Development of ERE-Transgenic Zebrafish for Studying Health Effects of Environmental Oestrogens

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

..... (Okhyun Lee)

ABSTRACT

It is now well established that there are a wide variety of known EDCs (Endocrine Disrupting Chemicals) in the aquatic environment, which include natural (e.g. oestrogen) and synthetic hormones (e.g. ethinyloestradiol), weak environmental oestrogens (e.g. bisphenol A and nonylphenol) and pesticides, fungicides and herbicides, that are able to alter the physiology of exposed wildlife and humans and have become a focus of increasing concern for human and environmental health over the past two last decades. However, the functional implications and potential significance of these findings has yet to be established. In this thesis, a transgenic fish system approach (using a new plasmid) was employed to investigate the effect pathways of EDCs in fish, using transgenic UAS-GFP zebrafish (*Danio rerio*) as a model species.

One of the most critical steps in this approach is the development of an effective construct for the development of sensitive transgenic zebrafish. The required ERE was cloned and three copies were incorporated into a construct. Three vector systems (pKS+, pCS2+ and pBR322-Tol2) were selected for making new plasmids, and investigated to improve the efficiency for sensitivity and tissue specificity. Three constructs were made and tested progressively in the development process. The plasmids contain three oestrogen response elements (3ERE), a TATA box and GAL4ff. The three constructs, pKS-ERE-Gal4ff, pCS2-ERE-Galf4ff and pBR-Tol2-ERE, were examined by injecting into zebrafish in a transient assay system. The pBR-Tol2-ERE-Gal4ff construct was selected for generating transgenic zebrafish because it showed tissue specific manner (i.e. reduce mosaicism) and *Tol2* transposon has the capacity to improving greatly the chances for generating TG fish such as high germline transmission frequency.

The construct pBR-Tol2-ERE-Gal4ff required to examine the functional capability of using transient expression assay before generating transgenic fish. The Gal4-UAS/GFP method was adopted and produced a two step amplification of the oestrogenic signal. A novel transient expression assay system was developed using a synthetic oestrogen responsive element, the *Tol2* mediated Gal4-UAS systems and the GFP reporter gene, which are responsive to environmental oestrogens using green fluorescent microscopy in zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*). The construct worked well and vector system in transient expression assays proved a rapid, sensitive, tissue specific system for the detection of oestrogenic EDCs in both the zebrafish and medaka, indicating the likely wide suitability for application to other fish species. These data indicate that this system would be a particularly powerful technique for use in species with long generation times and/or where there are other difficulties for generating transgenic lines in those species.

After the transient expression assay, the construct pBR-Tol2-ERE-Gal4ff with transposase mRNA was co-injected into early one cell stage and bred for 3 months. The embryos were collected from the founders (a male and a female) and exposed to EE2 to identify the transgenic zebrafish. An oestrogen responsive transgenic zebrafish (ERE-TG fish) was established under

an oestrogen inducible promoter derived from multiple tandem oestrogen responsive elements. Gal4ff-UAS system was adopted to generate ERE-TG fish to enhance the sensitivity, which has not been used for generating biosensor zebrafish yet. Oestrogen exposures were shown to induce specific GFP expression in the heart, muscle, otic vesicle, brain, neuromasts, eye/ear ganglions and fin, in addition to that in the liver and gonad, which has not been shown previously in other oestrogen responsive transgenic zebrafish, illustrating both the enhanced sensitivity of our detection system and the potential for these tissues as target sites for wider health effects for exposure to environmental oestrogens. Furthermore, oestrogen chemicals are converted to an oestrogenic signal in different tissues preference. Oestrogen receptors (ER α, β1 and β2) morpholinos (MOs) and exposures with oestrogen receptor antagonist were carried out to assess whether the GFP expression was mediated through the ER, regardless of the subtypes. GFP expression was inhibited through injection of the mixture of ERs MOs compared with uninjected transgenic fish larvae. This result indicated that GFP expression was mediated through the ERs. The lowest detectable concentration of EE2, E2, BPA and NP was 1 ngEE2/L, 5 ngE2/L, 100 µgBPA/L and 1 µgNP/L in ERE-TG fish embryos/larvae. These findings provide that ERE-TG fish embryos/larvae systems are useful both for studying physiological mechanisms and for detecting biological target sites of environmental oestrogens.

As a preliminary experiment, immature (40 day old) and mature (3 month old) ERE-TG fish were exposed to low concentrations of EE2 for 7 days to prove its potential for developing tissue-specific models of EDCs at different life stages. The lowest detectable EE2 effect concentration was 5 ngEE2/L in the liver and gonad and 10 ngEE2/L in the muscle in both immature and mature ERE-TG fish. These data demonstrate that muscle is as susceptible as liver and gonad to EE2 and ERE-TG fish (both immature and mature fish) would appear to be a useful system for both detecting target sites for oestrogen chemicals in these life stages fish.

In summary, the work undertaken in this thesis has developed transiently and stably transfected zebrafish as a tool for screening oestrogen chemicals to assess for the potential health impacts of oestrogens in the environment, provided novel insights into many tissue target tissues by oestrogen exposure, and has established different patterns of tissue responses for different environmental oestrogens tested suggesting differing functional implications.

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LIST OF GENERAL FIGURES AND TABLES

LIST OF GENERAL ABBREVIATIONS

AFP	Antifreeze protein
APEs	Alkylphenol polyethoxylates
AR	Androgen receptor
BBP	butylbenzyl phthalate
BPA	Bisphenol A
CMV	cytomegalovirus
DBDs	DNA-binding domains
DBP	Dibutyl phthalates
DES	Diethylstilbestrol
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dpf	Days post fertilisation
ds RFP	*Discosoma* red fluorescent protein
E1	Oestrone
E2	Oestradiol-17β
EE2	Ethinyloestradiol-17α
EDCs	Endocrine disrupting chemicals
EPA	Environmental Protection Agency
GSI	Gonadosomatic index
ER α	Oestrogen receptor α
ER β	Oestrogen receptor β
ERs MOs	Oestrogen receptors morpholinos
ERs	Oestrogen receptors
EREs	Oestrogen response elements
FSH	Follicle Stimulating Hormone
GH	Growth hormone
GtH	Gonadotropin
hpf	Hours post fertilisation
HSI	hepatosomatic index
MCSs	Multiple cloning sites
mM	Millimolar
mRNA	Messenger RNA
nM	Nanomolar
NP	Nonylphenol
OP	Octhylphenol
PAS	Polyaromatic hydrocarbons

PBDEs	Polybrominated dimethylethers
PCBs	Polychlorinated biphenyls
PCR	Polymerase chain reaction
PGCs	Primordial germ cells
Poly [A] tail	Polyadenine tail
RNA	Ribose nucleic acid
Rnase	Ribonuclease
sbERβ	Seabream β
SOC	Super Optimal Catabolite
STWs	Sewage treatment effluents
TBT	Tributyltin
tiGH	Tilapia growth hormone
TSH	Thyroid Stimulating Hormone
UAS	Upstream Activating Sequences
VEPs	Vitelline envelope proteins
VTG	Vitellogenin
w/v	Weight per volume
YES	Yeast oestrogen screen
Zrp	Zona radiata proteins
µM	Micromolar

LATIN NAMES OF SPECIES

African catfish	*Clarias gariepinus*
African clawed frog	*Xenopus laevis*
Atlantic salmon	*Salmo salar*
Coho salmon	*Oncorhynchus kisutch*
Copepod	*Acartia tonsa*
Chironomids	*Chironomus riparius*
Dog-whelk snail	*Nucella lapillus*
Viviparous eelpout	*Zoarces viviparus*
European flounder	*Platichthys flesus*
Fathead minnow	*Pimephales promelas*
Firefly	*Photinus pyralis*
Florida panthers	*Felis concolor coryi*
Fruit fly	*Drosophila melanogaster*
Goldfish	*Carassius auratus*
Jellyfish	*Aequorea victoria*
Leopard frogs	*Rana pipiens*
Medaka	*Oryzias latipes*
Merican leopard frogs	*Rana pipiens*
macrophytic algae	*Cladophora glomerata*
Mosquitofish	*Gambusia holbrooki*
Northern pike	*Esox Iucius*
Ocean pout	*Macrozoarces americanus*
Sand gobies	*Pomatoschistus minutus*
Seabream	*Sparus aurata*
Threespine stickleback	*Gasterosteus aculeatus*
Ramshorn snail	*Marisa cornuarietis*
Rare minnow	*Gobiocypris rarus*
Red eared turtles	*Trachemys scripta*
Roach	*Rutilus rutilus*
Zebrafish	*Danio Rerio*

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1: General introduction

1.1 The endocrine system

The endocrine system is one of the body's main systems in regulating and controlling the physiological mechanisms of reproduction, growth, development and many of the processes of metabolism. The major constituents of the endocrine system are reproductive tissues (i.e. ovaries in females and testes in males), the hypothalamus, pituitary, thyroid, and adrenal glands. Hormones are chemical messengers produced by endocrine cells, usually at very low concentrations, that are then transported throughout the body via the blood, eliciting responses only in target cells. The effects of hormones are slower and more general than those mediated via nerve action. Hormones affect control of long-term changes such as rate of growth, rate of activity and sexual maturity. Hormones bind specifically to target receptor sites on the cell surface (non-steroid hormones) or internal to the cell in the cytoplasm or to nuclear protein receptors (such as for the oestrogen receptor) and induce physiological changes through the release or inhibition of biochemicals, or regulation of genes. Nuclear hormone receptors are ligand-activated regulators effecting transcription of genes by interacting with specific DNA sequences upstream of their target genes (Katzenellenbogen and Katzenellenbogen, 1996).

There are two main classes into which hormones fall (Tsai and O'Malley, 1994): steroids (e.g. cortisol, oestradiol) and non-steroidal (e.g. amines, such as epinephrine and norepinephrine, peptides such as oxytocin, antidiurectic hormone, proteins, such as growth hormone and insulin, glycoproteins, such as Follicle Stimulating Hormone [FSH] and Thyroid Stimulating Hormone [TSH]). In vertebrates, steroids are synthesised from cholesterol in the adrenal cortex, testis, ovary and placenta. Steroid hormones are relatively small and lipid soluble. For this reason, they diffuse freely into and out of cells. Hormone-receptor complexes move into the nucleus where the complex binds to DNA and stimulates the transcription of specific genes (synthesis of mRNA). Peptide and protein hormones do not diffuse into cells and target cells have specific receptors for these hormones on the cell surface. The hormone binds to the receptor and causes a second messenger (i.e. cyclic nucleotide, calcium [Ca] ion) to be released within the cell.

Sex steroids, such as oestrogens, progestins and androgens, regulate gametogenesis, secondary sexual characteristics and have various other effects (Babin *et al*., 2007). They also exert a feedback effect on the hypothalamus and pituitary release of gonadotrophins. This effect can be negative or positive depending on the concentration of hormone needed to meet the physiological and reproductive needs. Oestrogens and androgens are involved in feminine development and masculine development, respectively (Wersinger *et al*., 1997). Progestins are involved in maturation of the mammalian oocyte (Thomas *et al*., 2006). While oestrogen primarily influences the female reproductive tract in development, maturation and function, but, they also play a role in male reproductive function (Brevini *et al*., 2005). There are three major steroid oestrogens; oestradiol-17β, oestrone and oestriol. Oestradiol-17β (E2) is the predominant oestrogen. Oestrogens are mainly produced in the ovaries and the placenta and in small amounts by the adrenal glands and by the male testes.

Much of this thesis work is focused around oestrogens and their biological effects, and thus here some elements relating to the functioning and complexity of the oestrogen receptor (ER) system(s) in vertebrates are now considered.

1.2 Estrogen receptors and their tissues distribution

In mammals, there are two ERs (oestrogen receptors), ERα and ERβ, which each display distinct expression patterns. Tissues that show a high level of expression of ERα include the uterus, mammary gland, placenta, central nervous system, cardiovascular system and bone (Nilsson *et al*., 2001). These tissues respond to E2 with increases in transcription of certain E2-responsive genes. ER β has a broader tissues distribution and is expressed in the prostate, testis, ovary, pineal gland, thyroid gland, parathyroids, adrenals, pancreas, gallbladder, skin, urinary tract, lymphoid, erythroid tissues, certain ERα-lacking brain regions and muscle (Ciocca and Roig, 1995; Gustafsson, 1999). Oestrogen binds to receptors in the nucleus, and the activated ERs as homodimers or heterodimers to oestrogen response elements (EREs) located in the

promoters of target genes (Bjornstrom and Sjoberg, 2005; Nilsson *et al*., 2001). ERα and ERβ bind to EREs (oestrogen responsive elements) with similar affinities (Loven *et al.*, 2001; Yi *et al*., 2002) possibly because they have high amino acid identity (96%) in their DNA-binding domains (DBDs) (Hall *et al.*, 2001; Li *et al.*, 2004; Nilsson and Gustafsson, 2002). Distinct structural characteristics that account for different transcriptional responses to ligands in a promoter- and cell-dependent manner also indicate functional differences between the receptor subtypes (Cowley and Parker, 1999; Hall and McDonnell, 1999; McInerney *et al*., 1998; Paech *et al*., 1997; Yi *et al*., 2002). 17β-oestradiol (E2) binds with similar affinity to both ERα and ERβ (K_a = 0.2 nM for ERα and K_a = 0.6 nM for ERβ) (Kuiper *et al*., 1996; Paech *et al*., 1997).

There have been few reports on ER in skeletal muscle. ERα expression in skeletal muscle however has been shown in several different animal species (mouse, rat, cows and human; Couse *et al*., 1997, Lemoine *et al*., 2002; Lemoine *et al*., 2003; Pfaffl *et al*., 2001). Pfaffl *et al*. (2001) found expression of both ERα and ERβ messenger RNA (mRNA) in skeletal muscle in cows. Similarly both ERα and ERβ receptors have been detected in mouse and pig skeletal muscle, as well as in myoblasts from rat and mouse (Barros *et al*., 2006; Kalbe *et al*., 2007). Research by Mahmoodzadeh *et al*. (2006) reported that ERα is involved in the development of myocardial hypertrophy and heart failure.

Three ER subtypes (ERα, β1 and β2) have been found in teleost fish, while only ERα and β subtypes have been found in mammals (Hawkins *et al*., 2000; Menuet *et al*., 2002). In fish, tissue expression of ERα and ERβ have been shown to differ between species, but generally they appear to be concentrated in the gonad and liver (Filby and Tyler, 2005; Ma *et al*., 2000; Menuet *et al*., 2002; Socorro *et al*., 2000; Tchoudakova *et al*., 1999). These tissue localisations are consistent with the pivotal role of oestrogens in gonadal sex differentiation and development (Devlin and Nagahama, 2002), and in the hepatic production of the egg yolk precursor, vitellogenin (VTG), and vitelline envelope proteins (VEPs) required for oocyte synthesis. This contrasts with ERγ, which in fish is predominantly expressed in somatic tissues (e.g. (Filby and Tyler, 2005)). In fish, research by Socorro *et al.* (2000) found that two receptors (ERα and β) in seabream (*Sparus aurata*) and showed they had different expression patterns. Sea bream ERα

(sbERα) occurred mainly in testis, liver and heart, while sbERβ was found in most tissues, being more abundant in ovary, testis, liver, intestine and kidney. Many studies have reported different ligand binding traits, oestrogen regulation, and expression patterns of the different ER subtypes and their genes in fish (Hawkins *et al*., 2000; Menuet *et al*., 2002; Patino *et al*., 2000; Tingaud-Sequeira *et al*., 2004; Xia *et al*., 1999). In adult, post-spawning roach, both ER mRNAs were detected in brain, gonad and liver, which are consistent with the broad range of tissue localisation and functions of oestrogens described for animals generally, including fish (Couse *et al*., 1997; Kuiper *et al*., 1997; Tchoudakova *et al*., 1999). In muscle only ERβ was detected and only in males. The absence of ERα expression in muscle differs from some other findings in fish (Andersen *et al*., 2001; Choi and Habibi, 2003; Teves *et al*., 2003), but is consistent with that for the fathead minnow (Filby and Tyler, 2005). High levels of ERα expression occurred in the female liver, the site for the oestrogen dependent synthesis of VTG and VEPs. Recent data suggest that ERα has the dominant role in vitellogenesis, whereas ERβ may have no functional role in this process (Menuet *et al*., 2004; Sabo-Attwood *et al*., 2004). Although the liver and ovary are major target tissues for oestradiol, the liver in post spawning female roach has been shown to contain higher ERα levels than in the ovary where ERβ appears to be more highly expressed. Menuet *et al*. (2001) similarly found higher levels of ERα expression in the liver compared with the ovary in pre-vitellogenic female trout. The highest expression level of ERα in both male and female goldfish (*Carassius auratus*) was found in pituitary and ovary and testis were found to have higher transcript levels of ERβ1 (Choi and Habibi, 2003). ERβ2 in goldfish (*Carassius auratus*) is expressed predominantly in pituitary, telencephalon and hypothalamus as well as in liver (Ma *et al*., 2000). ERα in African catfish (*Clarias gariepinus*) been reported to be expressed abundantly in pituitary (Teves *et al*., 2003). In contrast with these finding, tissue distribution pattern of ERα, β1 and β2 in zebrafish showed predominantly expressed in the brain, pituitary, liver and gonad (Menuet *et al*., 2002).

1.3 Endocrine disrupting chemicals

Thousands of chemicals are produced through manufacturing and other activities by man and they can be spread around world by prevailing air and water currents and via animal transportation. There has been increasing concern about the impacts of chemicals in the environment that have ability to disrupt the endocrine system and produce adverse developmental, reproductive, neurological, and immune effects in both humans and wild life (Crisp *et al*., 1998; reviewed in Tyler *et al*., 1998). These chemicals have been referred to as endocrine disrupting chemicals (EDCs), but there are many other terminologies for them, including endocrine modulating substances, endocrine active chemicals etc. (Goodhead and Tyler, 2009). An endocrine-disrupting compound has been defined by the U.S. Environmental Protection Agency (EPA) as *"an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process." or* by the European Commission as *"an endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny or sub population."*

A wide range of chemicals with very different structures have been identified as endocrine disrupters. Some of the chemicals listed as EDCs are illustrated in Table. 1.1 and include: natural steroidal oestrogens (17 β-oestradiol [E2], oestrone [E1], natural phyto-oestrogens (genistein), synthetic steroidal oestrogens (17 α-ethinyloestradiol [EE2], diethylstilbestrol [DES]) and androgens, polyaromatic hydrocarbons (PAS), alkylphenol polyethoxylates (APEs) and their breakdown products (4-tert-nonylphenol [NP], 4-tert-octhylphenol [OP]), dioxins and furans, brominated flame retardants, bisphenol A (BPA) (reviewed in Guillette *et al*. 1996; Vidaeff and Sever, 2005). Figure 1.1 illustrates some the chemical structures of these EDCs and their known modes of action (see next section). EDCs can be found in many products such as contraceptive pills, hormone replacement therapy, detergents, flame retardants, plastic bottles, metal cans, pesticides and cosmetics. Many EDCs are persistent in the environment and are resistant to degradation (see Table 1.2). Even exposure to low levels of many EDCs in the environment can result in high burdens in the body tissues of animals and humans, due to

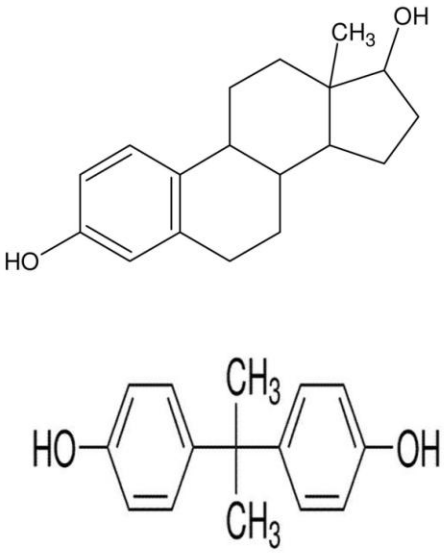
bioconcentration and bioaccumulation. Many EDCs, including alkylphenols, organochlorine pesticides and PCBs are lipophilic and thus become accumulated in fatty tissues. While other EDCs such as BPA and E2 do not bioaccumulate, they are constantly entering the environment and thus wildlife (and humans) are continuously exposed to them. It should be recognised that EDCs also include natural chemicals, as well as synthetic chemicals, for example phyto- and myco-oestrogens. All of these substances may enter the environment via sewage treatment works into rivers and estuaries or from agricultural runoff or landfill leachate. EDCs have been shown to cause harmful effects in wildlife and humans.

During the last 50 years, a number of examples of reproductive and developmental abnormalities in wildlife species have been reported as a consequence of exposure to EDCs (Kirk *et al*., 2002; Tyler *et al*., 1998; Vos *et al*., 2000). Despite the fact that most of the EDCs are less potent than natural steroid hormones, many of them are persistent and exist in environment as complex mixtures which may have additive or other interactive effects (Thorpe *et al*., 2003; Thorpe *et al*., 2001). There has been much debate on the potential risk of EDCs on both wildlife population and to the health of humans, but neither of these issues has been fully resolved.

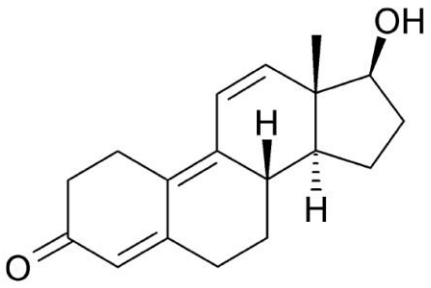
Table 1.1 Examples of endocrine disrupting chemicals (EDCs) found in the environment and their modes of action.

Chemical class	Examples of chemicals within class	Sources and uses	Mode of action
Naturally occurring chemicals			
Steroids - natural oestrogens and androgens	17β-oestradiol (E2), oestrone, oestriol, testosterone,	Released into STW and then discharge into rivers, lakes etc. Derived from human and animal excretion. Sex steroids play important roles in maturation, sex differentiation and reproduction	Oestrogenic, androgenic
Phyto-oestrogens	Geinistien, iosflavones, comumentrol	Human excretion, natural decay of plant material, pulp mill effluent. Enter soil and water via food processing and/or decomposition	Oestrogenic, anti-oestrogenic
Man-made chemicals			
Steroids-synthetic oestrogens and androgens	Ethinyloestradiol (EE2), dithylstilbesterol (DES), zeranol, trenbolone,	Human use, pharmaceutical products (i.e. contraceptive pill, hormone replacement therapies) or used in livestock farming. Detected in watercourses (i.e. rivers, lakes, sea etc.), mainly human and animal excretion	Oestrogenic or androgenic (trenbolone)
Agriculturally and Industrial chemicals			
Organo chlorinated pesticides	DDT, chlordane, endosulfan, kepone, atrazine, malathion	Agricultural use (i.e. insecticide). Many are banned, but still exist in the environment because of their persistence and tendancy to bioaccumulate	Oestrogenic, anti-oestrogenic, and anti-androgenic
Metals /Organometals	Cadmium, mercury, lead, tributyltin (TBT), triphenyltin	Atmospheric deposition, industrial discharge, Anti-fouling agents used on sailboat and ships to remove barnacles from the hull.	Oestrogenic, anti-oestrogenic
Alkylphenols	Nonylphenol (NP), octylphenol (OP), bisphenol A (BPA)	Industrial waste, pulp mill effluent, agricultural/forest management use. Enter landfills and soil and then end up in watercourses	Oestrogenic
Phthalates	Dibutyl phthalates (DBP), butylbenzyl phthalate (BBP)	Home use, personal care products, Industrial waste, landfills	Oestrogenic
Polyhalogenated organic compounds	Polychlorinated biphenyls (PBC), dioxins, TCDD	Many are banned, but are still present in the environment because of their persistence/ bioaccumulation	Anti-oestrogenic
Thyroid disrupting chemicals	benzophenone 2, dioxin, PCB polybrominated dimethylethers (PBDEs), polyphenols,	Industrial chemicals, electric equipment, natural substances, sewage treatment works	Negative effect on thyroid function

Oestrogens
e.g.17β-oestradiol
Bisphenol A



Androgens
e.g.Trenbolone



Thyroid hormone
e.g. PBDEs

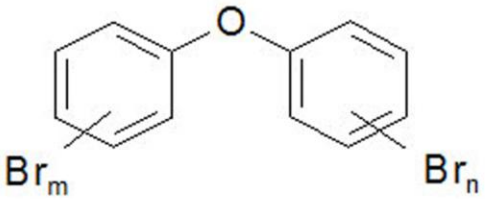


Figure 1.1 Chemical structures of some of the major classes of vertebrate hormones and some of their mimics. Examples shown are for oestrogens, androgens and thyroids hormones. The chemicals structures of oestradiol, testosterone and a thyroid hormone (T3 or T4) and then next to them some of their mimics (have more than one mimic for each class of these three hormones).

1.3.1 Mechanisms of endocrine disruption

EDCs can interfere with endocrine systems in a variety of ways. As a consequence, they may alter hormonal levels and functioning in the body, which in turn, may lead to adverse effects on health. Soto *et al*. (1995) reported that chemicals that can bind and activate the oestrogen receptor can have additive effects, so the effects of small quantities of a range of oestrogenic chemicals can add together into a much larger effect. Some EDCs are structurally similar to their endogenous hormone counterparts, and although they can bind to receptors they do not activate them, instead acting as hormone receptor antagonists. Examples of chemicals that can block the male sex hormone, the androgen receptor (AR), include the pesticides (i.e. vinclozolin and DDE - the latter is a breakdown product of DDT (Gray *et al*., 1994; Kelce *et al*., 1994; Kelce *et al*., 1995). Some EDCs are able to modify the metabolism of natural hormones. Chemicals such as lindane and atrazine are able to affect the metabolic pathway of oestradiol (Toppari *et al*., 1996). EDCs can modify the number of hormone receptors in a cell. Chemicals can affect natural hormone production by interfering in other signalling systems such as thyroid system, the immune and nervous systems (Figure 1.2). It is also the case that some EDCs can interfere with more than one hormonal system, causing multiple responses. The functioning of the endocrine system is very complex. For example, nonylphenol (NP) is acting as an ER agonist, a weak AR agonist and altering gonadotropin (GtH) synthesis and secretion (Harris *et al*., 2001; Sohoni and Sumpter, 1998). EDC can operate without acting through receptor binding, such as by interfering with receptor protein synthesis, by affecting the synthesis, metabolism, transport (on binding proteins), or excretion of hormones, by interfering with endocrine feedback mechanisms, or by damaging the endocrine organs directly (Matthiessen and Sumpter, 1998; Tabb and Blumberg, 2006).

There are a wide range of mechanisms of action for EDCs, as detailed above and a very wider range of chemicals that show EDC activity. The most common EDCs found in the environment are oestrogenic (McLachlan and Arnold, 1996) and feminisation of male fish has been reported in various wildlife species (Gross-Sorokin *et al*., 2006; Lange *et al*., 2008; Tyler *et al*., 2005). Intersex (the simultaneous presence of both males and female sex cells within a single gonad) is

the most researched case on the feminisation of wildlife. Reproductive success and possibly fish population stability are affected by the occurrence of intersex individuals. Induction of the aromatase enzyme can result in feminisation of males, as androgens are converted to oestrogens (Hayes *et al*., 2002; Sanderson *et al*., 2001; Sanderson *et al*., 2000). Aromatase inhibitors, such as fadrozole, can reduce the production of oestrogens form androgens which many result in masculinisation (Afonso *et al*., 1999; Fenske and Segner, 2004; Kwon *et al*., 2002). Natural and synthetic chemicals, including certain xeno-oestrogens, phyto-oestrogens, pesticides, and organotin compounds, are able to inhibit aromatase activity, both in mammals and fish (reviewed in Kazeto *et al*., 2004 and Cheshenko *et al*., 2008). Some EDCs such as bisphenol A, *o,p'*-DDT, and butyl benzylphthalate, have been shown to possess both oestrogenic and antiandrogenic activity, and can act both as an agonist at the oestrogen and antagonist at the androgen receptor (Sohoni and Sumpter, 1998).

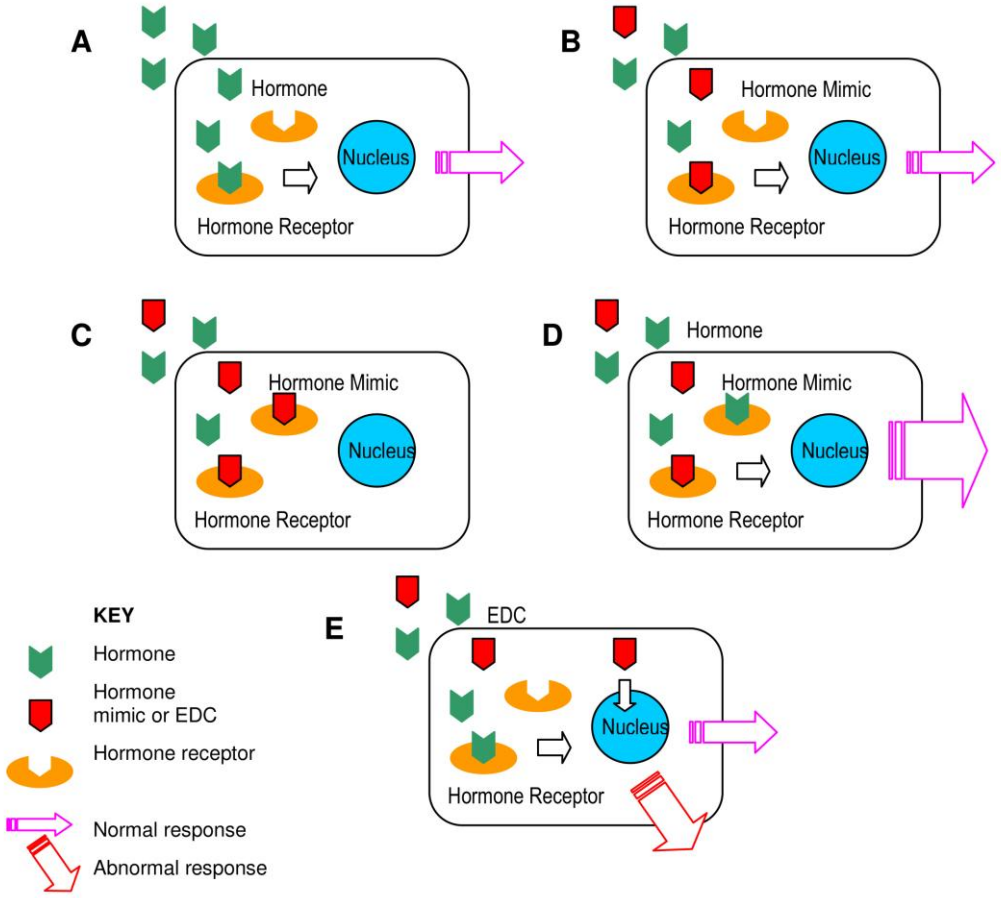


Figure 1.2 Effect pathways for Endocrine Disrupting Chemicals (EDCs). A: Normal hormonal and cellular response, B: Hormone mimic (EDC) eliciting an agonistic response, C: Receptor and EDC complex inhibiting a normal response (antagonistic response), D: Hormone and hormone mimic displaying a synergistic response, E: EDC eliciting an abnormal cellular response independent from receptor binding mechanism.

Although there are many chemicals with endocrine disrupting activity, this thesis work focuses on environmental oestrogens and studies 3 chemicals (or chemical classes) only, and thus detailed information on these only is now provided, specifically for steroid oestrogens (natural and synthetic), nonylphenol, and bisphenol A.

1.3.2 Steroid oestrogens

One of the major classes of EDCs that have been of increasing concern is the steroid oestrogens. Steroid oestrogens are female sex hormones that are synthesised predominantly in the ovary. However, they also can be found in the brain, testis and adipose tissue (O'Donnell *et al*., 2001). In addition to their reproductive roles, oestrogens affect the growth, differentiation and diverse target tissues throughout the body and are involved in numerous other physiological process in both male and female vertebrates, including bone metabolism (Manolagas, 2000), behaviour, the immune system, and the cardiovascular system (Harris and Bird, 2000; reviewed in Sharpe 1998).

Although E2 is the most potent and dominant oestrogen in humans lower levels of the oestrogens, oestrone and oestriol are also present. E2 is the major sex steroid and has an important role in inducing and maintaining ovarian development. In fish, a crucial role of E2 is in the liver, where it stimulates the production of VTG which is then transported by the blood to the gonads and taken up by the developing oocytes. Consistent with its role in ovarian development, plasma levels of E2 correlate well with the stage of ovarian development in synchronous fish species, increasing in the pre-vitellogenic phase and reaching a peak in the vitellogein phase (Rinchard and Kestemont, 1996; Singh and Singh, 1987). It has been reported that oestrogens also play a critical role at multiple levels of testicular function and in male fertility (Couse *et al*., 2001; Hess *et al*., 1997; O'Donnell *et al*., 2001). Furthermore, oestrogens are known to be regulators of cellular growth and differentiation in a variety of different target tissues, and are involved in many other physiological processes, such as behaviour, immune system, bone growth and mineralisation, fat deposition and cardiovascular system (Harris and

Bird, 2000; reviewed in Sharpe, 1998). Major oestrogenic contaminants of aquatic systems are natural steroids derived from humans and livestock. Sex steroids are mainly excreted as glucoronate or sulphate conjugates, and are thus inactive upon excretion. However, they are rapidly de-conjugated in the environment by bacteria, rendering them active again (Lintelmann *et al*., 2003; Panter *et al*., 1999; Ternes *et al*., 1999). Excretion rates of sex steroids depend on age, gender, reproductive state, diet and pregnancy (Reviewed in Lintelmann *et al*., 2003).

E2 concentrations have been reported at concentrations of between 1 and 88 ng/L in British sewage treatment effluents (STWs; Desbrow *et al*., 1998; Rodgers-Gray *et al*., 2001). Other natural steroidal oestrogens that have been detected in the environment include 17α-oestradiol and oestriol (Belfroid *et al*., 1999; Kolpin *et al*., 2002; Yin *et al*., 2002). However, these oestrogens have weaker oestrogenic potencies than E2 (Perusquia and Navarrete, 2005; Salste *et al*., 2007). While EE2 is the most potent oestrogen found in STW effluents, the most common steroid oestrogen found in the STW effluents is E1 (Katsu *et al*., 2007; Rodgers-Gray *et al*., 2001). Moreover, controlled exposure studies with both steroidal oestrogens and oestrogenic STW effluents have shown that environmentally relevant concentrations are sufficient to induce the abnormal levels of VTG synthesis (Routledge *et al*., 1998; Snyder *et al*., 2001; Thorpe *et al*., 2001) induce adverse effects on gonadal development and differentiation (Liney *et al*., 2005; Rodgers-Gray *et al*., 2001; van Aerle *et al*., 2002), reduce fecundity (Lange *et al*., 2001; Nash *et al*., 2004; Thorpe *et al*., 2009).

EE2 (17α-ethinyloestradiol) entering the environment is derived from pharmaceutical and veterinary products, for example being widely used in the contraceptive pill. Many studies have evaluated the oestrogenic activity of EE2 in both *in vitro* and *in vivo* assays. It has been reported that EE2 is 1.25 fold higher potency than E2 in yeast-based *in vitro* assays (Beck *et al*., 2006). In MVLN-assay (transformed MCF-7 human breast cancer cell line) (Van den Belt *et al*., 2004) EE2 was shown to be 1.6 times more potent and E1 about five times less potent than E2. For *in vivo* assays, EE2 has a greater relative potency with around a 30 times higher potency than E2 and E1 for induction of VTG in female zebrafish (Van den Belt *et al*., 2004). EE2 had been detected in surface water in concentrations ranging from below detection limits of 0.01 ng/L in

the river Rhine in Germany (Hintemann *et al*., 2006) to levels as high as 73 ng/L in U.S stream (Kolpin *et al*., 2002), but this latter figure is highly questionable. The high oestrogenic potencies of natural and especially synthetic steroid oestrogens, are sufficient to explain the phenomena of vitellogenin induction observed in male trout placed in cages downstream of sewage treatment effluents in UK rivers (Harries *et al*., 1997). The activated sludge treatment process is capable of removing over 85% of the steroid oestrogens, oestradiol, oestriol and ethinyloestradiol, but ethinyloestradiol might accumulate in sludge because of its recalcitrance and hydrophobicity (reviewed in Johnson and Sumpter, 2001).

1.3.3 Nonylphenol

Nonylphenol (NP) belongs to alkylphenols that are used mainly in the production of cleaning products, such as detergents, but also in many other products such as pesticides. Alkylphenols are mainly used to make alkyphenol ethoxylate (APE) surfactants (detergents) and these have been used for over 40 years in the manufacture of plastics, elastomers, agricultural chemicals, pulping and industrial detergent formulations. Alkylphenols are made of an alkyl group which can vary in size, branching and position joined to a phenolic ring. Alkylphenols have been found in the tissues of animals as well as in water and soil (Ahel *et al*., 1993; Bennett and Metcalfe, 1998; Bennie *et al*., 1997). Nonylphenols are long-lasting in the environment and can accumulate in the tissues of exposed organisms. NP enters rivers and estuaries via sewage effluent and spread onto agricultural land in sewage sludge. It has been reported that nonylphenol is detected at concentrations in some effluents sufficient to cause significant effects on reproductive development and function in fish (Blackburn and Waldock, 1995; Sheahan *et al*., 2002; Sole *et al*., 2000). Concentrations in UK rivers and sewage effluents have been reported in the range <0.2−12 μg/L (Blackburn and Waldock, 1995). The mean measured concentration of NP in sample taken from the final effluent of the Keighley STW in the UK was 63 μg/L (Sheahan *et al*., 2002). Concerns have focused on the potential for NP to contribute to feminisation in wildlife as well as being a potential factor in the increasing incidence of

reproductive organ disorder and decreasing sperm counts (Bian *et al*., 2010; Christiansen *et al*., 1998; Harries *et al*., 1997; Seki *et al*., 2003). NP has a bioconcentration factor of between 13 and 410, dependent on the species, with macrophytic algae, such as *Cladophora glomerata*, particularly able to bioconcentrate NP (Ahel *et al*., 1993). NP is considered to be an environmental oestrogen (xeno-oestrogen) and is also resistant to biodegradation. Soto *et al.* (1991) and White *et al.* (1994) showed that alkylphenols exert their oestrogen effect by binding to the ER; however, it was shown that NP binds also the androgen receptor in an agonistic manner, although only very weakly (Sohoni and Sumpter, 1998). However, it was only in 1991 that publication of the effects of nonylphenol on cultured human breast cells led to human health concerns (Soto *et al*., 1991). Oestrogenic effects have also been shown in rainbow trout hepatocytes, chicken embryo fibroblasts and a mouse oestrogen receptor (Sumpter and Jobling, 1993; White *et al*., 1994). Oestrogenic effects occur at tissue concentrations of 0.1 µM for octylphenol (OP) and 1 µM for nonylphenol (Soto *et al*., 1995). A recombinant yeast screen using the human oestrogen receptor has shown similar results (Routledge and Sumpter, 1996).

In a study on the partitioning of NP is the body of fish, most was found in bile and faeces after 144 h, but some was also found in the muscle (Coldham *et al*., 1998). NP has been shown to affect the first generation in medaka (*Oryzias latipes*) after parental exposure, reducing the embryo survival and development of sex characteristics at an exposure concentration of 17.7 µg/L (Yokota *et al*., 2001). Recent research found severe kidney lesions in NP-exposed (20 µg/L) fish and elevated VTG induction at exposures down to 5 µg/L; male rare minnow (*Gobiocypris rarus*) (Zha *et al*., 2008).

1.3.4 Bisphenol A

Bisphenol A (Figure 1.1) belongs to the phenol class of aromatic organic compounds and is composed of two unsaturated phenol rings. Bisphenol A (BPA) is one of the most important chemicals worldwide and is mainly used in the manufacture of polycarbonate plastic, epoxy resins (used to line metal food and drink cans), and production of polyester resins.

BPA is considered to be an environmental oestrogen able of causing hormonal dysfunction in body systems. Human exposure to BAP is primarily through ingestion (i.e. BPA can move from polycarbonate plastic bottles or food storage containers into food when the container is heated to high temperatures and some dental sealants can also release BPA). Ingested BPA is rapidly metabolised into water soluble and oestrogen inactive (non-hormonally active) compounds. BPA has an estimated half-live for biodegradation below 5 days in surface waters (Klecka *et al*., 2001). In mice, BPA exposure caused disruptions in puberty onset, regularity of oestrous cyclicity (Markey *et al*., 2002), and development of polycystic ovaries (Kato *et al*., 2003). Despite the fact that BPA binds to both ERα and β with low affinity (Gaido *et al*., 1997), BPA competes with [^{3}H]-oestradiol for binding to the ER from the rat uterus, induces the expression of progesterone receptors, and promotes cell proliferation in cultured human mammary cancer cells (MCF-7) (Krishnan *et al*., 1993; Lee *et al*., 2003). It has been reported that BPA has a higher affinity for ERβ in target cells; the binding affinity relative to E2 for BPA at ERβ was 6.6-fold higher than at ERα (0.33) (Kuiper *et al*., 1997; reviewed in Wetherill *et al*., 2007).

It has demonstrated that BPA binds to oestrogen receptor with an affinity approximately 1:2000 lower than that of 17β-oestradiol (E2) in oestrogen-sensitive MCF-7 cells (Gaido *et al*., 1997). Furthermore, it has been reported that BPA is 15,000 fold less active than E2 in yeast-based *in vitro* assays (Gaido *et al*., 1997). BPA has been shown to induce cell proliferation in MCF-7 cells (Brotons *et al*., 1995; Soto *et al*., 1995), and upregulate the expression of vitellogenin RNA in primary hepatocytes from the male *Xenopus laevis* (Kuiper *et al*., 1998). In addition, *Xenopus laevis* showed feminisation of sexual differentiation for an exposure dose of 10^{-7} mol/L BPA (Kloas *et al*., 1999). Melzer *et al*. (2010) found that higher BPA exposure was associated with heart disease, diabetes and abnormal liver enzymes in humans.

