Critical Reviews in Toxicology

http://informahealthcare.com/txc
ISSN: 1040-8444 (print), 1547-6898 (electronic)

Crit Rev Toxicol, 2014; Early Online: 1–18
 DOI: 10.3109/10408444.2014.965805

informa
healthcare

REVIEW ARTICLE

Transgenic fish systems and their application in ecotoxicology

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Abstract

The use of transgenics in fish is a relatively recent development for advancing understanding of genetic mechanisms and developmental processes, improving aquaculture, and for pharmaceutical discovery. Transgenic fish have also been applied in ecotoxicology where they have the potential to provide more advanced and integrated systems for assessing health impacts of chemicals. The zebrafish (*Daniorerio*) is the most popular fish for transgenic models, for reasons including their high fecundity, transparency of their embryos, rapid organogenesis and availability of extensive genetic resources. The most commonly used technique for producing transgenic zebrafish is via microinjection of transgenes into fertilized eggs. Transposon and meganuclease have become the most reliable methods for insertion of the genetic construct in the production of stable transgenic fish lines. The GAL4–UAS system, where GAL4 is placed under the control of a desired promoter and UAS is fused with a fluorescent marker, has greatly enhanced model development for studies in ecotoxicology. Transgenic fish have been developed to study for the effects of heavy metal toxicity (via heat-shock protein genes), oxidative stress (via an electrophile-responsive element), for various organic chemicals acting through the aryl hydrocarbon receptor, thyroid and glucocorticoid response pathways, and estrogenicity. These models vary in their sensitivity with only very few able to detect responses for environmentally relevant exposures. Nevertheless, the potential of these systems for analyses of chemical effects in real time and across multiple targets in intact organisms is considerable. Here we illustrate the techniques used for generating transgenic zebrafish and assess progress in the development and application of transgenic fish (principally zebrafish) for studies in environmental toxicology. We further provide a viewpoint on future development opportunities.

Keywords

biosensor, ecotoxicology, environmental, medaka, model, pollutant, review, transgenic, transgene, technique, zebrafish

History

Received 20 March 2014
Revised 11 September 2014
Accepted 11 September 2014

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Introduction

Since the early 1980, transgenic animals – those that carry foreign DNA derived from an exogenous source and deliberately inserted into their genome – have been created in a variety of animals including mammals, birds, amphibians, fish and invertebrates (Donovan et al. 2005, Fan and Watanabe 2003, Hamra et al. 2002, Hong et al. 2009, Hrytsenko et al. 2010, Huss et al. 2008, Mozdziak and Petitte 2004, Sinzelle et al. 2006, Tsai et al. 1997, Wang et al. 1995). Transgenic technology has been used in biological and medical research to find cures for cancers, diabetes, Huntington's disease and cardiovascular diseases and has contributed significantly to agriculture including improved milk production, and both increased growth rate and disease

resistance in farmed animals (Bader et al. 2000, Donovan et al. 2005, Fan and Watanabe 2003, Hansson et al. 1999, Houdebine 1995, Mozdziak and Petitte 2004, Reventos and Munell 1997, Thomas et al. 2001). Transgenic research applied to mammals was initiated in the 1980s and for fish somewhat after this in the late 1990s. Subsequently, there has been a rapid increase in the use of transgenic animals in research, illustrated by the number of academic papers published, increasing from around 280 in 1988 to almost 15,000 in 2013 (using the search term transgenic animal – PubMed, Figure 1a) and in fish from 8 in 1990 to around 300 in 2013 (using the search term fish + transgenic – PubMed, Figure 1).

Transgenic fish have been developed as model systems for understanding genetics mechanisms and developmental processes and for pharmaceutical discovery (target validation), safety assessment and bio-synthesis (Dunham 2009, Moro et al. 2012, Nguyen et al. 2012). They have also been created to study-specific processes in fish including cold tolerance and for enhancing both disease resistance and growth rate in aquaculture species (Devlin et al. 2004, Du et al. 1992, Dunham 2009, Wang et al. 1995). Recently, transgenic fish have also been developed for studies in ecotoxicology to screen and test chemicals where they can inform on tissue targets and effect mechanisms (Blechinger et al. 2002, Bogers et al. 2006, Gorelick and Halpern 2011, Lee et al. 2012a, Legler et al. 2000, Tong et al. 2009). Transgenic fish have been created in divergent species including the loach *Misgurnus anguillicaudtis*, mud loach *Misgurnus mizolepis*, goldfish *Carassius auratus*, common carp *Cyprinus carpio*, rainbow trout *Oncorhynchus mykiss*, Atlantic salmon *Salmo salar*, coho salmon *Oncorhynchus kisutch*, channel catfish *Ictalurus punctatus*, nile tilapia *Oreochromis niloticus*, zebrafish *Danio* rerio and medaka *Oryzias latipes* (Devlin et al. 2004, Dunham et al. 2002a, 2002b, Kinoshita 2004, Kobayashi et al. 2007, Lawson and Weinstein 2002, Maclean et al. 1987, Nam et al. 2001, Saunders et al. 1998, Uzbekova et al. 2000, Wang et al. 1995).

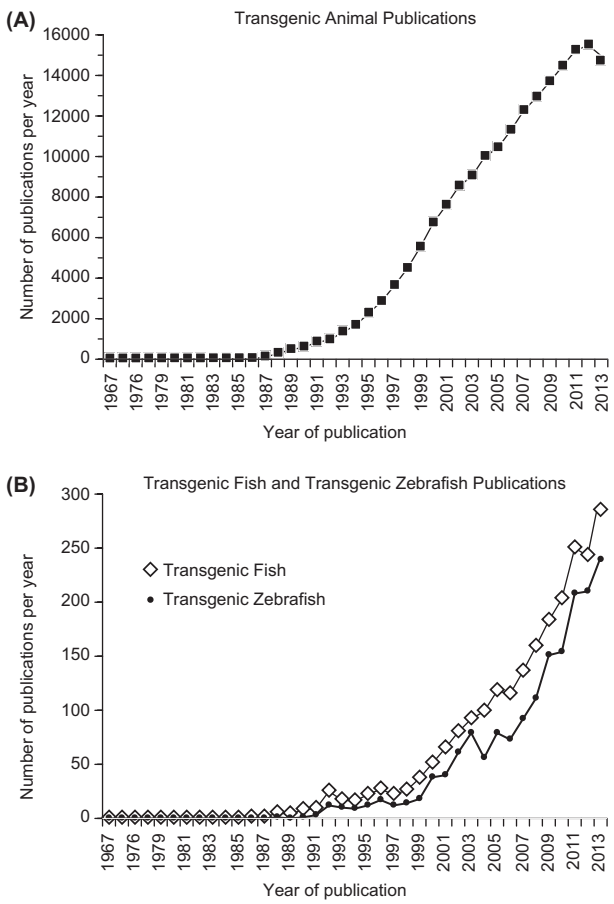

Figure 1. Number of publications between the years 1967 and 2013 that include 'transgenic animals' (1a) or transgenic (TG) fish/transgenic zebrafish as analyzed using PubMed. A search for 'transgenic (TG) zebrafish in ecotoxicology' returned only12 publications in the last 15 years.

Zebrafish and medaka have become the most popular model fish species for transgenic manipulations. Favorable features of these species for transgenic work include their ease of breeding in the laboratory, relatively low associated maintenance costs, high fecundity, transparency of the embryos and rapid organogenesis (attractive features for studying developmental processes) and the availability of sequenced genomes providing extensive genetic resources. Additionally, for the zebrafish, the chorion of the egg is relatively soft facilitating microinjection of DNA and genetic constructs. The availability of mutant lines for this species, such as casper (roy + nacre mutant zebrafish) that lack skin pigmentation also enables studies for observing effects in body tissues, for example tracing individual cancer cells as they spread through the body (Feitsma and Cuppen 2008, White et al. 2008). The zebrafish has high genome (approximately 70%), structural and physiological similarities with humans (Blader and Strahle 2000, Briggs 2002). High-quality genomic resources are also available for the medaka (Matsumoto et al. 2009), which has a large within-species genetic diversity. The medaka has a smaller genome, compared with the zebrafish (about half the size) and human (one-third the size) (Naruse et al. 2004).

The zebrafish has been used to advance understanding on formation of the embryonic axis, cell lineages, and formation of the central and peripheral nervous systems. It has also proven valuable for studies on human diseases, notably carcinogenesis, wound healing, immunological diseases, behavioral abnormalities, infection and Parkinson's disease (Blader and Strahle 2000, Bretaud et al. 2004, Burgess and Granato 2008, Feitsma and Cuppen 2008, Kelly et al. 1995, Kimmel and Warga 1987, Lieschke and Currie 2007, Martin and Feng 2009). The zebrafish and medaka have both become popular model organisms for studies on molecular mechanisms of chemical toxicity and analysis of behavioral outcomes (Hill et al. 2005, Nagel 2002, Puiseux–Dao and Edery 2006). Transgenic zebrafish and medaka employing fluorescent markers are used not only to identify chemically induced target gene activation but also to quantify relative uptake and accumulation of chemicals in those tissues. An advantage of the medaka over the zebrafish for studies into the effects of chemicals on sex is that it has an established XY/XX genetic sex determination mechanism, whereas the sex determining mechanism(s) in the zebrafish are not determined.

In this review, we first present a critical overview on the methods for producing transgenic fish, the recent progress made in different gene transfer methods, and illustrate their utility and applications. In latter part of this review, we detail progress in the development of transgenic zebrafish and medaka for studies in environmental toxicology. Finally, we

set out some thoughts on future prospects for the use of these small transgenic fish in environmental toxicology.

Methods for producing transgenic fish

Creating a transgenic fish first requires production of the gene of interest and then its introduction into the organism. The general method for generating the gene construct is shown in Figure 2.

