Transgenic Zebrafish Models for Understanding Retinitis Pigmentosa

Raghupathy RK, Patnaik SR and Shu X*
Department of Life Sciences, Glasgow Caledonian University, Glasgow, UK

Abstract
Retinitis pigmentosa (RP) is a heterogeneous genetic retinal disorder, characterized by impaired dark adaptation and progressive loss of photoreceptor cells. More than 46 genes have been identified to be responsible for RP, but the functional roles of RP-causing mutants are largely unknown. Due to the similarities of anatomy, physiology and functional signal pathways to human retina, the zebrafish has become a valuable model to study human retinal diseases. With the aid of improved techniques in transgenesis, the use of the zebrafish model has been accelerated, especially in the field of retinal degeneration. In this brief review, we will present an overview of the transgenic approaches in zebrafish and the utility of transgenic zebrafish for assessing the pathogenicity of RP-causing alleles.

Keywords: Zebrafish; Transgenic; Retinitis pigmentosa

Introduction
Retinitis pigmentosa (RP, MIM# 268000) is a heterogeneous group of genetic disorders, characterized by the degeneration of rod and cone photoreceptors [1]. The earliest clinical manifestation of RP is night blindness, progressing to reduction in mid-peripheral visual field. As the disease becomes worse, the patients develop tunnel vision and eventually lose central vision. Other clinical features include bone spicule fundus deposits, attenuated retinal arterioles, optic disk pallor, absent or subnormal electroretinogram (ERG) amplitudes [2] (Figure 1). The prevalence of RP is in the region of 1 in 3500–4000 in most populations. Most cases result from a series of monogenic disorders inherited in an autosomal-dominant, autosomal-recessive, or X-linked manner [3,4]. Oligogenic inheritance and mitochondrial inheritance have been established in a small proportion of RP cases [5,6]. More than 53 genetic loci are associated with RP, 46 genes have been identified, 18 genes for autosomal dominant RP, 26 for autosomal recessive RP and 2 for X-linked RP (http://www.sph.uth.tmc.edu/Retnet/). Most causing genes are only responsible for a small proportion of cases, but mutations in the rhodopsin gene (RHO) cause about 25% of autosomal dominant RP, the USH2A gene accounts for about 20% of autosomal recessive RP, and the RPGR gene accounts for about 60-75% of the X-linked RP families [1,2,7].

Zebrafish (Danio rerio) is a tropical freshwater fish, which has become the most commonly used and favourite vertebrate model in biomedical research in recent years. Ease of maintenance, optical clarity and rapid development of the embryos made zebrafish a favourite model for developmental biologists, but recent developments in transgenesis and other genetic manipulation tools make it a ubiquitous animal model in a wide range of fields from cardiovascular disease to visual disorders [8]. The zebrafish genome consists of 25 chromosomes with a total size of 1.412 gigabase. Zv9 assembly shows zebrafish have 26,206 protein-coding genes, the highest gene number of any sequenced vertebrate species. The zebrafish genome has its own features, including the highest repeat content in genome sequence, lower number of pseudogenes and higher number of species-specific gene when compared to the human genome. The zebrafish genome shares high homology with the human genome, with approximate 70% of human genes having at least one zebrafish orthologue [9].

The zebrafish neural retina is conserved in evolution, its anatomy, histology, and function closely resembling that of human retina (Figure 2A). Zebrafish retina develops very early, following an inner to outer retinal order, similar to that of other vertebrate species [10]. The retinal ganglion cells (RGC) begin to differentiate at around 28 h post-fertilization (hpf), and the differentiation of RGC spread rapidly into the temporal retina between 36 and 40hpf [10-12]. The inner nuclear layer starts to form at 38hpf and photoreceptors begin to differentiate at approximately 50 hpf [10]. Retinal lamination initiates at around 32 hpf and spreads across most retina at 48 hpf. The retina is fully laminated by 60 hpf [12] and the three neuronal layers are clearly distinguished by morphological criteria (Figure 2B). Zebrafish retinal photoreceptors contain one type of rod cell and four types of cones, which are sensitive to ultraviolet, blue, red and green wavelengths respectively [13]. Zebrafish cone-rich retina is similar to that of human retina, providing a wonderful model to study human retinal degeneration.