1.4 The evidence for endocrine disruption in wildlife

There has been an explosion in the number of scientific papers that have given rise to concern with regards to effects of EDCs to wildlife. Many studies have confirmed the links between

EDCs exposure during critical development life periods and subsequent health problems. For males, most of the health effects of EDCs have been poor semen quality (low sperm counts, low ejaculate volume, high number of abnormal sperm), increased testicular cancer and other male reproductive tract disorders (hypospadias) and in females (humans) increased breast cancer (Carlsen *et al*., 1992; Jacobson and Jacobson, 1996; Moller, 1998; Paulozzi, 1999; Swan, 2003; Warner *et al*., 2002).

In wildlife populations numerous effects of EDCs have been reported. Bryan *et al*. (1987) reported that populations of the dog-whelk snail (*Nucella lapillus*) were diminishing around the UK coast because of the effects of the tributyltin (TBT), a compound used in antifouling paints (to prevent colonisation of animals on the hulls of ships and in harbours). Furthermore, Gibbs *et al*., (1987) found that female dog whelks showed male characteristics (e.g. imposex) when exposed to TBT exposure. There is extensive evidence for effects of EDCs in wild/feral fish populations, and these effects are described later in this introduction. Populations of frogs, toads and salamanders have declined dramatically worldwide (EPA, 1997) and some of these effects have been linked with exposure to EDCs. Examples include feminisation of male American leopard frogs (*Rana pipiens*) through exposure to atrazine, a common herbicide, across the Midwest corn growing belt of North America. In reptiles, demasculinisation of male alligators contaminated with high levels of DDT, DDE, dicofol and related compounds has been reported in Florida (Guillette *et al*., 1994). Red eared turtles (*Trachemys scripta*) too in Lake Apopka were also observed to be demasculated in (Gross and Guillette, 1994). Further examples of effects associated with EDC exposure and effects in wildlife include demasculinisation and feminisation of male Florida panthers (*Felis concolor coryi*) in Southern Florida as a result of their contamination with a variety of chemical compounds including mercury, p,p'-DDE, and polychlorinated biphenyls (Facemire *et al*., 1995). There have also been numerous studies on the adverse effects associated with agricultural and industrial waste chemicals, including EDCs, on wild bird populations. Eggshell thinning and teratogenesis have been two major issues for chemical associated effects in wild birds (Kannan *et al*., 1998; Sheffield *et al*., 1998; Stephens *et al*., 2000). Fry *et al*. (1995) found that gulls were sensitive to the feminising effects of DDT.

Evidence for the ability of chemicals to induce endocrine disruption can be found from many laboratory studies with exposures conducted on single or simple mixtures of EDCs, including at environmentally relevant concentrations. The studies have been conducted a variety of taxonomic groups, including invertebrates, fish, amphibians, reptiles, birds and mammals (Coe *et al*., 2010; Fry, 1995; Hayes *et al*., 2006; Jobling and Tyler, 2003; Lange *et al*., 2008; Oehlmann *et al*., 2000; Solomon, 1998; Vos *et al*., 2000).

Several laboratory studies have also demonstrated effects of EDCs on invertebrates. Watts *et al*. (2003) found that deformities in the mouthparts of chironomids (*Chironomus riparius*) for exposures to 10 ngEE2/L. Moulting was also delayed and larval wet weight significantly reduced at the highest concentration (1 mg/L, for BPA also), but these exposure levels do not reflect environmental relevance conditions. Naupliar development of the copepod (*Acartia tonsa*) was delayed after exposure to EE2, NP and BPA exposure, at concentrations of 88 µg/L, 150 µg/L and 550 µg/L, respectively (Andersen *et al*., 2001). Again, however these exposures bear little environmental relevance. Furthermore, Oehlmann *et al*. (2000) found that two species of snail, the ramshorn snail (*Marisa cornuarietis*) and the dogwhelk (*Nucella lapillus*) were sensitive to BPA and OP (octylphenol). The female genital system was malformed while spawning mass and egg production were increased (5 µg/L, 25 µg/L and 100 µg/L) and they also showed increase in the frequency of imposex (100 µg/L).

1.4.1 Evidence for endocrine disruption in human health

A large number of studies have linked exposure to DDT with breast cancer (Colborn *et al*., 1993; Dewailly *et al*., 1994). Many studies have further reported reduced sperm counts and declining sperm quality in men due to exposure to DDT and other endocrine EDCs (Sharpe and Skakkebaek, 1993; Zinaman and Katz, 1997).

The ability of chemicals to have adverse effects on the reproductive system is highlighted by the case studies on the effects of diethylstilbestrol (DES), a synthetic oestrogen, prescribed to many women to prevent miscarriages in the 1950s and 1960s. This leads to adverse health

effects including birth defects in the uterus and ovaries and immune suppression in the offspring. Boys showed genital tract abnormalities and un-descended testes and girls had an unusual form of vaginal cancer. It is the case that many EDCs can be ingested by pregnant women and passed to the foetus and thus induced harmful effects in children (Kanja *et al*., 1992; Koopman-Esseboom *et al*., 1994; Lin *et al*., 2010; Rice, 1995). As an example, studies have shown that boys have slightly shorter penises at age 11-14 when their mothers were exposed to PCB-contaminated rice oil during their pregnancy in 1978-1979 (Guo *et al*., 1994; Guo *et al*., 1995).

EDCs have in fact been shown to effects on a wide range of physiological targets in animal models and humans, in both male and females, including on breast development and breast cancer, prostate cancer, neuroendocrinology, thyroid function, metabolism and obesity, and cardiovascular function. Reviews of the evidence for endocrine disruption in animal models and human clinical observations can be found in Toppari and Skakkebaek (1998), Safe (2000), Toppari (2002), Diamanti-Kandarakis *et al*. (2009). A recent study reported that higher urinary levels of the weak environmental oestrogen, bisphenol A (BPA) were associated with a higher risk of heart disease, diabetes and elevated liver enzymes in a human population (Melzer *et al*., 2010).

1.4.2 Endocrine disruption in fish

A number of studies have reported endocrine disruption in wild fish populations and reproductive effect in different fish species exposure to EDCs in the laboratory. Many examples of disruption have been associated with fish, likely because the aquatic environment receives most of the pollutants, natural or man-made, via STW effluents, industrial discharges or agricultural runoff (Sumpter, 1998). Fish are thus especially vulnerable to EDC exposure. Furthermore, fish can uptake EDCs from the water through several routes, including via skin and gills, via the diet, or via maternal transfer of EDCs that have accumulated in lipid reserves during ovarian development (reviewed in Jobling and Tyler, 2003).

Evidence for EDCs in wild fish populations has been found at many sites around the world. Examples include the European flounder (*Platichthys flesus*), roach (*Rutilus rutilus*) and eelpout (*Zoarces viviparus*). Wild European flounder caught from estuaries of the Mersey and Tyne, UK, showed altered spermatogenesis (Lye *et al*., 1997) and ovotestis (Allen *et al*., 1999; Matthiessen and Sumpter, 1998; Minier *et al*., 2000). In the eelpout (*Zoarces viviparus*) a relatively high prevalence of intersex has been reported in the coastal waters of north-eastern Germany (Gercken and Sordyl, 2010). A further study on endocrine disruption in marine fish has found that morphological abnormalities in the secondary sexual characteristics of male sand gobies (*Pomatoschistus minutus*) were much more frequent at sites receiving higher concentrations of STW effluents. Intersex was found to correlate with altered sexual behaviour in the mosquitofish (*Gambusia holbrooki*) where females at an androgen contaminated site displayed masculinised sexual behaviour and males were more aggressive and dominant than fish from a non-contaminated site (Howell *et al*., 1980).

The most detailed studies on effects of EDCs (oestrogens) in fish however come from studies on roach (*Rutilus rutilus*) in UK Rivers. In the history of these findings, concerns were first raised by the report of intersex in roach (*Rutilus rutilus*) living downstream from STW effluent discharges and this prompted further study by the Thames Water Authority (Larsson *et al*., 1999; Sweeting, 1981). Wild populations of roach caught downstream (the River Lea) from a STW effluent discharge showed a 5 % incidence of intersex (hermaphroditism; the simultaneous presence of both male and female germ cells). These findings were subsequently confirmed on a much wider scales in UK Rivers (Jobling *et al*., 2002a). The evidence that these feminised responses (VTG induction, feminised reproductive ducts and oocytes in the testes) have resulted from exposure to STW effluents has since been proven through controlled exposures to STW effluents. These controlled exposures (including for roach) have now induced all of the feminised responses seen in wild fish (including VTG induction, feminised gonadal ducts and even intersex; Harries *et al*., 1997; Lange *et al*., 2009; Lange *et al*., 2001; Larsson *et al*., 1999; Liney *et al*., 2005; Purdom *et al*., 1994; Rodgers-Gray *et al*., 2001; Sole *et al*., 2000; Van Aerle *et al*., 2002). Recent studies have shown intersex fish have a reduced

capability to produce sperm of good quality (Jobling *et al*., 2002a; Jobling *et al*., 2002b) and for moderately to severely intersex fish a reduced ability of the 'males' to compete with others to sire offspring (assessed via paternity analysis in breeding colonies; Harris *et al*., 2010).

A significant amount of work has been conducted to identify the causative EDCs in the effluent. STW effluents are highly complex mixtures of natural and man-made chemicals (domestic and industrial waste). Further complicating matters are that these chemicals have different rates and levels of degradation and metabolite pattern. Hence, identifying the specific chemical(s) causing the adverse effects detected in wild fish is severely difficult. Although a number of various chemicals with different modes of action have been implicated in the feminisation of fish in the wild, including oestrogens, and anti-androgens, most evidence to date supports the involvement of oestrogens as the key element in the effects seen. A range of oestrogenic chemicals has been identified and closely associated with the feminised responses seen in fish, and they include E1, E2, EE2 and NP, as detailed above. Steroid hormones (E1, E2 and EE2) and the alkylphenols, are some of the key oestrogenic contaminants in STW effluents (Blackburn and Waldock, 1995; Larsson *et al*., 1999; Routledge *et al*., 1998; Shore *et al*., 1993; White *et al*., 1994) E1, E2 and EE2 in domestic effluents in Britain range from 1-50ng/L, 1-80ng/L and 0.2-7.0 ng/L, respectively (Desbrow *et al*., 1998 ; Johnson *et al*., 2005). Johnson *et al*. (2005) studying E1, E2 and EE2 and NP in 17 different sewage treatment works (STW) across Europe (Norway, Sweden, Finland, The Netherlands, Belgium, Germany, France and Switzerland) found the following concentrations: 0.7-5.7 ng/L of E2, 3.0 ng/L of E1, 0.8-2.8 ng/L for EE2 and NP 0.05 to 1.31 µg/L for NP. Numerous laboratory exposures to some of these chemicals alone, and in combination have shown them able to induce feminised responses in fish, and in some cases as environmentally relevant concentrations.

Examples of these exposures and effects include for EE2 to cause feminisation of male roach (Lange *et al*., 2009). At an exposure of 4 ngEE2/L for three years there was even complete sex reversal (all males were phenotypic females; Lange *et al*., 2011). EE2 has also been shown to affect breeding behaviour and reproductive success in zebrafish (Coe *et al*., 2010; Nash *et al*., 2004). Indeed, many laboratory studies have shown the effect of steroidal oestrogens on male

reproductive behaviour. For example adult male threespine stickleback (*Gasterosteus aculeatus*) showed delayed onset of nest building for an exposure to 15 ngEE2/L (Wibe *et al*., 2002) and a reduced gluing frequency at the nest at an exposure at dose of 10 ng/L EE2 (Brian *et al*., 2006). Sand gobies (*Pomatoschistus minutus*) were less able to gain and keep a nest after exposure of adults to 4ng/L EE2, which reduced their display of sexual behaviour (Saaristo *et al*., 2009). Chronic exposure of fathead minnow (*Pimephales promelas*) to EE2 (5-6 ng/L) for 7 years in a whole lake experiment in Canada caused the feminisation of male fish (production of VTG and altered oogenesis in female fish; (Kidd *et al*., 2007)). It also caused the almost complete collapse of the fathead minnow fishery, which subsequently recovered after the oestrogen treatment was removed.

Alkylphenols have been known to possess weak oestrogenic activity, unlike steroidal oestrogen (Arukwe *et al*., 1997; Jobling *et al*., 1996; Sumpter, 1995). Jobling *et al*. (1996) reported that NP (as low as 20 µg/L) was able to induce VTG production and retard testicular growth. Research by Arukwe *et al*. (1997) found that NP induced VTG and zona radiata proteins (Zrp) in a dose-dependent manner. Japanese medaka (*Oryzias latipes*) exposed to 50 µgNP/L and 100 µgNP/L from hatching for 3 months caused some fish to develop ovotestis and the exposures also resulted in alteration in the ratio of males to females (Gray and Metcalfe, 1997).

The contribution of BPA to the feminisation of fish is less clear. Concentrations of BPA in STW effluents have been measured at 1.14 µg/L from a pharmaceutical plant (Eggen *et al*., 2003). Laboratory exposures of fish have shown that it induces VTG synthesis, but for the most part only at very high exposure concentrations (Lindholst *et al*., 2000; Lv *et al*., 2007; Park *et al*., 2003; Sohoni *et al*., 2001). Sohoni *et al.* (2001) showed that BPA altered male sexual development for an exposure to 16ug BPA/L in the fathead minnow.

1.5 Experimental approaches for assessing oestrogenic activity and effects of environmental oestrogens

A variety of experiment methods (i.e. *in vivo* and *in vitro* methods) have been developed to determine the effects of chemicals with oestrogen activity in fish. Many *in vitro* assays have been developed to assess the oestrogenic activity of a substance. *In vitro* assays usually involve a small amount of test chemical to assess dose-response relationships. Furthermore, *in vitro* approaches often have ethical (with respect to the use of less test animals), technical and economical advantages over *in vivo* methods. *In vitro* methods also tend to generate less waste (Baksi and Frazier, 1990). In addition, *in vitro* methods are often cheaper and more rapid than *in vivo* systems. However, one of the major drawbacks of using *in vitro* system is the cell systems used often do not account for metabolism of a chemical or they do not take into account bioconcentration, which can serve to increase the biological effect of an EDC (Folmar *et al*., 2002; Murk *et al*., 2002).

Several *in vitro* assays are available to detect oestrogenicity, including the yeast oestrogen screen (YES), and the oestrogen receptor-mediated, luciferase reporter gene-expression (ER-CALUX) assay. In the YES requires that the oestrogenic activity of the compound being tested is normalised to the E2 (17ß-oestradiol) and reported as E2 equivalents. The ER-CALUX assay is a more sensitive assay than YES (Murk *et al*., 2002). Both systems are capable of detecting oestrogens and anti-oestrogens. However, a major drawback is that they lack metabolic capabilities and cell wall permeability might limit compound uptake, thus limiting further extrapolation to effects that might occur *in vivo* (Zacharewski, 1997). There are also a wide range of cell lines responsive to oestrogens (e.g. MCF7 cells; (Byford *et al*., 2002; Charles and Darbre, 2009)). Further *in vitro* systems for assessing oestrogenic activity of chemicals in fish include fish ER reporter assays (Chakraborty *et al*., 2010; Chakraborty *et al*., 2011).

Various *in vivo* assessment systems for oestrogenicity of chemicals have been developed and they include both short and long-term systems. *In vivo* assays include induction of plasma VTG (Gunnarsson *et al*., 2007; Rodgers-Gray *et al*., 2000; Thompson *et al*., 2000). VTG induction (measured as a protein in the plasma or VTG mRNA in the liver) is extremely widely used for measuring oestrogenic responses in fish (Bogers *et al*., 2006; Cosnefroy *et al*., 2009; Craft *et al*.,

2004). The benefits include, sensitivity, rapidity of the response (hours), dynamic range (up to a million fold induction) and specificity for oestrogens (Thomas-Jones *et al*., 2003).

Other *in vivo* responses for oestrogens in fish include effects on the liver (increased mass), as measured by the hepatosomatic index (HSI) and gonad growth (as measured by the GSI) (Sindhe and Kulkarni, 2004; Yang and Baumann, 2006). The latter however, is not specific to oestrogens.

Recently, transgenic fish lines have emerged as a new biosensor system for ecotoxicological research. Transgenic fish can provide the advantages of both *in vivo* and *in vitro* systems. Throughput analysis can be rapid, and the systems can be sensitive and (relatively) cheap and set up costs (establishing the TG line, however, are expensive). In transgenic fish it is possible to visualise expression of the target gene of interest and associated cellular and physiological processes in live animals, using reporters, such as green fluorescent protein (GFP). In this way it is possible to develop an integrative analysis and understanding on the uptake, distribution, accumulation and action of the chemicals in the tissue of live fish. The next section details the process of generating TG fish and their applications to date.

1.6. Transgenic Fish

An overview of producing transgenic fish

Despite the fact that several methods are available to produce transgenic fish, all of them require a transgenic construct with a promoter and a gene. The foreign gene (transgene) is constructed using recombinant DNA methodology. The recombinant DNA carries the transgene of interest. Ectopic expression (the expression of a gene in an abnormal place in an organism) of transgenes and disruption of endogenous genes in transgenic animals has enabled us to understand mechanisms of development and developmental gene regulation and functional interactions between the nervous and immune systems (Grabher and Wittbrodt, 2008; Wei,

1997). The most common expression vector used is *E. coli* plasmid vectors that replicate to high levels in their host cells.

Once the construct is made, it is transferred into the early one cell stage fish eggs using microinjection and embryos carrying the transgene are bred and maintained to adulthood. The fish then need to be assessed to ensure they carry the transgene. Transgenes can be screened by cutting a small piece of fin tissue and using polymerase chain reaction (PCR) or a Southern blot analysis to show the foreign gene (Horvat *et al*., 1993). The fish that are injected and raised to adults are called founders (F0). The next generation of fish (F1) is derived from crosses between founder (transgenic fish) and wild-type fish (non-transgenic fish) and these needed to contain the inherited transgene. Progeny derived from crosses between the first filial generations (F1) is called the F2 and is screened as before. In the work in this thesis, instead of southern blotting to identify the transgenic fish from founders' embryos were exposed to EE2 to identify the transgenic zebrafish (Figure 1.3).

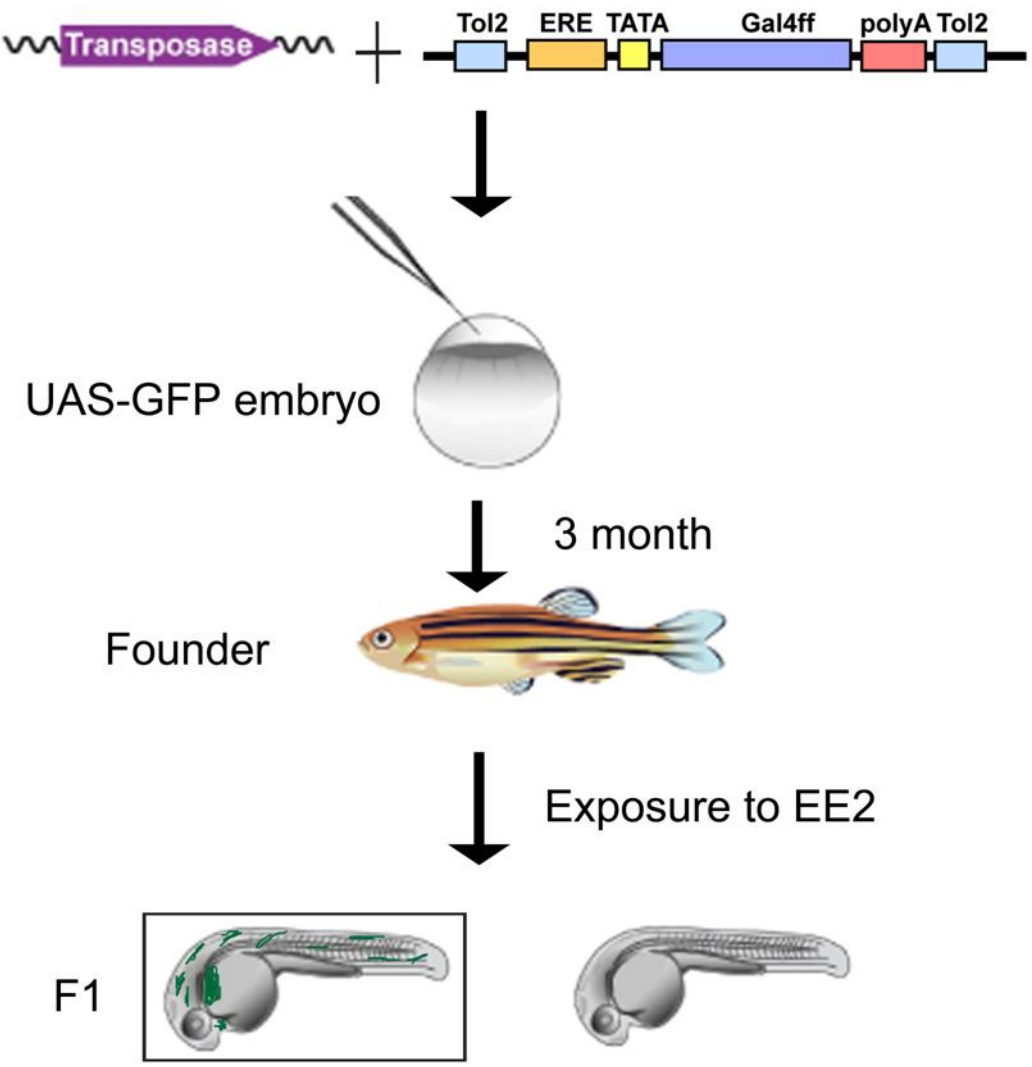


Figure 1.3 Procedure for generating transgenic zebrafish (as applied in this thesis). The plasmid is microinjected into early one cell stage and embryos subsequently derived from the adults of the founder population are exposed to oestrogen to identify positive trangenics.

1.6.1 Techniques for gene transfer

Transgenes have been introduced through several transfer methods into the fertilised eggs, via microinjection or electroporation of DNA in various fish species including salmon, carp, trout and tilapia. Gene transfer by microinjection is the most popular method to produce transgenic fish. Unlike microinjection for mammalian embryos, where the gene is injected into the pronuclei that are easily visible (Palmiter *et al*., 1982), the pronuclei in fish cannot be seen under the microscope after fertilisation and in most fish species eggs are opaque (Chen and Powers, 1990; Dunham *et al*., 1987; Hayat *et al*., 1991). Consequently, linearised DNA is microinjected into the cytoplasm of fertilised eggs. While Dunham *et al.* (1987) reported that microinjection showed high egg mortality, Hayat *et al.* (1991) found that the survival of fish embryos after DNA microinjection ranged between 35% to 80% and the integration rate was 2-17%. Thus, a wide range of success rates has been reported. Success rates might depend upon embryo stages and species. The amount of DNA for injection depends on the species and egg size, but the optimal amount of DNA is up to 10^6 copies (Dunham *et al*., 1987; Fletcher *et al*., 1988; Stuart *et al*., 1988). Stuart *et al.* (1988) reported that the amplification of the injected DNA occurred at between 5 hours and 10 hours after fertilisation (between the initiation of gastrulation and onset of somitogenesis).

Microinjection is difficult for some fish species as they have an extremely hard chorion. Furthermore, embryos in some fish species develop very quickly and the first cell stage is too short a time interval to allow the microinjection. However, zebrafish and medaka are suitable models for transgenic fish. Developmental processes in these species can also be seen due to the fact that their eggs are transparent. Furthermore, they lay many eggs each day (each female lays >200 eggs per week (Zon, 1999)). For zebrafish, the chorion is relatively soft so that injection is much easier than with many other fish species.

Electroporation has been developed as a new technique and as an alternative method for microinjection (Chen and Powers, 1990). In general, electroporation requires 2-4 cell stage eggs. The DNA is placed in buffer solution into a cuvette and eggs are electroporated to theoretically

make transient openings of the cell membrane which allows the transfer of genetic material from solution into the cell (Dunham, 2004). Many parameters (total voltage, buffer choice, buffer volume, quantity of DNA, number of bursts and frequency of wave) affect the efficiency of the electroporation. In some of the first research using electroporation (Inoue *et al*., 1990) the survival and integration rates for electroporation were similar to that for microinjection. The survival and integration rates of embryos in common carp and channel catfish after electroporation have subsequently been shown to be dependent on timing (the stage of development) (Powers *et al*., 1992). Electroporation with zebrafish embryos has not yet been successful as a technique for integrating the gene of interest (Kavumpurath *et al*., 1993). In addition, electroporation as a technique has considerable variation as mentioned above and has not been standardised (Powers *et al*., 1995).

Another approach is the use of primordial germ cells (PGCs). PGCs are the precursors of the germ cell lineage and it are committed to differentiate into either supermatogonia or oogonia after gonadal sex differentiation (Yoshizaki *et al*., 2003). A cell-mediated gene transfer system can deliver a foreign gene into the genome of cultured cells and isolate only the transformants by drug selection before they are converted into individual fish (Takeuchi *et al*., 2002). Germ-line contribution can be performed by microinjection into peritoneal cavity of hatched embryos with cultured PGC (Matsui *et al*., 1992). PGC-mediated gene transfer has been used in salmon and trout that have a thick chorion, a large body and a long generation time. PGC markers including alkaline phosphatase activity or antibodies against PGC-specific cell surface antigens are not available in fish but are available in mammals and birds (Timmermans, 1996). The germline marker *vasa* was characterised in zebrafish (Olsen *et al*., 1997; Yoon *et al*., 1997) and also a *vasa*-like homologue was found in rainbow trout (*Oncorhynchus mykiss*) (Yoshizaki *et al*., 2000).

1.6.2 Examples of transgenic fish

Many studies have been published describing the generation of recombinant fish that have been used in various fields of research including genetic and developmental research and chemical screening for ecotoxicological and pharmaceutical approach. The transgenic fish approach may allow correlation of molecular and integrated effects for the individual test organisms, thus it may prove useful for the measurement of immediate cellular responses. Transgenic research in mammals was popular in the 1980s and has more recently been developed and applied in fish. Fish have become popular in transgenic research for a number of reasons. One of these reasons is that fish are one of the most important human foods. Fish aquaculture provides an alternative approach without diminishing ocean resources and transgenic techniques have been applied to help boost this industry. Various transgenic fish have been developed using microinjection to increase growth rate in species including the Northern pike (*Esox Iucius*), Atlantic salmon (*Salmo salar*), Coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*) (Devlin *et al*., 1995; Du *et al*., 1992; Gross *et al*., 1992; Jonsson *et al*., 1996).

Transgenic research has been extended to study a number of physiological processes in fish. Examples include the study of cold tolerance and disease resistance in fish species. For example, Ocean pout (*Macrozoarces americanus*) antifreeze protein (AFP) genes were inserted into oocytes of goldfish (*Carassius auratus*) to show that transgenic goldfish were able to tolerate low temperatures compared with controls (non-transgenic goldfish) (Morvan *et al*., 1994; Wang *et al*., 1995). Their studies suggest that the AFP gene may offer cold tolerance in addition to freeze resistance for a variety of fish species. Morvan *et al*. (1994) investigated the effects of magainin 1, a peptide antibiotic originally extracted from the skin of *Xenopus laevis*, against *Bonamia ostreae* which infects the oysters. Viability of purified protozoa was assessed microscopically by the uptake of the vital dyes acridine orange and ethidium bromide. Their study suggested that antimicrobial peptides such as magainins may be utilised to develop disease resistant broodstock using transgenesis. In addition, another study showed that size increase did not report in the tilapia, therefore, transgenic tilapia were generated with the tilapia growth hormone (tiGH) cDNA was linked to the human cytomegalovirus (CMV) enhancer-

promoter (Martinez *et al*., 1996). Their results showed that transgenic F1 progeny were 82 % larger than non-transgenic fish.

Transgenic zebrafish research was introduced by Stuart *et al.* (1988). Zebrafish have been popular for transgenic research, such as studying integration and transmission of transgenes, promoter analysis (Caldovic and Hackett, 1995; Lin *et al*., 1994; Westerfield *et al*., 1992). The transgenic fish approach has become an established technique in developmental analyses, and generally involves using specific promoter and a green fluorescent protein (GFP) tagged reporter gene that allows for a signalling effect (expression of that gene) to be identified. Studies with tissue-specific promoters have become a powerful tool to recapitulate endogenous gene expression programs (Higashijima *et al*., 2000; Higashijima *et al*., 1997) and to analyse the function of gene promoters (Ju *et al*., 1999; Muller *et al*., 1999).

Various reporter genes have been utilised in transgenic zebrafish research. Reporter genes have identified which cells have taken up the gene and which have integrated it into their genome. The most common reporter genes used in the research are the green fluorescent protein (GFP) (see 1.7: specific information) and luciferase. GFP is a fluorescent protein isolated from the jellyfish *Aequoria victoria*. GFP shows a green fluorescence via microscopy. It also can be visualised directly in living cells and can be detected in zebrafish during both embryonic and larval development. In contrast, luciferase, which originates from *Photinus pyralis* (firefly) encodes the enzyme luciferase, and causes the cell that expresses it to catalyse luciferins and produce light (Contag and Bachmann, 2002). Although luciferase is sensitive and simple to use, it requires a costly substrate and is less stable (luciferase half-life varies from 95 m to 3 h as it depends on the host cell and the gene construct) (Goodman and Gao, 1999; Leclerc *et al*., 2000).

Transgenic fish have been developed using reporter genes driven by promoters that are responsive to various chemical exposures. GFP and luciferase, in combination with pollutant-responsive promoters, have been stably integrated into zebrafish and their progeny have been used successfully to detect for specific classes of chemicals. Various sub-acute effects have been determined using transgenic fish including mutagenic effects (Amanuma *et al*., 2000),

cadmium-toxicity by induction of heat-shock proteins (Blechinger *et al*., 2002), aryl hydrocarbon receptor-mediated toxicity (Mattingly *et al*., 2001), oestrogenicity (Chen *et al*., 2010; Legler *et al*., 2000; Salam *et al*., 2008; Zeng *et al*., 2005) and anti-oestrogenicity (Schreurs *et al*., 2004). In the work by Legler *et al.* (2000) transgenic zebrafish were developed with oestrogen responsive element (ERE) that expressed the reporter gene luciferase and using these animals they were able to show that the period of gonad differentiation was highly sensitive to oestradiol (E2). They exposed juvenile stages of transgenic zebrafish (35 dpf) to E2 for 96 h in order to determine the sensitivity and found that the induction of luciferase was detected at 0.1 nM E2.

Transgenic fish have great utility for measuring responses to endocrine disrupting chemicals, potentially allowing for more efficient experimentation (exposure times, effects analysis) compared with non TG animals. For example, screening for chemical effects with transgenic fish carrying GFP or luciferase could be conducted in 1-2 days, whereas traditional methods take a minimum of several days or even months from sampling to determination for pollutant effects. GFP responses (the intensity of the response) potentially allow for an assessment on bioconcentration of an oestrogen.

1.7 Green fluorescent protein (GFP)

As detailed above, one of the major detection systems now used for TG systems is GFP. GFP is composed of a chain of 238 amino acids and is roughly 27 kDa in size (Heim and Tsien, 1996; Shimomura, 1979). Compared with luciferase which is used as an enzymatic reporter gene, the GFP can be introduced into, and detected in, living cells without selection or staining. GFP was discovered by Osamu Shimomura and is a bioluminescent protein called Aequorin produced by the jellyfish *Aequoria victoria*. It emits green light when irradiated with blue or UV light (Chalfie *et al*., 1994; Johnson *et al*., 1962; Prasher *et al*., 1992; Shimomura *et al*., 1962). Johnson *et al* (1962) found that the emission spectrum of wild-type Aequoria GFP was at 395 nm, 470 nm and 508 nm (peaking at the latter wavelength). A significant breakthrough

came in the 1990s when Prasher *et al*. (1992) sequenced the gene for GFP. Studies by Chalfie *et al*. (1994) then inserted the gene for GFP into the bacterium *E. coli*, and subsequently showed it fluoresced with a green light in the presence of UV radiation. Since the mid 1990s, the properties of GFP have been improved by mutations in the gene such as spectral (colour) variants (RFP (red), YFP (yellow), CFP (cyan)). Many mutant forms of GFP have been created and developed to improve fluorescence brightness or altered excitation/emission spectra of the fluorescence, shifting it towards either red or blue. GFP has become widespread now for use to monitor gene expression and protein localisation of GFP-tagged proteins *in vivo* in many areas of cell and molecular biology research.

Enhanced GFP, or EGFP, is one of the GFP mutants that has been optimised based on preferred human codon usage and have been improved the utility of the protein. It produces a signal 35-fold brighter than wild type GFP in mammalian cells (Yang *et al*., 1996). Despite the fact that much research does not distinguish between GFP and EGFP, most studies now use a EGFP DNA construct as a reporter gene. GFP has now been used as a fluorescent genetic tag in cellular and molecular research and to develop transgenic animals and plants in scientific and medical research. However, weak auto-fluorescence of yolk is a drawback in its use in zebrafish early life stages and in embryos. Nevertheless, this drawback can be overcome using *Discosoma* red fluorescent protein (ds RFP) which is substantially shifted towards red in its extinction spectrum compared with the *Aequoria* GFP (Amsterdam *et al*., 1996; Tavare *et al*., 2001). Amsterdam (1996) reported that detection limits for GFPs were approximately 5×10^4 molecules of GFP per cell in zebrafish embryos.

GFP has had a major impact on the development of transgenic fish. GFP was first used in zebrafish in 1996 (Amsterdam *et al*., 1996), while Zhiyuan Gong's group developed colour transgenic zebrafish using living colour gene constructs (green, red, yellow and cyan) under a zebrafish muscle-specific promoter by injection (Xu *et al*., 1999; Xu *et al*., 2000). Subsequently, a range of other transgenic fish has been produced using GFP. Generation of GFP transgenic zebrafish using tissue-specific promoters has been powerful 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). In addition, another application of GFP in transgenic fish has been as biosensor systems to monitor chemicals such as heavy metals and oestrogens (Blechinger *et al*., 2002; Chen *et al*., 2010; Kurauchi *et al*., 2008; Salam *et al*., 2008; Zeng *et al*., 2005).

1.8 *Tol2* transposon (overcoming technical difficulties using *Tol2* transposon system)

Zebrafish have been developed as an important model organism for studying genetics and applied extensively for the generation of transgenic and mutant lines over decades of research (Burket *et al*., 2008; Moore *et al*., 2006; Stainier, 2001). When transgenic zebrafish were first developed by Stuart *et al*. (1988) using microinjection, integration of the transgene into the genome was inefficient (germ line transmission: 5%). In early studies, linearised plasmids were utilised for transgenesis which resulted in a low percentage of embryos as transgenic fish. Part of the reason for this was that the linearised plasmid was concatemerised (a long continuous DNA molecule that contains multiple copies of the same DNA sequences linked in series) at a high rate (Viret *et al*., 1991) after injection. Multiple gene copies are randomly integrated into the genome or placed as extrachromosomal DNA in the cell. Although microinjection with linearised plasmid has the drawback of such a low transgenic efficiency, it is commonly used to make transgenic zebrafish due to its simplicity. Since these early studies, microinjection technique has been improved and detailed protocols have been developed specifically for generating transgenic zebrafish. One factor improving the efficiency of transgenesis by microinjection is to use embryos at the early one cell stage. DNA transposons are efficient tools for gene delivery and expression and have therefore become popular in transgenesis work. A highly efficient transgenesis method (the *Tol2* transposon system) has been developed and is now widely used in zebrafish and other vertebrates, opening up new transgenic research opportunities because the *Tol2* system enhances integration (Hamlet *et al*., 2006; Kawakami, 2007; Kawakami *et al*., 2000; Sato *et al*., 2007).

The *Tol2* transposon, derived from medaka (*Oryzias latipes*), belongs to the hAT(hobo/Ac/Tam3) family of transposons (Kawakami *et al*., 2000). Transposons are sequences of DNA that are able to move directly from one site to another site within the chromosome or onto extrachromosomol DNA within the same cell. There are two types of transposons, autonomous and non-autonomous. An autonomous transposon contains the sequence to its own enzyme for transposition, which helps the transposon to move around. In contrast, a non-autonomous transposon cannot encode its own transposition proteins and it requires transposase activity, which can be supplied as mRNA, in order to relocate. The only non-autonomous transposon that requires artificially provided transposase can be utilised to produce stage transgenics (Kawakami *et al*., 2004; Ryder and Russell, 2003). The transposase should be introduced into early one cell stage eggs by microinjection with transposase mRNA.

Kawakami *et al.* (2004) reported that germline transmission frequency was approximately 50 % when *Tol2* construct was co-injected with transposase mRNA. Furthermore the use of the *Tol2* transposon system may not suffer from the problems of gene silencing effects that can occur with linear plasmid injection (Kawakami, 2005). In addition, a number of studies have been applied to examine *Tol2* transposon vector carrying GFP in a specific tissue or organ and rescue a mutant phenotype (Asakawa and Kawakami, 2009; Higashijima *et al*., 1997; Kawakami *et al*., 2004; Kobayashi *et al*., 2001; Taylor *et al*., 2005). Asakawa and Kawakami (2009) found that *Tol2*-mediated Gal4FF;UAS:GFP transgenic fish is reproducible and persists after passage through generation and did not show mosaic expression. Therefore, they reported that gene silencing can be solved by using and *Tol2*-mediated transgenesis.

1.9 GAL4- UAS system

The GAL4-UAS/GFP system allows for the amplification of the signal (primary response: oestrogen induces synthesis of Gal4ff protein, secondary response: Gal4ff protein induces synthesis of GFP) and was central in the process of developing the TG zebrafish in this thesis and thus its nature and use is now described.

The GAL4-UAS system (Figure 1.4) is widely used for the over expression of transgenes. The GAL4 gene encodes a protein of 881 amino acid (Johnston *et al*., 1982) and was identified in the yeast *Saccharomyces cerevisiae* as a regulator of the galactose-inducible genes (Laughon and Gesteland, 1984). The GAL4 protein binds to a sequence called Upstream Activating Sequences (UAS) element, 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) and used to analyse the function of developmental genes (Brand and Dormand, 1995; Brand *et al*., 1994). This system is based on a bipartite (two-part) expression system that uses the yeast transcription factor GAL4 and its target upstream activation sequence (UAS), to which Gal4 binds in order to activate gene transcription. The GAL4/UAS developed in *Drosophila*, introduced spatial and temporal control of transgene expression using two transgenic lines that were combined, one activator line and one effector line (Brand and Perrimon, 1993; Fischer *et al*., 1988; Fisher *et al*., 2006; Rorth, 1998). In an activator line the gene for the yeast transcriptional activator GAL4 is placed under the control of a desired promoter (the activator protein Gal4 may be present but has no target gene to activate) whereas the effector lines contain DNA-binding motif of GAL4-(UAS) linked the gene of interest. UAS is fused to an effector gene which is silent if GAL4 activator is absent. GAL4 can be expressed in many different patterns by placing it under the control of various '*Drosophila melanogaster*' tissue-specific promoter sequences, since UAS promoter sequences (CGGAGTACTGTCCTCC) are not found in *Drosophila* (Kramer and Staveley, 2003). When the GAL4 activator line and UAS effector lines are crossed, the target genes are turned on in the double-transgenic progeny, following the expression pattern of GAL4-dependent transgene in a tissue-specific manner (Davison *et al*., 2007; Halpern *et al*., 2008; Zhan and Gong, 2010).

Many GAL4 lines have been isolated by many laboratories and have been widely used for ectopic expression of genes of interest. Furthermore, the GAL4-UAS system has also been introduced into mice, *Xenopus* and zebrafish (Goentoro *et al*., 2006; Hartley *et al*., 2002; Ornitz

et al., 1991; Scheer and Campos-Ortega, 1999). As activator and effector strains can be freely combined, a large number of targeted transgene expression studies can be conducted from a limited number of transgenic strains (Distel *et al*., 2009). Studies with transgenic zebrafish have already confirmed that the GAL4-UAS system is useful in cell type-specific ablation studies, the mapping of neuronal circuits and the inhibition of neuronal activity in distinct neuronal populations (Asakawa *et al*., 2008; Davison *et al*., 2007; Distel *et al*., 2009; Kaiser, 1993; Scott *et al*., 2007).

The GAL4-UAS method for targeted gene expression offers considerable advantages for studies in zebrafish. For example, a steadily increasing number of genes cloned from zebrafish could be examined in more detail than is feasible with mRNA injections (Scheer and Campos-Ortega, 1999). In addition, the use of this method allows the expression of GAL4 in variety of stages and tissues since various promoters and tissue-specific enhancer sequences have been isolated from zebrafish (Scheer and Campos-Ortega, 1999). GFP can be used in GAL4-UAS systems in order to visualise where desired cells are expressed. To do so, a GAL4 line needs to be crossed with a GFP reporter line that expresses GFP. The desired subsets of cells are produced in the offspring, and in these cells the GAL4 binds to the UAS, and allows the production of GFP.