Production of the gene construct

Several methods are now used to produce transgenic fish, but all involve a transgenic construct with a promoter and a gene.

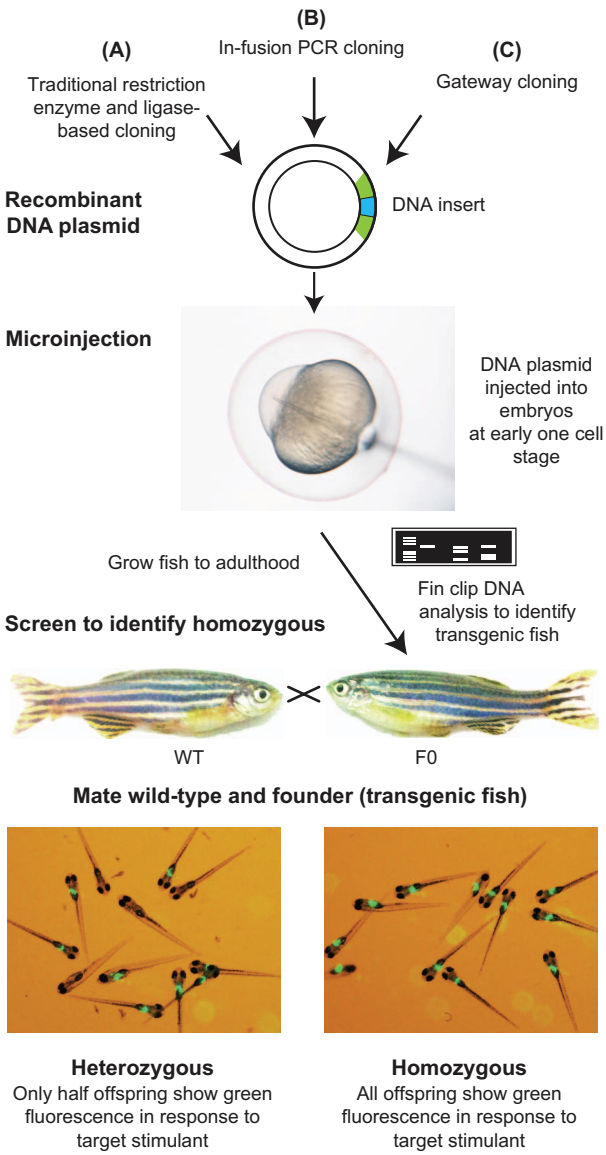

Figure 2. Procedure for generating transgenic fish. Once the construct is made by either restriction enzyme/ligase cloning (A), In-Fusion cloning (Clontech) (B), or Gateway cloning (Life Technologies) (C), the plasmid is microinjected into one cell stage embryos and the embryos are subsequently raised to adulthood. The presence of the transgene is confirmed by polymerase chain reaction (PCR) and/or Southern blotting on genomic DNA isolated from fin tissue. Single founder (F0) fish are mated with single wild-type (non-transgenic) fish and their offspring are mated with each other to confirm germ-line integration and to establish a homozygous transgenic line.

The foreign gene for transfer (transgene) is constructed using recombinant DNA techniques. Traditionally the gene of interest was most commonly expressed using *E. coli* plasmid vectors that replicate at high levels in their host cells. To clone the DNA of interest the sequence is inserted into a cloning or expression vector through the use of restriction enzymes to create compatible ends and ligase to seal the integration. This approach to cloning, however, is time-consuming and relatively inefficient, and it can be difficult to find suitable restriction enzymes in the target DNA. False-positive clones (vector without insert) can be common using this method due also to universal nucleotide ligation (Liu 2013). More efficient methods for cloning the DNA target of interest now include In-Fusion cloning (Clontech) and Gateway cloning (Life Technologies) which are also considerably faster too. In-Fusion cloning can ligate a DNA fragment with 15 bases of homology at their linear ends and a linearized vector with the use of their proprietary In-Fusion enzyme, a poxvirus DNA polymerase with 3′–5′ exonuclease activity. The Gateway cloning system is a site-specific recombination method and uses a set of the components of the λ (lambda) system for *in vitro* transfer of DNA, the λ integrase protein (Int), the λ excisionase protein (Xis), the *Escherichia coli* protein IHF and the *att* recombination sequences embedded in the DNA to be recombined (Marsischky and LaBaer 2004). There are many other cloning systems available from commercial suppliers, but In-Fusion and Gateway cloning systems have become especially popular for gene construct generation due to their ease of use, fewer number of cloning steps, and good reliability (Xiao et al. 2010).

Introduction of the gene construct into fish host

Having produced the construct, this is then transferred into the cytoplasm of fertilized fish eggs at the one cell stage, often using microinjection or electroporation (see next section). The injected DNA undergoes replication and some cells in the embryos will subsequently carry the transgene. Some integration events occur subsequent to DNA replication giving rise to mosaic fish, however, which may, or may not, contain the transgene in the germ line. Animals are then maintained to adulthood and confirmation that they carry the transgene is undertaken using polymerase chain reaction (PCR) or Southern blot analysis in an external tissue, such as fin (Horvat et al. 1993). Southern Blot is commonly used to determine transgene copy number and the number of integration sites in the transgenic founder. The identification of the integration sites and copy number is important for understanding the relationship between the integration site and the specific phenotype.

Founders containing the inherited transgene (F0) are identified by crossing founder (transgenic fish) and wild-type fish (non-transgenic fish) and assessing whether the transgene occurs in the F1 generation. The germ-line transmission rate from the F0 generation to the F1 generation is variable because F0 are mosaic (variable cell expression) and the transgene is unevenly integrated in their gonadal tissues. Progeny derived from the F0 fish are crossed with wild-type fish, and the resulting heterozygous offspring are crossed with each other to create a homozygous fish (i.e. both alleles are present in the same fish) (see Figure 2).

Gene transfer methods

Microinjection

Transgenes can be introduced into the fertilized eggs, via microinjection or electroporation, or via the use of primordial germ cells (PGCs). Gene transfer by microinjection is the most popular method. In mammals, the gene is injected into the pronuclei of the embryo, but in fish the pronuclei cannot be seen after fertilization and some fish eggs are opaque (Chen and Powers 1990, Palmiter et al. 1982). Consequently, in fish linearized DNA is microinjected into the *cytoplasm* of fertilized eggs. This approach in fish has been shown to induce high, albeit variable (35% to 80%), rates of mortality and relatively low integration rates (2–17%) (Hayat et al. 1991). Differences in success rates between the studies in fish probably include differences in the timing of the injections (embryo stage). The amount of DNA injected depends on the egg size of the fish species but often includes up to 10^6 copies (Culp et al. 1991, Stuart et al. 1988). Amplification of the injected DNA occurs in the embryo between the initiation of gastrulation and onset of somitogenesis (Stuart et al. 1988). Introducing DNA into the egg of fish species that produce eggs with a hard outer surface (chorion) is difficult and more likely induces significant damage. Embryos in some fish species also develop very rapidly, passing through the first cell stage in a very short time interval and making it extremely difficult to microinject a large compliment of eggs.

Electroporation

Electroporation, the application of short electrical pulses that result in an enhanced permeability of the cell membrane, is another method for introducing the DNA insert into the host embryo (Cerda et al. 2006, Chen and Powers 1990, Dunham 2003). *In vivo* electroporation has proved to be an efficient method for delivering expression plasmids to large numbers of cells in different regions of the developing nervous system in zebrafish embryos and adults and has also been used to study gain or loss of function in various tissues (Cerda et al. 2006, Kera et al. 2010, Rao et al. 2008, Teh et al. 2003).

Voltage, buffer choice, quantity of DNA, and the manner in which the voltage is applied (bursts and frequency of the current) can all affect the efficiency of the electroporation. A major drawback of electroporation as a technique is the considerable variation in the uptake efficiency of the DNA into the embryo, making standardization(s) difficult (Powers et al. 1995). Electroporation, however, can be used to target specific tissues and this has been shown for embryonic neural tube, retinal, and somatic tissue (Tawk et al. 2002, Teh et al. 2003, Vergara and Canto-Soler 2012).

Primordial germ cells

The use of PGCs is the most recently used method for gene transfer. PGCs are the precursors of the germ cell lineage which are committed to differentiate into either spermatogonia or oogonia after gonadal sex differentiation (Yoshizaki et al. 2003). PGC-mediated gene transfer is used to deliver a foreign gene into the genome of cultured cells and the transformants are isolated by drug selection before they are introduced into individual fish (Takeuchi et al. 2002). Germ-line contribution can be performed by microinjection into the peritoneal cavity of hatched embryos with cultured PGC (Matsui et al. 1992). PGC-mediated gene transfer offers the potential for producing transgenic fish of commercially valuable species that have a long generation time and produce low numbers of offspring. This method also allows for the generation of gene-targeted fish via homologous recombination. A method for the culture of PGCs obtained from zebrafish embryos has been established by Fan et al. (2008), but this method is limited because it is difficult to isolate PGCs in high quantities from early stage embryos. Furthermore, the information on the required *in vitro* culture conditions for enabling proliferation and survival of PGCs in fish is extremely limited.

Transgenesis vectors and systems

A number of different vectors have been used for the delivery and insertion of transgenes into the genome of zebrafish (and medaka) embryos. Commercially available *E. coli* plasmid constructs have most commonly been used due to their wide availability, ease of use and suitable size for carrying most transgene sequences. There are, however, other vectors that hold certain advantages over traditional *E. coli* plasmid constructs, which we now consider.

