothelial cell-derived factors and transplanted human HSCs differentiate into monocytic/myeloid lineage [58].

Human-induced pluripotent stem cells generated through reprogramming of somatic cells, in this case, skin fibroblasts turned into neural progenitor cells, have also been transplanted into embryonic and 72 hpf zebrafish larvae. These cells have been shown to differentiate into neurons and survive in the recipients for more than 2 weeks [59]. Cellular therapies like stem cell transplants hold great promise for regenerative medicine and zebrafish models are providing a faster and cheaper way to understand the factors involved in regulation, differentiation, trafficking, homeostasis, and fate of transplanted cells in real time. In addition to allowing the establishment of models to conduct high throughput screening of drugs to treat human diseases.

### Parenchymal cells

Normal allogeneic muscle cells have been transplanted into homozygous rag2 mutant fish. Normal skeletal muscles cells from an α-actin-RFP transgenic line were transplanted into rag2 and wild types. Wild type recipients failed to engraft muscle cells over a 30-day experimental period, whereas 64.3% of rag2 contained RFP-positive cells near the injection site that showed persistent and robust engraftment even 115 days after transplantation and differentiation into multinucleated fibers [60].

Endothelial cells produced from human-induced pluripotent stem cells derived from skin fibroblasts or blood outgrowth endothelial cells were injected into zebrafish embryos and 48 hpf larvae. Both cell types were highly proficient in developing into vessel-like structures and were able to functionally integrate into the vasculature of zebrafish embryos and larvae whereas HUVECs and $CD31^{-}$ control cells were not. Human endothelial cell vessel formation in the animals was confirmed by labeling with a human-specific antibody against platelet endothelial cell adhesion molecule-1 [61]. These and other tools for monitoring endothelial cell injury/biology would be a complimentary tool for organ transplantation, especially given the importance of endothelial cells throughout the organ transplantation process.

Cardiac regeneration is largely studied in zebrafish because of the species' natural capacity to replace lost ventricular area through proliferation of preexisting adult cardiomyocytes [62]. Researchers have also developed a model of partial heart transplantation in adult zebrafish whereby a donor heart is dissected, and a piece of the ventricle is transferred to the pericardial area of a recipient zebrafish after cryoinjury. In specimens that survived the surgery and had the expected healing at 30 days postinjury, donor cells were clearly visible and intermixed with the recipient's heart tissue [32]. This study was conducted with the aim of understanding heart regeneration in zebrafish, but it can also be credited as the first study showing the possibility to successfully perform a partial heart transplant in zebrafish, therefore, opening up possibilities to explore transplant of other solid organs using this animal model.

### Immune cells

Human monocytes/macrophages have been injected into 48 hpf zebrafish larvae, both directly into circulation and into the hindbrain parenchyma. They are shown to survive in the host for up to 2 weeks postinjection, interact with zebrafish astrocytes and become activated [63]. Zebrafish transgenic lines that express fluorescent markers in immune cells, such as astrocytes, macrophages, and neutrophils were used in the aforementioned study. Humanized zebrafish models have also been explored. A transgenic line expressing human CXCL12 – a chemokine normally expressed by stromal cells – has been developed to study T-cell acute lymphoblastic leukemia [64]. Transgenic zebrafish with neutrophils expressing human myeloperoxidase with a fluorescent tag have also been produced to facilitate in-vivo studies looking at neutrophil function and migration [65]. These lines constitute another example of a valuable tool that could be applied to transplantation in organ rejection studies.

## CONCLUSION

Here we reviewed several current uses of zebrafish as transplantation models. Although most of the literature focuses on stem cells and tumors, other types of transplants are slowly appearing (i.e. muscle, vessel, heart). However, it is important to point out that the primary focus of those studies was not organ transplantation *per se*, still organ transplantation researchers could adapt those models to study preservation and transplantation while taking full advantage of the many decades of knowledge and tools (e.g. transgenic and mutant lines) generated by the zebrafish research community. Another point to be made is that zebrafish could not fully replace rodent or larger animal models, but instead could be used as a starting point for screening and optimization so that only the most promising conditions would be carried forward in mammalian models.

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**KEY POINTS**

- Organ transplantation faces many challenges including, but not limited to, warm ischemia time and resuscitation of marginal organs, short preservation durations and allograft rejection.
- New approaches and solutions from several fields are needed in order to effectively address the organ shortage.
- Animal models are an essential part of organ transplantation and preservation research.
- Zebrafish have been used as animal models in biomedical research for many years, but the area of organ transplantation/preservation has yet to discover their utility.
- The variety of tools available for zebrafish research would make them a useful model to bridge the gap between in-vitro studies and larger animal models.