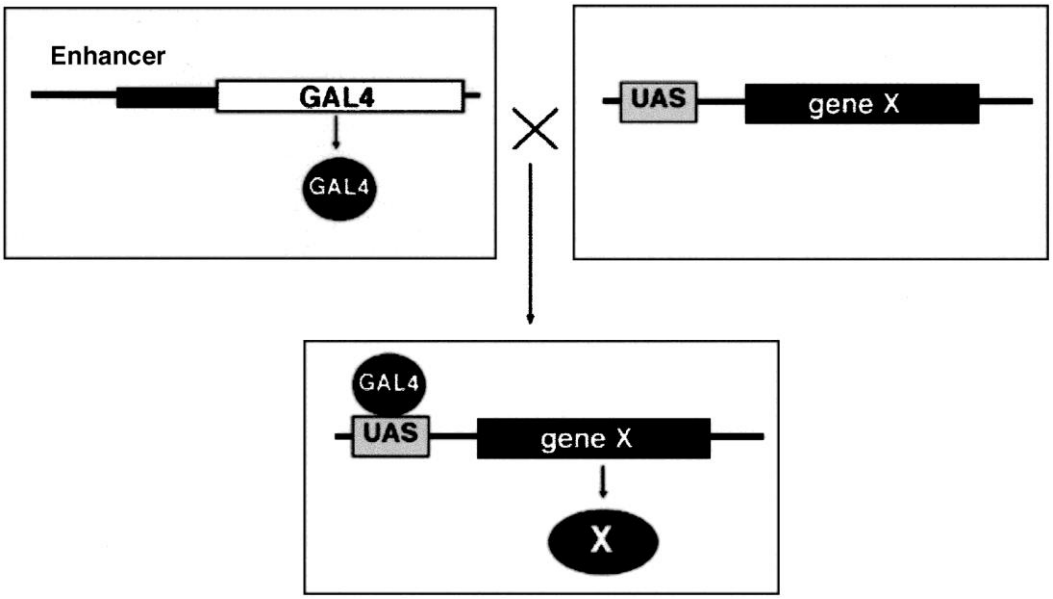


Figure 1.4 Diagram of the GAL4/UAS binary expression system. The yeast transcription factor GAL4 is crossed to effector line in which the gene of interest (gene *X*) is under the control of a UAS element (upper right box). In the progeny of this cross (lower box), GAL4 binds to the UAS sequence leading to the transcriptional activation of gene *X*. (Brand *et al*., 1994; Kaiser, 1993)

1.10 Zebrafish as a model system

Zebrafish (*Danio Rerio*) have only been used extensively as research models over the past three decades. The zebrafish is a small tropical freshwater fish that has become a popular model organism to study genetics and embryonic development. Attributes that favour its use include its high fecundity, the fact that many aspects of its physiological are similar to those in mammals, and the availability of powerful genetic tools (Briggs, 2002; Squire *et al*., 2008; Wienholds *et al*., 2003; Zon, 1999). As a model animal system the zebrafish is also easy to breed in the laboratory with relatively low associated maintenance costs. In addition, transparency of the embryos and rapid organogenesis offer very attractive features in this experimental model.

A large number of studies have used zebrafish to examine a wide range of aspects of embryonic development including determination of the embryonic axis, the differential regulation of gene expression, cell lineage analysis, formation of the central and peripheral nervous systems (Abdelilah *et al*., 1994; Blader and Strahle, 2000; Holth *et al*., 2008; Kelly *et al*., 1995; Kimmel and Warga, 1987; Tiso *et al*., 2009).

Zebrafish have also been proven to be a valuable model for human diseases and drug screening (Berghmans *et al*., 2007; den Hertog, 2005; Lieschke and Currie, 2007; Stern and Zon, 2003). In the past, drug screening has mainly been based on cell culture or protein-binding assay, but zebrafish offer an excellent whole-organism model system for preclinical drug screening. Increasingly the zebrafish are being used as a human disease model for studies on carcinogenesis, wound healing, immunological diseases, behavioural abnormalities, infection and Parkinson's disease (Bretaud *et al*., 2010; Burgess and Granato, 2008; Goessling *et al*., 2007; Lieschke and Currie, 2007; Martin and Feng, 2009; van der Sar *et al*., 2004; Yoder *et al*., 2002).

Given the concern regarding the presence of chemicals in the environment that can potentially induce adverse effects on health, there is growing public awareness of environmental impact of pollutants. The zebrafish are becoming a popular model organism in ecotoxicological studies and are now widespread in many laboratories. Numerous studies have been published

investigating various different oestrogen chemicals using the zebrafish model and these studies have not only identified effects on the morphology and physiology of zebrafish, but have also identified the molecular mechanisms of their toxicity (Birnbaum, 1994; Carney *et al*., 2006; Ung *et al*., 2010; Vosges *et al*., 2010) and shown behaviour outcomes (Coe *et al*., 2010; Salierno and Kane, 2009; Wibe *et al*., 2002).

Zebrafish have many advantages for creating transgenics including the relatively low costs (compared with mammals), the ability to rapidly introduce and test the efficiency of the introduction of foreign DNA. The transparency of zebrafish eggs and the large number of eggs that can be obtained from a single fish are further advantages. Transgenic zebrafish have now been produced and applied to study the function of a number of genes. Recently, zebrafish have been created that are transparent even as adults which enables their internal organs to be observed (White *et al*., 2008). In these transparent transgenic zebrafish it is possible to observe individual cancer cells as they spread through the body. These fish also offer potential advantages for studies on the effects of pollutants in fish as adults. The transgenic zebrafish model system generally offers sensitive and labour-efficient models to monitor uptake and biological effects of compounds in the aquatic environment (Carvan *et al*., 2000), but this possibility is only slowly being realised.

1.10.1 Medaka as a model system

Medaka (*Oryzias latipes*) have been used a model animal in developmental molecular biology and since the early 20th century. Medaka have been widely used to study in embryonic development, genetics, carcinogenic and ecotoxicological research (Ahsan *et al*., 2008; Hawkins *et al*., 2003; Patyna *et al*., 1999; Turksen *et al*., 2006). Medaka are the first vertebrate species in which successful sex-reversal was undertaken and the medaka genome has been sequenced (Kasahara *et al*., 2007; Yamamoto, 1953). Transgenesis and mutagenesis protocols for medaka have been established (Matsumoto *et al*., 2006; Naruse *et al*., 2004; Shima and Mitani, 2004; Wittbrodt *et al*., 2002). Medaka have a short generation time (approximately 3 months) and are

easy to handle and obtain in large quantities. In addition, medaka eggs are transparent which makes observation easily. It takes 11 days for the eggs to hatch and the mature females lay about 40 eggs per day (Iwai *et al*., 2009). Another advantage is the natural tolerance of medaka to low temperature, consequently they are able to survive at 40 ℃ in summer and 4 ℃ in winter without any thermostatic regulators (reviewed in Kinoshita, 2009).

1.11 Aims of this thesis (my PhD)

Despite the fact that there is a growing concern over the effects of EDCs in the aquatic environment, there are very few bioassay systems that are sufficient to effectively assess oestrogen signalling pathways and physiological impacts in an integrative manner and many of the assays available are expensive to run.

The overall aim of the work presented in this thesis was to develop transgenic zebrafish which were highly sensitive for the detection of exposure to oestrogenic EDCs. In theory the fish developed should allow the target tissues affected by the oestrogens to be identified and thus to assess tissue specificities and responsiveness for each of the EDC studied. Assessments were made both using transient expression and transgenic fish assays. Such a system should potentially be applicable for high throughput screening of oestrogenic chemicals, identifying the target tissue for oestrogens and for making better integrated health impact assessment of environmental oestrogens.

In order to achieve this overall aim the following experimental work was conducted with the following specific aims:

Aim 1 (Chapter 3): To develop new constructs, pKS-ERE-Gal4ff, pCS2-ERE-Galf4ff and pBR-*Tol2*-ERE, for generating transgenic zebrafish

The main questions in the first experimental chapter were to decide which vector system (pKS+, pCS2+ and pBR322-*Tol2*) might be the most appropriate for making transgenic zebrafish and investigate ways to improve the efficiency for sensitivity and tissue specificity. To answer these questions, three constructs were made and examined progressively in the development process. The plasmids containing three oestrogen response elements (3ERE), a TATA box and GAL4ff were made and these three constructs, pKS-ERE-Gal4ff, pCS2-ERE-Galf4ff and pBR-*Tol2*-ERE, were examined by injecting into zebrafish in a transient assay system. Finally, the pBR-*Tol2*-ERE-Gal4ff construct was produced that worked in a tissue specific manner and enabled the generation of TG fish lines with high frequency and optimised response to oestrogens, thus improving greatly the chances for generating TG fish.

Aim 2 (Chapter 4): To develop a quick and effective transient assay system to examine the effects of oestrogenic chemicals on embryo development in wide range of fish species

To achieve this aim, we developed a novel transient assay system using a construct containing a synthetic oestrogen responsive element (ERE) and a *Tol2* mediated Gal4-UAS systems linked with a GFP reporter gene. Key elements in this work were to assess the sensitivity of the TG and tissue specificity. It was also investigated whether this system could be applied to another fish species (the medaka). The transient assay involved injecting the construct into 1-2 cell staged zebrafish embryos, and the injected embryos were then exposed to various oestrogen chemicals.

Aim 3 (Chapter 5): To investigate sensitivity and tissue targets for environmental oestrogens, responses to oestrogens were assessed using oestrogen responsive transgenic (ERE-TG**)** zebrafish larvae.

In the first part of this work, ERE-TG zebrafish embryos were exposed to various oestrogen chemicals (EE2, E2, NP and BPA) for 96 hours to identify target tissues and receptor pathway

interactions. The work was also established to look at time-related responses to oestrogens across the different body tissues. A further goal was to investigate thresholds for responses to different oestrogens. To assess pathways of action of steroidal oestrogens, oestrogen receptors (ERα, β1 and β2) morpholinos (MOs) and exposures with oestrogen receptor antagonist were conducted.

Aim 4 (Chapter 6): A final analysis undertook preliminary studies to investigate which target organs were oestrogen sensitive for a short-term exposure to EE2 in immature (40 days old) and adult (3 month old) ERE-TG zebrafish to compare their responses to oestrogen across life stages.

Here immature and mature ERE-TG zebrafish were exposed different concentrations of EE2 for 7 days and GFP expression assessed via green fluorescent microscopy and quantified by western blot analysis.

CHAPTER 2

General Materials and Methods

CHAPTER 2: General Materials and Methods

2.1. General Approach

The main aim of this thesis was to develop oestrogen responsive transgenic (ERE-TG) zebrafish and assess them for screening and testing of environmental oestrogens. In this endeavour, a new construct was developed, tested in a transient expression assay and then applied to create transgenic zebrafish. The transgenic fish were created via injection of the construct into one cell stage embryos and the resulting fish grown to 3 months in age, when they were old enough to breed. From this founder population, three transgenic zebrafish lines were selected and used for breeding to create the F1, from which F2 fish were then generated for screening and testing of environmental oestrogens. Studies conducted with the transgenic fish included screening for the effects of oestrogenic chemicals during embryo development, assessing the most sensitive life stages and identifying target organs. This work also included studies with immature and mature ERE-TG zebrafish.

The methods required to generate and examine the ERE-TG fish were very wide ranging and included making plasmids, microinjection, western blotting, oestrogen receptors morpholinos injection, and chemicals tests. Major parts of the methodologies for creating the three plasmids, pKS+ERE-TATA-Gal4ff, pCS2+ERE-TATA-Gal4ff, pBRTol2-ERE-TATA-Gal4FF (Chapter 3), examining the final plasmid pTol2-ERE-TATA-Gal4FF in a transient expression assay (Chapter 4) and the development and application of the ERE-TG zebrafish (Chapters 5, early life stages; Chapter 6, later life stages, including adults) are covered in the respective chapters. Here the following general methods are described; purification, digestion, preparation of insert and the vector, running agarose gels, cutting a specific DNA band from a gel, ligation, transformation and LB broth culture medium, transgenic fish sample collections, image analysis, and generation of TG zebrafish line.

2.2. Preparation of the insert and vector

In the construction of pTol2-ERE-TATA-Gal4FF, the general method for plasmid generation and construct insertion is shown in Figure 1 (also see Figure 2.1).

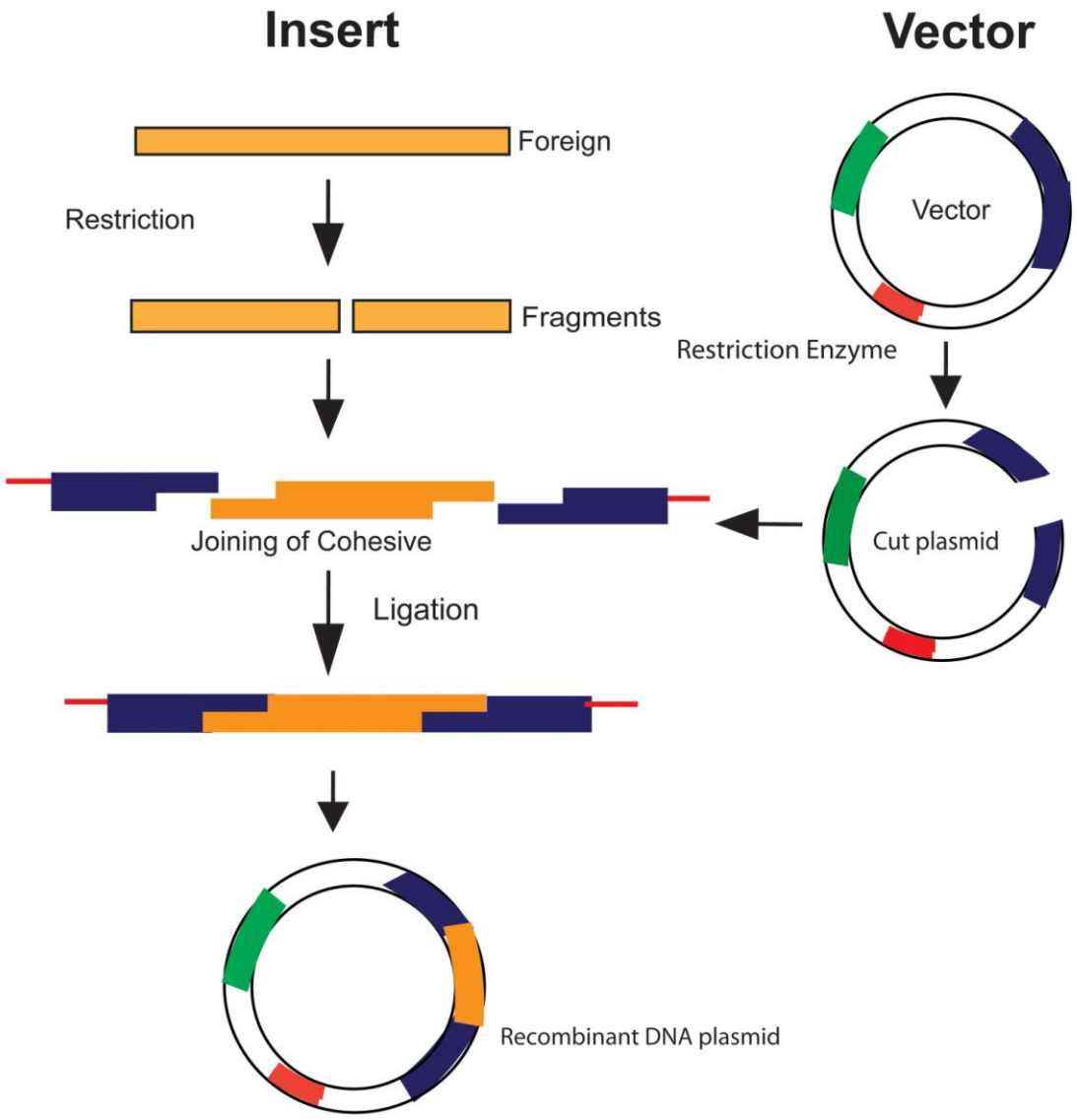


Figure 2.1 Illustration of the procedure to form recombinant DNA: A foreign DNA fragment can be inserted. The insertion of the fragment into the cloning vector is carried out by treating the vehicle and the foreign DNA with a restriction enzyme. Restriction enzymes are used to cut the DNA and vector so they can be joined multiple copies of the gene of interest. The multiple cloning sites (MCSs: blue colour) contains many restriction sites. MCSs allow for insertions of DNA into the vector to be targeted and possibly directed in a chosen orientation.

The required DNA fragments for insertion into the vector were generated by polymerase chain reaction (PCR) and using restriction enzyme digestion. For the insert (DNA fragment), specific primers (see Chapter 3) were designed with appropriate restriction sites. PCR reaction was carried out to generate EREs using specific primers. To reduce the risk of mutation, the cycling conditions of 3ERE and TATA were used for 8 cycles: denaturation at 96 ℃ for 1min, annealing at 60 ℃ and extension at 72 ℃ for 1 min. The following conditions were used to generate the Gal4-FF and polyadenine tail (poly [A] tail) ; 25 PCR cycles: denaturation at 96 ℃ for 1min, annealing at 60 ℃ and extension at 72 ℃ for 5 min. PCR amplifications were carried out in a reaction volume of 100 µl containing 54.5 µl of ddH_2O, 8 µl of 2.5 mM dNTP, 4 µl of each primer, 8 µl of 25 mM $MgCl_2$, 20 µl of 5X GoTq Flex buffer, 0.5 µl of *Taq* polymerase (Promega) and 1 µl template DNA (PCS2+Gal4ff). An aliquot of 10 µl of the PCR product from this reactant was used the check the concentration and the quality of PCR products (by agarose gel electrophoresis) and 90 µl of PCR product were purified using a phenol:chloroform extraction and ethanol precipitation as follows: An equal volume of phenol/chloroform was added to an aqueous DNA sample in a microcentrifuge tube and then vortexed for 30-60 seconds. The sample was then centrifuged for 2 minutes at room temperature to separate the phases. The aqueous phase was transferred to a new tube and 1 µl glycogen was added. This solution was then diluted with 1:10 volume in 3M sodium acetate and 3 volumes of ethanol. It was then left at - 80 ℃ for 1 hour and afterwards centrifuged at 14,000 rpm for 20 minutes at 4 ℃. The supernatant was removed and washed with 75% ethanol for 5 minutes. The DNA was then dried and subsequently re-suspended in water.

The insert (fragment) and vector (pKS+, PCS2+ or pBR plasmid) were digested with appropriate restriction enzymes (see chapter 3) to produce a DNA fragment that could be cloned directly into a vector (pKS+, PCS2+ or pBR plasmid). The resulting DNA digest for this was incubated at 37 ℃ for 2-4 hours in a reaction volume of 100 µl containing 20 µl of insert, 5 µl of vector, 10 µl of 10X restriction enzyme buffer (Promega), 62.5 µl of ddH_2O and 2.5 µl of restriction enzyme (Promega).

2.2.1. Preparing and running standard agarose gels

Agarose gels were used to check the size of insert and vector. To make the gels, 1g of agarose powder was mixed with 100 ml of electrophoresis buffer (Tris-acetate-EDTA [TAE]), then heated in a microwave for 1-2 min until it was melted completely. After cooling, ethidium bromide was added to the solution (final concentration 0.5 µg/ml). The gel was then poured into a casting tray containing a 10 well comb and left to become solidified at room temperature for 20 min. After the gel has set, the comb was removed. For the electropheoresis reactions, the gel in the casting tray was inserted horizontally into the electrophoresis chamber and covered with electrophoresis buffer. DNA ladder was loaded into the first well. Samples for analysis containing loading buffer were pipetted into the sample wells, the lid of the chamber closed and power applied to run the gel and separate the DNA fragments.

In order to visualise DNA or RNA, the gel was placed on an ultraviolet transilluminator, and a photographic record of each gel was taken using agarose gel photo machine.

2.2.2. Extracting DNA from the agarose gel

After running the agarose gel, the required DNA band was identified for removal. The gel was placed in a long wave length UV light box in the dark room to minimise the DNA damage and the specific DNA band was cut out using a sterilized scalpel blade. As little agarose was taken as possible in the extraction process. The band (DNA) removed was placed into 1.5 ml microcentrifuge tube. The microcentrifuge tube was weighted before receiving the DNA/agarose extraction and when containing the extracted gel/DNA before proceeding to purify the DNA from the gel using Qiagen Gel Extraction Kit.

2.2.3. Ligation and transformation

Ligation was conducted via incubation of the DNA fragment (insert) with the plasmid (vector) that has been linearised at 4 ℃ overnight or room temperature for 2-3 hours. Before initiating the ligation process, the insert and vector were run on a gel to enable the amounts of insert and vector to be quantified for use in the ligation. For the ligation, the reaction (5 µl of vector, 12 µl of insert, 2 µl of 10X DNA ligase buffer and 1 µl of T4 DNA ligase) was mixed gently by pipetting and incubating it at room temperature for 2-3 hours or 4 ℃ overnight.

The transformation step results in the incorporation of the ligated DNA into bacteria for propagation. The bacteria are then plated on selective agar to select for bacteria that have the plasmid of interest. Transformation was conducted following ligation. LB-Broth (Luria-Bertani) was prepared by adding 20g LB broth and making the volume up to 1000 ml with ddH_2O which was then mixed, autoclaved, and stored at 4 ℃, until required. Transformation was undertaken using 1 µl of ligated DNA (ligation reaction) and 10 µl of *E. coli* cells that were mixed and incubated on ice for 10 min. The cells were then heat shocked in at 42 ℃ for 42 sec. The heat will enlarge the pore on the cell membrane, allowing the plasmid to enter into the cell easily. Following heat shock, the transformation reaction was immediately placed on the ice for 5 min. 100 µl of SOC (Super Optimal Catabolite) was then added to the tube containing the transformation reaction and the tube was incubated for 30 min at 37 ℃ in a shaking incubator. The reaction was spread with a glass spreader on a LB-agar plate containing ampicillin (100mg/ml; Sigma) and put in the 37 ℃ incubator overnight. The next day, individual colonies were picked and added into the tube containing 2ml of LB-Broth with ampicillin.

2.2.4. Preparation of Plasmid DNA by Alkaline Lysis with SDS: Minipreparation

Transformed *E. coli* carrying the newly synthesised recombinant plasmid DNA contains many false positive (empty) plasmids. To select the right clone which carries the correctly recombinant plasmid, multiple (normally 8 to 12) colonies were amplified in a small scale and the plasmid was purified with Alkaline lysis method.

Alkaline lysis solution I consisted of 50 mM glucose, 25 mM Tris-Cl (pH 8.0) and 10 m EDTA (pH8.0) and was stored at 4 ℃. Alkaline lysis solution II contained 0.2 N NaOH (freshly diluted from a 10 N stock) was made up to 1% in SDS and was used at room temperature. Alkaline lysis solution III was composed 5 M potassium acetate(60.0 ml), Glacial acetic acid (11.5 ml) and H_2O (28.5 ml).

In the following transformation and culture, alkaline lysis is used to break cells open to isolate and purify plasmid DNA.

A single colony of transformed bacteria was inoculated into 2 ml of LB Broth. The culture was incubated overnight at 37 ℃ with vigorous shaking. 1.5 ml of the culture was poured into a microfuge tube and centrifuged at maximum speed for 30 seconds at 4 ℃. The supernatant from the microfuge tube was removed by aspiration, to leave the bacterial pellet as dry as possible. The bacterial pellet was resuspended by vigorous vortexing in 100 μl of ice-cold alkaline lysis solution I, and then 200 μl of freshly prepared alkaline lysis solution II was added to bacterial suspension. The tube was closed tightly and the contents mixed by inverting the tube rapidly five times. 150 μl of ice-cold alkaline lysis solution III was then added and the tube was closed and inverted several times. The tube was then stored on ice for 3-5 minutes. After this the bacterial lysate was centrifuged at maximum speed for 5 minutes at 4 ℃ in microfuge and the supernatant transferred into a fresh tube. An equal volume of phenol:chloroform was added and the aqueous phases was separated by vortexing and centrifuged at the maximum speed for 2 minutes at 4 ℃ in microfuge. The aqueous upper layer was transferred to a fresh tube. Next, nucleic acids were precipitated from the aqueous solution by adding 2 volumes of ethanol at room temperature. The solution was mixed by vortexing and then the mixture was allowed to stand for 2 minutes at room temperature. The precipitated nucleic acids were collected by centrifugation at 1,4000 rpm for 20 minutes at 4 ℃. The supernatant was removed and l ml of 70% ethanol was added and mixed with the pellet by inverting the closed tube several times. The DNA was recovered by centrifugation at maximum speed for 2 minutes at 4 ℃. The ethanol was removed and the tube left at room temperature until any remaining ethanol

evaporated (for 5-10 minutes). The nucleic acid was dissolved in 50 µl of H_2O. The DNA solution was vortexed gently for a few seconds and then stored at -20 ℃ until required.

2.2.5. Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit (Promega)

A miniprep procedure was required to isolate and purify the plasmid DNA from the recombinant *E. coli* cells. For this purpose, the QIAprep Spin Miniprep Kit (Qiagen) was used. Pelleted bacterial cells were re-suspended in 250 µl Buffer P1 (resuspension buffer) and were transferred to a microcentrifuge tube. The samples were added to 250 µl Buffer P2 (shake buffer) and mixed thoroughly by inverting the tube 4-6 times. 350 µl Buffer N3 (neutralisation buffer) was then added and mixed immediately and thoroughly by inverting the tube 4-6 times. They were centrifuged for 10 min at 13,000 rpm in a table-top microcentrifuge and the supernatants added to the QIAprep spin column by pipetting. These were then centrifuged again for 30-60 s and the flow through was discarded. QIAprep spin columns were washed by adding the 0.5 ml Buffer PB and centrifuged for 30-60 s. Next, they were washed by adding 0.75 ml Buffer PE (wash buffer) and centrifuged for 30-60 s. After that, they were centrifuged for an additional 1 min to remove residual wash buffer. The QIAprep columns were placed in a clean 1.5 ml microcentrifuge tubes. To elute DNA, 50 µl Buffer EB (10mM Tris·Cl, pH 8.5) or water was added to the tubes to the centre of each QIAprep spin column, and then were centrifuged for 1min.

2.3. Microinjections for transient assay and generating transgenic zebrafish

Injections of plasmid DNA into zebrafish embryos were accomplished with a pressure injector (INTRACEL, PICOSPRITZER® III) with Narishige manipulator under a dissecting microscope (NIKON SMZ1500). Injection needles were prepared from borosilicate glass capillaries (HARVARD APPARATUS, GC100F-15) using a micropipette puller (Narishige, model PC-10). Plasmid DNA for injection was prepared using the Qiagen Midiprep Kit

(Qiagen). DNA for injection was suspended in dH_2O. The injection solution consisted of 18 ng/µl plasmid DNA and 0.05% w/v phenol red (Sigma). For preparation of the injection solution, the mixture was centrifuged at the maximum speed (14,000 rpm) for 30 s. The microinjection mixture was then placed into the glass capillary which was placed in the injector. Using the 4X magnification on a dissecting microscope, the tip of the needle was gently cut with clean forceps. The injection pedal was pressed to ensure that the tip had been broken and DNA was seen to flow out of the needle. Fertilised eggs for injection were the aligned at the edge of the cover slide. The needle was carefully extended through the chorion and into the yolk and the injection pedal was depressed to inject approximately 1 nL of the mixture into the cytoplasm of the cell. The injected embryos were then incubated at 28 ℃.

2.4. Anaesthesia and Dissections

All manipulations of embryos, larvae and fish for observing green fluorescence were carried out under anaesthesia. Anaesthesia was conducted using 0.4% tricaine (3-aminobenzoic acid ethyl ester, pH 7.4; Sigma). Tricaine solution was prepared by adding 400 mg of tricaine powder to 97.9 ml of distilled water and approximately 2.1 ml of Tris (pH 9) was added to adjust the solution to pH 7. The stock was stored in the fridge.

For observations of green fluorescence in juvenile and adult fish, dissection was required. Fish were anesthetised to termination and then rinsed in ddH_2O to remove anaestheic residues The fish's abdomen was then opened using a scalpel blade and forceps. After observation of GFP under fluorescence microscopy, tissues (liver, gonad and intestine) were collected to perform western blot analysis for quantifying GFP expression (see Chapter 4, 5 and 6). Dissection was carried out under sterile conditions. The dissection instruments were soaked in 70% ethanol to sterilise them and then rinsed in ddH_2O. The dissection instruments were re-sterilised following the removal of each tissue.

2.5. Generation of ERE-TG zebrafish

For creation of transgenic zebrafish, the plasmid pTol2-ERE-TATA-Gal4ff with transposase mRNA was injected into 400 UAS-GFP transgenic zebrafish at the one cell stage.

Injected embryos were cultured for 4 day at 28 ℃, with the removals of any dead or abnormal embryos. After 4 days, the injected embryos (approximately 300 surviving) were put into 3 tanks to grow them to maturity. Unfortunately, all larvae in one tank died. The remaining 200 juvenile fish were raised and identified as either homozygous or heterozygous founders (possessing two different forms of a particular gene, one inherited from each parent). Fish were pre-treated with 100 ng/L of EE2, so in fact they may have been "primed" to respond differently to oestrogen exposure. Eggs were collected from founder colonies and exposed to 100 ng/L of EE2 for 3 days to observe the GFP fluorescent signal. From this we selected the GFP-positive embryos and rinsed them in the physiological saline 10 times to remove EE2 and then raised the fish to adulthood. 25 pairs of F1 transgenic fish were placed into separate spaning tanks and the next day eggs were collected from each chamber and exposed to 100 ng/L EE2 for 3 days to screen the expression of GFP (see Fig. 2.2). Overall, 12 zebrafish lines showed variation of the intensity of the GFP expression when they were exposed to EE2. Three oestrogen responsive transgenic fish lines were selected that showed a relatively strong GFP expression after exposure to 100 ng/L of EE2. Three transgenic zebrafish lines were subsequently established for the F2 generation.

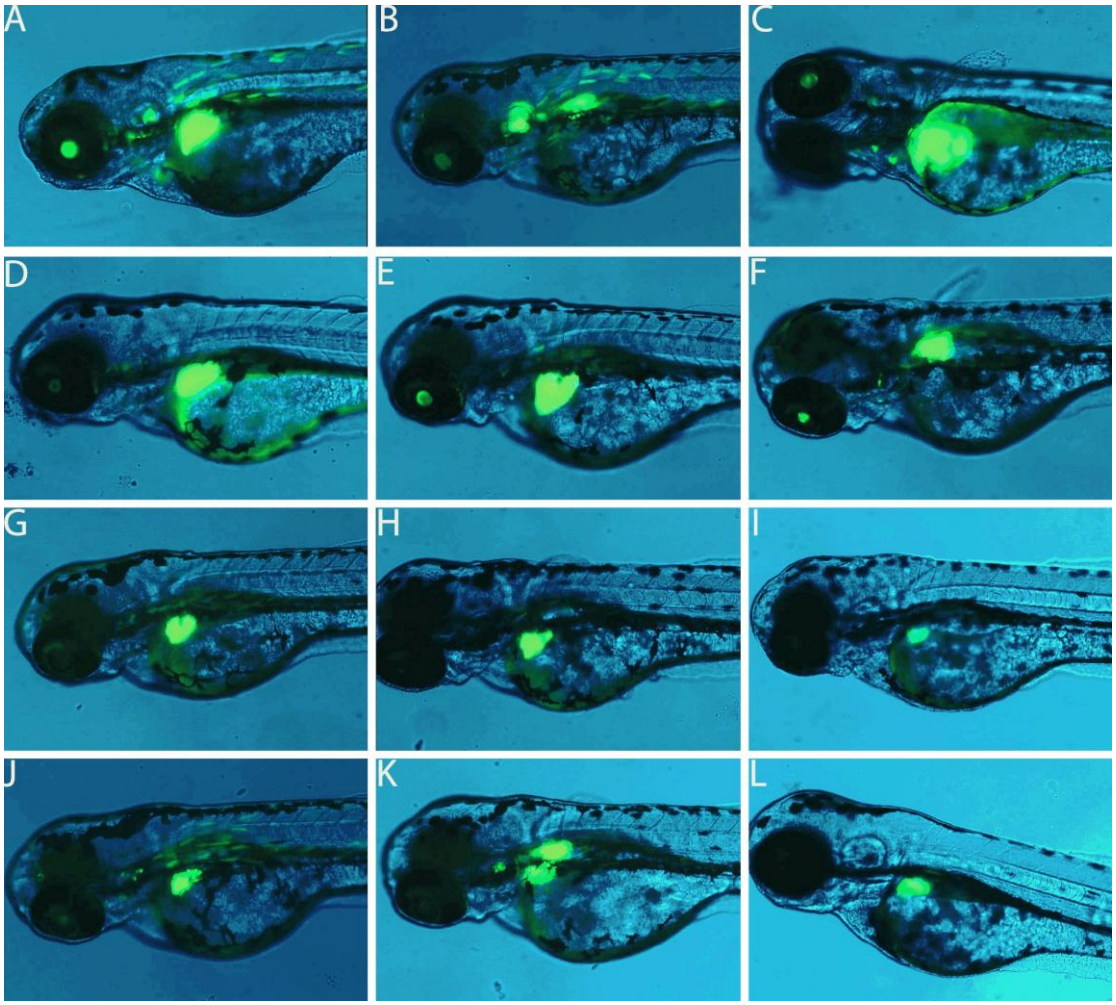


Figure 2.2 Establishment of oestrogen responsive F1 transgenic zebrafish (as assessed at 3 dpf). Fertilised eggs were collected from F0 breeding transgenic fish and exposed to 100 ng/L EE2 for 3 days to determine GFP expression. A, B and C were chosen to establish oestrogen responsive transgenic zebrafish lines.

2.6. Analysis of GFP expression

For compound microscopic analyses, the embryo was lifted into a pasteur pipette and gently placed onto a depression slide. A drop of 3% methyl cellulose was applied to the embryos. The embryo was then oriented with a fine pipette tip and observed under a fluorescent microscopy (LEICA DM1 4000 B). Microscope settings, including saturation (0.85), gamma (0.7) and gain (2.8x), were kept constant throughout the experimental analyses. 700 ms exposure time was used for fluorescent and auto-exposure was used for normal light. A magnification of X10 was used to view the presence of GFP in the specimens unless more detail was required, in which case the magnification was increased to X20. Identical pictures were taken both in normal light and fluorescent.

For the confocal microscopy (that has a greater resolution power), embryos were mounted in 0.7 % agarose (low melting point) and added to an embryo medium. The images of larvae oriented in a lateral, dorsal and ventral side were photographed under 10X magnification on a confocal-laser scanning microscope (ZEISS LSM510 Meta). Images were prepared with Adobe Photoshop 7 software to adjust light and contrast, as required.

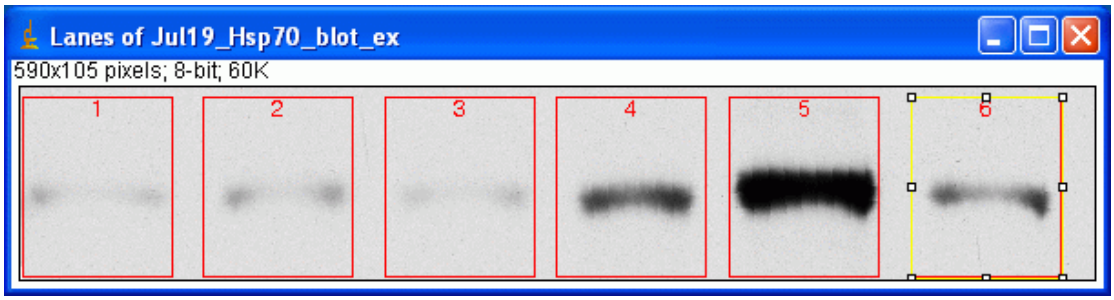
2.6.1. Analysis of GFP expression - Western Blot analysis

Western blot analysis was performed to quantify the expression of GFP. 10 embryos were transferred to a 1.5ml tube filled with 100 µl of lysis buffer (2 ml of 2x sample buffer, 500 µl of 2-Mercapto ethanol and 2.5 ml of distilled water), incubated at 95 ℃ for 5 minutes and the vessel tapped to get them to the bottom of tube. Next, the samples were homogenised five times and centrifuged for 1 min at 1000rpm. 50 µl of protein samples were applied to 5% polyacrylamide-SDS gel and subjected to electrophoresis at 110 V for 2 hour and separated proteins were transferred to nitrocellulose membrane. The membranes were blocked for 1 hour in blocking solution (5% skimmed milk in 1x phosphate buffered saline [PBS] + 0.1% Tween [PBSTx], and washed 3 times with distilled water (ddH_2O). The membranes were incubated overnight at 4 ℃ with primary antibody, rabbit anti-GFP (ams Biotechonology), diluted 1:2500

in blocking solution. The membranes were washed for 3 x 15 minutes in PBSTx. The membranes were then incubated with HRP-Goat Anti-Rabbit IgG (Invitrogen, Carlsbad, U.S.A) at 1:2000 in blocking solution for 2 hours and then washed 3 x 15 minutes in PBSTx. For detection, western blotting luminol reagent (Thermo Scientific) was used. The intensity of GFP was analysed using Image J (http://rsbweb.nih.gov/ij/), normalised to the intensity of alpha-tubulin band and indicated as fold increase in GFP over the level in control larvae.

2.6.2. Data analysis using Image J techniques

Western blot data was quantified using Image J t to compared the intensity of bands. To do this, X-ray film of western blot data was scanned as grayscale image and set to the highest resolution (600 dpi). The scanned film file was opened and converted the image to grayscale. The scanned image was vertically oriented and the rectangular selection tool palette was chosen and the rectangle was drawn around a first lane. The band of interest was encompassed in area of the band above and below. Arrow key was used to move the rectangle over the next lane.



Results

File Edit

	area	percent
1	344.6274	2.5892
2	495.5772	3.7233
3	242.2635	1.8202
4	2597.5980	19.5162
5	8089.5685	60.7782
6	1540.3553	11.5729

From the results window each of the measurements of the selected rectangle area was recorded, as above. The results were pasted into an Excel spreadsheet and the mean value for each band multiplied by the pixel value to provide an integrated measure of the intensity and size of the band. The absolute intensity of each sample band was divided by the absolute intensity of a standard to provide a relative intensity for each sample band. If the band had a relative intensity lower or higher than 1, it has less protein or more protein than the standard, respectively. The mean value was the average gray value (from 0-255) for the area inside selection. The pixels value is the number of pixels contained in selection area.

CHAPTER 3

Generation of plasmids constructs for detecting oestrogenic chemicals

CHAPTER 3

Generation of plasmids constructs for detecting oestrogenic chemicals

Abstract

In order to study the effects of oestrogenic endocrine disrupting chemicals (EDCs) in fish, a transient expression assay and transgenic zebrafish responsive to oestrogen were developed. These systems included the synthesis of a new plasmid containing three oestrogen response elements (3ERE), with a fused minimal promoter, a TATA box and the reporter, GAL4FF. This paper describes the sequential construction of this plasmid and its validation in the transient expression assay.

During the development of the plasmid we made five progressive constructs for investigation, namely pKS_ERE, pKS_ERE-TATA, pKS_ERE-TATA-Gal4ff, pCS2+_ERE-Galf4ff and pBR_Tol2-ERE-Gal4ff. When the pKS-ERE-TATA-Galf44 plasmid was injected into zebrafish embryos, Gal4ff mRNA was induced (detected via using *in-situ* hybridisation). In the next step, having constructed the linearised plasmid pCS2+_ERE-TATA-Gal4ff containing a polyadenilation signal, this construct was injected into transgenic UAS-GFP zebrafish embryos at the 1-2 cell stage and the GFP expression measured in response to the natural steroid oestrogen 17ß-oestradiol (E2) and the synthetic oestrogen 17α-ethynyloestradiol (EE2). After 24h no GFP expression was observed in embryos in unexposed control embryos, but GFP expression was strongly induced in oestrogen treated embryos. In embryos exposed to EE2 there was a concentration related expression of GFP in the skin epithelium (100 ng/L and 1000 ng/L), and a weak expression was detected at 10 ngEE2/L. For E2 exposure, specific and mosaic expression of GFP was detected in the lens, skin epithelium and many other cells in both 100 ng/L and 1000 ng/L E2 treated groups, but no expression was detected at 10ng E2/L. The final construct pTol2_3ERE-TATA-Gal4ff, containing *Tol2* to facilitate enhanced integration to the genome showed tissue specific GFP expression on exposure to EE2 (100 ng/L) in the heart, otic vesicle and somite muscles when injected into transgenic UAS-GFP zebrafish.

Our results show that the plasmid containing pTol2_3ERE-TATA-Gal4ff was responsive to oestrogen with potential use in a transient assay for screening chemicals with oestrogenic activity using embryos and early life stages. Our findings from the transient expression assays (response sensitivity) suggest that the use of the two step amplification system in the signalling process should enable the production of transgenic fish that are highly responsive to oestrogen exposure.

Keyword: Oestrogen response elements; oestrodaiol-17; *in-situ* hybridisation; Transient expression assay

Introduction

A number of studies have been reported a dramatic rise in the number of known endocrine disrupting chemicals (EDCs), as systematic processes for screening and testing chemicals for this activity have been implemented and more and more laboratories have entered into this research field. EDCs include various groups of chemicals, including synthetic and natural steroid oestrogens, that can alter the control of gene expression and interfere with homeostatic feedback loops to disrupt developmental and reproductive processes (Myers *et al*., 2003). Despite the fact that many EDCs identified are oestrogens, their interactions with the oestrogen receptor (ERs) have not been well established. This information is essential to unravel how oestrogenic EDCs interact within the body as a whole to better understand what the health implications for these exposures might be.

The oestrogen receptor (ER) is a member of the steroid/nuclear receptor superfamily of proteins that act as hormone-inducible transcription factors (Mangelsdorf *et al*., 1995) and can control a variety of physiological and developmental processes by regulating the expression of specific genes at their target sites (Sathya *et al*., 1997). 17β-oestradiol (E2) binds to the ligand-binding domain and the ER undergoes conformational changes leading to dimerisation. 17β-oestradiol (E2) binds in the ligand-binding domain and the ER undergoes conformational changes that lead to ER dimerisation. The E2-ER homodimer complex recognises and binds to oestrogen responsive elements (ERE) which are present in the regulatory regions of the promoter region of E2 target genes. The ERE is able to interact with other transcription factors, such as vitellogenin, ER, or progesterone receptor, to regulate oestrogen-target gene transcription (McKenna *et al*., 1999; Paige *et al*., 1999). The ERE was first identified by aligning the promoter regions of the *Xenopus laevis* genes A1, A2, B1 and B2 and chicken apo-VLDLII gene yielding a 13bp palindromic inverted repeat 5'GGTCAnnnTGACC3' (n, any nucleotide) (Klein-Hitpass *et al*., 1988; Stokes *et al*., 2004; Walker *et al*., 1984). Roughly twenty oestrogen responsive genes have been identified and their oestrogen responses characterised in transiently transfected cells (reviewed in Klinge, 2001; Stokes *et al*., 2004). Among these genes, only the vitellogenin A2 gene encodes the consensus palindromic ERE and

all other known natural oestrogen response genes contain imperfect EREs that differ from the consensus sequence by one or more base pairs (bp), and confer different levels of ER transcriptional activation compared with the vitellogenin ERE (reviewed in Klinge, 200l; Hyder *et al*., 1999; Stokes *et al*., 2004). ER α and β bind with high affinity to EREc38 (Tyulmenkov *et al*., 2000).