BAC clone and fosmids

Bacterial artificial chromosomes (BAC) have been used widely to study zebrafish gene function and regulation. BAC clones are able to hold large DNA insertions (up to 300 kb) and therefore have the potential to carry a complete gene structure, including any endogenous promoters associated with cell-type specificity or temporal expression of the gene. Another benefit of BACs is their apparent resistance to positional effects in comparison with smaller plasmid vectors (Suster et al. 2011). Positional effects are a category of transgene silencing incurred by undesirable chromosomal location and flanking DNA. An example of the application of BAC transgenesis is for a reporter system for estrogen-like substances in the medaka described in more detail below under *Transgenic Fish in Ecotoxicology* (Kurauchi et al. 2005).

An alternative but similar technique to BAC recombination is the use of fosmids. Fosmids are single-copy plasmids that act as a vector for large DNA insertions. This system has been applied successfully for the production of a transgenic *Cyp1a* reporter zebrafish investigating dioxin target tissues (Kim et al. 2013), also described below in the section *Transgenic Fish in Ecotoxicology*. The large size of BAC clones and fosmids, however, tend to suffer from low efficiency rates in producing transgenic lines. The use of *Tol2* transposon-assisted insertions (see *Transposons* section), however, in particular, an inverted *Tol2* cassette (*iTol2* cassette) is improving success rates considerably (Suster et al. 2011).

Retroviruses

The Moloney murine leukemia virus (MLV) is a viral vector that can be adapted (pseudotyped) to infect a range of different host cells, including zebrafish cells, for transgene delivery. Retroviral insertion has been used in the application of gene traps (GT), enhancer traps (ET) and protein traps (PT) in

conjunction with typical transgenic reporter sequences (Trinh le and Fraser 2013). These traps are an effective means for the identification of transcriptionally active genes and analysis of their function (reverse genetic screening). The MLV retrovirus has been used in a large-scale enhancer detection screen to insert an enhancer trap vector into zebrafish (Ellingsen et al. 2005). This technique involves the insertion of a zebrafish promoter and reporter sequence (such as GFP) into the genome via the MLV vector for the identification and characterization of regulatory gene activity during development.

The pseudotyped MLV system has proven to be one of the most efficient transgene insertion techniques available and can result in successful germ-line transmission of insertions to almost all F1 progeny, with an average of 10 copies per cell (Chen et al. 2002, Wang et al. 2007). Due to the difficulties associated with producing high-titer viruses and limitations in cargo sizes, however, transposable elements are the more popular approach (Trinh le and Fraser 2013). Nevertheless, the technique holds significant promise for use in reverse genetic screens in ecotoxicology and provides a strong alternative to genetic knockout technologies (Bedell et al. 2011, Meng et al. 2008).

Transposons

The frequency of germ-line transmission for conventional DNA microinjection is very low and results in mosaic expression in F0. Furthermore, gene silencing can occur over generations using conventional DNA microinjection methods. In the light of these problems, transposons have become popular as methods for gene transmission. Transposons are sequences of DNA that are able to move directly from one site to another site within the chromosome or onto extra chromosomal DNA within the same cell. There are two types of transposons, autonomous and non-autonomous. An autonomous transposon encodes its own enzyme (transposase) and can excise and transpose. In contrast, a non-autonomous transposon does not encode its own transposition proteins and requires transposase activity, which can be supplied as mRNA, in order to enable its relocation. Only non-autonomous transposons that requires artificially provided transposase can be utilized to produce stage transgenics (Kawakami et al. 2004, Ryder and Russell 2003). Use of transposons requires transposase introduction into early one cell stage eggs by microinjection together with transposase mRNA. *Tol2* transposon and *Sleeping Beauty* (*SB*) are the main transposons used currently in creating transgenic zebrafish. Both these methods use enzymes to facilitate the integration of foreign DNA into the host genome. The *Tol2* transposon system, derived from medaka and belonging to the *hAT*(*hobo*/*Ac*/*Tam3*) family of transposons, is now widely used in zebrafish, and in other vertebrates, due to its high efficiency for gene delivery and expression (Hamlet et al. 2006, Kawakami 2005, Kawakami et al. 2000, Sato et al. 2007). Using a *Tol2* construct co-injected with transposase mRNA, germ-line transmission frequency success rates can be as high as 50% (Kawakami et al. 2004). Furthermore the use of the *Tol2* transposon system tends to avoid gene silencing effects that can occur with linear plasmid injection (Kawakami 2005). This transposon vector system is useful for the generation of a stable transgenic fish or analyses of activation of a promoter, an enhancer, or a gene of interest in transient expression assay (Asakawa and Kawakami 2009, Asakawa et al. 2008, Lee et al. 2012a, 2012b). The *Tol2* transposon is derived from the medaka, so this system cannot be used in the medaka due to an endogenous activity.

Sleeping beauty is a reactivated transposon from the Tc1/*mariner* superfamily of transposable elements isolated from fish and is used regularly in the production of transgenic zebrafish models (Ivics et al. 1997, Miskey et al. 2005). The transgenesis rate using *sleeping beauty*, however, tends to lower than that for *Tol2* (at around 30% (Davidson et al. 2003, Thermes et al. 2002)), and so the latter has become the most favored transposon system (Balciunas et al. 2006).

Meganucleases

The traditional approach of microinjecting circular or linearized plasmid DNA into fertilized embryos can often result in concatemeric copies of transgenic DNA. This can lead to mosaicism or silencing in F0 generations, as well as reduced germ-line integration (Culp et al. 1991, Stuart et al. 1988, Westerfield et al. 1992, Winkler et al. 1991). To improve the specificity and rate of stable transgenesis, transposase and meganuclease *I-SceI* can be co-injected with plasmid DNA (Grabher et al. 2004, Kawakami 2007, Rembold et al. 2006, Thermes et al. 2002). Meganuclease is used to mediate a single insertion at a low copy number, whereas transposase is used to produce numerous single-copy insertions (Kawakami et al. 2004, Ogino et al. 2006, Rembold et al. 2006, Thermes et al. 2002).

Meganuclease *I-SceI* is an endonuclease that prevents concatamerization when co-injected with a plasmid containing *I-SceI* sites flanking the transgene (Jacquier and Dujon 1985). It is thought that meganulcease *I-SceI* remains bound to the flanking recognition sites, which may be how concatamerization is prevented, but in general the mechanism of meganuclease's success is still unclear (Soroldoni et al. 2009). For comparative studies on *cis*-regulatory sequences in a fish genome, a consistent and unique locus of transgene insertion is extremely important. Having meganuclease sites artificially engineered into the genome gives high specificity of a single insertion and is therefore particularly useful in these studies (Grabher and Wittbrodt 2008). However, *Tol2*-assisted insertion remains the most reliable and efficient systems in producing stable transgenic lines and hence remains a much more popular technique for mediating transgenesis.

PhiC31 integrase-based targeting method

Most established methods for transgenesis are not able to control copy number and integration sites leading to variable transgene expression caused by so-called position effects (Kirchmaier et al. 2013). The position effects refer to varying transcription of a transgene inserted into different chromosomal regions. An exception to this is the recently developed *PhiC31* integrase-based targeting method, developed in zebrafish and medaka, which enables pre-selection of successfully targeted integrations early on in the injected generation and provides highly reproducible patterns of transgene activity (Kirchmaier et al. 2013, Roberts et al. 2014).

Targeted genome editing

An emerging area of genome modification for insertion of a transgene is targeted genome editing. The process involves engineering of customised nucleases to induce DNA sequence-specific double strand breaks (DSBs) which are then exploited for sequence alterations. The three techniques gaining most attention currently are clustered regularly interspaced short palindromic repeats (CRISPRs), transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs), each differing in the ease of construction methods, potential off-target activities and their theoretical target range. ZFNs have been the most widely used to date but the relative difficulty and high cost of their construction have meant that CRISPRs and TALENs are now gaining the most attention.

CRISPRs

The highly adaptive CRISPR–Cas (clustered regularly interspaced short palindromic repeats–CRISPR-associated proteins) immune system occurs in bacteria and archaea to provide protection against viruses, plasmid DNAs and other disruptive mobile genetic elements (MGEs). Once exposed to a new MGE, spacer sequences derived from the MGE are incorporated into the CRISPR loci, separating a series of repetitive DNA motifs. Transcription leads to small CRISPR RNAs that are displayed on Cas protein complexes to provide RNA-guided targeting and degradation of foreign DNA by Cas nucleases (Bikard and Marraffini 2013).

Hwang et al. have shown that site-specific nucleotide deletions or substitutions in the zebrafish genome can be achieved using RNA-guided Cas9 nuclease (RGN) with germ-line transmission of the mutations reaching up to 100% (Hwang et al. 2013). Auer and co-workers have since demonstrated the use of the CRISPR–Cas9 RGNs to mediate the loci-specific insertion (or 'knock-in') of DNA cassettes (Auer et al. 2014). Using previously established transgenic zebrafish lines, Auer et al. (2014) were able to target GFP reporter sequences and knock-in a KalTA4 sequence (an alternate version of Gal4 (Distel et al. 2009)) resulting in the expression of KalTA4 in formally GFP-positive cells. The accuracy and efficiency of the CRISPR–Cas9 targeting system have made RGNs a promising tool as an alternative to the use of morpholinos for gene silencing as well as for creating transgenic zebrafish reporter lines. CRISPR RGNs are also relatively easy to construct (Joung and Sander 2013).

TALENs

TALENs comprise a non-specific *Fok*I nuclease fused to a customisable DNA-binding domain composed of highly conserved repeats derived from TALEs, proteins which are secreted by *Zanthomonas* spp. bacteria to alter gene transcription in host plant cells (Boch and Bonas 2010, Miller et al. 2010). Huang and co-workers first demonstrated that heritable mutations in zebrafish target genes could be achieved using TALENs, having successfully introduced indel mutations in *tnikb* and *dip2a* genes (expressed in blood cells and vasculature of zebrafish embryos) (Huang et al. 2011). Use of TALENs has also been extended to the knock-in of genes via homologous recombination (Hwang et al. 2014, Zu et al. 2013). Although slightly more complex to construct than CRISPRs, TALENs have a broader targeting range with almost no restrictions in target sequence (Reyon et al. 2012).