Early work of retinal degeneration in zebrafish was performed by a forward genetic approach. The first large-scale forward genetic screen identified 49 mutations causing retinal abnormalities, including loss of laminar pattern of neural retina, an abnormal specification of eye anlagen, cell death in outer nuclear layer, retardation of eye growth, nonspecific retinal degeneration, and retinal degeneration with pigmentation defect [14]. An oval zebrafish strain identified by the forward genetic screening exhibits photoreceptor degeneration and kidney cysts. A mutation (L260X) in ift88 gene was identified in...
we provide an overview of transgenic approaches in zebrafish and review the functional characterization of RP-causing mutant proteins in zebrafish.

Technologies for Zebrafish Transgenesis

The technologies for creating transgenic zebrafish have largely improved in the last 6 years. Early transgenic zebrafish were generated by injection of plasmid DNA containing the transgene into 1-4-cell-stage fertilized embryos, the transgene were integrated into the zebrafish genome and transmitted through the germ lines [21-24]. The efficiency of germ-line transmission for plasmid DNA injection is quite low, about 5% of injected fish produce transgenic lines. Due to the development of transposon-based tools for zebrafish, the efficiency of germ-line transmission is now significantly increased. Transposons, known as ‘jumping genes’, are the class of genetic elements which have the ability to move genetic material from one location to another location in the genome. When transposons are introduced into a target cell, they integrate into the host genome. There are three major transposon systems: PiggyBac (PB), sleeping beauty (SB), and Tol2, which are predominantly used for transgenesis in vertebrates. Every system has unique features such as SB has higher overexpression sensitivity, whereas Tol2 and PB systems have high tolerance for the large cargo and are found to be more active in particular species [25]. The Tol2 transposon system is a preferred choice for zebrafish transgenesis because of its high percentage of transmission to the F1 generation [26,27].

Generation of Tol2 vectors becomes much more efficient and rapid by the use of the gateway cloning system [28,29]. The gateway cloning system is based on the lambda phage based site-specific recombination. Reactions that facilitate the integration of bacteriophage λ occur by sequence specific sites ‘attP’ (from phage) and ‘attB’ (from bacterium) in the presence of phage-coded integrase (INT) and the bacterial integration host factor (IHF). The ‘attP x attB’ recombination reaction is termed an BP reaction and results in two hybrid sites ‘attL’ and ‘attR’ which enable excision in the presence of INT, IHF and excisase (XIS). This excision recombination reaction is termed an LR reaction [30]. The Tol2kit uses the three-insert multisite gateway system to clone three DNA fragments into a plasmid in a desired order (Figure 3). The three DNA fragments include a promoter for regulating expression, a gene of interest, and a polyadenylation signal coding fragment or a 3’tag [29]. To facilitate the screening of transgenic lines, the transgenes are co-expressed with the selection marker, EGFP, driven by an internal ribosomal sequence (IRES) or a separate cardiac myosin light chain2 (cmlc2) promoter. IRES: EGFP or cmlc2: EGFP is included in the vector backbone (Figure 3). After the co-injection of plasmid DNA of a desired expression construct with transposase RNA into one-cell stage embryos, the transgene were integrated into the zebrafish genome and transmitted through the germ lines [21-24]. The Tol2 transposon system is a preferred choice for zebrafish transgenesis because of its high percentage of transmission to the F1 generation [26,27].

Figure 2: (A) Similarities of retinal structure between zebrafish and human. Zebrafish cone cells are labelled with anti-Zpr 1 antibody (Red), human photoreceptor outer segments (OS) are labelled with anti-rhodopsin antibody (Yellow). INL: inner nuclear layer, IPL: inner plexiform layer, ONL: outer nuclear layer, OPL: outer plexiform layer, OS: photoreceptor cell outer segments, RGL: retinal ganglion cell layer, RGC: retinal ganglion cell, RPE: photoreceptor outer segments, RPE: retinal pigment epithelium.

Recently, the reverse genetic approaches have been used to directly characterize human retinal disease genes in zebrafish. The most widely used approach is the injection of morpholinos into one to two cell stage zebrafish embryos to prevent proper splicing or translation of the target genes. Although morpholinos are only effective for several days, their transient activities are sufficient to characterize the eye phenotypes of morphants with the depletion of target gene. For example, knock-down of X-linked RP gene, retinitis pigmentosa GTPase regulator (RPGR), in zebrafish by morpholino injection caused retinal developmental defects, including both laminating defects and abnormal photoreceptors with absence of outer segment, associated with increased cell death in the retina, suggesting RPGR has a role in retinal differentiation and laminating, and in preventing apoptotic retinal cell death [16,17]. Other gene target mutagenesis approaches, such as targeted induced local lesions in genomes (TILLING) [18], zinc-finger nucleases (ZFNs) engineering [19], and transcription activator-like effector nucleases (TALENs) engineering [20], make it possible for the knock-out of retinal disease genes in zebrafish.