Transgenic research has developed in fish since 1990 although transgenic mouse models been important in the study of gene functions in whole animals since the early 1980s. There have been a number of studies using constructs that describe the generation of recombinant fish in different fields of research. The transgenic fish technique requires a specific promoter and an easily assayable reporter gene such as a green fluorescent protein (GFP) that allows the expression of the transgenes to be identified. Studies with tissue-specific promoters have become a powerful tool to recapitulate endogenous gene expression programs (Higashijimas *et al*., 1997; Long *et al*., 1997) and to analyse the function of gene promoters (Ju *et al*., 1999; Muller *et al*., 1999). However, one of many difficulties of making a construct is that it is time consuming because it often produces insertion mutation.

The GFP gene was originally isolated from jellyfish *Aequorea Victoria* and is widely used as a reporter gene to investigate tissue-specific patterns of gene expression and cellular localisation of proteins. The fluorescence of its protein product, GFP, can be conveniently detected in living cells (Chalfie *et al*., 1994; Prasher *et al*., 1992; Tsien, 1998). A number of reports have demonstrated that GFP can be used effectively to illustrate protein expression in live embryos and juvenile zebrafish (Amsterdam *et al*., 1996; Peters *et al*., 1999). GFP and luciferase, in combination with pollutant-responsive promoters, have been stably integrated into zebrafish and progeny that have subsequently been used successfully to screen for several classes of chemicals. Various sub-acute effects have been determined using transgenic fish including mutagenic effects (Amanuma *et al*., 2000), cadimium-toxicity by induction of heat-shock proteins (Blechinger *et al*., 2002), aryl hydrocarbon receptor-mediated toxicity (Mattingly *et al*., 2001), oestrogenicity (Chen *et al*., 2010; Kurauchi *et al*., 2005; Legler *et al*., 2000; Salam *et al*., 2008; Zeng *et al*., 2005) and anti-oestrogenicity (Schreurs *et al*., 2002).

In the creation of transgenic (TG) animals various activation sequences have been employed (Distel *et al*., 2009). The Gal4-UAS (Upstream Activation Sequence) system is an activation sequence that has been widely used in the Drosophila (Duffy, 2002). It has also been tested successfully in mice (Ornitz *et al*., 1991), zebrafish (Scheer and Campos-Ortega, 1999) for the overexpression of transgenes. The yeast transcription activator Gal4 binds its target sequence UAS to activate target gene transcription. The Gal4-UAS system has been widely used to regulate gene expression in a cell-specific and temporally restricted manner and has provided a powerful tool to examine the function of genes during development and monitor subcellular structures and target tissues for selective ablation or physiological analyse (Halpern *et al*., 2008). This system has been popular in zebrafish in cell type-specific ablation studies, the mapping of neuronal circuits and the inhibition of neuronal activity in distinct neuronal populations (Asakawa *et al*., 2008; Davison *et al*., 2007; Distel *et al*., 2009; Scott *et al*., 2007).

Despite the fact that a number of studies have used tissue specific promoters using Gal4-UAS system in transgenic zebrafish, this has not been applied to the detection of endocrine disrupting chemicals (EDCs). The Gal4-UAS system has been improved by adopting the so-called *Tol2* transposon system (Kawakami *et al*., 2004; Kawakami *et al*., 2007). The *Tol2* transposable element is derived from the medaka and belongs to the hAT family (named for *hobo*, *Ac* and *Tam3*) and integrates into genome by a "cut-and-paste" mechanism (i. e. the *Tol2* transposon can move on its own, inserting or excising itself from the genome) (Koga, 2004; Kondrychyn *et al*., 2009). The *Tol2* transposon has become popular in the development of transgenic fish because it has been found to reduce mosaicism and to improve the germ-line transmission of transgenes (Hermanson *et al*., 2004).

The main aim of this thesis was to produce a transgenic fish capable of detecting oestrogenic EDCs with high sensitivity and in a stable transgenic line. In order to develop sensitive transgenic zebrafish, one of the most critical steps is the development of an effective construct. The first major step in the development of the ERE-TG zebrafish was the construction of a new plasmid pERE-TATA-Gal4ff that contained three copies of oestrogen response elements (3ERE) capable of binding all three ER subtypes, and Gal4ff. To enhance the sensitivity of our

TG fish, we used Gal4-UAS system that has not been used previously for generating biosensor zebrafish. The plasmid construction process is a long and tedious one and we therefore systematically investigated the functional capability of constructs in their incomplete forms (pKS_ERE-TATA-Gal4ff, pCS2+_ERE-TATA-Gal4FF). We examined the temporal expression pattern of Gal4ff during zebrafish development using a transient expression assay via *in situ hybridisation* and green fluorescence microscopy. In this paper, we explain the steps in the development of our plasmid and report the results of testing the plasmid. The final construct was tested in a transient expression assay in the next chapter.

Material and Methods

Construction of Reporter Gene Vectors with Oestrogen Responsive Promoters.

Construction of pKS-ERE

The transgenic plasmids were made in 5 progressive steps. The first construct (pKS+_3ERE) was first made containing an oestrogen responsive element (ERE; see figure 1). The ERE was based on the sequence of EREc38 (EREc38 5'- CCAGGTCAGAGTGACCTGAGCTA AAATAACACATTCAG-3') published by Sathya *et al* (1997).

The construct was generated by Polymerase Chain Reaction (PCR) using specific primers (Forward primer: CCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAGCCAG GTCAGAGTG and reverse primer: CTGAATGTGTTATTTTAGCTCAGGTCACTCTGACCT GGCTGAATGTGTTAT). The following conditions were used for 8 PCR cycles : denaturation at 96 ℃ for 1 min, annealing at 60 ℃ for 30 sec and extension at 72 ℃ for 1 min. *Pfu* DNA polymerase was used instead of *Taq* DNA polymerase to remove the A at the 3' end. Multiple tandem copies of EREc38 (nEREc38, where n = number of ERE copies) were obtained by PCR. The EREc38 was prepared for insertion into the plasmid as follows. 100 µl of PCR products were purified by phenol: chloroform extraction and ethanol precipitation (see. Chapter 2). After purification, purified DNA was separated on a 2.5 % agarose gel and the bands that representing 2ERE, 3ERE and 4ERE were separately cut using a laser scalpel under UV light in the dark room and put into eppendorf tubes in the dark. Extraction of the DNA from the gel was performed using gel extraction kits (QIAquick Gel extraction kit, Cat. No 28704). EREc38 oligomers (20 µl) and the reporter gene vectors pKS + (pBluescript II KS+) were prepared by restriction digestion of the 3ERE with *Sma*I for 2-3 hours at 37 ℃. Both fragments, the linearised vector and the 3ERE (insert), were purified and 1 µl of purified DNA (both insert and vector) were run on an agarose gel and then, ligated for 2 hours at room temperature or overnight at 4 ℃. The ligation product was transformed using competent cells (Competent cells, $>10^7$ cfu/µg, Promega). The transformed cells were grown up in overnight cultures of *E. coli* in LB (Luria-Bertani) medium (specific information written in the chapter 2). Purification of the

plasmid was performed using the QIAprep Spin Miniprep Kit (Qiagen) or Alkaline lysis method. Insertion of the ERE promoter region was confirmed by digestion with *Xho*I and *Xba*I for 1 hour at 37 ℃ and the fragments were run on a 2% agarose gel. The orientation and nucleotide sequence of the 3EREc38 was confirmed by sequencing using T3 primer. The 3ERE fragment obtained was inserted into a pKS+ plasmid following the protocol.

Construction of the pKS-ERE-TATA

After obtaining the 3ERE fragment, a second fragment called TATA was constructed following the same protocol using the following primers: (Forward primer: GGCGTCGACTCTAGAGGGTATATAATAGATCTGCGATCTAAGTAAGCTTGG and reverse primer: CGCGGGCCCGGCTTTACCAACAGTACCGGAATGCCAAGCTTACT TAGATCG). The forward and reverse primers, respectively, contained *Sal*I, *Xba*I, *Bgl*II and *Apa*I sites. 5 PCR cycles were used for the generation of the TATA fragment generation (denaturation at 96 ℃ for 1min, annealing at 60 ℃ for 30 sec and extension at 72 ℃ for 1 min). TATA PCR products were purified by phenol: chloroform extraction and ethanol precipitation. After purification, 20 µl of purified DNA (TATA) were first digested with *Sal*I and then with *Apa*I for 4 hours at 37 ℃ and 5 µl of the plasmid, pKS+_ERE were digested with *Sal*I and then with *Apa*I for 4 hours at 37 ℃. The insert (a second fragment, TATA) and vector (pKS+ plasmid with 3ERE) were purified and ligated overnight at 4 ℃. The ligation product was transformed into *E. coli* competent cells (JM109 competent cells). The transformed cells were grown overnight on LB agar plates containing ampicillin at 37 ℃ in the incubator. 24 clones were randomly selected for overnight culture. Successful insertion of TATA was confirmed by digestion with *Kpn*I and *Sac*I for 1 hour at 37 ℃ and run on a 1% agarose gel.

Construction of the pKS+_ERE-TATA-Gal4ff

A third fragment called Gal4ff was amplified by PCR using primers (Forward primer: GCCGGGCCCGCCACCATGAAGCTACTGTCTTCT and reverse primer: CGCGGTACCGA TTAGTTACCCGGGAGC) and pCS2+Gal4ff as template. The following conditions were used for 25 PCR cycles : denaturation at 96 ℃ for 1min, annealing at 60 ℃ for 30 sec and extension at 72 ℃ for 1 min. Gal4ff PCR products were purified by phenol : chloroform extraction and ethanol precipitation. PCR product and the plasmid, pKS+_ERE-TATA, were first digested with *Apa*I for 2-3 hours at 37 ℃, purified and then digested with *Kpn*I for 2-3 hours at 37 ℃. The insert (the third fragment, Gal4ff) and vector (pKS$_+$_ERE-TATA) were purified and ligated overnight at 4 ℃. The ligation product was transformed into *E. coli* competent cells (JM109 competent cells). The cells were grown overnight on LB agar plates containing ampicillin at 37 ℃. Twenty-four clones were randomly taken for overnight culture. Successful insertion of Gal4 was confirmed by digestion with *Kpn*I and *Sac*I for 1 hour at 37 ℃ and assessing the products on a 1% agarose gel. Positive clones were sequenced using T7 primers.

Construction of the pCS2+ERE-TATA-Gal4ff

The plasmid pKS+_ 3ERE-TATA-Gal4ff lacked a polyadenine (Poly-A) tail. To add the poly-A tail at the end of the Gal4ff transgene, the DNA fragment containing ERE-TATA-Gal4ff was transferred into pCS2+ vector. The plasmid pKS+_3ERE-TATA-Gal4ff was then digested with *BamH*I and *Xho*I for 2-3 hours at 37 ℃. The *BamH*I and *Xho*I fragments of pKS+_ ERE-TATA-Gal4ff were inserted into a PCS2+ plasmid by digestion with *BamH*I and *Xho*I. This plasmid was named pCS2+ ERE-TATA-Gal4ff.

Construction of pTol2-ERE-TATA-Gal4ff

To move all DNA elements into a vector which has *Tol2* transposon system, The DNA fragment containing 3ERE-TATA-Gal4ff including the polyadenine (Poly-A) tail in the pCS2+_ERE-TATA-Gal4ff was digested and was inserted into the *BamH*I and *Not*I sites of the plasmid pBR322 vector, the latter of which already contained the *Tol2* transposable element. The insertion of 3ERE-TATA-Gal4ff including the poly-A tail was confirmed by digestion with

XhoI. Positive clones were sequenced by specific primers which we designed (upstream primer CGATGATTTCGATCTCGA downstream primer ATCGCATGCTTGTTCGAT). This plasmid was named pTol2_3ERE-TATA-Gal4ff. When each fragment was inserted into the vector, the sequence was checked each time. All procedures (gel extraction kit, QIAprep Spin Miniprep Kit (Qiagen), Alkaline lysis, restriction digestion, ligation, transformation and culture) are provided in the general materials and methods chapter (Chapter 2).

2-2. In-situ hybridisation

We examined the effectiveness of the pKS+_ERE-TATA-Gal4ff using *in situ* hybridisation on embryos to detect induction of Gal4 as a probe to ensure the plasmid was functional. There are two different ways to generate templates for RNA probe synthesis: One is digestion of DNA and the other is to use PCR. In case of DNA digestion, T7, T3 or Sp6 primer sequences are required in the 3' side of the gene in the plasmid. Otherwise, we amplified the cDNA using PCR with gene specific primers with T3 primer sequence combined at the end of the gene-specific R-primer. PCR reaction was carried out using specific primers as follows:

ER α forward primer: GGATCCATTAACCCTCACTAAGGTCAGGGGTCAGGGCTATG

ER α reverse primer: CCGTCGACGCCACCATGTACCCTAAGGAGGAGCAC

The cycle conditions for Gal4 were: 25 cycles, denaturation at 96 ℃ for 1 min, annealing at 60 ℃ for 30 sec and extension at 72 ℃ for 1 min. 100 µl of the PCR products were purified by phenol: chloroform extraction and ethanol precipitation. The probe was then prepared using the following protocol: 20 µl reaction mixtures were prepared and included Dig labeling mix, RNAse inhibitor and T3 polimerase. The mixture was incubated for 5 hours at 37 ℃ and then, 2 µl of DNase was added and incubated for 1 hour at 37 ℃. Next, 30 µl of water were added. 4M LiCl and ethanol were added to the mixture and then, the mixture was centrifuged for 5 minutes. After centrifugation, the pellet was dried for 10 minutes at room temperature and 20 µl of water added. 1 µl of RNA was run on an agarose gel to check the quality of the RNA and the

remainder was dissolved in 20 µl of hybridisation buffer (50 % formamide, 5X SSC, 5 mM EDTA, 0.1 % Tween 20, Heparin (50 µl/ml) and Torula RNA 1mg/ml). Lastly, the ERα probe was diluted 100X in hybridisation buffer just prior to use.

In order to detect Gal4 expression in zebrafish embryos, *in-situ* hybridisation was performed using the following method (devised in the laboratory of Toyama, Itoh and Steve). Embryos were fixed in 4% Paraformaldehyde (PFA)/ Phosphate buffered saline (PBS) and stored overnight at 4 ℃. The chorion was removed the following day using Phosphate buffered saline Tween-20 (PBST) and the de-chorionated embryos were placed into methanol at -20 ℃ until analysis. Embryos were washed in PBST for 1 hour at 65 ℃. 1 µl of the solution containing Digoxigenin labelled ER α probe (100X) was added to 200 µl of hybridisation buffer and incubated at 80-90 ℃ for 10 minutes, then the reaction was placed on ice for 5 minutes. Pre-hybridisation buffer was removed from embryos, and the ER α probe was diluted 200X in the hybridisation buffer. The embryos were incubated with the probe overnight at 65 ℃. The next day the embryos were washed with buffer containing 2X sodium chloride-sodium citrate buffer (SSC), 50% formamide and 0.1% Tween 20 for 1 hour, then with 2X SSC containing 0.1% Tween 20 for 30 minutes, and 0.2X SSC including 0.1% Tween20 twice for 30 minutes. The embryos were then washed with Malic Acid Buffer (MAB) for 10 minutes. Blocking solution (2.5 ml of Goat serum, 47.5 ml MAB+ 1 g blocking reagent) was subsequently added for 1h hour and then anti-Digoxigenin Antibody (AB) was added for 3-4 hours at room temperature. Anti-Digoxigenin antibody stock was diluted 50X with blocking solution and diluted 5000X for use. AB was removed and the embryos were washed PBST twice for 30 minutes. They were incubated overnight at 4 ℃ and the next day they were washed with PBST for 30 minutes. After washing with PBST, Alkaline Phosphatase (AP) buffer was added to wash for 10 minutes. For staining, AP buffer was removed, and the embryos added to BM purple (AP substrate) to visualise signals. This was done in to 24 well plates and after staining, the embryos were washed with PBST for 10 minutes and then, washed with PFA and stored at 4 ℃.

Chemicals

17β-oestradiol (E2) (98% purity) and 17α-ethynyloestradiol (EE2) (≥98% purity) were purchased from Sigma Chemical Co.Ltd. The stock solutions of EE2 and E2 were prepared in acetone at a concentration of 1 µg/ml and diluted as required for the embryo exposures. Chemical stock solutions were stored at 4 ℃, until required for the exposures. Prior to exposure, the solvent was evaporated under a stream of nitrogen and the working solutions made up with water and stirred vigorously for 1 day.

Chemicals were added to glass Petri dishes in the treatment solutions. Forty embryos per concentration were exposed for up to 72 hours at 28 ℃. All experiments were run in duplicate and were repeated at least five times.

Microinjection and exposure

UAS-GFP Zebrafish were maintained at 28 ± 1 ℃ under a 14h light/ 10h dark photoperiod and were fed Tetramin dry tropical flake food (Tetramin; Tetraweke, Melle, Germany) twice a day. Fertilised UAS-GFP zebrafish eggs were collected within 10 minutes of fertilisation. The plasmid pKS+_ERE-TATA-Gal4ff was digested with *BamHI* for 2 hours and then, purified by phenol: chloroform extraction and ethanol precipitation. The concentration of DNA (20ng/µl or 80ng/µl of linearised plasmid pCS2+_ERE-TATA-Gal4ff) was examined for injection to embryos without being toxic. We also examined the percentage survival between wild type embryos and the embryos with the plasmid. After finding an ideal concentration for injection, the linear plasmid was injected into 1-2 cell staged transgenic UAS-GFP (green fluorescent protein) zebrafish embryos. The concentration of plasmid was 18ng/µl and phenol red dye (red dye is used for visualisation) was added to make the solution visible to aid the injection work. Embryos were microinjected at the 1-2 cell stage with 2nl DNA using microinjector (INTRACEL, PICOSPRITZER® III) under a dissecting microscope (NIKON SMZ1500), and the injected embryos were exposed to E2 (1000 and 100 ng/L) or pure water for 24 hours at

28 ℃ for *in-situ* hybridisation. The plasmid pCS+_ERE-TATA-Gal4ff was digested with *EcoRV* for 2 hours and then, purified using phenol: chloroform extraction and ethanol precipitation. The linear pCS+_ERE-TATA-Gal4ff was injected into 1-2 cell stage which were then treated with E2 (1000, 100 and 10 ng/L), EE2 (1000, 100 and 10 ng/L) or pure water for 24 hours at 28 ℃ after which green fluorescence was observed under a microscope. The pTol2_ERE-TATA-Gal4ff was injected into 1-2 cell stage embryos and the injected embryos were exposed to 100 ng EE2/L for 72 hours at 28 ℃ and the green fluorescence observed under a microscope

Results

The progressive construction of the five constructs called, pKS-3ERE, pKS-3ERE-TATA, pKS-3ERE-TATA-Gal4ff, PCS-ERE-TATA-Gal4ff and pBR_3ERE-TATA-Gal4ff, the final of which was used in a transient assay and for the generation of TG fish is shown in Figure1.

Construction and testing of the pKS2+_ERE-TATA-Gal4ff

In the first instance, specific primers of EREc38 were used in PCR reactions to produce the required EREs (Figure 2). The centre-to-centre spacing of the consensus EREs and the distance between adjacent half-EREs in these constructs was 38 nucleotides. We optimised and found 8 PCR cycles was the most effective condition to generate the 3ERE. DNA bands representing 2ERE, 3ERE and 4ERE were produced successfully (Figure 3) and were cut from the gel and then inserted into pBluescript II KS+ [pKS(+)] with *SmaI* (Figure 1B). The constructs containing EREc38 were called pKS(+) 2EREc38, pKS(+) 3EREc38, pKS(+) 4EREc38 (Figure 4). After checking the three different ERE constructs, the 3ERE sequence was chosen to make our plasmid because it has been shown to be more sensitive to oestrogenic compounds compared to the other ERE repeats (2ERE and 4ERE) in a cell culture system (Santhya *et al*., 1997).

A total of 14 clones were subcloned and 10 clones were positive (i.e. the vector pKS(+) contained the insert 3ERE.). Four of them were sequenced and mutations had occurred in some of these, but we found some constructs with no insertion mutation. After the 3ERE fragment was subcloned into the vector (pKS-ERE, Fig. 1B), a second fragment, TATA, was inserted into pKS-ERE (Figure 1C). TATA was inserted into pKS-ERE in the *Sal*I and *Apa*I sites. Many mutations occurred as did inappropriate orientations by inserting the TATA fragment. A total of 22 clones were subcloned and 19 clones were positive. Ten clones were found to have the insertion in the right direction. The right orientation of the TATA fragment in pKS-ERE was confirmed by digestion with *Kpn*I and *Sac*I. The size of the insert when it was incorporated in the right direction was 288bp (204bp corresponded to an insertion in the wrong direction) (Figure 5).

After successful insertion of TATA, Gal4ff was inserted into pKS2+_ERE-TATA at the *Apa*I and *Kpn*I sites (Figure 1D). A total of 8 clones on the LB plate were subcloned and successful insertion of Gal4 was confirmed by digestion with *Kpn*I and *Sac*I. There were 4 positive clones and these were checked and the sequence and two of them had no mutation. The plasmid which contained the oestrogen responsive element (ERE), TATA and Gal4ff was called pKS-ERE-TATA-Gal4ff (Figure 1E, and 6).

In situ hybridisation

There was no expression of the Gal4 gene in the control group. However, there was expression in both of the E2 treated groups. The expression of the Gal4 gene in the embryos which were exposed to a high E2 concentration showed mosaic patterns in the skin and yolk (i.e. they showed ubiquitous expressions in the yolk) (Figure 7). The whole-mount embryos showed a E2-concentration dependent induction of Gal4 mRNA-expression.

Construction and testing of the pCS2+_ERE-TATA-Gal4ff

After the construction of the pKS+_ERE-TATA-Gal4ff, we still needed to add two more elements (poly A and Tol2) to generate an effective transgenic vector. For that reason, the plasmid pKS+_ 3ERE-TATA-Gal4ff was, after digestion with *BamH*I and *Xho*I, inserted into a pCS2+ plasmid. (Figure 1F). A total of 22 clones were subcloned and 8 clones were positive (i.e. the vector pCS2+ contains the insert ERE-TATA-Gal4ff.). All of them were checked for the appropriate sequence. The rate of insertion mutation was in 1 out of 8 and eventually we found a clone that contained no insertion mutation. It was called pCS2+_ERE-TATA-Gal4ff (Figure 8).

95 % of the embryos injected with 80 ng/µl of DNA (the plasmid) died, but those alive showed an abnormal development (Figure 9). The injection amount was thus reduced to 20ng/µl., and this resulted in no adverse effects. Both groups (wild type embryos and the embryos with the plasmid) showed that the injection process itself had no harm (Figure 9). Our

results show that injection of 20 ng/µl of the plasmid was not toxic and provided an adequate concentration of DNA in the embryos.

Transient expression detected via green fluorescent protein

There was no GFP expression detected in embryos in unexposed controls (Figure 10A), but GFP expression was strongly induced in the anterior and posterior parts of the body in 24h embryos after EE2 exposure at 100 ng /L and 1000 ng /L (Figure 10C-D). The number of cells expressing GFP showed a concentration related response across the different oestrogen treatment groups. A weak expression only of GFP was observed in the 10 ngEE2/L treatment (Figure 10B). In contrast with EE2, no GFP expression was detected in the low E2 concentration group (10 ngE2/L) (Figure 10F). However, specific and mosaic expression of GFP was detected in the lens, skin epithelium and many other cells in both the low (100 ngE2/L) and high (1000 ngE2/L) treatment groups after 24h exposure (Figure 10G-H). There was no morphological effect and mortality due to oestrogen treatment and/or the microinjection procedure. Yolk region showed a low background autofluorescence in all treatment groups.

Construction and testing of the pTol2_ERE-TATA-Gal4ff

The final construct contained *Tol2* (pTol2_ERE-TATA-Gal4ff) (Figure 11) in an attempt to enhance the frequency of transgenesis and to improve the sensitivity of the oestrogen detection system (Figure 1G and 12). We used the transient expression assay in response to oestrogen in UAS-GFP transgenic zebrafish to assess whether this construct could reduce mosaicism and show tissue specific expression. GFP expression occurred at 24 hours post fertilisation (hpf) in embryos injected with the construct pTol2_ERE-TATA-Gal4ff, but it was still in a mosaic expression pattern in the skin. In embryos injected with the construct pTol2_ERE-TATA-Gal4ff there was specific tissue expression at 72 hpf (Figure 12). This was not the case with the injections with the linear plasmid pCS2+_ERE-TATA-Gal4ff (Figure 8). No GFP expression was detected in the unexposed controls, but GFP expressing cells were observed in the heart, otic vesicle, somite muscle in embryos exposed to 100 ngEE2/L (Figure 12). GFP expression in

the heart was confirmed by the periodic contractile movement of the GFP expressing cells in synchrony with the heart beat in the live injected larvae.

Discussion

The main aim of this study was to generate and validate (test progressively) several transgenic plasmid vectors (in transient assays) for the eventual production of a ERE TG zebrafish. In this process we used all of the available information to decide on what insert features would likely make the most efficient construct. Using MCF-7 human breast cancer cells, Sathya and co worker's research (1997) demonstrated that three copies of the ERE produced an enhanced expression compared with 2 or 4 EREs, especially in response to E2. These data (Sathya *et al*., 1997) suggested that 3EREs are optimal for induction of an oestrogenic response. Similarly, Tyulmenkov *et al*. (2000) reported that both ERα and ERβ induce transcriptional synergy where there are three or four tandem copies of EREc38, but not with two copies of an ERE. Given these findings, we therefore adopted the same formula (i.e. 3EREs) in our construct. We used Gal4ff despite many previous studies using the Gal4-VP16/UAS system because Gal4ff is less toxic and more tolerable than GAL4-VP16 (Asakawa *et al*., 2008). Previous studies have developed their own constructs for developing oestrogen response systems such as pEREtata-LUC (Legler *et al*., 2000), pEGFP-ChgL (Ueno *et al*., 2004), pZVTG1-EGFP (Chen *et al*., 2009). Luciferase is very difficult to reliably measure in live organisms (pEREtata-LUC) and the others (pEGFP-ChgL, pZVTG1-EGFP) are liver specific genes. In addition, those systems also did not use the *Tol2* vector system which has the capability to enhance significantly the genomic integration of introduced DNA.

Construction and testing of the pKS+_ERE-TATA-Gal4ff

This *in-situ* hybridisation findings (for the plasmid pKS+_ERE-Gal4ff injected embryos stained with the Gal4 specific *in situ* probe) suggested the suitability of our plasmid for making transgenic zebrafish, although expression was weak in these preliminary studies possibly due to the use of the incomplete plasmid without the poly A component. Weak ER gene expression was observed in the low E2 concentration exposure from *in-situ* hybridation studies possibly due to lack of poly-A which is important for RNA stabilisation.

Construction and testing of the pCS2+_ERE-TATA-Gal4ff

Injection of embryos with the linear plasmid pCS2+_ERE-TATA-Gal4ff resulted in a strong ectopic GFP expression at the 24 h stage. This expression was also observed in later embryo stages. Such high and ectopic expression (the expression of a gene in an abnormal place in an organism) might be due to the general promoter SV40 in the pCS2+ (Hyatt and Ekker, 1999). Also, possibly because of this, pCS2+ has a high basal expression of GFP after injection. The pCS2 vector is frequently used as a backbone for misexpression (wrong expression) experiments (Rupp *et al*., 1994; Turner and Weintraub, 1994). The poly-A tail is added to ensure a proper translation of the protein and is an important feature for both RNA stabilisation as well as translation efficiency (de Moor *et al*., 2005; Fink *et al*., 2006).

The injection of linear plasmid in a transient assay has several drawbacks. For example, they normally showed high mosaicism and variable ectopic expression and a very low percent of integration. The reasons for this might include that the linearsied plasmid is concatemerised at a high rate after injection (Viret *et al*., 1991). Multiple gene copies are randomly integrated into the genome or placed as extrachromosomal DNA in the cell. To improve these problems, *Tol2* transposon system has been adopted in zebrafish and other vertebrates and opened new opportunities for transient assay and transgenic fish (Hamlet *et al*., 2006; Kawakami, 2007; Kawakami *et al*., 2000; Sato *et al*., 2007). The *Tol2* transposon system has been proved to improve the low efficiency of integration of germline and reduce high mosaicismis (Kawakami *et al*., 2000). The *Tol2* system was therefore applied in the next step of construction to improve mosaicism and efficiency of integration.

Construction and testing of the pTol2_ERE-TATA-Gal4ff

Injection of embryos with the plasmid pTol2_3ERE-TATA-Gal4ff showed tissue-specific expression at the 72 h stage compared to injection of linear plasmid (pCS2+_ERE-TATA-Gal4ff). These data proved that the *Tol2* transposon system enhanced tissue-specific expression. As a preliminary study, we tested the final plasmid pTol2_3ERE-TATA-Gal4ff and showed the

final plasmid (pTol2_3ERE-TATA-Gal4ff) system worked well detecting oestrogenic responses. We did not however, inject transposase mRNA with pTol2_3ERE-TATA-Gal4ff which would likely have resulted in a stronger and more specific tissue expression. Co-injection with the plasmid containing the *Tol2* element and transposase mRNA produce more consistent expression as the foreign DNA integrates very efficiently during early development (Kawakami *et al*., 2000). Thus further investigation on this examining the responsiveness of the completed construct pTol2_3ERE-TATA-Gal4ff with *Tol2* transposase mRNA was conducted using the transient expression assay in the next chapter. We also tested the construct in exposures to a variety of oestrogenic endocrine chemicals. The construct was applied to generate transgenic fish for studies in Chapter 5 and Chapter 6.

Concluding this chapter, results show that the plasmid containing pTol2_3ERE-TATA-Gal4ff was responsive to oestrogen with potential use in a transient assay for screening chemicals with oestrogenic activity using embryos and early life stages and with good potential to generate ERE-TG fish.

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List of Tables

Table 1. Sequence of 3ERE_TATA_Gal4FF (Red : 3ERE, Grey : TATA, Blue : Gal4ff, pink highlights: start codon (ATG) and stop codon (TAA).)

GGATCCCCCCTGAATGTGTTATTTTAGCTCAGGTCACTCTGACCTGGCTGAATGTGT
TATTTTAGCTCAGGTCACTCTGACCTGGCTGAATGTGTTATTTTAGCTCAGGTCACT
CTGACCTGGGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACTCTAG
AGGGTATATAATAGATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGTA
AAGCCGGGCCCGCCACCATGAAGCTACTGTCTTCTATCGAACAAGCATGCGATATT
TGCCGACTTAAAAAGCTCAAGTGCTCCAAAGAAAAACCGAAGTGCGCCAAGTGTC
TGAAGAACAACTGGGAGTGTCGCTACTCTCCCAAAACCAAAAGGTCTCCGCTGACT
AGGGCACATCTGACAGAAGTGGAATCAAGGCTAGAAAGACTGGAACAGCTATTTC
TACTGATTTTTCCTCGAGAAGACCTTGACATGATTTTGAAAATGGATTCTTTACAGG
ATATAAAAGCATTGTTAACAGGATTATTTGTACAAGATAATGTGAATAAAGATGCC
GTCACAGATAGATTGGCTTCAGTGGAGACTGATATGCCTCTAACATTGAGACAGCA
TAGAATAAGTGCGACATCATCATCGGAAGAGAGTAGTAACAAAGGTCAAAGACAG
TTGACTGTATCGCCGGAATTCCCGGCCGACGCCCTGGACGACTTCGACCTGGACAT
GCTGCCTGCTGATGCTCTCGATGATTTCGATCTCGATATGCTCCCGGGTAACTAATC
GGTACC

List of Figures

Figure 1. Construction of Reporter Gene Vectors with Oestrogen Responsive Promoter.

The first fragment containing 3ERE was inserted into a pBluescript II KS+ (pKS+) plasmid by digestion with *Sma*I (A). The vector, pKS+ plasmid containing 3ERE, was inserted with a second fragment TATA, through digestion with *Apa*I and *Sal*I (B). A third fragment to be inserted, Gal4ff, and vector (pKS+ plasmid with 3ERE and TATA) were digested with *ApaI* and *KpnI* (C). Three oestrogen response elements (3ERE), TATA and Gal4ff were thus inserted in the multiple cloning site (MCS) of the pBluescript II KS+ (plasmid pKS_ 3ERE-TATA-Gal4ff) (D). The *BamH*I and *Xho*I fragments of 3ERE-TATA-Gal4ff were inserted into a PCS2+ plasmid by digestion with *BamH*I and *Xho*I (E). The *BamH*I and *Not*I fragments of pCS2+_3ERE-TATA-Gal4ff including polyadenine (Poly-A) tail were inserted into the *BamH*I and *Not*I sites of the plasmid pBR322 vector containing a *Tol2* transposable element (plasmid pBR_Tol2_3ERE-TATA-Gal4ff) (F).

Figure 2. Scheme to generate multiple EREs by PCR. To generate 3 tandem repeats of EREc38, the designed forward and reverse PCR primers encoded the full EREc38 sequence and an additional first 13 base of EREc38. Therefore these two primer overlap with 26bp in a complementary manner that allows annealing of these two primers. The first extension reaction would generates a 2 tandem repeated ERE. The second PCR reaction allows some of the 2EREs to anneal in a manner shown in the diagram that allows the extension to form 3ERE. By optimising the cycle number of PCR (8 cycles), 3ERE was effectively amplified and the appropriately sized PCR product was confirmed by agarose gel electrophoresis (Fig. 3)

Figure 3. Multiple ERE products derived from PCR of the EREc38 DNA sequence. Multiple tandem copies of EREc38 (nEREc38, where n=number of ERE copies) were obtained by PCR. Lane 1; 1 kb ladder (Promega), Lane 2 and Lane 3; ERE products. The dashed lined red

rectangles represent the different multiples of the ERE. The sizes of the 2, 3 and 4EREs were 76 bp (base pairs), 114bp and 152 bp, respectively. A 100 bp DNA ladder was used as a molecular weight marker.

Figure 4. pBluescript II KS+ (pKS+) containing 2ERE, 3ERE and 4ERE which were digested *Xba*I and *Xho*I, respectively. The dashed lined red rectangle represents the 3ERE fragment. The dashed lined green and yellow rectangles represent the 2ERE and 4ERE, respectively. Lane 1, PCR product containing different number of EREs without digestion of *Xba*I and *Xho*I.

Figure 5. The vector, pBluescript II KS+ (pKS+) containing 3ERE, and a second fragment TATA, were digested with *Apa*I and *Sal*I. Lane 1, 1kb ladder, Lanes 2-11, individual clones. Successful insertion of TATA was confirmed by digestion with *Kpn*I and *Sac*I. The green lined rectangle represents the vector (pKS+) and the insert (3ERE+TATA). Red rectangles illustrate where the insert was in the right orientation (288 bp). Wrong orientation size: 204 bp

Figure 6. The pKS+_3ERE3TATA-GAL4ff construct: The insert (a third fragment, Gal4ff) and vector (pKS+ plasmid with 3ERE and TATA) were digested with *ApaI and KpnI*. Successful insertion of Gal4ff was checked by digestion with *Kpn*I and *Sac*I. The first and third lanes represent where the pKS+ plasmid did not contain the insert, 3ERE and TATA. The size of this digested fragment was 836 bp. Lane 1; 1 kb ladder, Lanes 2-5; individual clones.

Figure 7. Transient Gal4 expression assay using *in-situ* hybridisation.

Linearised plasmid pKS+_ ERE-TATA-Gal4ff was injected into 1-2 cell stage zebrafish embryos and the embryos were exposed to E2 at 100 ng/L or 1000 ng/L (B,C) or to

water (A). Embryos were fixed 24 h after injection. (Ai-Aii) None exposed injected embryos (Bi-Bii) 100ng E2/L-exposed injected embryos. (Ci-Cii) 1000ngE2/L-exposed injected embryos. The expression of the Gal4 gene in the embryos exposed to 1000 ngE2/L-showed mosaic pattern in the skin and yolk.

Figure 8. The pCS2+_3ERE-TATA-GAL4ff construct: 3ERE-TATA-Gal4ff was inserted into a PCS2+ plasmid by digestion with *BamH*I and *Xho*I. The dashed green and red rectangles represent the vector (pCS2+) and the insert (3ERE+TATA+Gal4ff), respectively. Lane 1, 1 kb ladder, Lane 2-7, individual clones. The insert size was 437 bp.

Figure 9. Survivorship of embryos without (A) or with (B) injection of linear plasmid pCS+_3ERE-TATA-Gal4ff after exposure to 17β-oestradiol (E2). Embryos were collected and exposed to 100 ng/L, or 1000 ng/L of E2, or to pure water. Embryos (n=50) were incubated at 28+/- 1 ℃ for 24 h.

Figure 10. Transient expression assay using the linear plasmid pCS2+_ERE-TATA-Gal4ff. Fertilised UAS-GFP transgenic zebrafish eggs were microinjected at the 1-2 cell stage with the plasmid pERE-TATA-GalFF. Injected embryos were exposed for 24 h to increasing concentrations of the natural steroid oestrogen 17ß-oestradiol (E2) (E-H) or the synthetic oestrogen 17α-ethynyloestradiol (EE2) (A-D). These embryos showed ectopic mosaic expression of GFP. The expression of GFP was concentration-dependent for the steroid oestrogen exposures. A-H: 24 hpf-embryos injected with PCS2+_ERE-TATA-Gal4ff.

Figure 11. The pBR322Tol2_3ERE-TATA-Gal4ff construct: The insert (3ERE, TATA, Gal4ff and polyadenine (Poly-A) tail) were inserted into the *BamH*I and *Not*I sites of the plasmid vector pBR322, the latter of which already contained the *Tol2* transposable element. The insertion of 3ERE-TATA-Gal4ff, including the poly-A tail, was confirmed by digestion with *XhoI*. The dashed green and red rectangles represent the vector (pBR322_Tol2) and the insert (3ERE+TATA+Gal4ff+ Poly-A tail), respectively. 1 kb ladder, Lane 2-7, individual clones. The insert size was 1076 bp.