The GAL4–UAS transgene expression system

Various manipulated systems have been introduced to improve efficiency, sensitivity, tissue specificity and ease of generation transgenic fish, as described above. A system that has greatly enhanced transgenic models in zebrafish (and *Xenopus*), and is now being employed for studies in ecotoxicology, is the GAL4–UAS system (Hartley et al. 2002, Scheer and Campos-Ortega 1999). This is comprised of a two-part expression system using a yeast (*Saccharomyces cerevisiae*) transcription activator protein GAL4 and its target sequence UAS (Upstream Activated Sequence) (Duffy 2002, Hartley et al. 2002). The GAL4 gene encodes a protein of 881 amino acid and in the yeast is a regulator of the galactose-inducible genes (Johnston and Hopper 1982, Scott et al. 2007). The UAS element is analogous to an enhancer element defined in multicellular eukaryotes, which is essential for the transcriptional activation of GAL4-regulated genes (Brand and Perrimon 1993, Duffy 2002).

The GAL4–UAS system was first introduced into *Drosophila* by Brand and Perrimon (1993) to analyze the function of developmental genes (Brand and Dormand 1995, Brand et al. 1994). GAL4/UAS introduced spatial and temporal control of transgene expression using two transgenic lines, one activator line and one effector line, that were combined (Brand and Perrimon 1993, Fischer et al. 1988). In an activator line, the gene for the yeast transcriptional activator GAL4 is placed under the control of a desired promoter, whereas the effector lines contain DNA-binding motif of GAL4–-UAS linked the gene of interest (Scheer and Campos-Ortega 1999). UAS is fused to an effector gene which is silent if the GAL4 activator is absent. GAL4 can be expressed in many different patterns through placement under the control of various '*Drosophila melanogaster*' tissue-specific promoter sequences (Kramer and Staveley 2003). Many GAL4 lines have been developed and widely used for ectopic expression (the expression of a gene in an abnormal place in an organism) for genes of interest. When the GAL4 activator line and UAS effector line are crossed, the offspring expresses a GAL4-dependent transgene in a tissue-specific manner (Distel et al. 2009).

The GAL4–UAS system was first applied in zebrafish and has been used since the generation of various transgenic zebrafish models including studies into cell type-specific ablation and in the mapping of neuronal circuits and neuronal activity (Asakawa et al. 2008, Davison et al. 2007, Distel et al. 2009, Scott et al. 2007). GAL4FF is an engineered transcriptional activator consisting of the DNA-binding domain from GAL4 fused to a duplicated portion of the VP16 transcriptional activation domain. GAL4FF is reported to be less toxic to zebrafish embryos (Asakawa et al. 2008) and aGAL4FF–UAS system (Figure 5) has been successfully applied for chemical effects studies in ecotoxicology (Lee et al. 2012a).

A drawback in the generation of transgenic lines using the UAS systems is that these sequences can be prone to CpG methylation and thus silencing, especially with high UAS copy number (Goll et al. 2009, Subedi et al. 2013). Attempts

have been made to solve this problem by modifying the UAS to mitigate for silencing (Akitake et al. 2011). Recently, alternative bipartite reporter systems have developed in the zebrafish that are not prone to transcriptional silencing, including the Q transcriptional regulatory system and tryptophan repressor (Subedi et al. 2013, Suli et al. 2014). The Q transcriptional regulatory system, derived from genes in *Neurospora crassa*, is similar to GAL4–UAS system. The transcriptional activator QF binds to the QUAS upstream regulatory sequence and induces the expression of target genes. Transcriptional silencing of Q system does not occur because the QF binding site does not carry essential CpG dinucleotide sequences that are subject to DNA methylation (Subedi et al. 2013).

Recently, the *E. coil* tryptophan repressor was used in stable zebrafish transgenic lines and the tryptophan repressor system showed no transcriptional silencing in subsequent generations of zebrafish (Suli et al. 2014). A regulatory protein called a repressor can bind to the operator site of the tryptophan operon and becomes active only when it is associated with tryptophan. The binding of tryptophan to the tryptophan repressor protein causes a change in conformation in the repressor.

Seeing the response - reporter genes

Reporter genes are used to quantify expression of the transgene and can also be used to rapidly determine success of gene transfer techniques and the tissue location of their expression in the host organism (Alam and Cook 1990). Most reporter genes are placed downstream of the promoter of the inserted transgene. Common reporter genes used in transgenic research include *E. coli* β-galactosidase (lacZ), chloramphenicol acetyltransferase (CAT), neomycin phosphotransferase (NPT), luciferase (Luc), green fluorescent protein (GFP), Kaede, YFP, DsRed and mCherry (Ando et al. 2002, Culp et al. 1991, Dunham et al. 1999, Leclerc et al. 2000, Nagai et al. 2002, Razak et al. 1999, Uzbekova et al. 2003). The most common reporter genes used in transgenic fish research to date are GFP and Luc (Figure 3).

Luciferase (Luc) originates from *Photinuspyralis* (firefly) and encodes the enzyme luciferase and causes the cell that expresses it to catalyze luciferins and produce light (Contag and Bachmann 2002). The luciferase gene is commonly used in cell-base reporter assays because it has high sensitivity. Furthermore, there is a high signal intensity associated with the luciferase molecule which allows for rapid measurements. It is also suitable as a reporter gene for the quantitative measurement of gene expression. The luciferase assay is widely used in pharmaceutical research (drug screening) for high-throughput analysis of gene transcription in living cells because the procedure is simple and requires only a small volume of test material. Luciferase as a method, however, requires a costly substrate and is not stable (luciferase half-life varies from 1.5 to 3 h depending on the host cell and the gene construct) (Goodman and Gao 1999). Furthermore, the image integration time is long to compensate for the low signal intensity (Vooijs et al. 2002, Xiong et al. 1999). Recently, several research groups have shown that luciferase can be detected in live zebrafish embryos, larvae and adults (Chen et al. 2013, Weger et al. 2012).

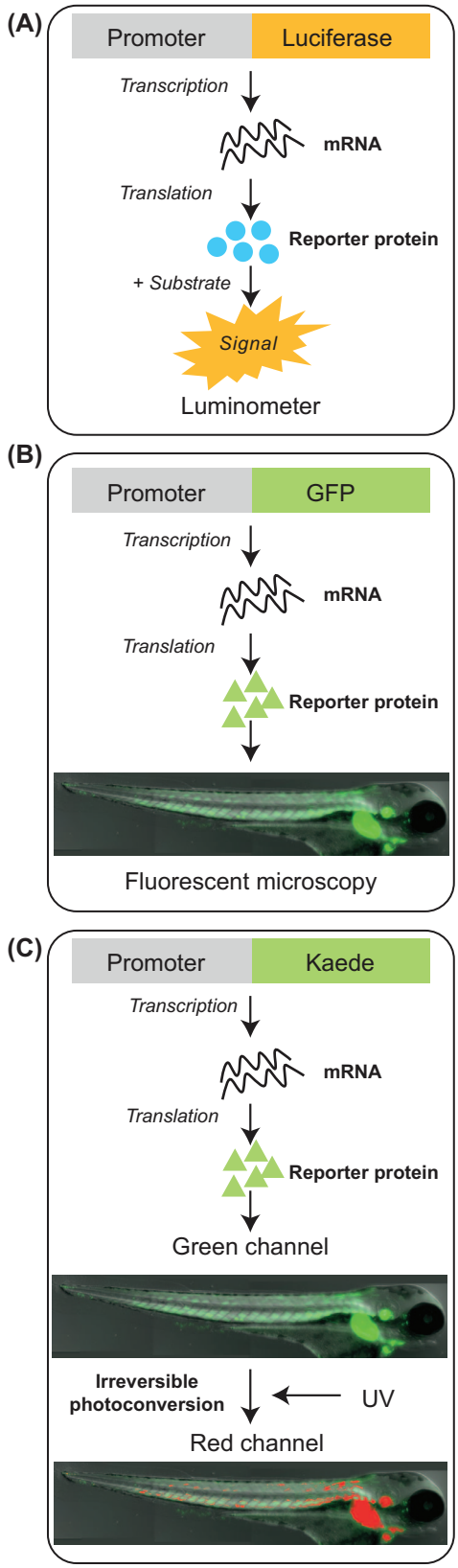

Figure 3. Principles of how reporter genes (luciferase (Luc) (A), green fluorescent protein (GFP) (B) and kaede (C) enable visualizing target gene expression. The reporter genes (Luc, GFP and Kaede) are expressed when DNA is transcribed into messenger RNA (mRNA) and then translated into a reporter protein. In a reporter gene assay using luciferase, a luminescence signal is generated by the reaction of luciferase's substrate, luciferin (A). GFP-expressing cells are detected by fluorescence microscopy (B). Kaede protein, when exposed to UV or violet light, is capable of irreversible photo-conversion from a green to a red fluorescent form (C).