Zebrafish have also been used to investigate the function of human disease-causing mutant proteins through transgenic approaches. Here...
rhodopsin gene are the most common cause of autosomal dominant RP, with more than 120 point mutations identified (see rhodopsin mutation database: http://www.retina-international.org/files/sci-news/rhomut.htm). Most mutations cause autosomal dominant RP, while others cause recessive RP and stationary night blindness. According to the biochemical properties, autosomal dominant RP-causing rhodopsin mutations are classified into two major types. For class I mutations occurring in rhodopsin C-terminal, both their translocation to the plasma membrane and their formation of a functional chromophore with 11-cis-retinal resembled that of the wildtype protein [31-33].

In vivo studies in animal models showed those C-terminal mutations impaired the targeting to the photoreceptor outer segments [34,35]. Class II mutations occur in the rhodopsin transmembrane and cytoplasmic domains, and these mutant proteins are misfolded and fail to form a functional chromophore with 11-cis-retinal [31-33]. Rhodopsin knock-out mice do not form rod outer segments; the rod photoreceptors have no ERG response and degenerate over 3 months, suggesting rhodopsin is essential for phototransduction, development of rod outer segments, and maintenance of rod photoreceptors [36].

Transgenic animal models have been made to characterize the functional role of rhodopsin mutant proteins. The rhodopsin
transgenic models, such as T17M transgenic mouse [37], P23H transgenic mouse, rat and pig [37-39], P347L transgenic rabbit and pig [40,41], and S334ter transgenic rat [42], exhibit photoreceptor degeneration, a similar phenotype presented in RP patients. Recently, zebrafish has been used to characterize human rhodopsin mutant, rhodopsin wildtype and Q344X transgenic zebrafish strains were made using the Tol2 transposon system, with expression of the human mutant rhodopsin controlled by the zebrafish rhodopsin promoter [42]. The Q344X mutant proteins were found to be mislocalized in rod photoreceptors of Q344X transgenic fish. There was no significant difference of rod photoreceptor number between wildtype and Q344X transgenic fish retina at 3dpf, but the number of rod photoreceptors of Q344X animals is significantly reduced compared to that in wildtype rhodopsin transgenic fish retina at 5dpf and 7dpf. The reduction of rod photoreceptors is caused by increased apoptotic cell death. The cone photoreceptors did not decrease in Q344X transgenic zebrafish retina at 5dpf, 7dpf and even 1.5mpf, which mimic rhodopsin Q344ter mutation, caused RP. When the Q344X transgenic fish were continuously exposed to light, the number of rod photoreceptors was significantly reduced at 5dpf when compared to that of untreated controls. Morpholino mediated knock-down of transducin-a in the Q344X transgenic fish significantly increased the number of surviving rod photoreceptors at 5dpf. However, suppression of the rod cGMP-phosphodiesterase β subunit (PDE6β) in the Q344X transgenic fish did not rescue the death of rod photoreceptors. When activities of adenylyl cyclase (ADCY) were inhibited with 100µM of SQ22536, an ADCY inhibitor, in the Q344X transgenic fish, the death of rod photoreceptors was suppressed. All the data suggested that the mislocalization of Q344X caused rod photoreceptor cell death through phototransduction and ADCY signalling pathways, which may aid development of potential treatments for RP [42].

Conclusion

The zebrafish represents an increasingly favoured model for understanding the pathogenesis of retinal diseases. The improvement of transgenic techniques offers opportunities to create more transgenic fish lines for retinal diseases. Recent studies demonstrate that transgenic zebrafish can help to functionally characterize RP-causing alleles. Future research efforts to make more transgenic zebrafish lines will provide a solid platform to assess the function of different RP-causing alleles, which will shed light on the disease mechanisms of RP and enable development relevant therapeutic strategies.

Acknowledgements

The work in Dr Shu’s laboratory is supported by the Royal Society London, Rosetrees Trust, National Eye Research Centre, UK Fight for Sight, TENOVIUS Scotland, Carnegie Trust for the Universities of Scotland, and EU INTERREG IVb (2012) no PILLs in Waters.

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