Figure 12. Transient expression assay using the plasmid pTol2-ERE-TATA-Gal4ff. Fertilised UAS-GFP transgenic zebrafish eggs were microinjected at the 1-2 cell stage with the plasmid pTol2-TATA-Galff. Injected embryos were exposed for 72 h post-fertilisation to the synthetic oestrogen 17α-ethynyloestradiol (EE2) at 100 ng/L or to embryo culture water. There was no GFP expression in the control whereas tissue specific GFP expression was observed in the heart (h), otic vesicle (ov) and somite muscles (sm) by EE2 exposure. Ai and Aii: anterolateral view (control) Bi and Bii: anterolateral view (EE2)

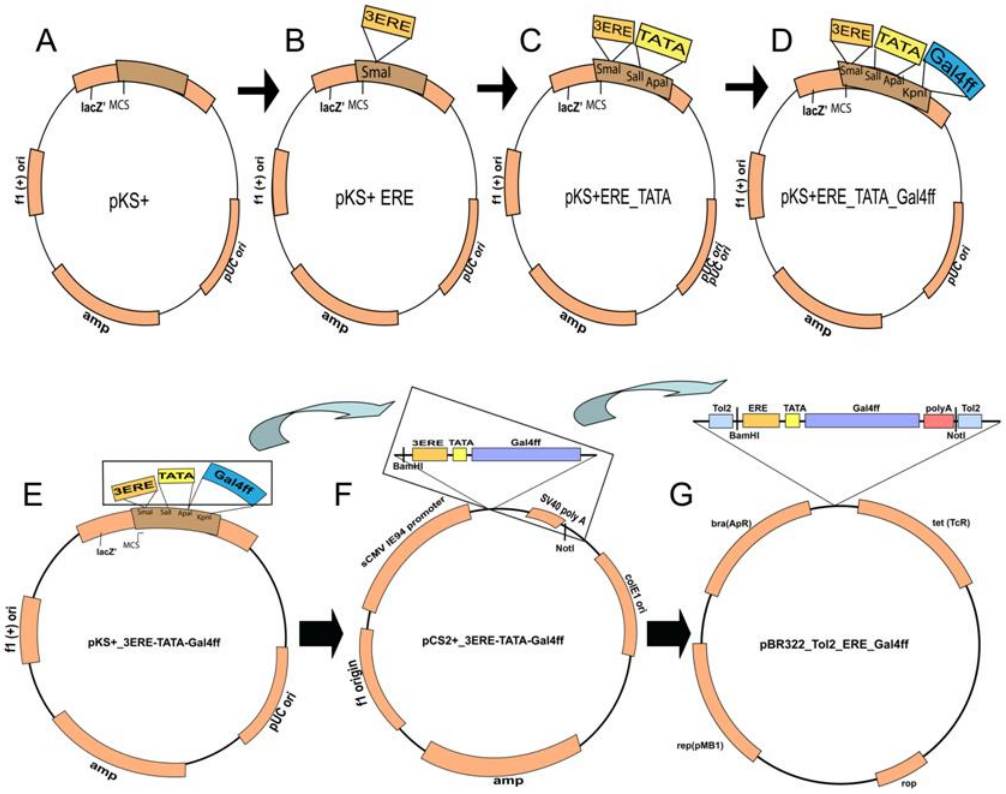

A
B
C
D
3ERE
TATA
Gal4ff
lacZ' MCS
SmaI
SalI
ApaI
KpnI
f1 (+) ori
pUC ori
amp
pKS+
pKS+ ERE
pKS+ERE_TATA
pKS+ERE_TATA_Gal4ff
E
F
G
BamHI
NotI
Tol2
ERE
polyA
pKS+_3ERE-TATA-Gal4ff
pCS2+_3ERE-TATA-Gal4ff
pBR322_Tol2_ERE_Gal4ff
sCMV IE94 promoter
SV40 poly A
f1 origin
colE1 ori
bra(ApR)
tet (TcR)
rep(pMB1)
rop

Figure 1

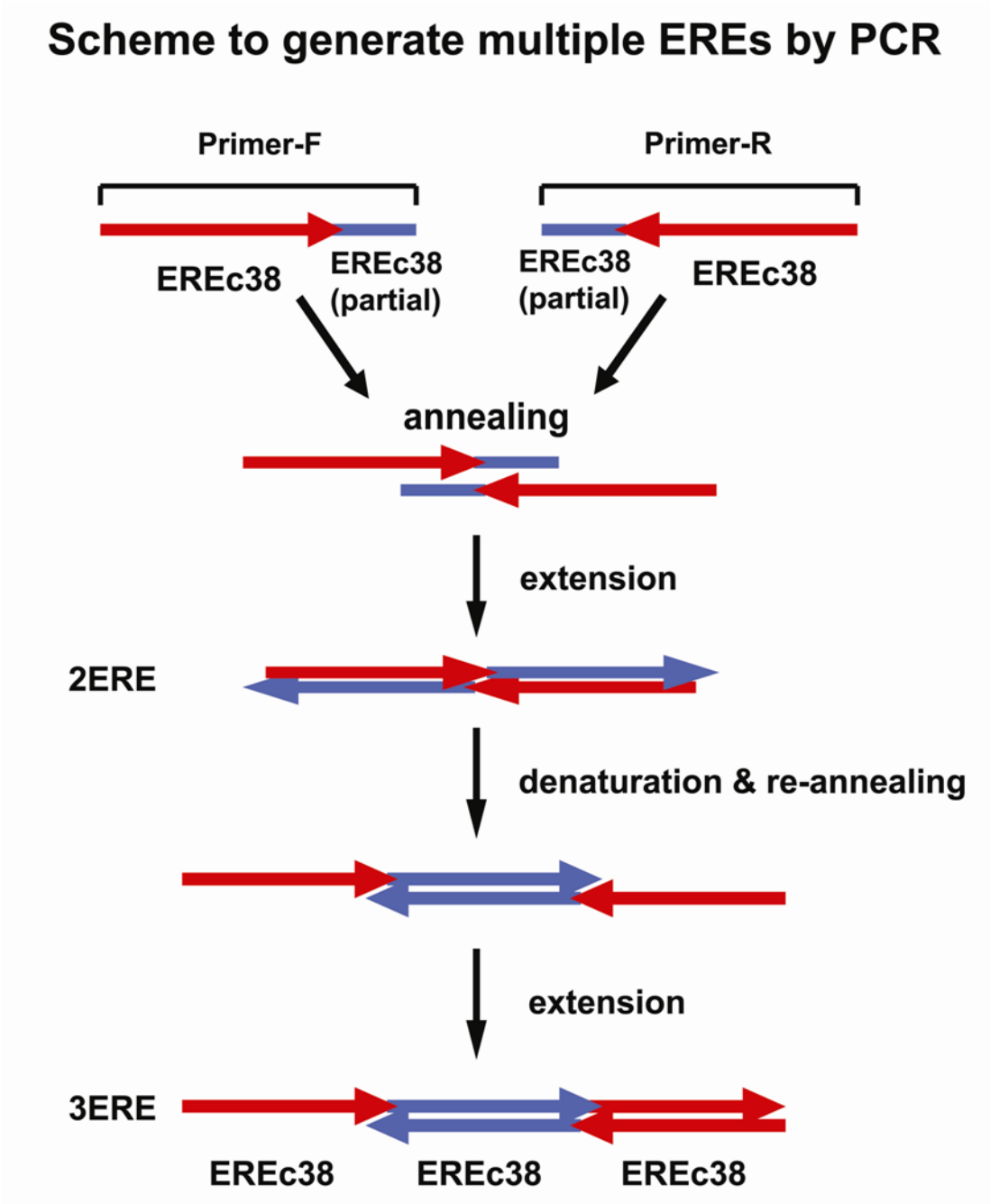
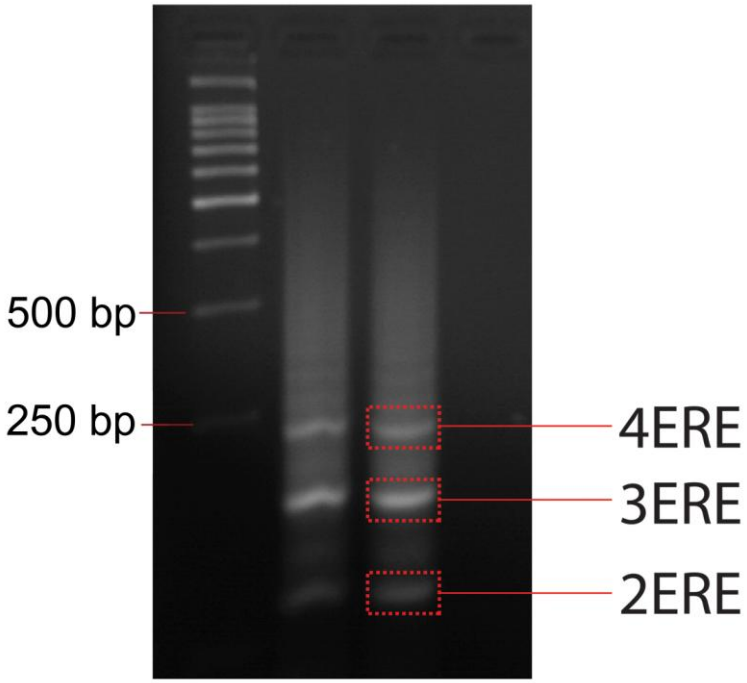

Scheme to generate multiple EREs by PCR
Primer-F
Primer-R
EREc38
EREc38 (partial)
EREc38 (partial)
EREc38
annealing
extension
2ERE
denaturation & re-annealing
extension
3ERE
EREc38
EREc38
EREc38

Figure 2



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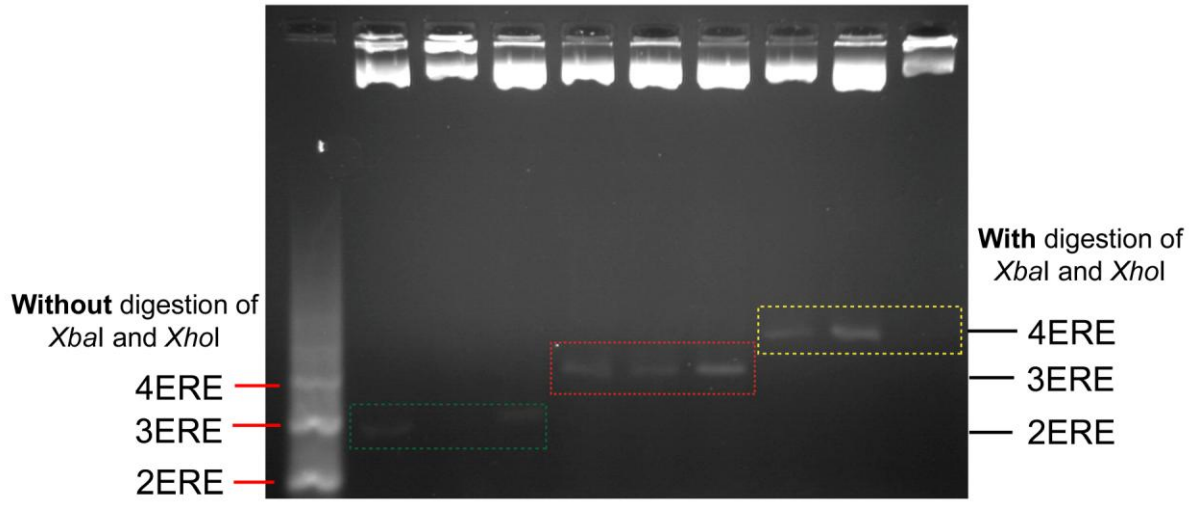

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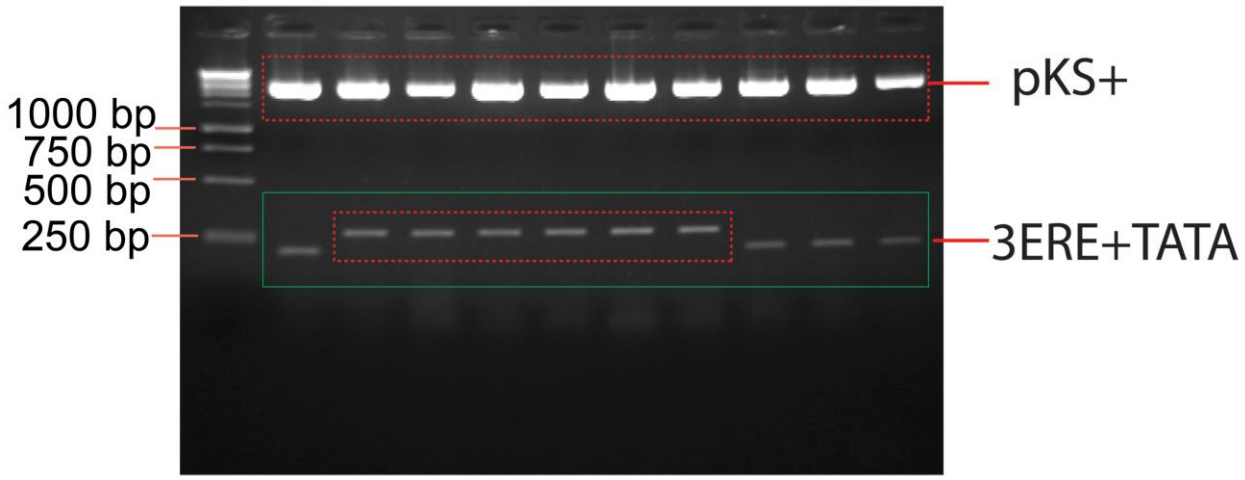

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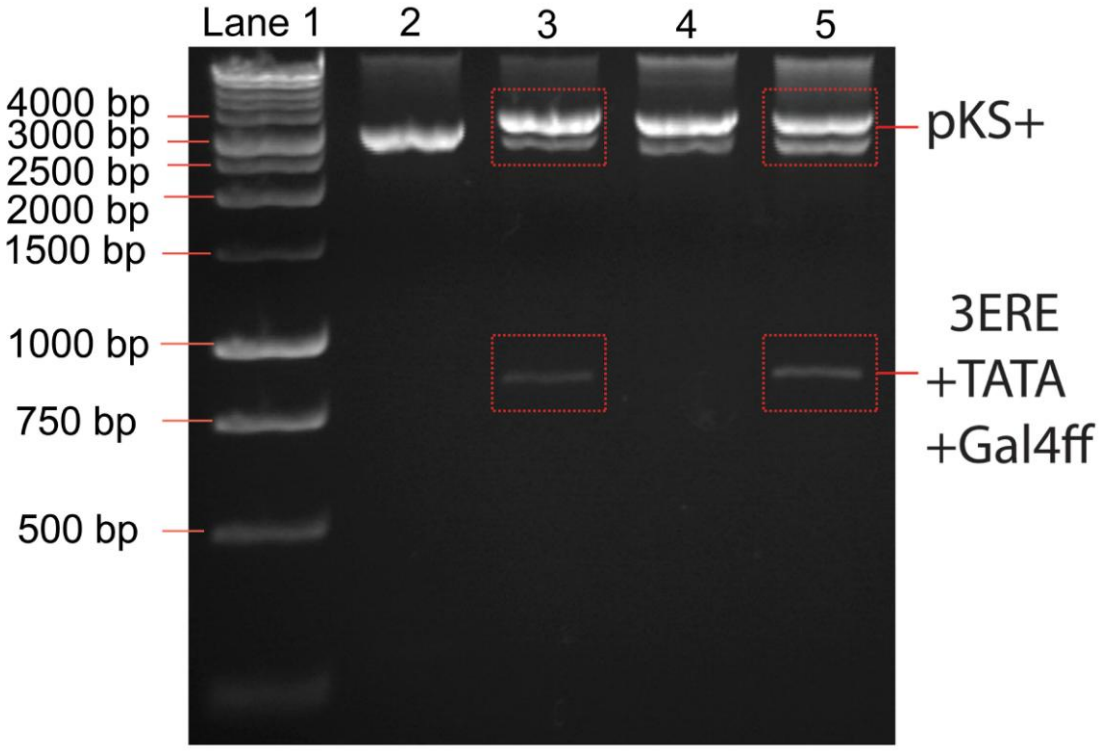
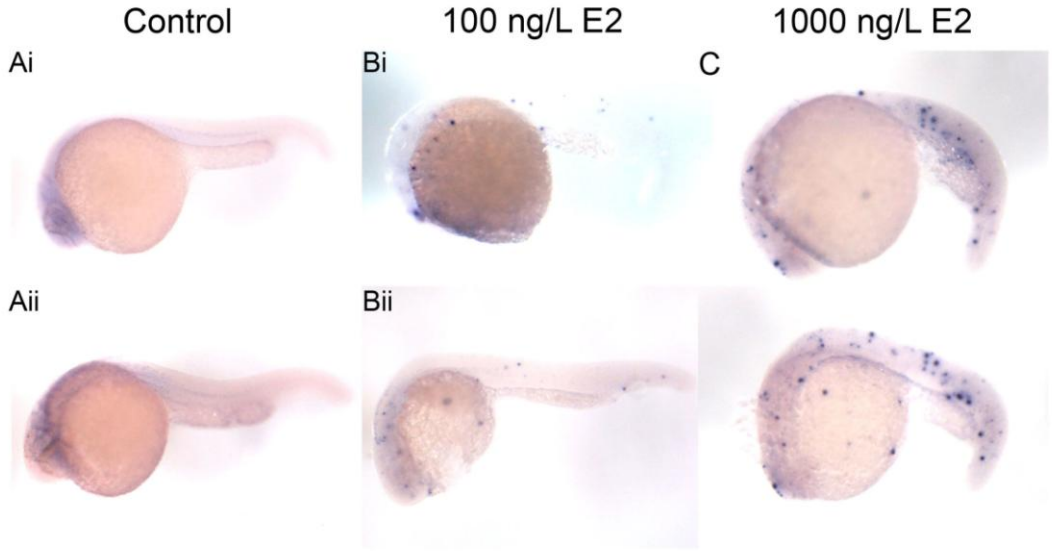


Figure 6



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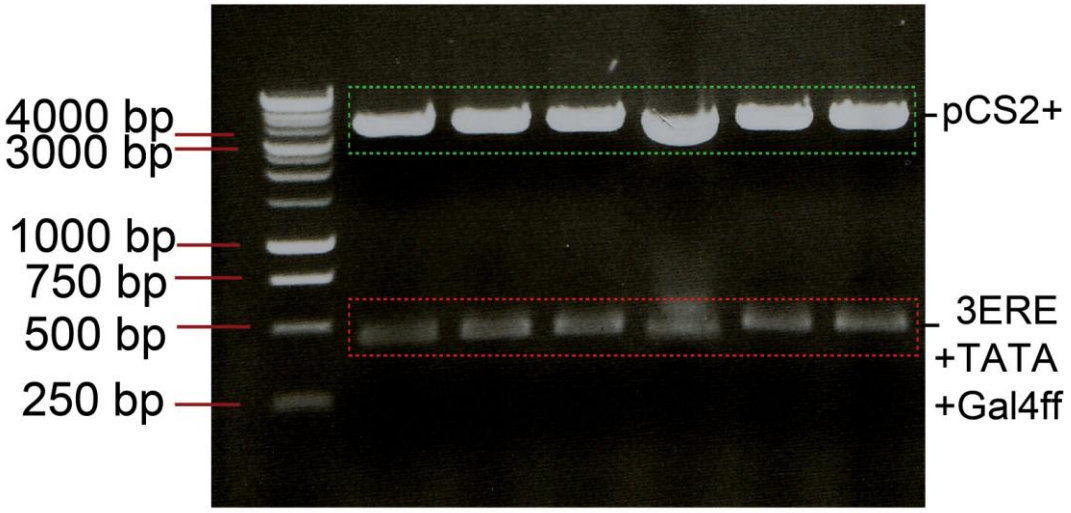

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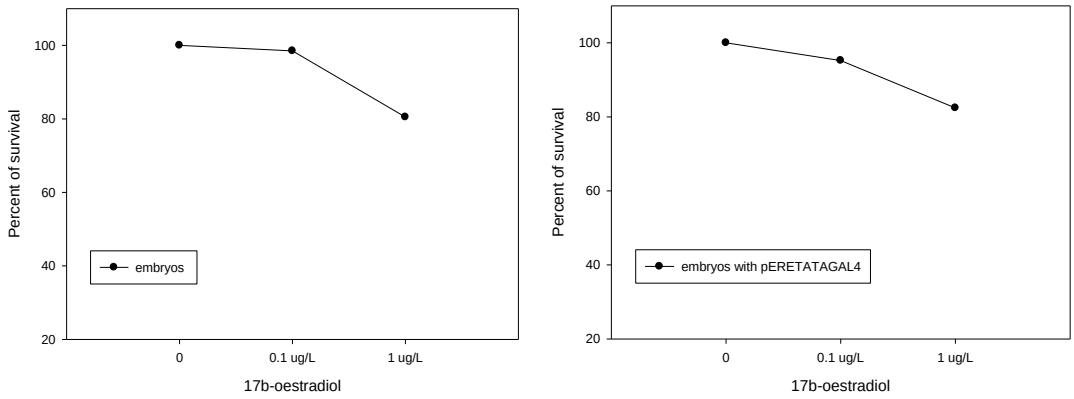

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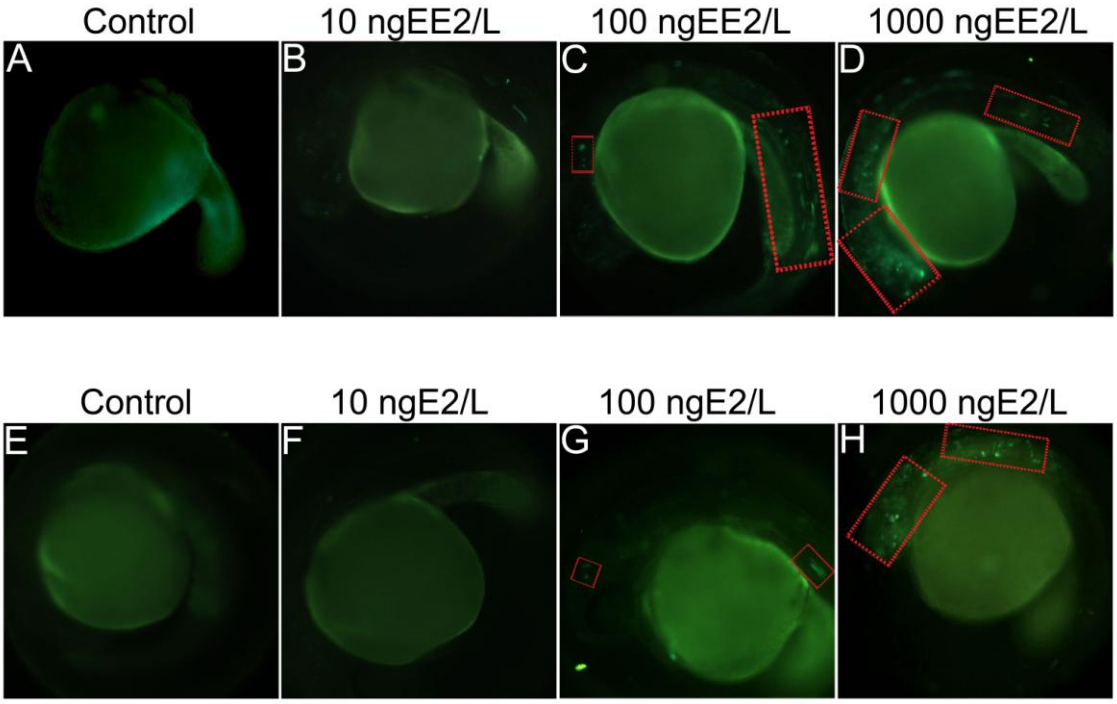

Figure 10

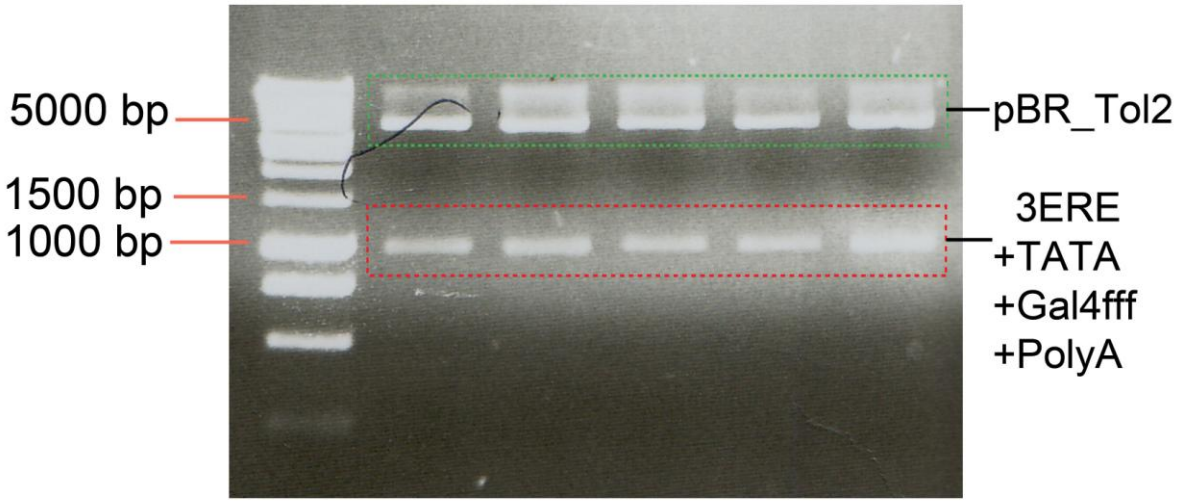

Figure 11

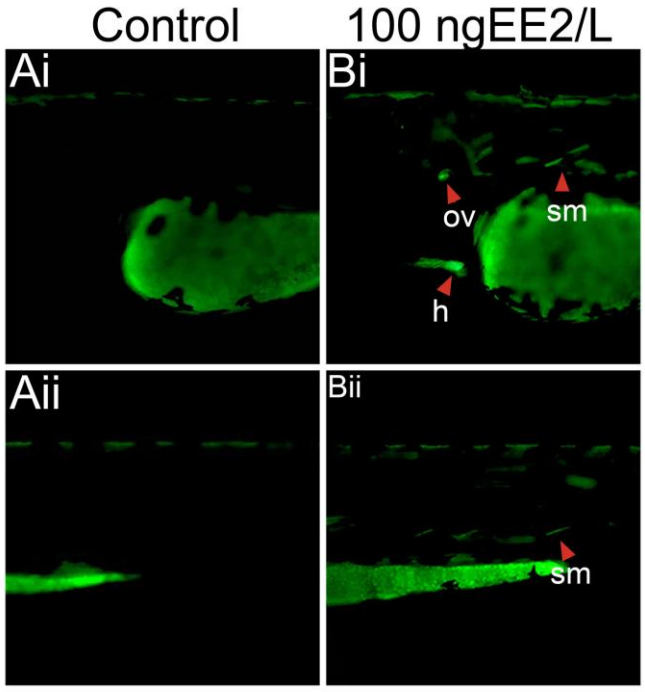

Figure 12

CHAPTER 4

Development of a transient expression assay employing a Gal4ff-UAS system for detecting environmental oestrogens in zebrafish and medaka embryos

Paper in preparation for submission to BMC Biotechnology

CHAPTER 4

Development of a transient expression assay employing a Gal4ff-UAS system for detecting environmental oestrogens in zebrafish and medaka embryos

Abstract

Oestrogenic contaminants are widespread in the aquatic environment and diverse in nature, and include both natural and synthetic chemicals. Environmental oestrogens have been shown to induce adverse effects in exposed fish, particularly affecting reproductive development and function. Here we developed a transient expression assay to investigate the effects of oestrogenic chemicals in fish early life stages and to identify sensitive target organs for oestrogenic effects. In an attempt to enhance the response sensitivity to oestrogen, we adopted the use of multiple tandem oestrogen responsive elements (EREc38) in a *Tol2* transposon mediated Gal4ff-UAS system. The plasmid constructed (pTol2_ERE-TATA-Gal4ff), contains three copies of oestrogen response elements (3ERE) that on exposure to oestrogen induces expression of Gal4ff which in turn binds Gal4-responsive Upstream Activated Sequence (UAS) elements, driving the expression of a second reporter gene, EGFP (Enhanced Green Fluorescent Protein).

We examined the response of our construct to oestrogen exposure in zebrafish embryos using a transient expression assay. The plasmids were injected separately into 1-2 cell staged zebrafish embryos, and the embryos were exposed to various oestrogens including the natural steroid oestrogen 17ß-oestradiol (E2), the synthetic oestrogen 17α-ethynyloestradiol (EE2), and the weak environmental oestrogen nonylphenol (NP), and GFP expression was examined in the subsequent embryos using a fluorescent microscopy. There was no GFP expression detected in unexposed embryos, but specific and mosaic expression of GFP was detected in the liver, heart, somite muscle and many other cells for both steroid oestrogen treatments (EE2; 10 ng/L, E2; 100 ng/L, after 72h exposures). For the NP exposures, all embryos died at the highest

concentration tested (100 µg/L) after 72h, but GFP expression was observed at an exposure of 10 µgNP/L. We also showed that our construct works in medaka, another model fish test species, suggesting the transient assay is applicable for screening of oestrogen chemicals in fish generally. Our results indicate that the transient expression assay system can be used as a rapid integrated screening system for environmental oestrogens and to detect the oestrogenic effects on embryo development in fish.

Keywords: Oestrogenic endocrine disrupting chemicals; Oestrogen response elements; UAS-GAL4; transient expression assay, zebrafish.

Introduction

Decades of research have shown that a number of natural and man-made chemicals interfere with the endocrine system and can result in adverse health effects in humans, mammals and fish (Andersen *et al*., 2003; Carlsen *et al*., 1992; Christin *et al*., 2003; Jobling *et al*., 2006). Wildlife living in, or closely associated with the aquatic environment have been shown to be especially impacted by these so-called endocrine-disrupting chemicals (EDCs), because our freshwaters, estuaries and oceans act as sinks for chemical discharges (Goodhead and Tyler 2009). Oestrogenic EDCs in the environment are of particular concern. Endogenous oestrogens are a group of closely related steroid hormones essential in the development and functioning of the reproductive system. Extensive work has been conducted on natural (e.g. 17β-oestradiol) and synthetic oestrogens (e.g. 17α-ethinyloestradiol from the contraceptive pill) and their interactions in the vertebrate body, including their tissue distributions, mechanisms of action and pathways of elimination (Blechinger *et al*., 2002). Furthermore, adverse effects of exposure to environmental oestrogens have received considerable research attention in a number of animal species, but this work is largely restricted to adults and juveniles (Filby *et al*., 2010; Garcia-Reyero *et al*., 2009), and there has been little study of effects on embryos. Nothing is known regarding the relative sensitivities of the different cell types and tissues to oestrogenic EDCs, or other contaminants, during embryogenesis. In spite of the widespread concern for EDCs in the aquatic environment, there are very few bioassay systems that are sufficiently sensitive for accurate prediction of adverse biological effects. Moreover, conventional methods of EDCs detection such as tissue culture (Bruschweiler *et al*., 1996) and *in vitro* techniques (Chen *et al*., 2009; Mazurais *et al*., 2000) are limited in their capacity to elucidate oestrogen signalling pathways and physiological impacts.

Teleost fish have three oestrogen receptors (ER), ERalpha, ERbeta-1 and ERbeta-2, that show tissue specific patterns of expression and function in adults (Hawkins *et al*., 2000; Kuiper *et al*., 1998; Sun *et al*., 1999). The different ER subtypes are also widely expressed in body tissues in early life stages, from embryos to young larvae (Tingaud-Sequeira *et al*., 2004), suggesting crucial roles of these signalling pathways in early development. Indeed, recently it

was found that knockdown of ER-beta2 in the zebrafish suppressed normal development of the lateral line neuromast cells (Froehlicher *et al*., 2009).

Endogenous oestrogen receptors activated by oestrogenic chemicals bind to these oestrogen response elements within regulatory regions of oestrogen-responsive genes. Response elements are recognized by nuclear transcription factors, including members of the steroid/nuclear receptor super family that then, together with various other regulatory factors mediate transcription of the associated downstream genes (Lefstin and Yamamoto, 1998).

The adopted model in this work, the zebrafish (*Danio rerio*) has become one of the most commonly used animals for examining effects of aquatic pollutants (Hallare *et al*., 2004). Furthermore, with the available genomic resources and suitability of this species for molecular manipulations, the zebrafish has been adopted more widely for research in developmental biology and understanding of disease processes. The medaka (*O.latipes*) is another model species widely used in ecotoxicology research and for the development of transgenic techniques. These two species are easy to breed in the laboratory, have a short life cycle, and their embryos are transparent. Many studies have shown that early life stages of fish have the greatest sensitivity to environmental contaminants (Hamm *et al*., 2001; Panter *et al*., 2006; Purdie *et al*., 2009).

Zebrafish and medaka are highly suitable for GFP expression studies, and the use of tissue-specific promoters has become a powerful tool for studies on endogenous gene expression (Higashijima *et al*., 1997) and to analyse the function of promoters (Hall *et al*., 2007; Mattingly *et al*., 2001; Zhang and Xu, 2009). The transgenic fish has become an established technique in developmental analyses, and generally includes using a specific promoter and a green fluorescent protein (GFP) or a luciferase reporter gene. To improve the efficiency of the sensitivity, tissue specificity and ease of generating transgenic fish, various manipulated gene systems have recently been introduced. One of these is the Gal4-UAS system. This is now used wildly for the overexpression of transgenes in various transgenic animals, including zebrafish (Asakawa and Kawakami, 2008). This system comprises a two-part expression system that

utilises the yeast transcription activator protein Gal4 and its target sequence UAS (Upstream Activated Sequence), to which Gal4 binds to activate gene transcription (Duffy, 2002; Hartley *et al*., 2002). It has also been reported that use of *Tol2* can increase transposition efficiency enhancing the generation of transgenic fish (Kawakami *et al*, 2004). The *Tol2* transposon system, originally identified in the medaka (Koga *et al.,* 1996), has been used to enhance the success rate of DNA integration into the zebrafish genome (Kawakami *et al*., 2000, 2004). This is illustrated in the work of Kawakami *et al*. (2004) and Stuart *et al*. (1988) where used of *Tol2* increased the transgenesis rate of linear DNA from 5% to 50 %.

Transgenic zebrafish have considerable potential for use in aquatic ecotoxicology to screen and test for hormone mimics and potentially to develop a more advanced system for assessing health impacts of chemicals. As a consequence there has been considerable activity in a number of laboratories to develop transgenic zebrafish as tools for screening and testing of chemicals. Only a small number of studies, however, have used transgenic fish for investigating the effect of real world environmental pollution mixtures. Transgenic fish have the advantage that tissue specific effects of EDCs can be identified to allow for more directed and detailed studies to inform on health outcomes. However, it is time consuming, both to produce and maintain the stable transgenic lines. There are also moderately high associated costs with maintaining those lines. As a consequence, a number of studies have investigated the use of transient expression assays to examine the spatial and temporal expression of reporter genes that are fused to the regulatory regions of various genes in zebrafish embryos. To date, biosensor TG fish have only been generated in the zebrafish and medaka, and such technology has not been applied widely to other fish species. In theory, however, having developed the technology for these model species, it would be possible to develop a functional 'transient assay', by which vector DNA is transiently introduced into the fish embryo, in almost any fish species, and thus examine the effect of chemical exposure specifically in those fish species.

To date, there have not been any reported transient expression assays for the detection of EDCs. The transient expression assay process normally involves the injection of fertilised embryos with a construct and followed by assaying of the response (e.g. GFP or Luciferase)

once the embryos/larvae have reached the desired stage of development. A major advantage of the transient assay is the rapidity of analysis and the potential for high analysis throughput.

Here we developed a novel transient assay system using a synthetic oestrogen responsive element, *Tol2* and Gal4-UAS systems and the GFP reporter gene, responsive to environmental oestrogen and investigated the functional capability of this construct using a transient expression assay using green fluorescent microscopy in zebrafish embryos/larvae. The aim of this study was to develop a quick and robust transient assay system to examine the oestrogenic activity of various EDCs using fish embryos. Here we provide the proofing of our vector system in transient expression assays in two different fish species, the zebrafish and medaka.

Materials and Methods

DNA construct for developing the transgenic zebrafish

The construct, called pTol2_3ERE-TATA-Gal4ff was based on the sequence of EREc38 (EREc38 5'- CCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG-3') published by Sathya *et al*. (1997). The construct was generated by Polymerase Chain Reaction (PCR) using primers (upstream primer CCAGGT CAGAGTGACCTGAGCTAAAATAACACATT

CAGCCAGGTCAGAGTG and downstream primer CTGAATGTGTTATTTTAGCTCAGG

TCACTC TGACCTGGCTGAATGTGTTAT). The following conditions were used for 8 PCR cycles : denaturation at 96 ℃ for 1min, annealing at 60 ℃ and extension at 72 ℃ for 1 min. Multiple tandem copies of EREc38 (nEREc38, where n = number of ERE copies) were obtained by PCR. EREc38 oligomers were prepared by restriction digestion of the 3ERE with *SmaI*. The reporter gene vector pKS(+) was digested with *XhoI* and *XbaI*. After obtaining the 3ERE fragment, a second fragment called TATA was constructed following the same protocol, using the following primers: (upstream primer GGCGTCGACTCTAGAGGGTAT ATAATAGATCTGCGATCTAAGTAAGCTTGG downstream primer CGCGGGCCCGGC TTTACCAACAGTACCGGAATGCCAAGCTTACTTAGATCG). TATA was digested with *ApaI* and *SalI*. Gal4-ff (amplified by PCR using primers: upstream primer GCCGGGCCCGCCACCATGAAGCTACTGCTGTCTTCT downstream primer CGCGGTA

CCGATTAGTTACCCGGGAGC) was inserted into the vector which already contained the 3ERE and TATA. The *BamHI* and *NotI* fragments of p3ERE-TATA-Gal4ffwere inserted into the *BamHI* and *NotI* sites of the plasmid pBR322 vector, the latter containing the *Tol2* transposon. The positive clones were confirmed by sequencing. The resulting plasmid was called pTol2_3ERE_Gal4ff.

Fish maintenance and microinjection of embryos with DNA

Zebrafish were maintained at 28 ± 1 ℃ under a 14h light/10h dark photoperiod and were fed Tetramin dry tropical flake food (Tetramin; Tetraweke, Melle, Germany) twice a day. Fertilised zebrafish eggs were collected within 20 minutes post-fertilisation. Medaka were maintained at 28 ℃ on a 14:8 light:dark cycle. All adult medaka in this study were fed *Artemia* twice a day. The concentration of plasmid was 20 pg/nl and 0.05% phenol red was added to make the solution visible. Under a dissecting microscope, fertilised eggs were placed in injection plate and eggs were microinjected with a syringe into the centre of the cell over the yolk-cytoplasm boundary. Embryos were microinjected at the 1-2 cell stage with 2 nl DNA using a microinjector (INTRACEL, PICOSPRITZER® III). Some of the embryos were also exposed to various chemicals as detailed below.

Chemicals

17β-oestradiol (98% purity), 17α-oestradiol (≥98% purity)) and 4-Nonylphenol (Acros Organics) were purchase from Sigma Chemical Co. Ltd. The stock solutions of EE2, E2 and NP were prepared in acetone and subsequently stored at 4℃, until required for the exposures. The working solutions were prepared 3 days before use in the exposures. Prior to exposure, the solvent was evaporated under a stream of nitrogen and the working solutions made up with water and stirred vigorously for 1 day.

The nominal concentration of treatment solutions were as follows:

17β-oestradiol (E2): 1000 ng/L, 100 ng/L and 10 ng/L

17α-oestradiol (EE2): 1000 ng/L, 100 ng/L and 10 ng/L

4-Nonylphenol (NP): 100 μg/L and 10 μg/L

Chemicals were dissolved in Fish water. Chemicals were added to glass Petri dishes from treatment solutions. Fifty embryos per concentration were exposed for up to 72 hours at 28 ℃. All experiments were run in duplicate and were repeated at least seven times.

The transient expression assay using fluorescent microscopy detection

The plasmid pTol2_ERE_ Gal4ff was mixed in a 1:1 ratio with the plasmid pTol2_UAS-GFP and then mixed with transposase mRNA in a ratio of 1:2. The mixture was microinjected (20pg/nl) into 1-2 cell stage embryos of zebrafish. Injected zebrafish embryos were cultured for up to 72 hours (zebrafish) with and without exposure to E2, EE2 and NP, and GFP expression examined by fluorescence microscopy (Leica DMI 4000 B). In the studies with medaka, the plasmid pTol2_ERE_ Gal4ff with transposase mRNA was mixed in a 1:1 ratio with the plasmid pTol2_UAS_GFP and this was then injected into 1-2 cell stage medaka embryos (20 pg/nl). Injected medaka eggs were exposed to EE2 (100 ng/L) and embryo culture water for 11 days at 28 ℃ (±1℃). GFP expression was observed under a fluorescent microscope (NIKON SMZ1500).

Synthesis of transposase mRNA

Transposase mRNA was synthesised *in vitro* using pCS-TP which carries *Tol2* transposase cDNA. The plasmid pCS-TP was digested with *NotI* and purified by phenol : chloroform extraction and ethanol precipitation. RNA synthesis was performed using mMessage mMachine SP6 kit (Ambion). The synthesized mRNA was purified with Quick spin column for radiolabeled RNA purification (Roche), purified by phenol : chloroform extraction and by the subsequently ethanol precipitated. The RNA precipitated was suspended in distilled water and used for co-microinjection with pTol2_ERE_Gal4ff.

Western blot analysis

Western blot analysis was employed to quantify GFP expression. Embryos were transferred to a 1.5ml tube filled with lysis buffer (2x sample buffer, 2-Mercapto ethanol and distilled water), incubated at 95 ℃ for 5 minutes and the vessel tapped to get them to the bottom of tube. Samples were homogenised five times and centrifuged for 1 min at 1000 rpm. Protein samples were applied to 5% polyacrylamide-SDS gel and subjected to electrophoresis at 110 V for 2 hour and separated proteins were transferred to nitrocellulose membrane. The membranes were

blocked for 1 hour in blocking solution (5% skimmed milk in 1x phosphate buffered saline (PBS) + 0.1% Tween (PBSTx)), and washed three times with distilled water (ddH_2O). The membranes were incubated overnight at 4 ℃ with primary antibody, rabbit anti-GFP (ams Biotechonology), diluted 1:2500 in blocking solution. The membranes were washed for 3 x 15 minutes in PBSTx. The membranes were incubated with HRP-Goat Anti-Rabbit IgG (Invitrogen, Carlsbad, U.S.A) at 1:2000 in blocking solution for 2 hours and then washed 3 x 15 minutes in PBSTx again. For detection, Western blotting luminol reagent (Thermo Scientific) was used. The intensity of GFP was analysed using Image J (http://rsbweb.nih.gov/ij/), normalised to the intensity of alpha-tubulin band and indicated as fold increase in GFP over the level in control larvae.

Results and Discussion

Construction of Reporter Gene Vectors with Oestrogen Responsive Promoters

With a plan to develop a rapid and sensitive transient expression assay system to evaluate the oestrogenic activity of environmental chemicals in different fish embryos, we utilised a Gal4ff-UAS system that incorporates a two-step signal amplification process and a synthetic oestrogen responsive element with 3EREs. A major aim in this initial work was to make a construct that was highly responsive to oestrogenic EDCs and in doing so, establish the optimal oestrogen response elements (EREs) for the most effective promoter. In this work, we then examined the responsiveness of the oestrogen response elements during specific stages of development in zebrafish using a transient expression assay. Finally, we tested our plasmid in another fish species, the medaka, to show that transient expression assay is suitable for observation of effects of oestrogen compounds in other fish test species.

The construct produced for detecting oestrogen is shown in figure 1. Specific primers of EREc38 were run in PCR reactions to produce the EREs. The EREs were 38 base pair (bp) sequences that consisted of a 17-bp inverted repeat 5'-CAGGTCA nnn TGACCTG-3' followed by an AT-rich sequence. A few studies have made TG fish for screening of oestrogen chemicals using different promoters, including, vitellogenin(Vtg), choriogenin L (ChgL) gene and aromatase promoters. Legler *et al*. (2000) developed a transgenic zebrafish line that expressed the reporter gene luciferase (LUC) for examining estrogenic compounds. Although the construct they used, pERE*tata*-LUC, was sensitive, luciferase cannot be measured in live organisms, therefore the fish had to be killed to assay for the response. In contrast, GFP can be detected in living cells/organisms. The use of GFP in intact fish also has advantages over use of *in vitro* transfected cell cultures, because it allows for integration of toxicodynamic and toxicokinetic factors into the response (Scholz *et al*., 2005). The constructs that have been used in previous studies for developing oestrogen response systems, such as pEGFP-ChgL (Ueno *et al*., 2004), pZVTG1-EGFP (Chen *et al*., 2010) are liver specific genes. Those systems also did not use

Tol2 vector system, which, as mentioned above, has the capability to enhance significantly genomic integration of introduced DNA.

In a mammalian cell culture system, Sathya *et al*. (1997) showed that a construct with 3EREs produced an enhanced expression compared with 2EREs and 4EREs, especially in response to E2. Therefore, we similarly adopted three copies of ERE in our construct. For the transcriptional initiation, the TATA box sequence was inserted at 158 bp upstream of the Gal4ff start codon and the Tol2 transposon system was adopted to provide a more efficient transient reporter analyses. After the sequential synthesis of five successive constructs, we generated a novel plasmid vector system, p3ERE-TATA-Gal4ff containing, *Tol2* and a poly A tail and employing two steps for amplification of the signal (Fig. 1) where Gal4ff activates "UAS (Upstream Activated Sequence)-GFP (Green Fluorescent Protein)". In the activation process, the first step binding of estrogens to ER and subsequent binding to ERE induces the synthesis of GAL4FF protein and the second step Gal4ff protein induces synthesis of GFP. This two-step process allows amplification of the resulting signal which, in theory, should give rise to a more "sensitive" transient expression assay system for detecting oestrogen chemicals than has been developed previously. In our approach, we used Gal4ff rather than the more commonly used Gal-VP16/UAS system, because GAL4ff is reported to be less toxic than GAL4-VP16, which generates the activator domain of the herpes simplex virus VP16 protein in zebrafish cells (Asakawa *et al*., 2008).