GFP is a fluorescent protein isolated from bioluminescent jellyfish *Aequorea victoria* and can be visualized by microscopy. It can be seen directly in living cells and intact organisms and responses can be measured in real time. Furthermore, when used in conjunction with zebrafish mutant lines that lack skin pigmentation, GFP can be detected in fish for all life stages. Advantages of GFP over luciferase include superior brightness, innate fluorescence and relatively high photostability. GFP was first used in zebrafish in 1996 (Amsterdam et al. 1996) and has since been used widely in the study of gene expression patterns, analysis of tissue-specific promoters/enhancers, tissue/organ development, cell migration and mutagenesis screening (Driever et al. 1996, Gong et al. 2001, Haffter et al. 1996, Udvadia and Linney 2003). GFP has also been applied in the development of biosensor transgenic zebrafish to monitor chemicals such as heavy metals and estrogens (Blechinger et al. 2002, Chen et al. 2010, Gorelick and Halpern 2011, Lee et al. 2012a, Tong et al. 2009). RFP (red fluorescent protein) has the advantage over GFP in that there is less background interference. DsRed, red fluorescent protein from *Discosoma sp.*, can be used together with other GFP variants for multicolor imaging (Dietrich and Maiss 2002). mCherry is the best general-purpose red monomer due to its superior photostability (Shaner et al. 2005).

Kaede is another fluorescent protein applied as a reporter in transgenic animals. The encoding gene for this protein was isolated from the stony coral *Trachyphylia geoffroyi* (Ando et al. 2002). The kaede protein is capable of irreversible photoconversion from green to red fluorescence under UV or violet light illumination (Figure 3). This lends itself, for example, to studies on assessing how stimulation of a gene response subsequently affects its response for a subsequent stimulation of that gene as the events can be separated by the different color patterning in the organism (Ando et al. 2002). Kaede is also a useful tool for sequentially tracking selectively labeled cells in fish embryos (Sato et al. 2006).

Recently, a system has been developed to manipulate further the production of incorporated fluorescent proteins, called kaloop. It involves autoactivation (self-perpetuation) of the fluorescence, and this is being applied as a powerful tool for spatiotemporal genetic fate mapping of specific cell types in zebrafish (Distel et al. 2009).

Transgenic fish and ecotoxicology

Transgenic zebrafish have considerable potential for use in aquatic ecotoxicology as biosensors and as more effective models for informing on health impacts of chemical exposure (Blechinger et al. 2002, Bogers et al. 2006, Gorelick and Halpern 2011, Lee et al. 2012a, Legler et al. 2000). Biosensor fish work on the premise that specific genes, often enzymes or receptors, are inducible by certain chemicals/pollutants. Exposure of the fish to the pollutant of concern, or a natural water containing that pollutant, induces the activation of an inducible promoter that in turn triggers expression of the reporter (e.g. GFP) (Figure 4). Transgenic fish developed to study contaminant and other environmental stressors include for cadmium and copper toxicity via induction of heat-shock protein gene, oxidative stress - through the induction of an electrophile-responsive element (EpRE), for various organic

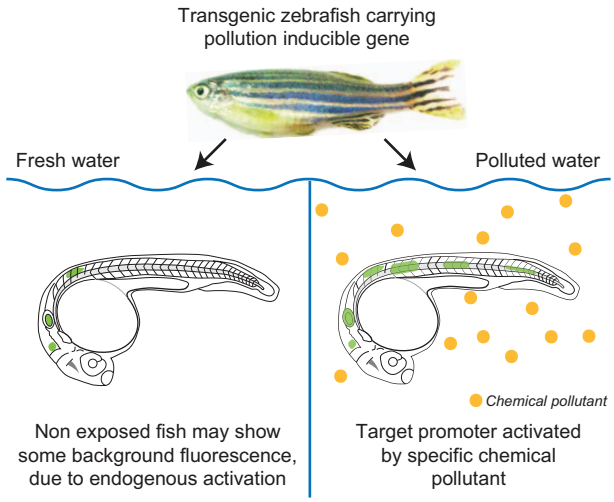

Figure 4. Schematic of a biosensor transgenic zebrafish system for detecting exposure to aquatic pollutants. The transgenic zebrafish containing DNA elements responsive to a toxicant is placed in the exposure water and as the contaminants enter and reach the body tissues, activation of the response elements occurs that induces the production of GFP in that target tissue.

chemicals interacting with the aryl hydrocarbon receptor-mediated toxicity (measured via *cyp1a1*), thyroid and glucocorticoid response pathways, and estrogenicity (*vitellogenin*, *choriogenins*, estrogen receptor-responsive elements) generally employing either luciferase or GFP as reporter genes (Almeida et al. 2010, Blechinger et al. 2002, 2007, Bogers et al. 2006, Chen et al. 2010, Gorelick and Halpern 2011, Ji et al. 2012, Kurauchi et al. 2008, Kurauchi et al. 2005, Kusik et al. 2008, Lee et al. 2012a, Legler et al. 2000, 2002, Mattingly et al. 2001, Petersen et al. 2013, Salam et al. 2008, Tong et al. 2009, Zeng et al. 2005) (See Table 1 for a summary of the different transgenic fish lines developed).

Heat-shock protein

Transgenic biosensor fish have been developed that employ the heat-shock protein (*hsp*) promoters, for 70 (*hsp 70*) and 27 (*hsp 27*) that are readily induced by various environmental stressors including increased temperature and heavy metals. In a transgenic zebrafish created using eGFP (enhanced green fluorescent protein) as the reporter under the control of the *hsp 70* gene promoter, exposure to cadmium (Cd, for 96 h) found a response at exposure concentrations as low as 0.2 µM (22.5 µg/L) (Blechinger et al. 2002). This is a more sensitive response system than for Cd induction of *hsp 70* expression in cultured cells (0.5–50 µM (0.06–5.6 mg/L)) (Ait-Aissa et al. 2000, Braeckman et al. 1999). In the *hsp 70-eGFP* transgenic zebrafish, cadmium treatment induced GFP expression in tissues in a dose-dependent manner with a tissue sensitivity order of gill > skin > olfactory organ > digestive tract > liver > pronephros. Subsequent studies identified responses in olfactory sensory neurons (OSNs) for a brief (3 h) 5 µM Cd (562.1 µg/L) exposure and responses in the lateral line neuromasts after 24 h for a 0.2 µM Cd (22.5 µg/L) exposure (Blechinger et al. 2007). In addition, cell death in the olfactory placode was observed following a 3 h 125 µM Cd (14.1 mg/L) exposure.

DOI 10.3109/10408444.2014.965805

Table 1. Summary of the different transgenic zebrafish and transgenic medaka lines developed for ecotoxicology. The table includes the transgene sequences and insertion techniques used to generate the individual transgenic lines as well as the chemicals used in exposures.

Signaling pathway	Chemicals	Fish	Reporter transgene	Insertion	Vector	Transposon	References
Estrogen Anti-Estrogen	E_1, E_2, EE_2, NP (Nonylphenol) AHTN and HHCB	Zebrafish	3×ERE:Luc	Microinjection	Linearized DNA	None	Bogers et al. (2006), Legler et al. (2000), Legler et al. (2002), Schreurs et al. (2004)
Estrogen	E_2, Environmental water samples	Zebrafish	*5 × ERE-GFP*	Microinjection	Circular plasmid	*Tol2*	Gorelick and Halpern (2011), Gorelick et al. (2014)
Estrogen	EE_2, E_2, BPA (Bisphenol A), NP	Zebrafish	*3 × ERE:Gal4ff - UAS:GFP*	Microinjection	Circular plasmid	*Tol2*	Lee et al. (2012a)
Estrogen + *Vitellogenin*	EE_2, E_2, $CdCl_2$ (Cadmium chloride), zearalenone, E_3, DES, BPA	Zebrafish	*ERE-zvtg1-GFP*	Microinjection	Linearized plasmid	None	Chen et al. (2010)
Vitellogenin	EE_2, E_2 and BPA	Medaka	*Mvtg1-GFP*	Microinjection	Linearized plasmid	None	Zeng et al. (2005)
Choriogenin	E_2	Medaka	*ChgL:GFP-emgb:RFP*	Microinjection	Plasmid	None	Salam et al. (2008), Ueno et al. (2004)
Choriogenin	EE_2, E_2 and E_1	Medaka	*ChgH-GFP*	Microinjection	BAC clone	None	Kurauchi et al. (2008), Kurauchi et al. (2005)
Choriogenin	E_2, EE_2, E_1, BPA and DES	Medaka	*ChgH-RFP*	Microinjection	Linearized plasmid	None	Cho et al. (2013)
42Sp51	EB (estradiol-benzoate)	Medaka	*42Sp50-GFP/RFP*	Microinjection	Circular plasmid	None	Kinoshita et al. (2010)
Cyp19a1b	E_2 More than 30 different estrogenic chemicals	Zebrafish	*Cyp19a1b-GFP*	Microinjection	Linearized plasmid	None	Brion et al. (2012), Tong et al. (2009)
Cyp1a1	Polychlorinated biphenyl (PCB)	Zebrafish	*Cyp1a-GFP*	Microinjection	Circular plasmid	None	Hung et al. (2012)
Cyp1a1	TCDD (2,3,7,8-Tetrachlorodibenzo-p-dioxin)	Zebrafish	*Cyp1a:nls-EGFP*	Microinjection	Fosmid	None	Kim et al. (2013)
Cyp1a2	TCDD, 3-MC (3-methylcholanthrene) and BaP (benzo[a]pyrene)	Medaka	*Cyp1a-GFP*	Microinjection	Circular plasmid	None	Ng and Gong (2013)
TSHβ	T3 (Triiodothyronine) and T4 (Thyroxine)	Zebrafish	*TSHβ:EGFP*	Microinjection	Linearized plasmid	None	Ji et al. (2012)
Hsp70	$CdCl_2$	Zebrafish	*Hsp70:EGFP*	Microinjection	Linearized plasmid	None	Blechinger et al. (2007), Blechinger et al. (2002)
Hsp27	$Na_2HAsO_4 \bullet 7H_2O$ (Sodium arsenate) and $CdCl_2$	Zebrafish	*Hsp27:GFP*	Microinjection	Linearized plasmid	None	Wu et al. (2008)
EpRE	$HgCl_2$	Zebrafish	*EPRE:LUC-GFP*	Microinjection	Linearized plasmid	*I-Sce I* meganuclease	Kusik et al. (2008)
EpRE	$CuSO_4$	Zebrafish	*EPRE:LUC*	Microinjection	Circular plasmid	None	Almeida et al. (2010)
GRE	DEX (Dexamethasone), HC(Hydrocortisone), BM(Betamethasone) and etc.	Zebrafish	*4 × GRE:LUC*	Microinjection	Circular plasmid	*Tol2*	Weger et al. (2012)
GRE	HC Fluticasone propionate	Zebrafish	*6 × GRE:d4EGFP*	Microinjection	Circular plasmid	*Tol2*	Krug et al. (2014)
GRE	DEX	Zebrafish	*9 × GCRE-HSV. U123:EGFP*	Microinjection	Circular plasmid	*Tol2*	Benato et al. (2014)
Osp1	EE_2, E_2, NP	Medaka	*Osp1-EGFP*	Microinjection	Linearized plasmid	None	Yanbin et al. (2014)