Induction of GFP in the zebrafish embryo transient expression assay

We used the transient expression assay to examine oestrogenic activity of various oestrogenic EDCs in zebrafish and also in medaka embryos to assess whether this system is applicable to other fish species. Live embryos injected with the mixture of plasmids, transposase mRNA and exposed to the selected environmental oestrogens were examined for expression of GFP under fluorescence microscopy. A transient expression analysis for detection of EDCs in fish has not been reported previously. Furthermore, a *Tol2* mediated

Gal4-UAS system has not been used for transient expression assay for screening oestrogens. After microinjection, expression of embryos was mosaic because the microinjected recombinant DNA was not integrated to all cells. We solved this problem by flanking the transgene with a Tol2 transposon and we were then able to identify target tissues for oestrogens in early life stages.

There was no significant mortalities or a delayed hatching rates in either zebrafish or medaka embryos exposed to the oestrogen chemicals. Survival rates in control, EE2 and E2 were 97%, 98 % and 96 %, respectively. Similarly, survival rate of 97% were observed after exposure to 10 µgNP/L. These survival rates did not differ from controls. In contrast, all embryos died when exposured to 100 µgNP/L. Scholz and Gutzeit (2000) similarly reported no differences in survival or growth rates for medaka exposed to 1, 10 and 100 ngEE2/L over a period of 2 months from hatching. However, eggs exposed to 1 µgEE2/L or 1 µgE2/L showed slightly delayed hatching rates after 72 h. Similarly, Kishida *et al*. (2001) reported that there were no morphological defects, no differences in hatching rate and mortality in zebrafish embryos exposed to 0.1 and 1 µM E2 (approximately 27 µg/L and 270 µg/L) for 120 h compared to control (non-exposure).

After exposure to EE2 (data not shown), GFP expression was observed at 24 hpf in the injected embryos with low expression, whereas there was no GFP expression detected in the controls at this time. However, there was stronger and more specific tissue GFP expression in EE2 treated embryos at 72 hpf, mainly in the liver, muscle and heart. We therefore adopted the use of 3 day-old embryos for assessing tissue specific effects of oestrogens in the transient assay. There was no morphological effect or mortality for the treatments with the environmental oestrogens, with the exception for exposure to the highest exposure concentration of NP (see below).

At 72hpf there was no GFP expression detected in embryos in the unexposed controls (Fig. 2, Ai-Aiii), but GFP expressing cells were seen clearly after exposure to EE2 (Fig. 2, Ci and Diii). No specific GFP expression was observed in the liver at the lowest exposure concentration of

EE2 (10 ng/L), but weak GFP expression signals were detected in the heart and somite muscle for this exposure concentration (Fig. 2, Bi-Biii). GFP expression was clearly seen in the higher EE2 exposures. For exposure to 100 ngEE2/L GFP was detected in a feint and dispersed manner in the liver and there was strong GFP expression in the heart and somite muscle. GFP expression in the heart was confirmed by the periodical contractile movement of the GFP expressing cells with each heart beat in the live injected larvae. Strong GFP expression was observed in the anterior somite muscles. For exposure to 1000 ngEE2/L GFP expression was induced strongly in the liver, somite muscle and heart (Fig. 2, Ci-Ciii). Moreover, many GFP expressing cells were observed in the anterior lateral part of the injected embryos (Fig. 2, Di). The number of cells expressing GFP appeared to be positively associated with the concentration of the EE2 exposure.

Exposure to the natural steroid oestrogen, E2, resulted in similar responses to that seen for EE2. No GFP expression was observed in unexposed controls (Fig. 3, Ai-Aiii). Similarly, no GFP expression was observed for the lowest E2 exposure concentration (10 ngE2/L, data not shown). No GFP expression was observed in the liver at 100 ng E2/L, but was seen in the muscle (somite and cranial) and heart (Fig. 3, Bi-Biii). Exposure to 1000 ngE2/L strongly induced GFP expression in the heart, otic vesicle and somite muscle and there were some GFP expressing cells observed in the liver (Fig. 3, Ci-Ciii). For the NP exposure, all embryos died at the highest concentration tested (100 µg/L). There were no mortalities, however, in the 10 µgNP/L exposure, nor any signs of developmental abnormalities. Exposure to 10 µgNP/L did however, induce low level GFP expression in the heart and somite muscle (Fig. 3, Di-Diii). The reason for weak GFP induction by NP might be due to a lower sensitivity of early development stages to this chemical in the zebrafish, as has been suggested previously (Segner *et al*., 2003). In addition, it has been reported that NP might be extensively transformed in fish to less potent metabolites (Bone, 1995; Lewis and Lech, 1996). We found specific GFP expression in heart and somite muscle in response to NP and they have not been reported previously as targets for oestrogenic chemicals. These data indicate that different xeno-oestrogens have different target tissue responsiveness and therefore potentially different health effects.

In general, the expression of GFP appeared to be a concentration-dependent response for exposure to the test oestrogens, as quantified via western blotting. Yolk region showed low background autofluorescence for all groups. The highest EE2 exposure group (1000 ng/L) indicated a twenty-fold increase in GFP levels in whole embryos compared with untreated controls. Exposure to 10 ngEE2/L resulted in a 2.5-fold increase in GFP compared with the control (Fig. 4A). Exposure to E2 at 1000 ng/L induced a 14-fold increase in GFP above controls and a 2.5-fold increase above controls for exposure to10 µgNP/L (Fig. 4B-C). For the highest exposures to EE2 and E2 (1000 ng/L), the level of GFP induction for EE2 was stronger than for E2, indicating that EE2 was more potent than E2. Legler *et al*. (2002) similarly reported that 17α-ethinyloestradiol (EE2) was the most potent (xeno) oestrogen compared with 17β-oestradiol (E2), nonylphenol (NP) and di(2-ethylhexyl)phthalate (DEHP) in their transgenic zebrafish assay. Thorpe *et al*. (2003) using a VTG induction assay, reported that EE2 was between 11 to 27 times more potent than E2.

Results for the transient expression assay with medaka are shown in Fig. 5. Medaka has four types of pigment cells that produce auto-fluorescence in the skin, namely melanophores, xanthophores, leucophores and iridophores (Nanda *et al*., 2002). To avoid interference of their natural autofluorescence with detection of GFP, we imaged the embryos using normal light, green (GFP) and red (RFP). This was done because pigmentation (auto-fluorescence) is fluorescent both in green and red spectra, and therefore by overlaying these different images we were able to isolate the signal due to GFP. There was no GFP expression detected in controls but for exposure to 100ngEE2/L GFP expression was detected in the heart and somite muscle. Strong ubiquitous GFP expression was observed in the early stage of embryos at 3 dpf in response to the oestrogen exposures, whereas no GFP expression was detected in the control (unexposed embryos). At 3 dpf, injected medaka embryos showed GFP expression in the skin in the anterior dorsal part and trunk. The transient assay showed mosaic expression. For this reason, the injected embryos were exposed for 10 days (until hatching) to observe any further specific tissue expression. Tissue specific expression became obvious in the liver, heart, gallbladder and somite after 10 days of development (stage 40; (Kinoshita *et al*., 2009)). EE2

treated embryos exhibited GFP production in the liver, heart and muscle. Although medaka showed less muscle expression compared with zebrafish, GFP expression was observed in the gallbladder in medaka. This might suggest that oestrogen receptor expression is varies between fish species or estrogen receptor binding affinities for different fish species might be different in the different embryos.

Our data have shown that our vector system worked well and effectively detected oestrogenic responses in body tissues in the both test fish species. Despite the fact that transient expression analysis has been used in various areas of developmental biology, there has been no report using transient expression analysis for detection of oestrogen compounds. Our transient assay system is therefore novel and can be applied to screen chemicals to test for body systems wide effects. Previous transgenic fish developed as biosensor systems for oestrogens have shown GFP expression in the liver at exposures down to 10 ngE2/L (Bogers *et al*., 2006) , and possibly 0.1 ngEE2/L ((Chen *et al*., 2010); these data were not clear).

In summary, we have designed and developed a plasmid system responsive to oestrogen and oestrogenic EDCs and incorporated this into an effective transient assay system. The vector system developed worked well for the detection of oestrogenic EDCs in both the zebrafish and medaka, indicating the likely wide suitability for application to other fish species. This system would be a particularly powerful technique for use in species with long generation times and/or where there are other difficulties for generating transgenic lines in those species. The transient expression assay further provides a novel in vivo system for investigating oestrogenic effects on embryo development in fish.

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Figure 4. Western blot analysis of 72 hour old embryos injected with pERE-TATA-Gal4FF containing the Tol2 transposon. Whole-body homogenate samples of 40 embryos exposed to 17β-oestradiol (E2), 17α-ethynyloestradiol (EE2) and nonylphonol (NP) at various exposure concentrations (via the water) are shown. Western blot analysis showed no detectable expression of GFP (Green fluorescent protein) in controls. The level of GFP expression was induced in a concentration dependent manner for the various oestrogenic chemicals (EE2, E2 and NP). GFP expression level was quantified by using Image J (http://rsb.info.nih.gov/ij/index/html). Error bars represent the standard deviation. This experiment was run in duplicate.

Figure 5. Analysis of transient expression of GFP in response to oestrogens in medaka embryos. The plasmid pERE-TATA-Gal4FF was injected into 1-2 cell stage medaka embryos and the embryos were exposed to 17α-ethynyloestradiol (100 ngEE2/L) via the water. No GFP expression was detected in controls. The injected medaka embryos exposed to EE2 showed strong GFP expression in the skin in the anterior dorsal region and in the body trunk (B). At 11dpf, fish exposed to EE2 showed GFP expression in the heart, liver, somite muscle and gallbladder (D, F and H).

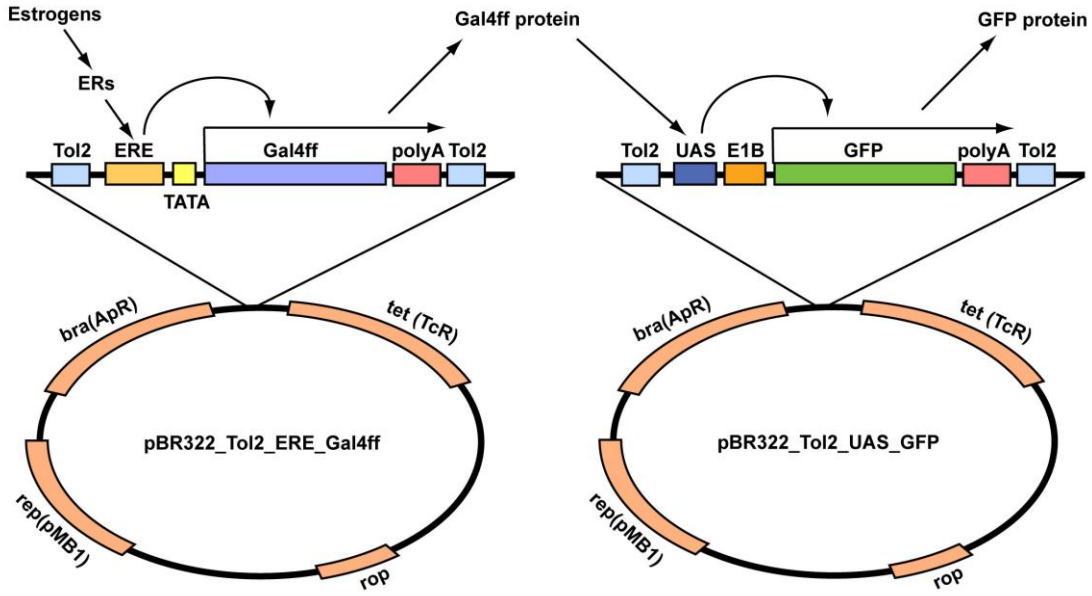
Estrogens
ERs
Tol2
ERE
TATA
Gal4ff
polyA
Tol2
Gal4ff protein
bra(ApR)
tet (TcR)
pBR322_Tol2_ERE_Gal4ff
rep(pMB1)
rop
GFP protein
Tol2
UAS
E1B
GFP
polyA
Tol2
bra(ApR)
tet (TcR)
pBR322_Tol2_UAS_GFP
rep(pMB1)
rop

Figure 1

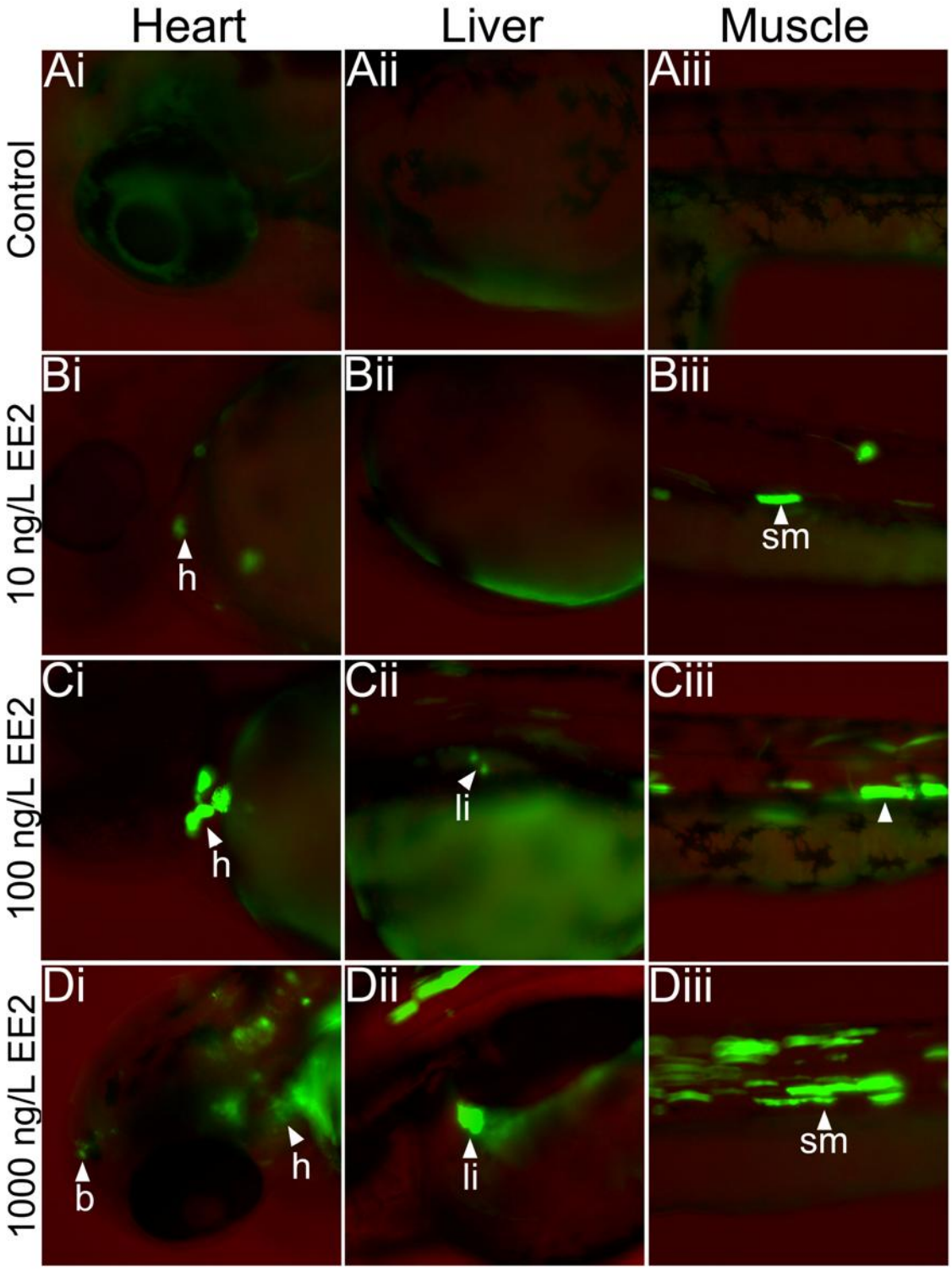
Heart
Liver
Muscle
Control
10 ng/L EE2
100 ng/L EE2
1000 ng/L EE2
Ai
Aii
Aiii
Bi
Bii
Biii
Ci
Cii
Ciii
Di
Dii
Diii
h
sm
li
b

Figure 2

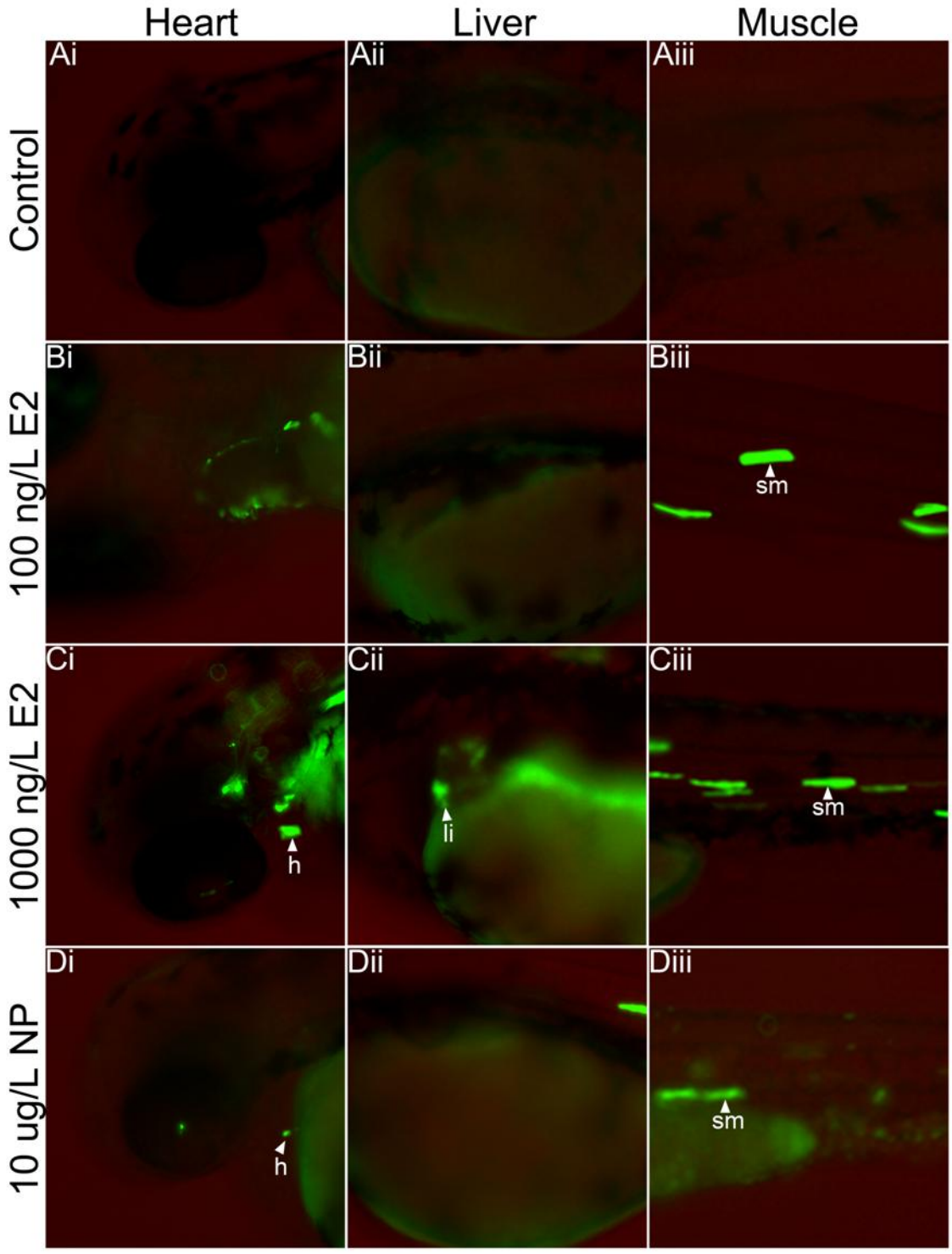
Heart
Liver
Muscle
Control
100 ng/L E2
1000 ng/L E2
10 ug/L NP
Ai
Aii
Aiii
Bi
Bii
Biii
Ci
Cii
Ciii
Di
Dii
Diii
sm
li
h

Figure 3

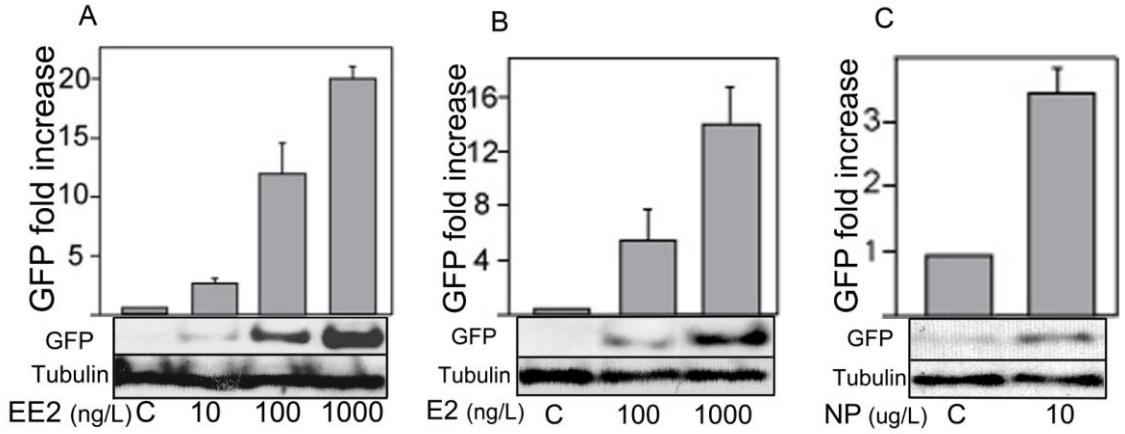
A
GFP fold increase
20
15
10
5
GFP
Tubulin
EE2 (ng/L) C 10 100 1000
B
GFP fold increase
16
12
8
4
GFP
Tubulin
E2 (ng/L) C 100 1000
C
GFP fold increase
3
2
1
GFP
Tubulin
NP (ug/L) C 10

Figure 4

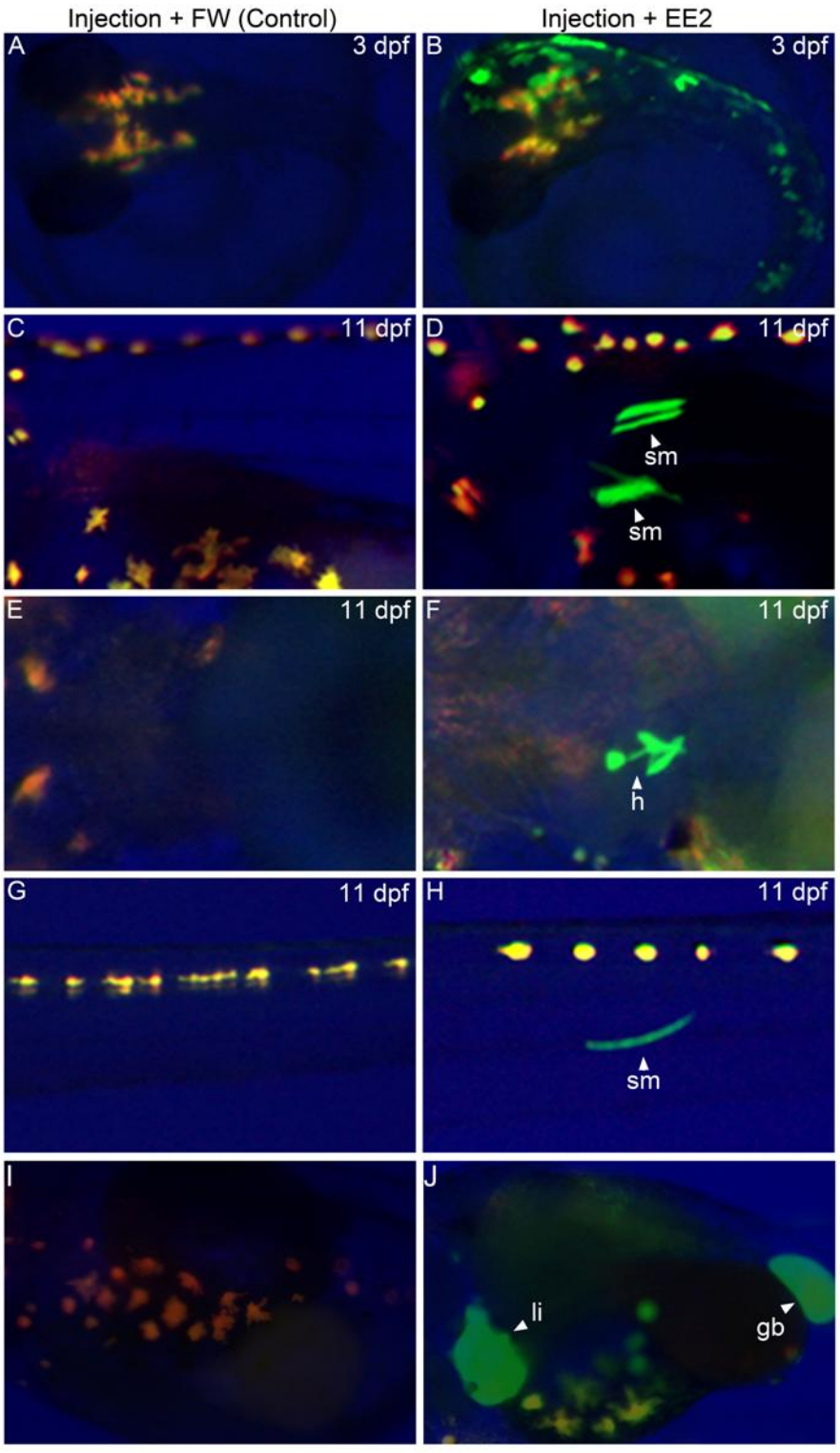
Injection + FW (Control)
Injection + EE2
A
3 dpf
B
3 dpf
C
11 dpf
D
11 dpf
sm
sm
E
11 dpf
F
11 dpf
h
G
11 dpf
H
11 dpf
sm
I
J
li
gb

Figure 5

CHAPTER 5

Biosensor zebrafish provide new insights into body target tissues for environmental oestrogens

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of the United States of America

CHAPTER 5

Biosensor zebrafish provide new insights into body target tissues for environmental oestrogens

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Abstract

We have developed a novel Gal4ff-UAS mediated estrogen responsive transgenic (TG) zebrafish for identifying body system targets of environmental oestrogens in real time. Exposure of the TG fish to oestrogenic endocrine disruption chemicals (EDCs) induced specific patterns of GFP expression in a wide variety of tissues including the liver, heart, skeletal muscle, ear, forebrain, lateral line and ganglions, most of which have not been established previously as targets for oestrogenic chemicals. Furthermore, we found that different EDCs induced GFP expression with different tissue response patterns and time trajectories suggesting different potential health effects. Our TG zebrafish model provides a new prospect for understanding toxicological effects and health impacts of environmental oestrogens in vertebrates.

Keywords: Gal4, UAS, endocrine disrupting chemicals (EDCs), oestrogen receptors (ERs), biosensor

Introduction

Environmental oestrogens are the major group of so-called Endocrine Disrupting Chemicals (EDCs) that can alter hormone signalling in the body and exposure to these chemicals in humans has been associated with decreases in semen quality/sperm count, increased incidences of breast cancer and testicular germ cell cancer, and urogenital tract malformation (Carlsen *et al*., 1992; Mocarelli *et al*., 2008). A recent study reported that higher urinary levels of the weak environmental oestrogen, bisphenol A (BPA), taken into the body via the diet, were associated with a higher risk of heart disease and diabetes in a human population (Melzer *et al*., ; Melzer *et al*., 2010). In fish, exposure to environmental oestrogens has been shown to impact on reproductive development and cause feminisation of males (Lange *et al*., 2009) and alter sexual behaviours (Van den Belt *et al*., 2004; Vos *et al*., 2000). Many thousands of chemicals have now been identified with oestrogenic effects and major international programmes have been established to screen and test for oestrogenic activity to avoid potential human and environmental health risks associated with exposure (Reif *et al*., 2010). Critically, however, available screening and testing systems for oestrogens are focused on specific individual mechanisms (e.g. oestrogen receptor activated cell lines) or in-life studies that assess effects on reproduction only.

The roles of steroid oestrogens in reproductive development are well established and many of these roles are common across the vertebrate phyla. Oestrogens are fundamental in the growth and development of the ovary in females (Richards *et al*., 1976) and they are also required for spermatogenesis in males (Mahato *et al*., 2001; O'Donnell *et al*., 2001). In addition, in mammals oestrogens are known to play key roles in a wide range of other physiological functions including immune responses, the central nervous system, and in normal somatic cell growth (Gustafsson, 2003). In mammals oestrogen signalling operates through two different oestrogen receptors (ERs), ER α and ER β, that show differences in their tissue distributions and which are thought to regulate different oestrogen responses (Pettersson and Gustafsson, 2001). In fish, three ERs, ER α, ER β1 and ER β2 are expressed with different tissue specificities (Hawkins *et al*., 2000; Kuiper *et al*., 1998; Menuet *et al*., 2002; Sun *et al*., 1999).

Understanding the physiological effects of oestrogenic chemicals would be greatly enhanced with *in vivo* models capable of detecting tissue specific effects of oestrogens with high sensitivity. Transgenic (TG) zebrafish have considerable potential for screening and testing EDCs, for understanding their pathways of effect and assessing for their potential health impacts both in animals and humans (Chico *et al*., 2008; Moss *et al*., 2009; Raldua and Babin, 2009). Here we established an oestrogen responsive transgenic zebrafish for detecting oestrogenic signalling of natural (endogenous) hormones and for exposure to exogenous oestrogens, including EDCs, in both embryonic and early larval stages, in real time. The system contains an oestrogen inducible promoter derived from a short stretch of multiple tandem oestrogen responsive elements (EREs) and devoid of any tissue specific enhancer/suppressor elements. To enhance the system's response sensitivity, we used a Gal4ff-UAS system, not previously applied in fish. The TG zebrafish produced are highly responsive to environmental oestrogens and identify a wide range of target tissues, most of which have not been reported previously. The TG fish further showed that different EDCs induced different tissue patterns and response time trajectories. The Gal4ff-UAS zebrafish thus provides a highly effective system for studying the potential for the health effects of exposure to oestrogenic EDCs and provide a new and enhanced capability for screening and testing of environmental oestrogens.

Results and Discussion

In creating these fish two transgenic vectors were synthesised that contained ERE-Gal4ff and UAS-GFP (Fig. 1). Three tandem repeats of ERE drive the 1st reporter Gal4ff that is a modified form of Gal4 transcription factor providing high transcriptional activation with low toxicity. Gal4ff transcription factor binds to UAS and activates GFP and the sequential activation of two reporters amplifies the signal. Double-transgenic fish (ERE-TG fish) were generated by injecting ERE-Gal4ff vector into UAS-GFP TG fish (Kajita *et al*., 2009) using a Tol2 transposon system (materials and methods) (Kawakami *et al*., 2004; Urasaki *et al*., 2006).

To examine the ability of our ERE-TG zebrafish to detect various oestrogens, we investigated for GFP expression in response to a series of selected oestrogens of environmental concern. In TG fish larvae (4 days old) without chemical exposure, a weak basal GFP expression was observed in the otic vesicle with a 57 % frequency and also in the heart with lower (40%) frequency. The variation in this GFP expression in the unexposed larvae may suggest differences in oestrogen levels in fish embryos and larvae occur from an early developmental stage. Whether these differences relate to sex has not been determined as there are no sex specific probes available for this species.

Exposure to different oestrogens induced different tissue patterns of GFP induction (Fig. 2). In the larvae exposed to EE2 (Fig. 2B), GFP expression was observed in the liver and forebrain, as has been reported with other oestrogen-responsive TG fish lines, such as *vitellogenin* promoter- and *cyp19a1b* promoter-derived GFP lines, respectively (Chen *et al*., 2010; Legler *et al*., 2000; Tong *et al*., 2009). However, we also found specific and strong GFP expression in other tissues that have not been identified previously as targets for oestrogen from other biosensor studies. The tissues include the heart, skeletal muscle, neuromast, and ear/eye and lateral line ganglions. GFP expression in the heart was confirmed by the periodical contractile movement of the two GFP expressing domains in the artery and in the ventricle, synchronizing with the heart beat in the live fish. The GFP expression in somite muscle was more intense in the anterior somites at early larval stages compared with in the posterior somites and posterior expression gradually increased in later larval stages. Each expression domain outlined the

shape of a single muscle myotube. Interestingly, in low EE2 concentration exposures the muscle somite GFP expression was often observed as a mosaic pattern, as it was expressed in only some of the myotubes. At high exposure concentrations GFP expression was observed in a uniform manner in myotubes. Specific GFP expression was also observed in the neuromasts of the lateral line located in the head and in ganglions adjacent to the ears and eyes, that showed typical large masses of neurons and neurite (e.g. Fig 2Ci, Dii). Exposure to the natural steroid oestrogen (E2) induced a similar tissue expression pattern of GFP as for EE2 (Fig. 2B, C).

In the exposures to BPA, a strong GFP expression signal was observed in the heart, cranial muscle, and ear and eye ganglions with long neurites (Fig. 2Di, Dii). In contrast with the EE2 and E2 exposures, GFP expression in the liver and somite muscle in the BPA exposures were relatively weak. GFP expression induced by NP was strongest in the liver and somite muscle, and was less intense in the otic vesicle and heart (Fig. 2E). These expression patterns suggest different oestrogens have common target tissues such as the liver, heart, muscle, otic vesicle, but they also have different degrees of effect on the different tissues (i.e. tissue specific responsiveness), with different potential health effect outcomes.

To understand the stage dependent tissue responses to oestrogen, the response dynamics of the different tissues were examined in exposures to EE2 (100 ng/L) and extending from 1hour post fertilisation (hpf) to 96hpf (Fig. 3I). Expression of GFP was seen at 24 hpf in the liver, heart and muscle, and increased progressively to 96 hpf, when it reached a maximal expression level (Fig. 3I). In contrast, GFP expression in the nervous system was observed later in development: GFP in the otic vesicle was seen only after 48 hpf, and subsequently in the lens and forebrain after 72hpf (Fig. 3I). These data indicate oestrogenic exposures affect different tissues in life stage dependent manners, and illustrate the capability of the ERE-TG fish model for understanding time related effects of estrogens in an intact animal.

ERE-TG fish embryos were exposed to various concentrations of the selected EDCs (Fig.3) to examine if they were capable of detecting responses for exposures to environmentally relevant concentrations. Responses to the most potent oestrogen, EE2, were titrated down to

1ng/L, where a low level GFP response was induced in cells in the liver. Higher exposure concentrations resulted in progressively higher GFP expression levels and at 10 ng/L EE2 induced very considerable expression throughout of the whole liver, accompanied with other GFP expressed domains in the muscle (somite and cranial muscle), heart and otic vesicle (Fig. 3D). The concentration-dependent GFP expression was further confirmed quantitatively via western blot analyses (Fig. 3K-N). The level of GFP expression increased dramatically and in a concentration dependent manner for both EE2 (1-10ng/L) and E2 (5-10 ng/L) (Fig. 3B-F). In contrast with the responses to EE2 and E2, the threshold concentrations for NP and BPA (1 µg/L and 100 µg /L, respectively), were significantly higher, suggesting relatively weak activity for both of these environmental oestrogens.

To demonstrate oestrogen-induced GFP expression in the ERE TG fish was mediated via ERs, we conducted further experiments to specifically suppress ERs activities in the ERE TG zebrafish. We used two strategies to do this. In the first the oestrogen receptor antagonist ICI 182,780 (ICI) was added to the embryo culture, and in the second approach antisense morpholino oligonucleotides for ERα, β1 and β2 receptor were injected into the TG zebrafish to suppress the expression of these receptors. Exposure of ERE-TG zebrafish larvae to ICI abolished the GFP expression seen in the otic vesicles in non-exposed fish (Fig 4B). Moreover, exposure to ICI greatly reduced the EE2-induced-GFP expression in all of the responding tissues including in the liver, heart, muscle, neuromasts and ganglions (Fig 4D). Consistent with the fluorescent imaging data, Western blot analysis confirmed that the ICI treatment led to a drastic inhibition in both the basal GFP expression in non-oestrogen exposed fish and GFP expression in the EE2-treated fish (Fig. 4E).

We observed that the EE2-induced GFP expression was also greatly inhibited in the TG larvae injected with the mixture of morpholinos against all ERs (ERα, β1 and β2) in both fluorescent microscopy and Western blot analyses (Fig. 5). Similar levels of suppression of GFP in the skeletal muscle and otic vesicle were observed for treatment with a combination of morpholinos for ER β1 and β2 as for treatment with a mixture of morpholinos against all ERs, whereas GFP expression in the liver and heart was still visible in the morphants (Fig.5E). In the

morphants for ERα, there was no clear suppression in the tissues examined (Fig.5F). These data suggest that ERβ1 and β2 play crucial roles in transducing the oestrogenic signal in the skeletal muscle, but the individual roles of ERα, β1 and β2 for the oestrogen signal transduction in the liver and heart are less clear; collectively the individual morphants suppressed GFP expression in these tissues, but separately (ERα, or ERβ1 and β2 combined) they did not. This would suggest that a level of cross-talk is required between these receptor sub-types and/or involvement of other factors for oestrogen signalling in the liver and heart. Further study in control experiments of MOs might be needed to observe the toxic effects of MOs. In the heart, ERα , ERβ1 and ERβ2 appeared to have some redundancy in oestrogen signalling. Oestrogen signalling in the heart might therefore involve oestrogen related receptors, a subfamily of orphan nuclear receptors, but this has yet to be investigated.

The functional consequences of exposure to one of the most important groups of contaminants affecting endocrine function, environmental oestrogens, especially during early life, and the molecular mechanisms underlining oestrogen-mediated physiological/ pathological consequences caused by these exposures are lacking. Transgenic zebrafish and medaka (*O. latipes*) have been developed previously to detect for exposure to environmental oestrogens, but expression of the reporter genes in these fish has been shown to be restricted to the liver and gonad. This is most likely due to the molecular properties of the chosen gene specific promoter (e.g. *vitellogenin*) which predominantly function in these tissues (Legler *et al*., 1999; Salam *et al*., 2008; Zeng *et al*., 2005). Our system employed a synthetic ERE lacking any additional enhancer/suppressor elements that could bias and restrict the oestrogen response in specific tissues and has allowed for the detection of estrogenic responses in a wider range of tissues in our TG fish, compared with other available TG fish systems. Our manipulations allow for considerably enhanced chemical detection sensitivity in a variety of tissues, and including for environmentally relevant chemical exposures.

Specific cell types in liver, gonad and forebrain are responsive to oestrogen (Chen *et al*., 2010; Hano *et al*., 2007; Legler *et al*., 1999; Tong *et al*., 2009) and neuromast cells have elevated expression levels of ERβ genes (Froehlicher *et al*., 2009). In addition to these target

tissues, here we demonstrate for the first time that muscle (somite and cranial), heart and many ganglion cells also respond to oestrogen exposure, thus activating oestrogen signalling cascade in these cells. Identifying the target genes of oestrogenic signalling in these tissues and the processes affected will help in understanding the functional significance of the responses.

Despite the fact that three ERs are known to be expressed very widely in the embryo and larvae of fish, oestrogenic GFP responses in our TG fish were detected in a limited number of tissues, including in the heart, muscle and brain, and this may imply that oestrogen signalling requires tissue specific co-factors/co-receptors to activate the oestrogen cascade. Oestrogenic responses differed in the tissues that were affected for different environmental oestrogenic chemicals. For instance, BPA caused a strong response in the heart whereas NP caused a more potent response in the skeletal muscle. These findings would suggest that although these chemicals have both been labelled as environmental oestrogens, they may have different health implications and outcomes. Having identified this, investigations into the tissue specific responses in the future should help resolve whether this is indeed the case.

The fact that our TG system effectively detected responses to environmental oestrogens (NP and BPA) of a comparatively weak potency compared with steroidal oestrogens and tissue response patterns differed for the different oestrogens tested shows that the system has considerable potential for the screening and characterising for estrogenic properties of chemicals and offers new prospect for understanding effects mechanisms. Applied to embryos/larval stages this system could be developed with high throughput, as is occurring for the use zebrafish embryo development in the testing of drugs in the pharmaceutical industry (Chakraborty *et al*., 2009; Xu *et al*. 2010).

We have thus developed a powerful model system for application to both screening and testing of environmental oestrogens and for intelligent targeting of tissue studies to identify the molecular mechanisms underlying estrogen signalling pathways and understanding their physiological /pathological impacts.

Acknowledgement

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Materials and methods

Generation of ERE-GFP transgenic fish

Specific primers (5'-CCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAGC CAGGTCAGAGTG-3' and 3'-CTGAATGTGTTATTTTAGCTCAGGTCACTCTGACCT GGCTGAATGTGTTAT-5') for EREs (Sathya *et al*., 1997) were run in a PCR reaction to produce a template (in 8 cycles) from which a series of different numbers of tandemly repeated EREs were generated. From the ladder of tandem EREs generated by PCR, the DNA bands for 3EREs were cut and inserted into *XhoI* and *XbaI* fragments sites of pBluescriptKS+. The resulting sequence was combined with a TATA sequence (upstream primer GGCGTCGACTCTAGAGGGTATATAATAGATCTGCGATCTAAGTAAGCTTGG downstream primer CGCGGGCCCGGCTTTACCAACAGTACCGGAATGCCAAGCTTAC TTAGATCG), Gal4ff (amplified by PCR using primers: upstream primer GCCGGGCCC GCCACCATGAAGCTACTGTCTTCT downstream primer GCGGTACCGATTAGTTACC CGGGAGC) and poly A sequences and inserted into pBR 322 Tol2 vector (Kawakami *et al*., 2004; Urasaki *et al*., 2006). The pBR Tol2-ERE-Gal4ff plasmid (18 ng/µl) was injected into one cell stage embryos for UAS-GFP transgenic zebrafish (Kajita *et al*. 2009) together with transposase mRNA (36 ng/µl), and the injected embryos were raised to adult stage. Eggs were collected from founders and exposed to 100 ng/L of EE2 for 3 days to detect for GFP fluorescent signal. We selected the GFP-positive embryos and raised them to adulthood.