Transgenic zebrafish developed with GFP driven by the regulatory region of *hsp 27* (also known as *HspB1*- one of the most widely expressed and distributed small heat-shock protein; Tucker et al. 2009) have also been shown to be responsive to Cd exposure with GFP expression occurring in heart, skin and muscle (Wu et al. 2008) after a 24 h heat-shock treatment. This model (*hsp 27-GFP* transgenic zebrafish), however, was far less sensitive to Cd compared with the *hsp 70-eGFP* transgenic zebrafish (Blechinger et al. 2002), with GFP expression detected in embryos only at high Cd exposure 320 μM Cd (36 mg/L) but not for exposures between 1.35 and 135 μM (0.2–15.2 mg/L).

Cyp1a1 promoter

The promoter of the *cyp1a1* gene has been used to drive a GFP reporter gene for measuring exposure to organic chemicals in both transgenic zebrafish and medaka (Hung et al. 2012, Kim et al. 2013, Ng and Gong 2013). *Cyp1a1* is a cytochrome P450 enzyme involved in the oxidative metabolism of various organic substances including polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). *Cyp1a1* also acts as a marker of the activation of the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor known to mediate the toxic effects of several classes of environmental contaminants including polychlorinated biphenyls (PCBs), polycyclic or halogenated aromatic hydrocarbons (PAHs) and dioxins (e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD) (Mattingly et al. 2001).

Transgenic *Cyp1a-GFP* medaka embryos (72 hpf) exposed to TCDD (for 24 h) showed GFP expression in kidney, liver and gut at an exposure concentration of only 0.005 nM (1.6 ng/L) (Ng and Gong 2013). Other responding tissues at a higher TCDD exposure concentration (0.5 nM (161 ng/L)) included olfactory pits, caudal fin, gills, and neuromast cells. This transgenic medaka was also shown to be highly responsive to other PAHs including 3-methylcholanthrene (3-MC) and benzo[a] pyrene (BaP) with expression in the liver and kidney. A similar transgenic *Cyp1a-GFP* zebrafish was shown to be responsive to PCBs in liver and intestine at exposure concentrations down to 0.04 μg/ml (40 μg/L) (Hung et al. 2012). In the most recently developed transgenic *cyp1a* reporter zebrafish, incorporating eGFP, responses to TCDD were detected in brain vessel, heart, gut, retinal bipolar cells, otic vesicle, lateral line, cloaca and pectoral fin bud down to 1 nM TCDD (322 ng/L) (Kim et al. 2013). The transgenic *cyp1a-eGFP* zebrafish embryos were also responsive to the AhR agonists benzo[a]pyrene (B[a]P), 3-methylcholanthrene (3-MC) and β-naphthoflavone (β-NF) at exposure concentrations of 1 μM (268.4 μg/L) and 10 μM (2.7 mg/L), respectively.

Electrophile-responsive element

Transgenic zebrafish models developed for studying oxidative stress, where chemical modification of DNA, proteins and lipids can alter their biological functions, have incorporated an EpRE, with luciferase and GFP as reporters (Kusik et al. 2008). In the first zebrafish model developed of this kind *EpRE-LUC-GFP*, a Luc-GFP protein fusion protein was used (Kusik et al. 2008). This combination of GFP and luciferase allowed for both visualizing the location of the tissue responses (GFP) and quantifying transgene expression (via luciferase) in the same fish. In the transient assay for this model (embryos injected at the one cell stage with a plasmid pEpRE-LUC-GFP), GFP expression was observed in the skin for exposure to 0.1 μM (27.2 μg/L) mercuric chloride ($HgCl_2$) and in many other cells, including neurons, muscle and the notochord, for exposure to 0.3 μM (81.45 μg/L) $HgCl_2$. In the stable transgenic *EpRE-LUC-GFP* zebrafish line that was created, there was a $HgCl_2$ concentration-dependent induction of luciferase activity in embryos (LOEC 0.2 μM (54.3 μg/L)) but no GFP expression, the reason for which was unclear. In a similar *EpRE* transgenic zebrafish developed with luciferase only as a reporter, embryos were not found to be especially responsive when challenged with copper sulfate limiting their use for environmental studies. There are other responsive elements mediating oxidative stress such as Nrf2 (nuclear factor E2-related factor 2)-antioxidant response element (ARE) and metal response elements (MREs) (Chu et al. 1999, Nguyen et al. 2009), but these have not yet been applied in transgenic fish models.

Endocrine disrupting chemicals

There is increasing concern about the impacts of chemicals in the environment that have ability to disrupt the endocrine system which can result in adverse effects on developmental, reproduction, neurological and/or immune function in both humans and wildlife (Chia et al. 2010, Goodhead and Tyler 2009, Li et al. 2011). These chemicals have been referred to as endocrine disrupting chemicals (EDCs) and they are derived from both natural and anthropogenic sources. Transgenic zebrafish models have been developed for studying the structure or function of thyroid, corticosteroid, androgen and estrogen systems.

A single zebrafish transgenic model has been developed for studying thyroid hormone function by Ji et al. (2012). They generated a transgenic zebrafish with eGFP driven by the TSHβ (glycoprotein hormone - thyrotropin β) promoter. Exposure to thyroid hormones, thyroxine (T4) and tri-iodothyronine (T3) decreased GFP expression in the pituitary gland (20 nM (13 μg/L) for T3, 90 nM (69.8 μg/L) for T4) for a 3-day exposure. Exposure to goitrogens (3.96 mM (548.9 mg/L) $KClO_4$ and 1.3 mM (221.3 mg/L) PTuracil) for 3 days induced activation of TSHβ expression and enhanced GFP expression in the pituitary gland. This transgenic *TSHβ-eGFP* zebrafish could usefully be applied for studies on pituitary TSHβ mRNA expression and thyroid function but not as an effective biosensor for assessing the effects of thyroid disrupting chemicals due to the high levels of GFP expression resulting from endogenous TSHβ.

Transgenic zebrafish lines have been developed for measuring responses to glucocorticoids. Glucocorticoids are important modulators of energy metabolism and a number of studies have showed that some EDCs might be able to stimulate glucocorticoid signaling through effects on the glucocorticoid receptor (GR) or by disturbing glucocorticoid synthesis or function (Atanasov et al. 2005, Sargis et al. 2010). The first transgenic zebrafish model developed for GRs contained four glucocorticoid response elements (GRE) upstream of TATA minimal promoter and luciferase as the reporter (Weger et al. 2012). The *GRE-LUC* transgenic zebrafish larvae were shown

to respond in a concentration-dependent manner to dexamethasone (DEX) treatment (2.5–10 μM (1~3.9 mg/L)) and glucocorticoid signaling was blocked by exposure to the GR antagonist, mifepristone (Weger et al. 2012). Exposure of these transgenic zebrafish larvae to tributyltin (TBT), an antifoulant used to prevented growth of marine organisms, inhibited ligand binding to GR and blocked GR activation. The metabolite of TBT, dibutyltin (DBT) is known to be responsible for this GR interaction. GC signaling in GRE-LUC larvae was inhibited with environmentally relevant exposure concentrations for TBT (20 nM (6.5 μg/L)). Very recently, two further GRE transgenic zebrafish lines have been developed by different research groups (Benato et al. 2014, Krug et al. 2014). One of these models (Krug et al. 2014) contains six GREs and a short half-life green fluorescent protein (6XGRE:d4EGFP). For acute and chronic exogenous glucocorticoid treatment, strong GFP was observed in a variety of tissues, including the brain after exposure to 10 μM (3.6 mg/L) hydrocortisone and 10 μM (5 mg/L) fluticasone propionate (Krug et al. 2014). This model was also shown to be applicable for studies into the stress response with elevated cortisol. In the GRE model developed by Benato et al. (2014), nine GRE tandem repeats were included to drive eGFP expression (Benato et al. 2014). In untreated transgenic zebrafish, there was ubiquitous expression of eGFP in the head, posterior trunk and tail bud at 14 hpf and subsequently in the head and caudal trunk around the yolk sac and its extension at 24 hpf. GFP intensity was then reduced in the head and tail regions at 72 hpf and become more localized in internal organs at 6 dpf. Exposure of 48 hpf embryos to DEX induced a concentration-related response with a LOEC of 100 nM (39.3 μg/L). At an exposure of 10 μM (3.9 mg/L) DEX, eGFP signal was enhanced in pectoral fins, dermal mesenchymal-like cell, muscle fibers, pituitary, pineal gland and blood vessels and blood cells. Reporter activity in this transgenic line was decreased by GR knockdown and RU486 treatment. GFP expression was also observed in several organs in untreated (natural expression) adult males and females. After 10 μM (3.9 mg/L) DEX treatment for 24 h, GFP signal was increased in the brain, liver, intestinal muscosa, kidney, splanchnocranium, spinal cord, eye and skin in both male and female in this transgenic line. This model therefore looks to have good utility for studies into GC functions in both early and adult life.

Various transgenic fish lines have been developed with an estrogen responsible promoter derived from the *vitellogenin* (*vtg*) or *choriogenin* (*chg*) genes. *Vitellogenin* (*vtg*) is an egg yolk precursor protein, normally synthesized in the liver of females, but also inducible in males in response to estrogen exposure, and is the most widely used biomarker for exposure to estrogenic chemicals in aquatic ecotoxicology. *Choriogenins* are egg envelope proteins that are similarly synthesized in the liver of maturing female fish in response to estrogens. Both *vitellogenin* and *choriogenin* respond to estrogens in low ng/L exposure regimes and at sub-nanogramme exposure concentrations for the synthetic estrogen ethinyl estradiol (Thomas-Jones et al. 2003).

Some of the first transgenic models employing promoters for the vitellogenin and *choriogenin* genes with GFP reporters were developed in medaka. These models, however, were not sufficiently sensitive for detecting exposure to estrogens for environmentally relevant exposures. Examples of this include a *vitellogenin*1 (*vtg*1) gene promoter in a transgenic medaka developed by Zeng et al. (2005) that required 500 ng E_2/L (17β-estradiol), 50 ng EE_2/L (17α-ethinylestradiol) or 1 mg BPA/L (bisphenol A) exposures to induce detectable levels of GFP. Similarly, a transgenic medaka developed with gene regulatory elements of *choriogenin H* (*chgH*) detected responses in the liver for exposure concentrations of 0.63 nM for E_2 (171 ng E_2/L), 0.34 nM for EE_2 (100 ng EE_2/L) and 14.8 nM for estrone (E_1, 4 μg E_1/L) after a 24 h exposure (Kurauchi et al. 2008). Kinoshita et al. (2010) applied this model to detect levels of estrogen-like substances in waters in Thailand and Malaysia, but its use in this regard would be for sites extremely heavily polluted with estrogen only. A further transgenic medaka incorporating a *chgH* gene promoter and a red fluorescent protein reporter (RFP) gene was similarly shown to have a relatively low detection sensitivity for estrogens, with responses to 1 μg E_1/L, 200 ng E_2/L, 1 μg DES/L and 10 mg BPA/L, for 7-day exposures (Cho et al. 2013). Although not sufficiently sensitive to detect estrogens that occur for most environments, the species of medaka used was euryhaline allowing for comparative effects analysis on chemicals in aqueous environments of differing salinity. The most sensitive transgenic medaka produced using the *choriogenin L* (*chgL*) gene with a GFP reporter was responsive to 25 ng E_2/L (for a 6-day exposure) (Salam et al. 2008). A single GFP transgenic zebrafish line has been developed with an estrogen-inducible promoter for a *vitellogenin* gene (*vtg1*) (Chen et al. 2010) that was reported to detect GFP in the liver at 0.1 μg E_2/L, 1 μg estriol/L, 1 mg BPA/L, and 10 mg nonylphenol (NP)/L. The lack of appropriate controls in this work, however, makes it difficult to provide a robust evaluation of this model.

The transgenic medaka and zebrafish lines developed incorporating *vitellogenin* and *choriogenin* genes in the reporter systems reported above vary in their responsiveness to estrogen and generally they are limited in the use for environmental monitoring as they lack the required sensitivity. Responses in all of these transgenic medaka and zebrafish for *vtg* and *Chg* genes are also restricted to the liver, the site of *vitellogenin* and *choriogenin* synthesis. They do nevertheless provide systems for screening chemicals for estrogenic activity *in vivo* in real time and can be applied to inform on cumulative responses to estrogens.

Of a slightly different nature, but nevertheless relevant for informing on the feminizing effects of estrogens in fish, transgenic medaka containing the regulatory promoter of the *42Sp50* gene driving GFP or RFP have been developed for detecting testis ova, a condition induced in response to estrogen exposure in males. *42Sp50* is abundantly expressed in oocytes (Kinoshita et al. 2009). In these studies, induction of testis ova occurred for a 2-week exposure to 830 ng estradiol-benzoate/L. Although this model was not sufficiently sensitive for detecting effects of estrogens on germ cell development for environmentally relevant exposures, it has utility for studying the mechanisms controlling oogenesis and germ cell differentiation.

There have been a number of transgenic lines developed in zebrafish for detecting estrogens using estrogen response elements (ERE), which binds the estrogen receptor–ligand complex activating estrogen-responsive genes. The first of these transgenic biosensor zebrafish developed by Legler et al.

(2000) incorporated 3 × EREs inserted upstream of TATA minimal promoter and luciferase as the reporter. Juveniles of this zebrafish transgenic model were responsive to E_2 at concentrations down to 0.1 nM (27.2 ng/L) (for a 96-h exposure) and the testis was the most responsive target tissue. This transgenic zebrafish was subsequently applied to show anti-estrogenic effects of the polycyclic musks (such as 6-acetyl-1,1,2,4,4,7-hexamethyltetraline (AHTN) and 1,2,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-ç-2-benzopyran (HHCB)), commonly used in the fragrance industry) (Schreurs et al. 2004). Limitations of this transgenic model include that measurement of the reporter luciferase required termination of the fish and identifying the specific responding tissues required their individual dissection and analysis. Work using this transgenic model did, however, show that the main target tissues (the liver and gonad) were responsive down to exposures of EE_2 (3 or 10 ng/L) and demonstrated also that responses to estrogenic EDCs differed for different developmental stages (Bogers et al. 2006).

Two subsequent transgenic zebrafish models developed incorporated EREs with GFP. In 2011, Gorelick and Halpern (2011) reported on a transgenic zebrafish containing five tandem consensus EREs upstream of a mouse *c-fos* minimal promoter and the GFP gene. The transgenic zebrafish embryos were exposed to a range of estrogenic chemicals including E_2, DES (diethylstilbestrol), BPA, EE_2 and NP, with responses seen in the heart, brain, liver, aorta and ventral fin for exposure to E_2 (with a LOEC of 100 µg/L), liver and heart for exposure to EE_2 (with a detection down to 10 ng/L). No GFP expression was detected in embryos exposed to NP. Use of this transgenic model indicated that different estrogenic compounds induced tissue-specific differences in their activity. A drawback of this model was the relatively low sensitivity - very high exposure concentrations of estrogens (1–100 µg E_2/L) were employed to induce these responses. However, in their more recent work with this model, they were able to observe tissue-specific GFP expression for exposure to wastewater effluents (Gorelick et al. 2014).

The most recently developed ERE transgenic zebrafish developed by Lee et al. (2012a) is currently the most sensitive transgenic zebrafish system developed for detecting environmental estrogens. This model includes a 3 × tandem ERE and a *Tol2*-mediated GAL4FF–UAS system. The zebrafish embryos and larvae had response sensitivities to the EDCs tested of 1 ng EE_2/L, 5 ng E_2/L, 100 µg BPA/L and 1 µg NP/L. Responses in this model were detected in a wide variety of tissues including the liver, heart, skeletal muscle (somite and cranial), ear/eye ganglions, brain, otic vesicle, lens and neuromasts (Figure 5). Skeletal muscle cells, cranial muscle cells, heart cells and neuromast cells were especially responsive to estrogen. There were tissue-specific expression patterns for the different environmental estrogens indicating differences in tissue toxicities (Lee et al. 2012a).

Life stage differences in responses to estrogens and differences in health effects for different developmental stages most likely relate to the expression patterns of the different estrogen receptor (ER) subtypes. However, it is not yet known how the estrogenic response pattern observed (via GFP expression) relates to the expression of ERs, although this could now be studied in this model using morpholino knockdown or CRISPR

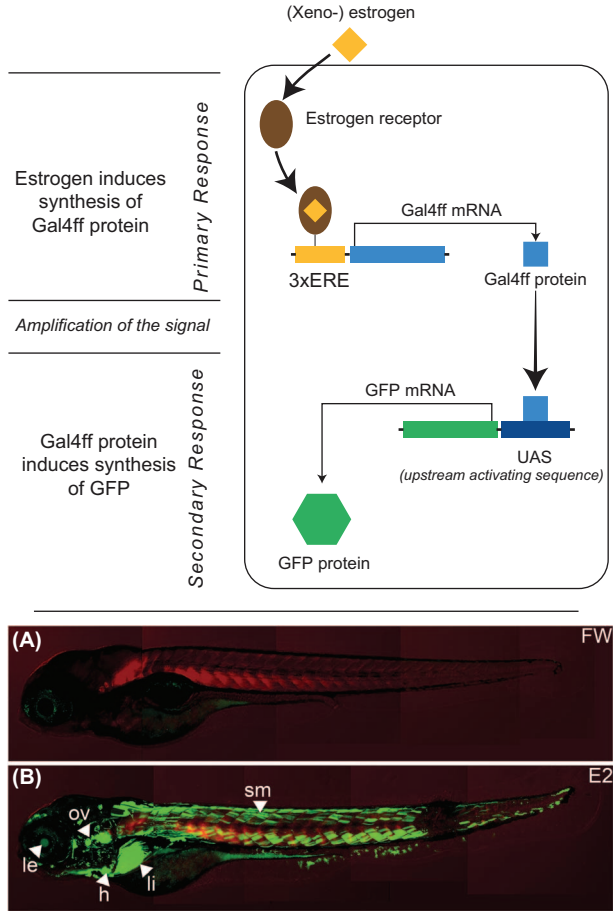

Figure 5. Mechanism of amplification of the estrogenic signal in a GAL4FF–UAS transgenic zebrafish. Within the transgenic fish, estrogen-responsive elements (EREs) respond to the estrogenic signal to drive the first reporter, Gal4ff. Gal4ff binds to UAS in the 2nd transgene to drive the 2nd reporter, a green fluorescent protein (GFP). This two-step reporter system amplifies the signal and enhances the sensitivity of the biosensor. Induction of GFP in *3 × ERE:GAL4FF/UAS:GFP* transgenic zebrafish exposed to estrogen. Four-day-old larvae exposed to clean water alone (control: A) or 17β-estradiol (100 ng E_2/L: B). GFP expression is observed in the cranial muscle (cm), heart (h), lens (le), liver (li), neuromast (n) and somite muscle (sm) by confocal microscopy (Zeiss) with a × 10 objective lens.

of the different estrogen receptor sub-types in organisms and in real time.