Zebrafish embryo chemical exposures

A series of chemicals was selected to assess the responsiveness of our TG zebrafish to estrogens. These included the natural steroid estrogen, 17β-oestradiol (E2), 17α-ethinyloestradiol (EE2), used in the contraceptive pill and hormone replacement therapy treatments, bisphenol A, used very widely as a plasticizer, and 4-nonylphenol, used as an industrial surfactant (Goodhead and Tyler, 2009). All of these chemicals have been shown to

contaminate the aquatic environment and induce feminised responses in fish (Goodhead and Tyler, 2009; Tyler *et al*., 1998). 17β-oestradiol (98% purity), 17α-oestradiol (≥98% purity), bisphenol A (99+%) and 4-nonylphenol (Acros Organics) were purchased from Sigma Chemical Co. Ltd and stock solutions of EE2, E2, NP and BPA were prepared by dissolving them in acetone and subsequently storing them at 4 ℃, until required for the exposures. The working solutions were prepared 3 days before use in the exposures. Prior to exposure, the solvent was evaporated under a stream of nitrogen and the working solutions made up with water and stirred vigorously for 1 day. ICI 182,780 (ICI), an oestrogen receptor antagonist, was purchased from Tocris Bioscience and dissolved in ethanol at a stock concentration of 50 mg/L. All experiments (fifty eggs per treatment) were run in duplicate and were repeated at least five times.

To confirm the oestrogenic activity (E2 equivalent) in the working solution of the EE2 exposure, 1L of working solution was collected and analysed using the recombinant yeast oestrogen screen. The result is shown in Table 1.

Table 1. Oestrogenic activity of EE2 working solution

nominal EE2 (ng/L)	E2 equivalent (ng/L)
0	0.59
0.1	0.89
1	2.31
10	7.89

Quantification of EGFP expression using Western blot analysis

4 day-old larvae were dissolved in reduced 2x LDS sample buffer (Invitrogen) and homogenised using a hand homogeniser. Samples were boiled for 15 min at 70 ℃, then analysed by electrophoresis using NuPAGE NOVEX 4-12% Bis-Tris gel (Invitrogen). Separated proteins were transferred onto a nitrocellulose membrane. The membranes were blocked with 5% milk in 1x phosphate buffered saline (PBS) + 0.01% Tween (PBSTx) at room temperature for 1 hour, and subsequently incubated overnight with rabbit anti-GFP antibody

(1:2500) (ams Biotechonology) at 4 ℃. The membrane was then washed three times with 1x PBSTx at room temperature for 15min, and incubated with HRP-conjugated goat anti-rabbit IgG (1:2000) at room temperature for 1 hour. The membrane was washed three times with 1xPBSTx as described above. For detection, western blotting luminol reagent (Thermo Scientific) was used. The intensity of GFP was analysed using Image J (http://rsbweb.nih.gov/ij/), normalised to the total α-TUB level and indicated as fold increase in GFP over the level of unexposed embryos.

Injection of estrogen receptor α, β1 and β 2 morpholinos

An ER α morpholino (ER α MO) was designed against the zebrafish ER α translational start site with the sequence 5'-AGGAAGGTTCCTCCAGGGCTTCTCT-3'. The ER β1 MO and the ER β2 MO had the sequences 5'-GAGTTCAGAGCTGTCGCATCAGTAA-3'and 5'-AGCTCATGCTGGAGAACACAAGAGA-3', respectively The MOs were obtained from Gene Tools, LLC (Philomath, Oregon, USA) and diluted in sterile water with phenol red. A single or mixture of ER α MO, ER β1 MO and/or ER β2 MO (each at 2 ng/nl) was injected into 1 to 4 cell stage embryos.

Image analysis

Live larvae were anesthetised with 0.4% tricaine and mounted in 0.7 % low melting agarose in zebrafish embryo medium and placed onto a glass-bottom 35mm dish (MatTek). Images of stained larvae oriented in lateral, dorsal and ventral views were obtained using an inverted confocal microscopy (Zeiss), with a x10 objective lens using LSM510 Meta program. Z-stacks of line-averaged (4 lines) sections were obtained by scanning the area of 102.4 x102.4 µm (0.1 µm pixel^{-1}) and 6 µm steps over a total vertical distance of 180-240 µm, and re-constituted using LSM510 Meta program. Re-constituted images from parts of the body of larvae were aligned and the contrast was adjusted using Adobe Photoshop 7, keeping the same intensity of the adjustment for control and all different chemical treated samples. Fluorescence microscope (Leica DMI 4000 B) was used to examine tissue response dynamics and sensitivity for the EE2 exposure.

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Figure legends:

Fig. 1.Transgenes used to generate the ERE-Gal4ff/UAS-GFP system. The transgene, ERE-Gal4ff (A) contains 3 synthetic EREs, TATA, Gal4ff reporter and two Tol2 elements. The transgene, UAS-GFP (B) contains UAS Gal4 binding sequence, E1B minimal promoter and EGFP reporter. Oestrogenic signal is detected by chemical/ER complex binding to ERE that activates Gal4ff. Subsequently Gal4ff protein binds to UAS to induce EGFP.

Fig. 2. Expression of GFP in different tissues in the transgenic zebrafish larvae exposed to various oestrogenic chemicals. Transgenic zebrafish larvae without chemical exposure (A), or exposed to the synthetic oestrogen, 17 α-ethinyloestradiol (100 ng/L EE2) (B), natural oestrogen 17β-oestradiol (100 ng/L E2) (C), environmental oestrogen bisphenol A (1 mg/L BPA) (D) or 4-nonylphenol (10 µg/L NP) (E) for 4 days. Head with lateral (L) and ventral (V) views (i and ii) and trunk (iii) with lateral view. The induction of GFP was observed in the otic vesicle (ov) in the unexposed larvae (A). Different oestrogenic chemicals induced different tissue patterns of GFP expression (B-E). EE2 and E2 induced GFP expression in liver (li), heart (h), somite muscles (sm), otic vesicle (ov), lens (le), ear ganglions (eag), eye ganglions (eyg), cartilage (c), cranial muscles (cm) and neuromasts (n) (B,C). In BPA exposed larvae, heart and cranial muscle expression was enhanced (D). In NP exposed larvae strong GFP expression was detected in the muscle (both somite and cranial) (E).

Fig. 3. Detectable concentrations of different oestrogenic compounds in the transgenic zebrafish embryos; unexposed (A) and exposed to EE2 (1, 2.5 and 10 ng/L) (B-D), E2 (5 and 10 ng/L) (E,F), BPA (100 µg/L) (G) and NP (1µg/L) (H). Images were focused on liver (i) (lateral view), heart (ii) ventral view, and somite muscle (iii) (lateral view). GFP expression was detected in the liver in the 1 ng EE2/L exposure (Bi), and in liver, heart and somite muscle treated with 2.5 and 10 ng EE2/L (C,D). In the E2 exposure, a few GFP expressing cells were

observed at 5 ng E2 /L (E) whereas strong GFP expression was detected in the liver and heart at 10 ng E2 /L E2 (F). In the low concentration BPA exposure, GFP was preferentially expressed in the heart (Gii), but not in the liver or somite muscle (Gi and Giii). Weak GFP expression was detected in the heart and somite muscle in the NP treatment (Hii and Hiii). Time related (I) analyses and concentration-dependent (J) of GFP expression were performed for 4 days for EE2 exposure. K-N. Dose response of GFP induction by various EDCs using Western Blotting (EE2: K, E2: L, BPA: M and NP: N). Alpha-Tubulin was used as a loading control.

Fig. 4. Oestrogen receptor inhibitor suppression of GFP expression in the ERE TG fish. A. Unexposed control fish. B-E. Transgenic zebrafish were exposed to ICI 182,780 (1ug/L) (B) or EE2 (20 ng/L) (C) alone, or to ICI and EE2 as a mixture (D). ICI suppressed GFP expression in the otic vesicle of non oestrogen exposed fish (Bi). Strong GFP expression was observed for EE2 exposure (C) whereas GFP expression was suppressed in the treatment with ICI / EE2 as a mixture (D). Western Blotting further confirmed that the GFP expression was significantly suppressed by ICI in a quantitative manner (E).

Fig. 5. Oestrogen Receptor knockdown effects on estrogen induced GFP expression in ERE-TG zebrafish. A-D, Transgenic zebrafish eggs were injected with single or mixtures of ER α, β1 and β2 morpholinos (MO) and cultured with and without EE2 (20 ng/L). A, B, no EE2 exposure, C, D, E, F exposed to EE2. B, D. α+β1+β2 MO injected. E, β1+β2 MO injected. F. α MO injected. Western Blotting further confirmed that the GFP expression was suppressed by α+β1+β2 MO injection (G).

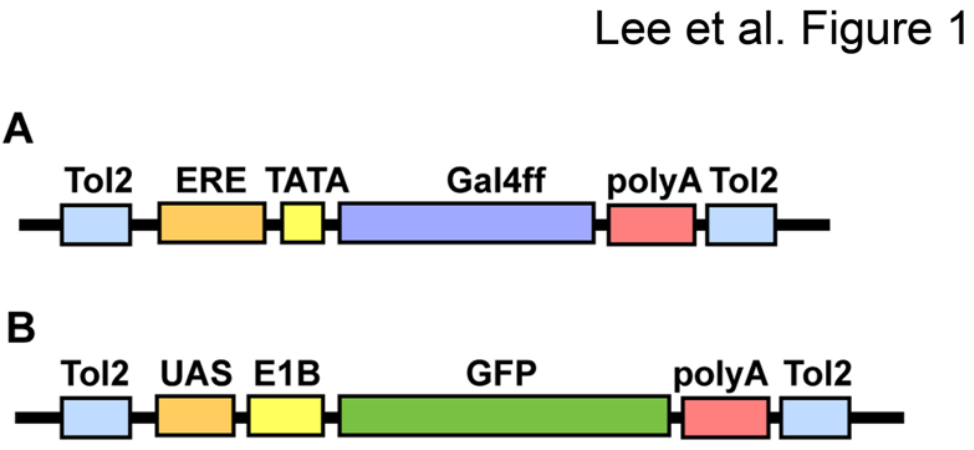
Lee et al. Figure 1
A
Tol2
ERE
TATA
Gal4ff
polyA
Tol2
B
Tol2
UAS
E1B
GFP
polyA
Tol2

Figure 1

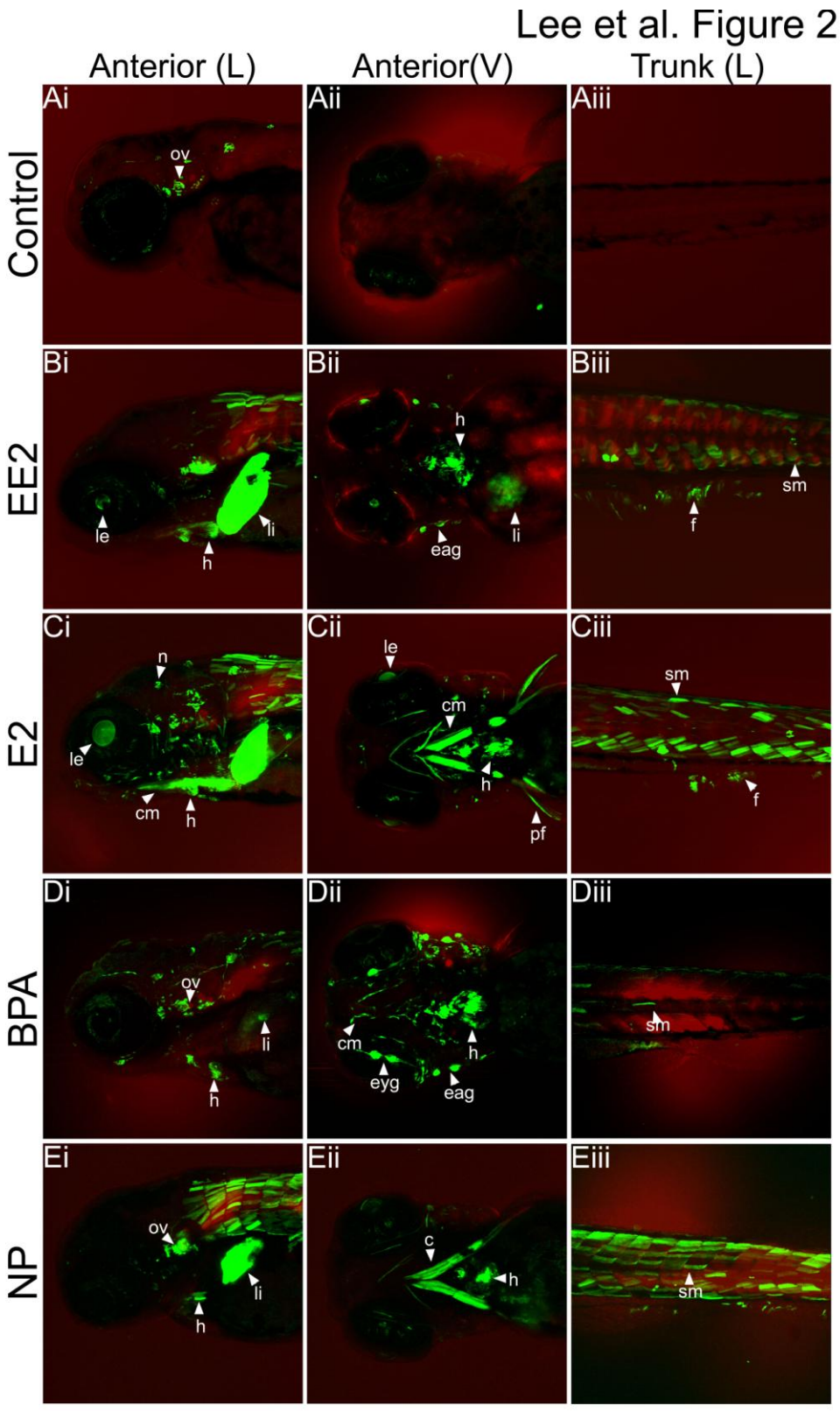
Lee et al. Figure 2
Anterior (L)
Anterior(V)
Trunk (L)
Control
EE2
E2
BPA
NP
Ai
Aii
Aiii
Bi
Bii
Biii
Ci
Cii
Ciii
Di
Dii
Diii
Ei
Eii
Eiii
ov
le
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Figure 2

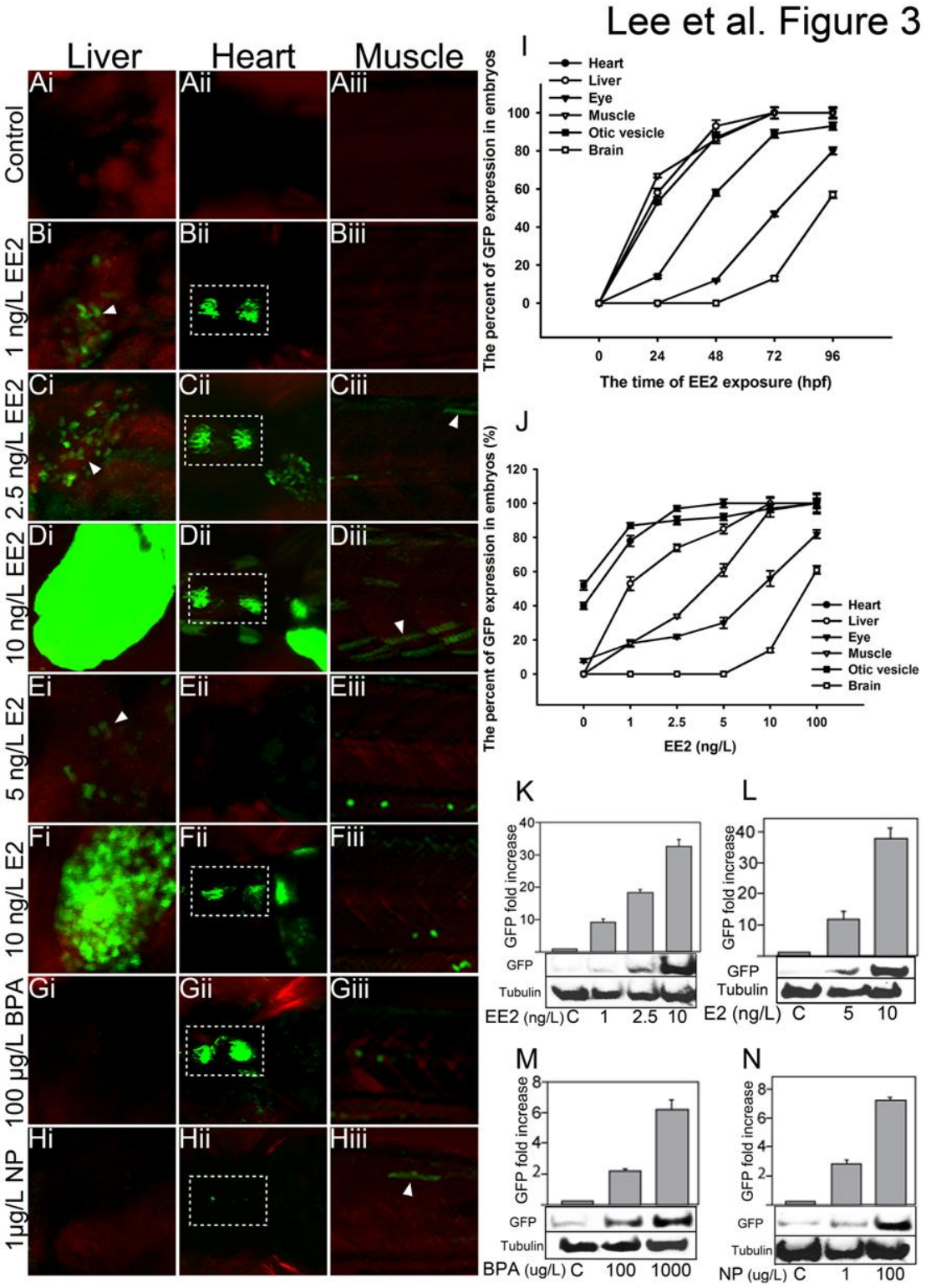
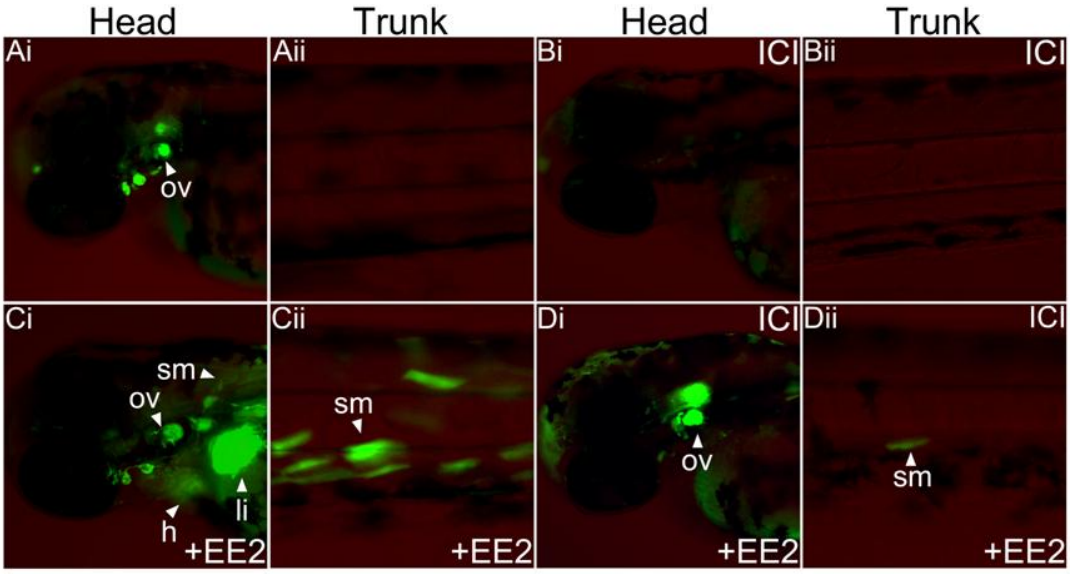

Lee et al. Figure 3
Liver
Heart
Muscle
Control
1 ng/L EE2
2.5 ng/L EE2
10 ng/L EE2
5 ng/L E2
10 ng/L E2
100 µg/L BPA
1µg/L NP
Ai
Aii
Aiii
Bi
Bii
Biii
Ci
Cii
Ciii
Di
Dii
Diii
Ei
Eii
Eiii
Fi
Fii
Fiii
Gi
Gii
Giii
Hi
Hii
Hiii
I
Heart
Liver
Eye
Muscle
Otic vesicle
Brain
The percent of GFP expression in embryos
The time of EE2 exposure (hpf)
J
The percent of GFP expression in embryos (%)
EE2 (ng/L)
K
L
GFP fold increase
GFP
Tubulin
EE2 (ng/L) C 1 2.5 10
E2 (ng/L) C 5 10
M
N
BPA (ug/L) C 100 1000
NP (ug/L) C 1 100

Figure 3

Lee et al. Figure 4



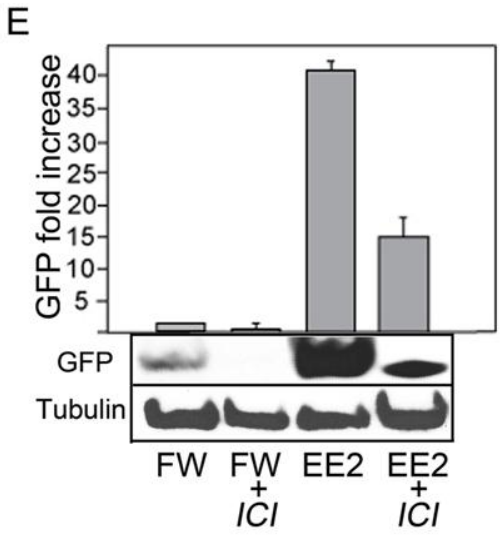
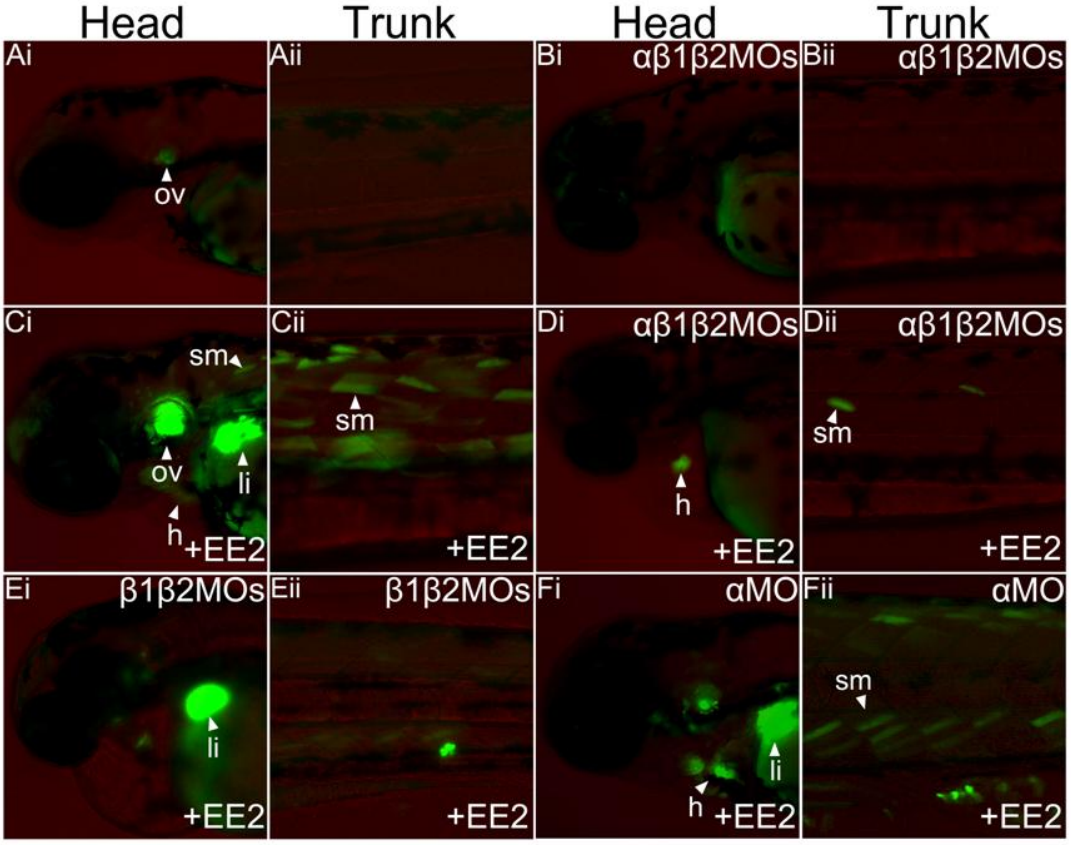


Figure 4


Head
Trunk
Head
Trunk
Ai
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Bi
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Bii
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Ci
sm
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h
+EE2
Cii
sm
+EE2
Di
αβ1β2MOs
h
+EE2
Dii
αβ1β2MOs
sm
+EE2
Ei
β1β2MOs
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li
h
+EE2
Fii
αMO
sm
+EE2

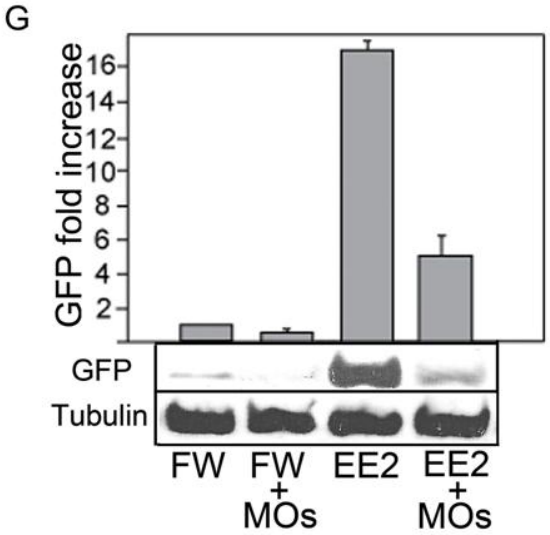
G
GFP fold increase
16
14
12
10
8
6
4
2
GFP
Tubulin
FW
FW + MOs
EE2
EE2 + MOs

Figure 5

CHAPTER 6

Target tissues responsive to 17α-ethyloestradiol in immature and adult ERE TG zebrafish- A Preliminary Analysis

CHAPTER 6

Target tissues responsive to 17α-ethyloestradiol in immature and adult ERE TG zebrafish- A Preliminary Analysis

Abstract

We have developed a sensitive oestrogen responsive transgenic zebrafish (ERE-TG fish) under an oestrogen inducible promoter with multiple tandem oestrogen responsive elements (EREc38) and a *Tol2* transposon mediated Gal4ff-UAS system. Here we assessed its potential for use in detecting responses to oestrogenic chemicals (ethinyloestradiol) in specific body tissues (liver, muscle, gonad and brain) in immature (40 day old) and mature (3 month old) ERE-TG fish for a short term (7 day) exposure. In both life stages, the lowest detectable effect concentration using conventional fluorescence microscopy to visualise GFP induction was 5 ngEE2 /L in the liver and gonad and 10 ngEE2 /L in the muscle (both nominal exposure concentrations). GFP expression was also detected in the brain but only at an exposure concentration of 25 ngEE2/L. This is the first report on the development of a transgenic zebrafish able to detect responses to oestrogen in these tissues for these life stages. These preliminary experiments support our previous finding for responses in early life stage fish and show that muscle is responsive, and thus likely affected by exposure to EE2.

The ERE-TG fish (both immature and mature fish) would appear to be a useful system for both detecting target sites and the effects of oestrogen chemicals in these life stage fish. A major limitation of this system, however, is the need to terminate the fish to allow for measurement of the oestrogen induced GFP. The microscopy technique used can also affect thresholds for detectable effects. In future we aim to develop the ERE-TG fish model in a pigment-free zebrafish, which would allow for response assessments in live fish, and to conduct more detailed analyses on the responsiveness of these fish to oestrogens, using multiphoton microscopy as a more sensitive detection method.

Keywords : EE2, transgenic zebrafish, oestrogen receptors (ERs)

Introduction

The presence of endocrine disrupting chemicals (EDCs) in the environment has become a major concern because of the potential risk to wildlife (Kolpin *et al*., 2002; Vos *et al*., 2000) and human health (Bencko, 2001). There is some evidence linking exposure to specific EDCs and their mixtures and to the decline in population levels of some wildlife species (Christin *et al*., 2003; Christin *et al*., 2004), but such examples are rare. The aquatic environment is impacted by many EDCs because these chemicals are actively discharged from industrial and wastewater treatment effluents and via agricultural run off into rivers (Leusch *et al*., 2006; Suzuki *et al*., 2004). Among these oestrogenic chemicals, synthetic birth control contraceptive 17α-ethinyloestradiol (EE2) is commonly detected and is a major contributor to the oestrogen activity in sewage effluents and thus their receiving waters (Rodgers-Gray *et al*., 2001; Tyler *et al*., 1998).

In mammals, oestrogen plays a critical regulatory role in development, reproduction, growth, and maintenance of a diverse range of mammalian tissues, including cardiovascular, immune and the central nervous system (Filby and Tyler, 2005; Korach, 1994). Oestrogenic effects are mediated via oestrogen receptors (ERs) that regulate the transcription of target genes via binding to specific DNA target sequences (Jin *et al*., 2010). Tissue- and cell-type specific effects of oestrogens are mediated by the differential expression of ER subtypes (Matthews and Gustafsson, 2003; Moggs and Orphanides, 2001). In fish, three ERs subtypes exists, ERα, β1 and β2 (Hawkins *et al*., 2000). These receptors show different in their capacities to bind to natural oestrogens and subtype-specific ligand interactions have been reported (Kuiper *et al*., 1998; Menuet *et al*., 2002; Sun *et al*., 1999). In addition, it has been reported that ER is upregulated by exposure to oestrogenic chemicals (Nimrod and Benson, 1997). ERα and β have shown distinct expression patterns in fish, but these patterns can differ between species. The highest expression of ERα in both male and female goldfish (*Carassius auratus*) has been found in the pituitary, while the ovary and testis were shown to have higher transcript levels of ERβ1 (Choi and Habibi, 2003). In goldfish (*Carassius auratus*) ER β2 is expressed predominantly in the pituitary, telencephalon and hypothalamus, as well as in liver (Ma *et al*., 2000). In the

African catfish (*Clarias gariepinus*) ERα has also been reported to be expressed abundantly in the pituitary (Teves *et al*., 2003). In contrast with these findings, the expression of ERα, β1 and β2 largely overlap in zebrafish and are predominantly expressed in the brain, pituitary, liver, and gonads (Menuet *et al*., 2002). There have been reports that ER subtypes are not modulated in the same manner by oestrogen chemicals such as EE2, NP and BPA, and ERs may not contribute equally to the transcriptional regulation of genes involved in fish development and reproduction (Wang *et al*., 2010).

Induction of vitellogenin (VTG), a precursor yolk protein, occurs in the liver, one of the most sensitive organs and a specific target for oestrogen chemicals. VTG is a widely used for detecting EDCs in fish and some other animals (e.g. frogs) (Nichols *et al*., 2001; Tyler *et al*., 1996; Versonnen and Janssen, 2004). There have been reports that sex steroids and glucocorticoid pathways are two of the primary target pathways for EE2 exposure effects in fish gonads (Flores-Valverde *et al*., 2010). In the work reported earlier in this thesis, we have shown that different oestrogenic EDCs induced specific GFP expression in a wide variety of tissues including the liver, heart, skeletal muscle, ear, forebrain, lateral line and ganglions in early life stage larvae ERE-TG zebrafish.

One of the greatest difficulties for predicting the effects of oestrogens is identifying the target tissues within the body. There are very few intact experimental systems that allow for this. An exception to this has been in the development of transgenic fish using fusion genes including luciferase (luc) from the firefly (Muller *et al*., 1993) and green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Amsterdam *et al*., 1995). The GFP gene allows for the detection of tissue-specific gene expression and cellular localisation of proteins in living cells (Chalfie *et al*., 1994; Prasher *et al*., 1992; Tsien, 1998). The use of GFP with tissue-specific promoters has become a powerful tool for studies on endogenous gene expression (Higashijima *et al*., 1997) and for analysing the function of gene promoters.

Transgenic fish have been a widely used for understanding both development and disease processes in humans and also for ecotoxicological research. Transgenic fish systems can provide advantages of both *in vivo* and *in vitro* systems, such as rapidity, sensitivity and

accuracy, and they can also be relatively cheap to use. Transgenic fish can enable visualisation of gene expression and cellular and physiological processes in live animals using GFP. This allows for integrated analyses for assessing uptake, distribution and accumulation of the chemicals in the tissues of live fish.

Here, we have developed transgenic zebrafish to screen and test for oestrogenic EDCs, to understand their pathways of effect and to assess to their potential health impacts. Levels of endogenous oestrogens differ during different life stages of zebrafish, as they serve different functional roles during development. Consequently, the effects of oestrogenic EDCs might be different in different life stages. The liver is known to be a highly sensitive target organs for oestrogenic chemicals, both in juvenile and adult fish. However, little is known about effects of oestrogenic chemicals in muscle or the brain, identified in our previous studies with larval ERE-TG zebrafish as target tissues. Thus, in these studies we examined the induction of GFP in muscle, liver, intestine and brain in immature (40 day old) fish, at the time of sexual differentiation and in mature (3 month old) fish, to compare life stage responses to EE2 exposure.

Material and methods

Fish Maintenance and Breeding

Adult oestrogen responsive transgenic (ERE-TG) zebrafish were bred in the Hatherly laboratories at Exeter University, UK. TG fish were maintained at 28 ± 1 ℃ under a photoperiod of 14 h of light and 10 h of darkness, and were fed Tetramin dry tropical flake food (Tetramin; Tetraweke, Melle, Germany) twice a day. A total of 300 fertilised zebrafish eggs were collected (within 20 minutes post-fertilization) and kept at 28 ℃ in the incubator until day 5. The larvae were then transferred to 3 aerated aquaria. Overall, 90 larvae were raised for 40 days in duplicate tanks, and 90 larvae were kept in duplicate tanks under the same conditions for 3 months for subsequent EE2 exposure experiments.

Sample Collection

40 day old fish and adult male fish (3 months old) were sampled for analysis using fluorescence microscopy (NIKON SMZ1500) (GFP expression, see experimental protocol below). For these analyses fish were anesthetised with 0.4% tricaine (3-aminobenzoic acid ethyl ester, pH 7.4; Sigma). After being anaesthetized, the fish were subsequently rinsed in ddH_2O to remove anaesthetic residues. In order to observe the GFP expression in the body tissues of immature and adult zebrafish, the fish's abdomen was opened using a scalpel blade and forceps (pigmentation and the thickness of the skin prevent live imaging analyses in the ERE-TG zebrafish at these life stages). After fluorescence microscopy analysis of GFP expression, body tissues (liver and muscle) were dissected out for use in western blot analysis. Dissection was carried out under sterile conditions (dissection instruments were soaked in 70% ethanol to sterilise and rinsed in ddH_2O). The dissection instruments were re-sterilised following the removal of each tissue.

Exposure experiments

Two exposure experiments were conducted, one with immature fish and the other with the 3 month old fish. All of the adult female ERE-TG adult female zebrafish expressed GFP in the controls (non-exposure), therefore, only male ERE-TG zebrafish were used in second experiments (mature fish). The exposure experiments were performed under flow-through conditions, with a total exchange of the test solution every two days. The experimental tanks used were 30×30×30 cm (length×depth×height; total volume= 18L). In order to determine oestrogen sensitive target tissues in the immature (40 day old) ERE-TG zebrafish we included a high EE2 exposure concentration (25 ng/L) in our analyses to ensure we would be able to detect responses in the target tissues. The immature fish were exposed to nominal test concentrations of 0, 5, 10, 25 ngEE2/L for 7 days. The mature (3 month old) male TG fish were treated with 0, 1, 5, 10 ngEE2/L for 7days. We used 1 ngEE2/L (an environmentally relevant concentration) to assess the sensitivity of the transgenic fish at these life stages. Each tank exposure tank contained 12 ERE TG zebrafish and all experiments were run in duplicate.

Western blot analysis

Western blot analysis was used to quantify GFP expression in the body tissues. Protein was extracted from liver and muscle tissue of transgenic zebrafish using freeze-thaw method with SDS sample buffer (Sambrook and Russell, 2000). Tissues were transferred to a 15 ml tube filled with buffer (10 mM Tris pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.5% Triton-X 100, 0.02% NaN3, 0.2 mM Phenylmethylsulfonyl fluoride (PMSF), 0.5 mM β-mercaptoethanol) and homogenised 6 times. After homogenisation, the samples were incubated for 30 m on ice and centrifuged for 30 m at 14,000g at 4 ℃. The supernatant was stored for western blot analysis. Protein samples were applied to 5% polyacrylamide-SDS gel and electrophoresed at 110 V for 2 h and separated proteins were transferred to nitrocellulose membrane. The membranes were blocked for 1 h in blocking solution (5% skimmed milk in 1x phosphate buffered saline (PBS) + 0.1% Tween (PBSTx)), and washed with distilled water (ddH_2O) three times. The membranes were incubated overnight at 4 ℃ with primary antibody, rabbit anti-GFP (ams Biotechonology), was diluted 1:2500 in blocking solution. The membranes were washed 3 x 15 m in PBSTx. The membranes were incubated with HRP-Goat Anti-Rabbit IgG (Invitrogen,

Carlsbad, U.S.A) at 1:2000 in blocking solution for 2 h and then, were washed 3 x 15 m in PBSTx again. For detection, western blotting luminol reagent (Thermo Scientific) was used. The intensity of GFP was analysed using Image J (http://rsbweb.nih.gov/ij/), normalised to the intensity of the alpha-tubulin band and indicated as fold increase in GFP over the level observed in the controls.

Statistical analyses

The data are expressed as mean values (± SE) and statistical analyses testing for significant differences were obtained by t-test using Sigma Plot. A value of $P<0.05$ was considered as significant.

Results

Tissue expression of GFP in response to EE2 in immature (40 day old) ERE-TG zebrafish

No GFP expression was detected in the tissues such as liver, intestine and muscle in unexposed immature ERE-TG zebrafish. GFP expression, however, was strongly induced in the liver tissue at an exposure concentration of 25 ng EE2/L (Fig. 1D). No GFP expression detected in the intestine at 25 ng EE2/L (Fig. 1D). Strong GFP expression was observed in the outer regions of the liver after exposure to 10 ngEE2/L, but this was much weaker in the central region of the liver (Fig. 1C). Weak GFP expression was also observed in the liver at 5 ngEE2/L. For brain tissue, GFP expression was observed only at an exposure to 25 ngEE2/L, with no responses in the 5 and 10 ng/L of EE2 treatment groups.

Western blot analysis found that exposure to EE2 at 25 ng/L induced a 13.4 fold increase in GFP levels in the liver compared with controls (Fig. 3A). Exposure to 10 ngEE2/L and 5 ngEE2/L in this tissue induced 8.7 and 3.7 fold increases in GFP, respectively, compared with controls (Fig. 3A). No GFP expression was detected in the muscle in the control or the lowest EE2 exposure concentration (5 ng/L) (Fig. 2A, B). However, GFP expression was strongly induced in the muscle fibre both at 25 ngEE2/L and at 10 ngEE2/L (Fig. 2C, D). In western blot analysis for the muscle, exposures to 25 ngEE2/L and 10 ngEE2/L induced 6.3 and 3.2 fold increases in GFP, respectively, compared with controls (Fig. 3B).

Threshold concentration for EE2 induction of GFP in immature ERE-TG zebrafish was 5 ng EE2/L in the liver (Fig. 1) and 10 ngEE2/L in the muscle (Fig. 2).

Tissue expression of GFP in response to EE2 in mature (3 month old fish) ERE-TG zebrafish

An adjustment to the EE2 exposure regimes compared with the study on the immature fish was adopted in adult ERE-TG male zebrafish (1, 5, 10ng EE2/L, for a period of 7 days) to establish the sensitivity of the target organs for responses to EE2 in this fish life stage. High

concentrations (25 ng/L) EE2 exposures were not adopted in these experiments as we had confirmed from the previous studies that good responses occurred (in immature fish) at lower EE2 exposure concentrations. No specific GFP expression was observed in the controls. The GFP expression pattern in the liver in mature ERE-TG zebrafish was similar to that seen in immature ERE-TG zebrafish. Strong GFP expression occurred across a major part of liver for an exposure to 10 ngEE2/L (Fig. 4A,D) and GFP expression was also seen in the liver and gonad at 5 ngEE2/L (Fig. 4D). U-shaped GFP expression patterns were seen in some parts of liver in the exposures to 5 ngEE2/L. There was no GFP expression detected in the liver in the lowest concentration studied (1ng EE2/L) (Fig. 4B). Threshold concentrations for the induction of GFP expression in the liver and gonad in adult ERE-TG zebrafish for EE2 was therefore 5ngEE2/L. Expression of GFP in response to EE2 was concentration-dependent in the liver as quantified via western blotting; exposures to 10 ngEE2/L and 5 ngEE2/L resulted in 9- and 5.9-fold increases in GFP, respectively, compared with the control.