Transgenic zebrafish have also been developed to examine the effects of estrogenic chemical exposure on development of the brain using the promoter of a *cyp19* gene. *Cytochrome P450 aromatase* (*cyp19*) is enzyme complex that catalyses the synthesis of estrogens, thereby controlling many different physiological processes of estrogens and is mainly expressed in the gonad (*cyp19ala*) and brain (*cyp19a1b*) (Chiang et al. 2001). These are estrogen-sensitive target genes. Tong et al. (2009) generated a transgenic zebrafish line that expresses GFP under the control of the brain aromatase *cyp19a1b* promoter. In this line, GFP expression occurred in the radial glial cells in response to estrogen and was associated with endogenous aromatase B expression (Tong et al. 2009). Exposure of embryos to a variety of different estrogenic chemical classes including natural and synthetic steroids, alkylphenolic compounds and phyto- and myco-estrogens (for 5 days) induced strong GFP expression in the region between the anterior telecephalon and

caudal hypothalmus and most of these responses were concentration dependent (Brion et al. 2012). The effective concentrations (EC_{50}) for inducing these responses were 0.013 nM (4 ng/L) EE_2, 0.01 nM (2.7 ng/L) DES, 0.48 nM (130.8 ng/L) E_2, 3303 nM (0.8 mg/L) BPA, 2501 nM (0.7 mg/L) genistein. This zebrafish line provides a very useful model for studies into the roles of natural estrogens, and effects of environmental estrogens, on brain development and function. Models using the *cyp19a1a* gene promoter are likely to be forthcoming in the very near future for studies into the effects of estrogens on gonadal development.

Benefits, limitations and future application of transgenic fish in ecotoxicology

Transgenic fish systems provide the advantages of both *in vitro* and *in vivo* systems for testing chemicals. As embryos/larvae they offer as reasonably rapid screening systems with high sensitivity (for some models) and good repeatability. They can also be relatively cheap to use, once the models have been established, and especially so when compared with similar mammalian models. Equally however, there are reasonably high costs, most notably in skilled time, to develop fish transgenic models and then in maintaining the lines. Transgenic fish allow for effective toxicodynamic studies for assessing uptake, distribution and accumulation of chemical ligands/activators in the tissues of live fish and this can be combined with biological effects analyses in a targeted manner. The ability to visualize tissues responsive to chemicals for exposures during early life offers the possibility also to track subsequent effects in those specific tissues in later life. Furthermore, transgenic fish could be usefully applied to study how different chemicals (single chemicals or chemical mixtures) interact within the body to affect different body tissues to assess the effects of sequential exposures and investigate effects of real world mixtures (e.g. effluent discharges) on developmental processes and health. The availability of fluorescent markers such as kaede offer exciting opportunities to help identify responses and effects for sequential exposures to the same, or to different chemicals. Transgenic fish systems also offer the potential for studying detailed effect mechanisms and signaling pathways. As an example, application of morpholinos/CRISPRs could be applied in combination with chemical exposures to ablate specific receptors and to identify roles of receptor subtypes in mediating the toxicant response. The transgenic models published to date are relatively simple and combining different reporter systems in the same animals (and using different fluorescent markers) in the future should allow for multiple toxicological responses in the same animal. As an example, incorporating both the ERE and cyp19 gene promoters that are linked with different fluorescent proteins in the same transgenic fish will more fully inform on the roles and effects of estrogens and estrogen mimicking chemicals in fish. Novel automated systems are now being developed to scan and analyze responses in transgenic zebrafish larvae in medium throughput systems (Chang et al. 2012), and this will enhance further small fish transgenic for chemicals screening and testing. A further major benefit of transgenic fish systems is they could lead to a significant replacement and reduction in animal in chemical testing, as they allow for better effects targeting and analysis across multiple organ systems in individual organisms in real time. Use of transgenic embryos rather than adult fish could also reduce substantially the numbers of fish in chemical testing. Equally however, it has to be recognized that substantial validation of these transgenic models is required before this is possible. Life stage-dependent effects may occur for specific chemicals and thus responses in embryos may not always be representative of responses in later life stages. This in turn will, in the short term, demand use of more intact animals for the validation process. Furthermore, to date there has been little attempt to compare directly responses in transgenic animals with wild type lines, embryos of otherwise, and this warrants further study. Confidence that transgenic fish are truly representative of the more standard lines of fish currently used in chemical testing will be required before they are taken up widely in standardized chemical testing. Indeed, it could be argued that a standardized validation process needs to be established for transgenic lines before they are accepted into standardized regulatory guideline tests for chemicals.

Although transgenic fish have been developed and applied successfully for detecting responses to a range of chemicals, there are also a number of inherent difficulties/limitations of the models. Most of these, however, should be relatively easy to resolve, in the near future with scientific endeavor and the appropriate resources to do so. It is clear from the transgenic fish models reported upon in the previous section that considerable differences occur in their sensitivity and efficiency. Some of these differences in transgene expression may be due to differences in the test chemicals/chemical formulations used from different manufactures in the different laboratories (rarely are details given in this regard). This, however, is unlikely to result in the orders of magnitude differences seen in sensitivity between some experimental models for the same promoter. More likely, these differences are due to differences in the targeting vector, the number of responsive sequences used, promoters adopted, and/or reporter gene and the site of integration. Differences in the exposure duration and fish life stages exposed may also account for some of the differences in sensitivity reported. It is difficult to reconcile these differences for recommending an 'optimized approach' and in fact it is unlikely that one approach will be optimal for all genes of interest. Nevertheless, there are some general features that will help in creating both a stable and responsive transgenic fish. From the published information, *Tol2* transposon and *I-SceI* meganuclease-mediated transgenesis protocols appear to be the more reliable (and stable) systems for creating transgenic biosensor fish as they have been successfully applied to a wide range of molecular sequences and both are suited to microinjection methods. The insertion of multiple response elements, rather than a single element, also appears to result in a more sensitive transgenic model. An area of research that could provide fruitful dividends is the efficacies of the different types (i.e. natural vs synthetic) and the nature of promoter sequences.

Pigmentation in skin has been a major limitation for using juvenile and adult transgenic fish. Embryos and early life stages of zebrafish, and many other fish species, lack significant skin pigmentation and induction of GFP and other fluorescent markers is easily observed even in relative deeply seated tissues. In

juveniles and adults, however, this is not the case and animals have to be dissected to view responses in internal organs. A way to circumvent this problem is to develop the transgenic systems in skin pigment free lines (e.g. *casper*, as one example for zebrafish). It is also the case that for models developed for understanding chemicals interacting with endogenous hormone receptors, the endogenous hormones will affect which life stages and possibly which sex can be usefully applied. As an example for studies into the effects of environmental estrogens, sexually maturing females will contain varying (but significant) levels of endogenous circulating estrogen, making any chemical effects analyses extremely difficult, and in adults such studies may only be practicable with males.

It should be recognized that to produce and maintain stable transgenic fish lines is a considerable undertaking and careful planning is required to ensure valuable lines can be maintained and secured for future studies. Separate lines of transgenic zebrafish should be bred and subsequent generations tested on a regular basis through routine screening of their responses to ensure consistency and also to avoid potential problems associated with inbreeding, such as a reduction of reproductive fitness. Consistency in responses of specific transgenic lines is crucial for any standardized chemical testing. Thus, the development and maintenance of transgenic fish lines are not a trivial exercise and more easily undertaken in large, well-funded laboratories. This being the case, transgenic lines created should be made available, on request, to other research laboratories to maximize their utility and model uptake and independent validation.

In conclusion, transgenic fish (principally zebrafish) have been applied widely in studies in developmental biology and medicine and provided systems for exploring many biological questions. Recent developments show that transgenic zebrafish offer promise as biosensors in ecotoxicology, but to date only a few models have been developed that have both the required specificity and sensitivity for application to understand environmental health effects. Most of the fish models developed, however, have good utility for investigating effect mechanisms of chemicals and in a highly integrative manner. Transgenic fish line may prove particularly beneficial in elucidating adverse outcome pathways. With the technology established for the creation of stable and sensitive transgenic fish for studying chemical effects and in real time there are exciting possibilities for ecotoxicologists.

Acknowledgements

We wish to thank Drs. Ross Brown and Jason Snape (AstraZeneca) and Dr. Tetsuhiro Kudoh (University of Exeter) for their constructive comments on the manuscript.We also thank Susan Rouillard (Drawing Service, University of Exeter) for supporting in the production of the figures.

Declaration of interest

The author's affiliation is as shown on the cover page and the authors have sole responsibility for the writing and content of the paper. Okhyun Lee was funded on a NERC grant (reference NE/I014470/1) and Jon Green on a BBSRC CASE studentship with Astra Zeneca (reference 620033640) and a BBSRC grant (reference BB/L01548X/1). Astra Zeneca was provided with the opportunity to comment on the paper. The literature review conducted, the interpretations made and the conclusions drawn are exclusively those of the authors. None of the authors has any actual or potential competing financial interests.

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