In the muscle no GFP expression was observed in the control, but GFP expression was detected in the muscle for an exposure to 10 ngEE2/L (Fig. 4D). By western blotting exposure to 10 ngEE2/L induced a 6.9 fold increase in GFP in the muscle, but no GFP expression was detected in the control, 1ngEE2/L or 5 ngEE2/L. For brain, intestine and gonad, no GFP expression was observed in the all EE2 treated groups including control.

Discussion

Transgenic fish (zebrafish and medaka) have been developed previously to detect and assess for potential health effects of exposure to environmental oestrogens. Most of those systems however, have focused only on expression of ERs in the liver of adult fish (Menuet *et al*., 2002). Studies on other tissues affected by environmental oestrogens using transgenic fish have been largely ignored. This is surprising given the diverse roles of oestrogens in vertebrate systems. Furthermore, to date transgenic fish have not been applied for assessing sensitivity of different life stage to environmental oestrogens. It is likely that these gaps in studies with transgenic fish may be explained by the lack of sensitivity of the available transgenic lines to detect such oestrogenic responses.

Transgenic fish (zebrafish and medaka) systems recorded have largely been restricted to responses in the liver only. In contrast in the ERE-TG zebrafish developed here, we found GFP expression in multiple tissues (liver, muscle, goand and brain) in both immature and adult zebrafish for short term exposures to EE2. We were able to detect oestrogens in the water at a concentration of 5 ngEE2/L (same concentration as responses in the liver were seen), using conventional fluorescence microscopy and western blot analysis. This is an exposure level that can occur in the wild, but only in some of the most polluted environments (Jobling *et al*., 2006).

Here we compare the sensitivity of the different transgenic fish systems developed for measuring estrogens with our ERE TG fish. The fish developed by Legler *et al* (2000) were responsive (luciferase production) to approximately 300 pM E2 (approximately 81 ngE2/L) for a 96h exposure in transgenic zebrafish 4-5 weeks in age (Legler *et al*., 2002). In their studies, exposure of transgenic zebrafish aged 35 days (and exposed for 96 h) the strongest luciferase induction was observed at the dose 1000 nM E2 (approximately 272 µgE2/L) and 1000 nM E1 (roughly 270 µgE2/L) and weak induction was found in the lowest exposure concentration (0.1 nM E2: roughly 27 ngE2/L). This study suggested that juvenile fish undergoing sexual differentiation may be especially sensitive to EE2. In another study, using the same transgenic zebrafish exposure of fish from 0 to 30 dpf (days post fertilization) or for 4 days as immature

fish (from 26 to 30 days) luciferase activity was induced at the nominal EE2 concentration of 10 ng/L for both exposure periods (Bogers *et al*., 2006).

In studies by Zeng *et al*. (2005) where the *gfp* reporter gene was under the control of medaka *vitellogenin*1 (*mvtg*1) gene promoter, exposure of the transgenic medaka to E2 for 30 days found responses down to 0.5 μgE2/L. In their work they also reported GFP expression for exposures to other oestrogens including EE2 (0.05 μgEE2/L), bisphenol A (1 mgBPA/L) and oestriol (10 μgE/L). Also in transgenic medaka with choriogenin H gene regulatory elements and a GFP reporter construct responses in the liver were detected at concentrations in the liver of 0.63 nM for E2 (roughly 171 ngE2/L), 0.34 mM for EE2 (approximately 100 ngEE2/L) and 14.8 mM for E1 (approximately 4 gE1/L) (Kurauchi *et al*. 2005).

In the transgenic medaka developed by Salam *et al*. (2008) harbouring choriogenin L (ChgL) tagged with GFP exposure, responses to E2 down to 25 ngE2/L (6 days exposure) were detected. Recently, Chen at al. (2009) has reported a GFP transgenic zebrafish line under an estrogen-inducible promoter from zvtg1 gene. Although they reported GFP expression in the liver at 0.1 ngEE2/L in their transgenic fish, there was a lack of comparable controls and the reported expression was questionable.

Although our previous results with ERE-TG fish larvae showed specific GFP expression in a wide variety of tissues including the liver, heart, skeletal muscle, ear, forebrain, lateral line and ganglions as targets for exposure to EE2 down to a concentration of 1 ng/L, in our studies with immature and adult ERE-TG fish we found responses only in the liver, muscle, gonad and brain (25 ngEE2/L) at higher threshold exposure concentrations. One possibility for these findings is that these life stage fish are less sensitive to oestrogen and there are wider tissue targets compared with larval stages. A more likely explanation however relates to the microscopy applied. In the work on immature and adult fish conventional fluorescence microscopy was applied, whereas in studies on the larval fish confocal microscopy was used. Confocal microscopy can build up a very clean three-dimensional (3D) image of the sample and have better resolution horizontally as well as vertically relative to conventional microscopy. Thus the standard microscopy approach would have a significantly lower threshold detection capability

(Two-sample t-test, $p<0.05$). Western blotting analysis in the immature and adult ERE-TG zebrafish was only applied to samples for fish where we saw GFP expression. This would have answered the question on whether the differences seen between the GFP expression in the larvae compared with the immature and adult fish was in fact due to the detection systems adopted. This work needs to be done in the future. Weak GFP expression was observed in the gonads of the mature male fish by 5 ngEE2/L exposure (Fig. 4C) as it is known that the testis contains ERs (Legler *et al*., 2000). The reason for this might be that a 7 day exposure may be too short to observe an effect on gonads. A number of studies has been reported on the reproductive effects of EE2, but these studies observed the gonadal effects after at least 3 weeks exposure (Maack and Segner, 2004; Van den Belt *et al*., 2001). Again, however, this may simply relate to the need to have a higher detection capability (i.e. confocal microscopy or multiphoton microscopy).

It is possible however, that some of the differences in response thresholds to oestrogen in the different life stages relate to differences in ER expression. Wang *et al*. (2010) reported ERs may not contribute equally to the transcriptional regulation of genes involved in fish development and reproduction. In their study, they reported that *ER* α transcript was up-regulated by EE2 (0.01, 0.1 and 1 nM: roughly 3 ngEE2/L, 30 ngEE2/L and 300 ngEE2/L), but *ER* β1 mRNA expression was suppressed by these concentrations of EE2. However, ER β2 transcript was up-regulated at the low concentration (0.1 nM: approximately 3 ngEE2/L) and was down-regulated in the high concentration (1 nM: roughly 300 ngEE2/L). Moreover, different life stages might have differential responsiveness under different oestrogen compounds. These factors also need more thorough investigation to clearly establish possible differences in estrogen responsiveness between the different fish life stages studied.

A major limitation of our system with the immature and adult ERE-TG zebrafish is that we can not detect exposure effects to oestrogens in real time. The reason for this is because of the barrier of thick skin in these life stages and the skin's pigmentation. A future possibility is to create an ERE-TG fish in a pigment free strain of zebrafish (e.g. caspers or absolute). This is a project that will shortly be undertaken in the laboratory at the University of Exeter.

In summary, the experiments in this chapter were conducted as a preliminary investigation to prove juvenile and adult ERE-TG fish were capable of detecting oestrogens. They proved to be able to do so. The detecting capabilities are likely to be higher with the use of confocal microscopy or multiphoton microscopy, but this has yet to be investigated. Future studies are required to more fully assess the response capabilities for immature and adult ERE-TG fish using confocal microscopy or multiphoton microscopy and applying western blotting across a wider (and lower) ranges of oestrogen exposure. In the future too the problems associated with pigmentation that restrict analyses of GFP in these fish in real time may be overcome by creating the ERE-TG system in a pigment free line of zebrafish.

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List of Figures

expression was detected for exposure to 5ngEE2/L and 10ngEE2/L (C, D). Lateral views are shown. The abdomen of the TG was opened for observation.

Fig. 5. GFP expression in the muscle in response to 17α-ethinyloestradiol (0, 1, 5 and 10 ngEE2/L) exposure for 7 days in adult ERE TG zebrafish. No GFP expression was detected in the muscle fibre in the control, 1 ngEE2/L and 5 ngEE2/L exposures (A, B and C). Strong GFP expression was seen in the muscle fibre for exposure to 10 ngEE2/L (D). Skin was removed to observe GFP expression in the muscle fibre. Figure shows lateral view.

Fig. 6. Western blotting was analysed to quantify the green fluorescent protein. There was no GFP detected in the liver in the control and 1ngEE2/L (A). Weak GFP expression was detected at 5 ngEE2/L (A) and strong GFP expression was induced at 10 ngEE2/L (A). For muscle, no GFP expression was detected in control, 1 ngEE2/L and 5 ngEE2/L. There was strong GFP expression detected in the muscle fibre at an exposure concentration of 10ng EE2/L (B). GFP expression was thus induced in a concentration dependent manner for EE2. GFP expression level was quantified by using Image J (http://rsb.info.nih.gov/ij/index/html). Error bars represent the standard deviation.

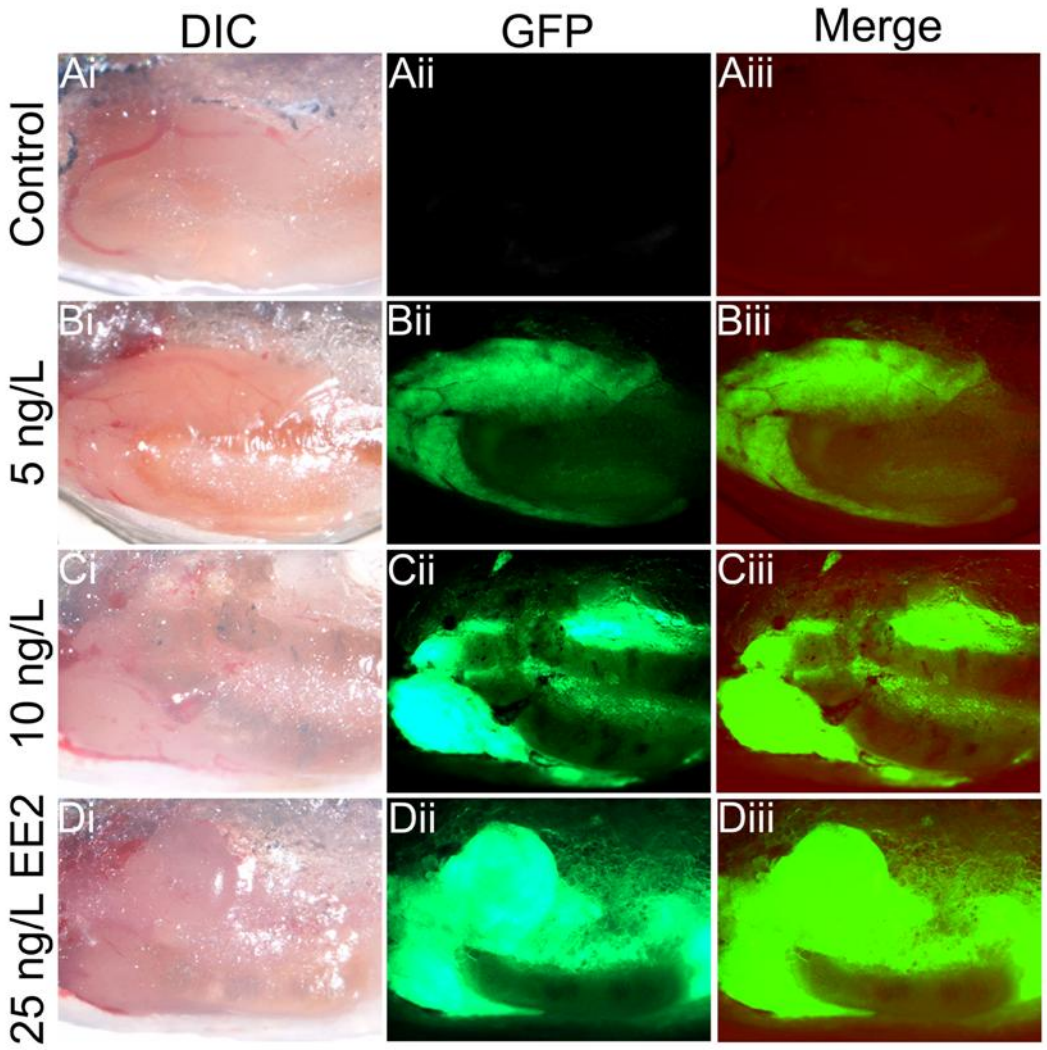
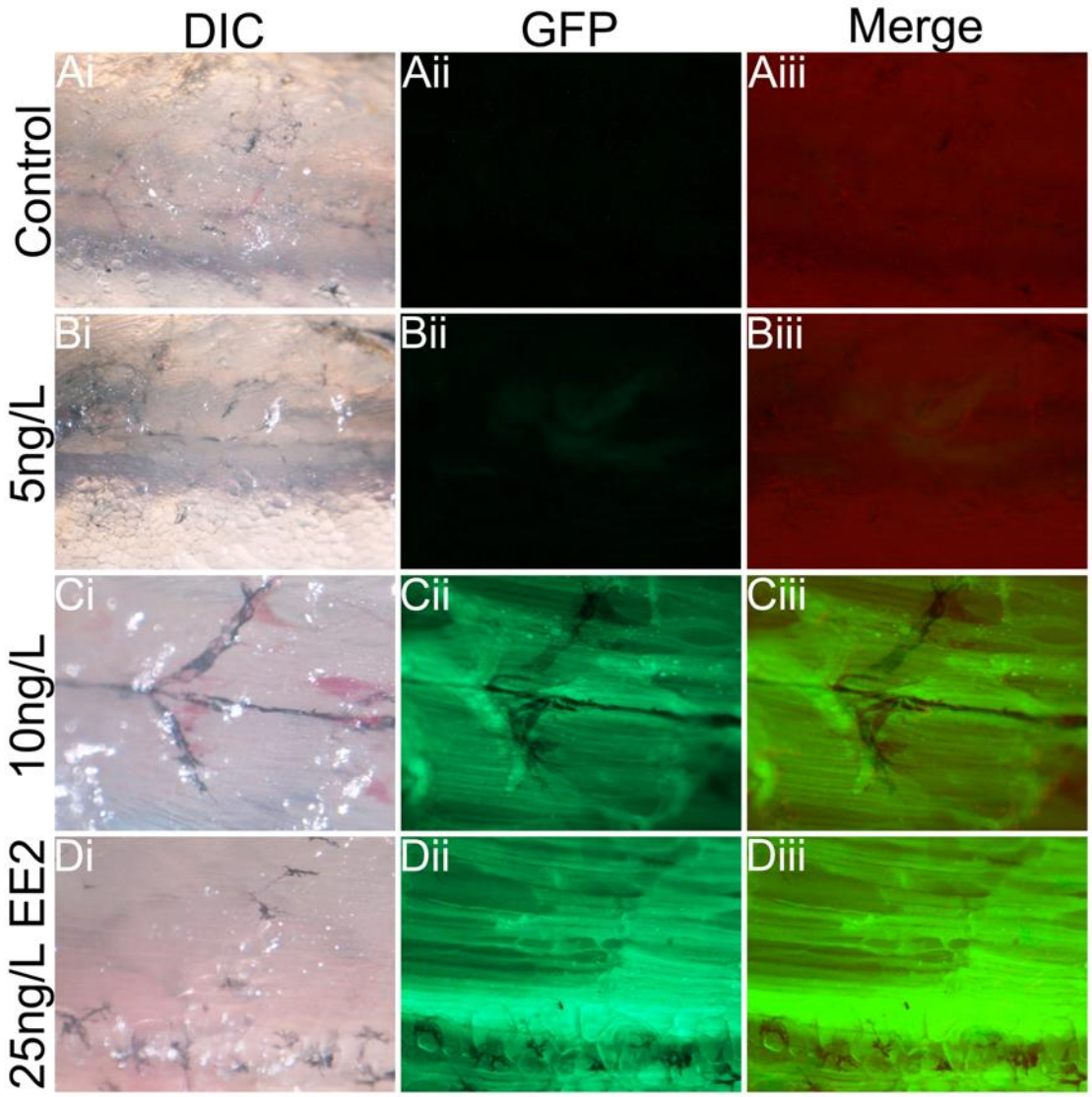

DIC
GFP
Merge
Control
5 ng/L
10 ng/L
25 ng/L EE2
Ai
Aii
Aiii
Bi
Bii
Biii
Ci
Cii
Ciii
Di
Dii
Diii

Figure 1


DIC
GFP
Merge
Control
5ng/L
10ng/L
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Biii
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Cii
Ciii
Di
Dii
Diii

Figure 2

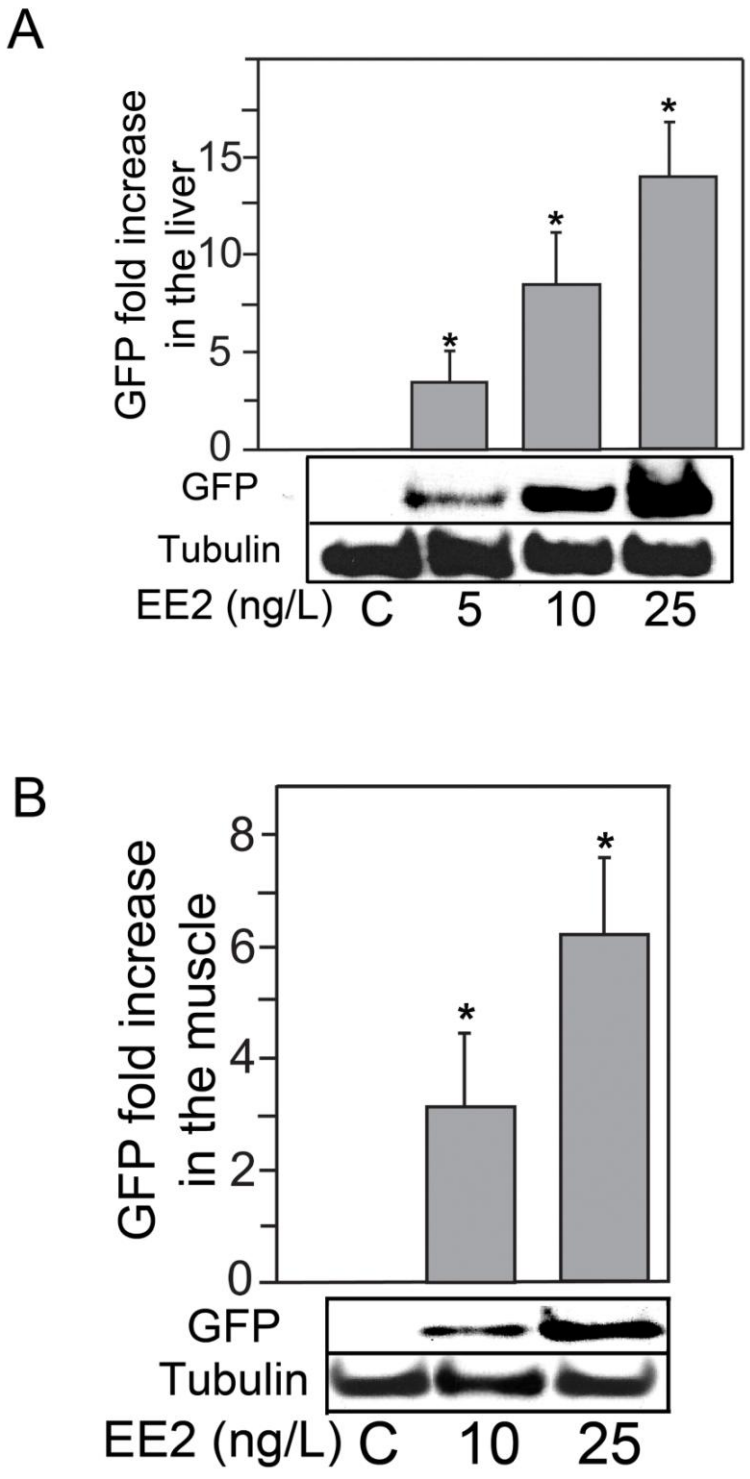
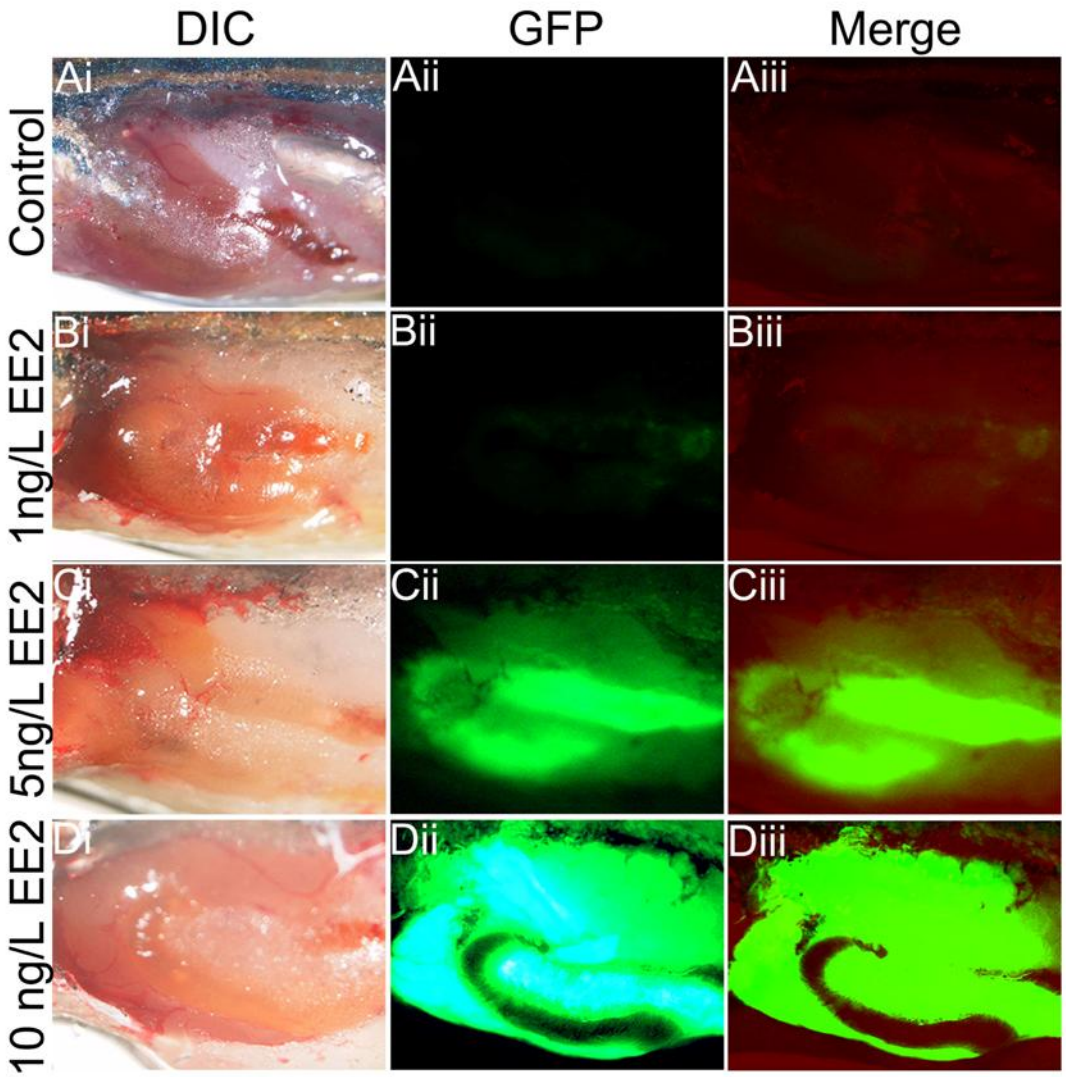

A
GFP fold increase in the liver
0
5
10
15
*
*
*
GFP
Tubulin
EE2 (ng/L) C 5 10 25
B
GFP fold increase in the muscle
0
2
4
6
8
*
*
GFP
Tubulin
EE2 (ng/L) C 10 25

Figure 3


DIC
GFP
Merge
Control
1ng/L EE2
5ng/L EE2
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Ai
Aii
Aiii
Bi
Bii
Biii
Ci
Cii
Ciii
Di
Dii
Diii

Figure 4

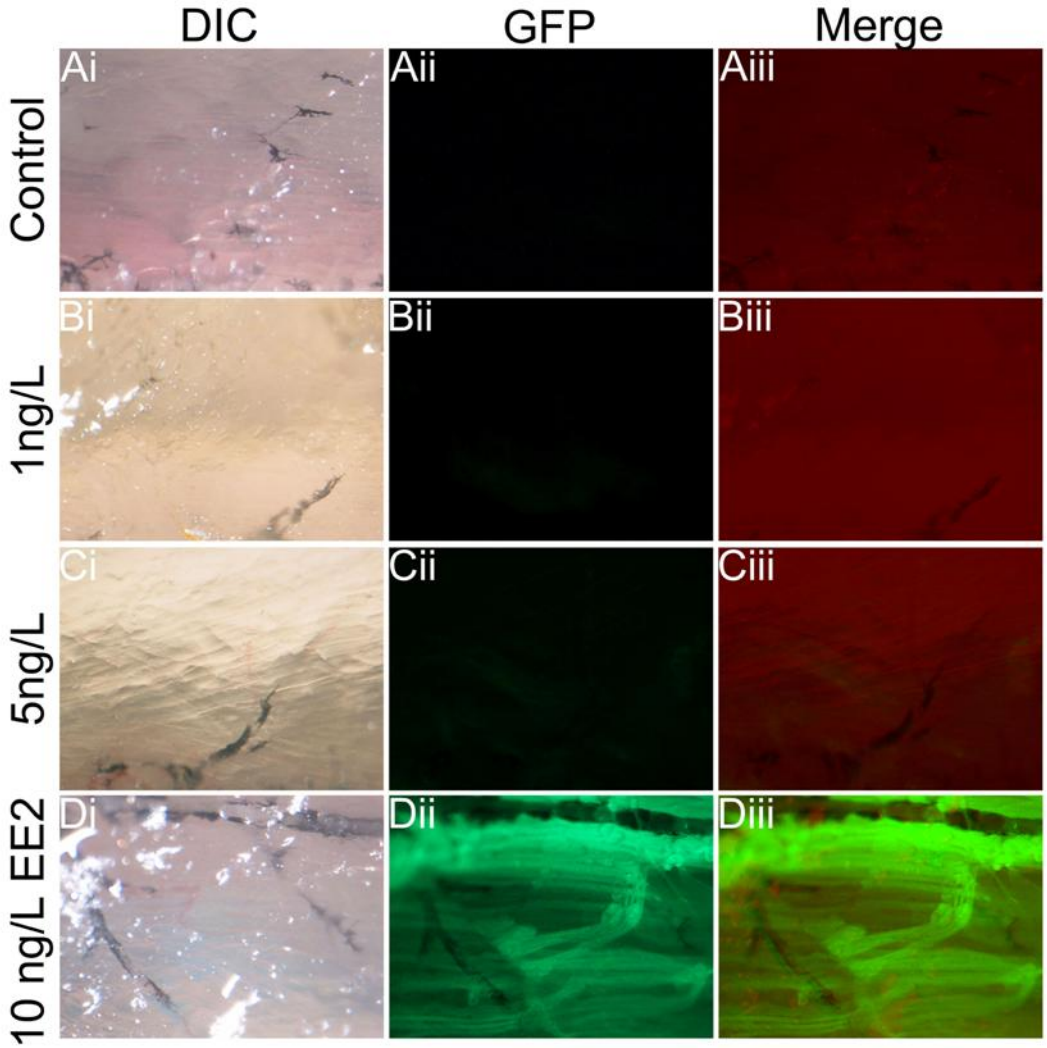
DIC
GFP
Merge
Control
1ng/L
5ng/L
10 ng/L EE2
Ai
Aii
Aiii
Bi
Bii
Biii
Ci
Cii
Ciii
Di
Dii
Diii

Figure 5

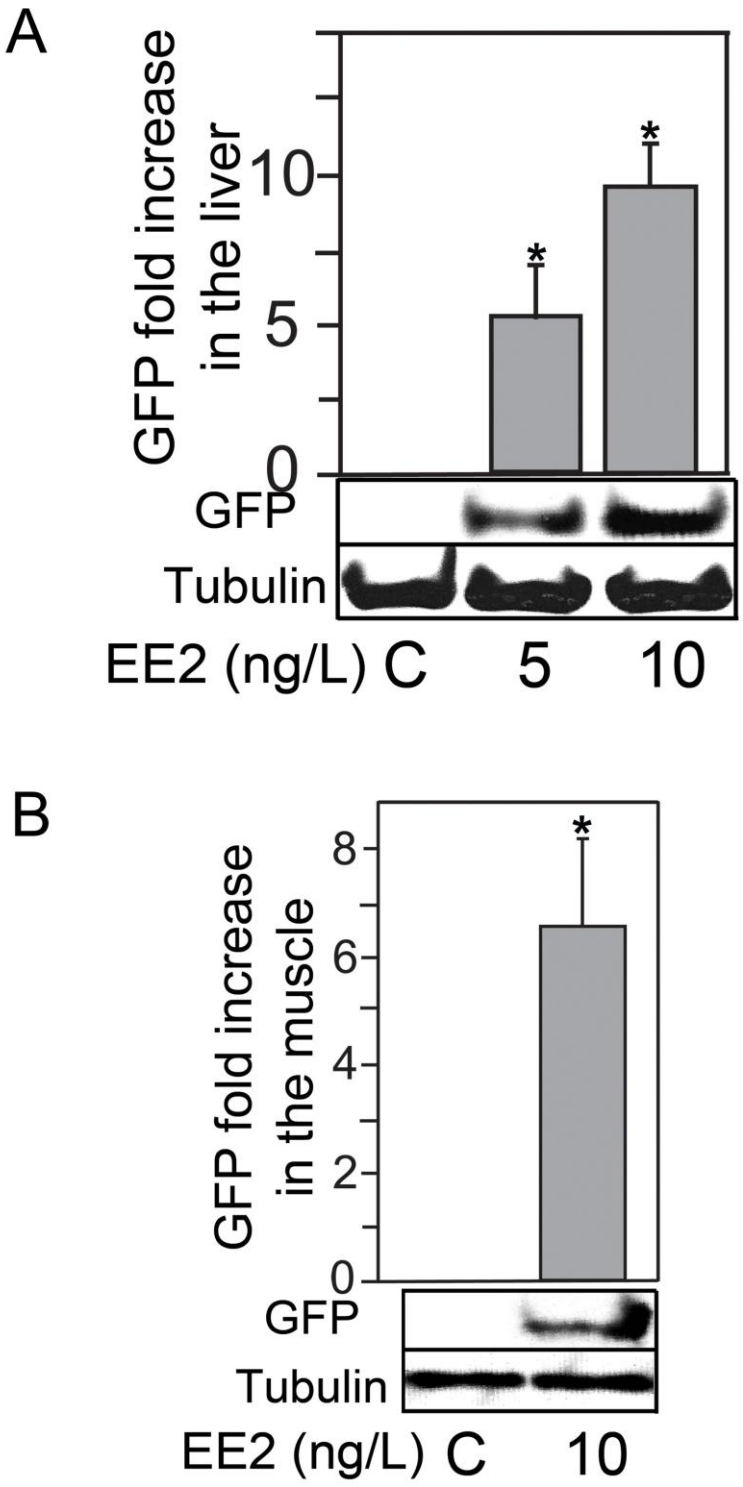
A
GFP fold increase in the liver
0
5
10
*
*
GFP
Tubulin
EE2 (ng/L) C 5 10
B
GFP fold increase in the muscle
0
2
4
6
8
*
GFP
Tubulin
EE2 (ng/L) C 10

Figure 6

CHAPTER 7

GENERAL DISCUSSION

CHAPTER 7: General Discussion

7.1 Overview of the ERE-TG zebrafish developed

It is well established that there is a wide range of chemicals that can mimic hormones and disrupt physiological function in an organism. One of the most commonly occurring group of hormone mimics are environmental oestrogens and they have been found to induce significant disruptions in reproductive function in a range of wildlife species and in humans too (Guo *et al*., 1995; Melzer *et al*., 2010; Zinaman and Katz, 1997)). Indeed, it is principally environmental oestrogens that have driven a heightened awareness of the need to more thoroughly understand the possible health implications associated with EDCs. As a consequence a wide range of screening and testing systems for environmental oestrogens, have been developed, including both *in vitro* to *in vivo* systems. All have their advantages and disadvantages, but few offer truly integrative systems for probing for the possible wider health effects associated with EDCs of any nature.

Transgenic fish, in contrast, have huge potential as test systems as they provide an integrative system that allows for comprehensive effects assessment of oestrogens across multiple tissues in the body. The main aim of this thesis was to develop transgenic zebrafish for the detection of oestrogens allowing further assessment of the potential health impacts of oestrogens in the environment. Three classes of oestrogens were tested, including steroids (natural and synthetic), an alkylphenol (NP) and a plasticizer (BPA) in both transiently and stably transfected zebrafish.

Various TG fish have been constructed previously, including for measurement of exposure to, and effects of, environmental oestrogens, and thus, the endeavour in this thesis may not seem to be especially novel. The TG systems available, however, have suffered a number of limitations, including the detection methodology for responses to oestrogens (e.g. luciferase; Legler *et al*., 2000), not being able to be undertaken in live fish. Other constructs developed, such as pEGFP-ChgL (Ueno *et al*., 2004), pZVTG1-EGFP (Chen *et al*., 2010)) have been based on liver specific genes, that although of use as biomonitoring systems for environmental oestrogens, do not allow for the wider health effects of oestrogens to be assessed.

In this thesis work, therefore, we set out on an ambitious task to develop a TG zebrafish that was able to assess for the effects of oestrogens across multiple tissues and, critically, using a marker system (GFP) that could be monitored in live fish. To facilitate this work, new plasmids had to be constructed which included cloning and incorporation of EREs. Importantly, the ERE would bind all ER sub-types and thus in the TG fish capture responses via all ER mediated pathways. This proved to be successful as in the final testing of our TG fish morpholinos for the different ER subtypes ablated oestrogen induced responses differently in the different tissues.

The development of the required construct took many attempts and a considerable period of time (18 months) and progressed through a series of sequential constructions using three different plasmid vectors, pBluescript KS+, PCS2+ and pBR322_Tol2. A major obstacle in the development of TG has been the successful genome integration of the plasmid construct. To enhance our chances of doing so we applied a *Tol2* vector system, that had been reported to provide germline transmission frequency of up to 50 % (Kawakami *et al*. 2000). This proved to be a successful approach in the synthesis of the ERE-TG zebrafish.

The ERE-TG fish in this thesis were constructed using the Gal4-UAS/GFP signalling system. This was adopted to produce a two step amplification of the oestrogenic signal, with the desire to produce a sensitive responding system for detecting exposure to oestrogens. In this system, the sequence of activation events that occur is; the oestrogens bind to ER and that complex subsequently binds to ERE inducing the synthesis of Gal4ff. This in turn binds to its target sequence UAS to activate gene transcription and induce synthesis of GFP. Again, this proved to be a highly successful with the ERE-TG produced having a response sensitivity to EE2 of 1 ng/L. In adopting Gal4ff rather than the more commonly used Gal-VP16/UAS we found no toxicity associated issues at all.

A major concern for any TG fish system is that is needs to be a reproducible system that persists across generations. We have found this to be the case for our ERE-TG zebrafish. Over 3 generations (F0-F3) the ERE-TG fish are showing a consistent response to oestrogens. Response patterns in GFP for exposure to oestrogens are proving to be consistent across tissues

in 4 day old larvae tissues. Sensitivity thresholds for steroid oestrogens are also proving to be highly consistent, as confirmed in very recent exposure studies with F3 generation ERE-TG zebrafish. Importantly, we have not found any evidence for gene silencing.

The ERE-TG zebrafish developed respond to environmental oestrogens that are diverse in nature (spanning steroidal oestrogens to an alkylphenol [NP] and a plasticizer [BPA]) sufficiently sensitive for environmentally relevant exposures. They show tissue specific responses, identify target tissues sensitive for potential health effects and ER morpholinos prove that at least some of the responses seen (GFP induction) operate through the ERs. The ERE-TG zebrafish therefore provides a model system for both probing for the wider health effects of oestrogens across different life stages and for screening and testing chemicals for possible oestrogen activity, in real time. They thus represent a considerable advancement for studies into the physiological processes and systems affected by oestrogens.

The following sections provide a critical analysis on the methodological approaches used and the new systems developed, focusing on the transient expression assay and the ERE-TG. Opinion is provided on the utility of the systems for future work in developing other TG fish and for application of the ERE-TG zebrafish to assess the associated health effects for exposure to environmental oestrogens.

7.2 The transient expression assay

A major challenge in creating TG fish lines of zebrafish is the time that it takes to do so. A vital element in the development of the ERE-TG fish was to test the functionality of the plasmid in a step-wise manner to avoid a TG fish that was subsequently not fit for purpose (i.e. specific and sufficiently sensitive to oestrogens). This was undertaken through use of a transient expression assay and detecting GFP induced by oestrogen via *in-situ* hybridisation or green fluorescent microscopy. This approach allowed me to ensure that the DNA elements integrated in the vector were indeed working and sensing the oestrogen during the long construction processes. In developing such a transient assay, many features of the injection process needed to

be optimised. This included the embryo stage, incubation temperature, amount of DNA and a size of needle. The following were found to be optimal for this work: 1-2 cell staged embryos, 28 ℃, approximately 20 ng/L of DNA and the gauge of the needle finally adopted was for microinjection. We would recommend these conditions for future work for transient assays with zebrafish.

In this thesis work was undertaken to investigate for the optimal life stage for use in screening for oestrogen responses in the transient expression assay. We found that different tissues showed different temporal responses to oestrogen exposure (as measured by GFP induction post oestrogen exposure; see Chapter 5). At 72 hpf however, there were both strong and specific tissue GFP expression patterns (in response to EE2) that were especially well defined in the liver, muscle and heart. This was also the case for the work with medaka. It is recommended therefore that this life stage is optimal for screening for oestrogenic activity of chemicals in the ERE-TG zebrafish.

Results with our transient assay in assessing the relative potencies of various environmental oestrogens were consistent with those reported in the literature with EE2 being the most potent compared with E2 and with NP and BPA. Legler *et al*. (2002) similarly showed that 17α-ethinyloestradiol (EE2) was the most potent (xeno) oestrogen compared with 17β-oestradiol (E2), and nonylphenol in their transgenic zebrafish assay. Relative potency estimates for these chemicals vary depending on the assessment method. For example EE2 was shown to be 1.25 fold more potent than E2 in yeast-based *in vitro* assay (Beck *et al*., 2006) but had around a 30 times higher potency than E2 and E1 for induction of VTG in female zebrafish (Van den Belt *et al*., 2004) in *in vivo*. Thorpe et al. (2003) similarly reported that EE2 was between 11 to 27 times more potent than E2 using a VTG induction assay *in vivo*. Comparing the relative responses to the different oestrogens in the MorDarT assay using developing zebrafish the relative abundance of VTG1 mRNA, EE2 was 100 times more potent than E2, and 90000 times more potent than BPA (Muncke *et al*., 2006; MorDarT is *D. rerio* teratogenicity test).

The transient assay developed is as sensitive as some of the various available TG fish (although it is questionable whether they should be compared directly) or some other oestrogen detection systems (e.g. VTG induction). For example, the most sensitive TG fish developed have been shown to respond to EE2 at 10 ngEE2/L (Bogers *et al*., 2006), but the lowest detectable concentration in the transient assay for EE2 was 10 ngEE2/L. Detection threshold for EE2 (as an example), *in vivo* for VTG induction in adult male rainbow trout; (Purdom *et al*., 1994)) is between 0.1 and 1.79 ngEE2/L. EE2 concentrations required for the induction of VTG in *in vitro* hepatocyte cultures for medaka and common carp are considerably higher (Bickley *et al*, 2007; Kordes *et al*., 2002).

A limitation of the transient expression system is that it requires microinjection of the embryo and this is affected by many factors, especially the expertise of the worker. Our transient assay system, nevertheless is novel - there are no reports previously using transient expression analysis for detection of oestrogen compounds - and can be applied to screen chemicals to test for full body systems wide effects in early life stage fish.

7.3 ERE-TG zebrafish larvae

The ERE-TG zebrafish larvae are the most sensitive TG fish system developed for detecting environmental oestrogens. This higher sensitivity is likely due, at least in part, to the multiple tandem EREs incorporated and the *Tol2* mediated Gal4ff-UAS system. The responses to oestrogen in the ERE-TG fish system equate favourably with that found for a number of other *in vivo* systems and *in vitro* test systems for oestrogens. The lowest detectable concentrations in the ERE-TG fish for EE2, E2, BPA and NP were 1 ngEE2/L, 5 ngE2/L, 100 µgBPA/L and 1 µg NP/L). The detection limit for EE2 induced responses in the TG zebrafish using luciferase as a reporter was 10 ngEE2/L (Bogers *et al*., 2006). Thus, although favourable, the response detection for oestrogen exposure in our ERE-TG fish does not reach that for VTG induction (as a simple biomarker for exposure) where responses down to between 0.1 to 1.79 ngEE2/L have been reported in adult male rainbow trout (Purdom *et al*., 1994). Rainbow trout however are

well established to be more sensitive to oestrogens compared with fish belonging to the cyprinid fish family (Tyler *et al*., 2005). Oestrogenic activity for NP has not been detected in other oestrogenic TG fish previously for environmentally relevant exposures. NP concentrations down to 0.65 µg/L for 3 weeks exposure have been shown to induce VTG synthesis (Harries *et al*., 2000).

A potential drawback with the ERE-TG fish developed is that the transgenic zebrafish can show high inter-individual variation in their response to oestrogens. GFP expression was observed in the otic vesicle and also in the heart in some ERE-TG fish larvae without oestrogen exposure. The differences seen in background expression of GFP (and potentially also contributing to inter-individual response variability) might relate to the sex of the fish. We were unable to prove this however because, as yet, there is no available sex probe for the zebrafish.

Without doubt one of the greatest assets of the newly developed ERE-TG zebrafish is that in early life stages they can be applied to identify the range of body tissues responding to oestrogen exposure. We found that our TG fish detected oestrogenic activity in a wide variety of tissues including the liver, heart, skeletal muscle (somite and cranial), ear/eye ganglions, brain, otic vesicle, lens and neuromasts. In addition, the somite muscle cells, cranial muscle cells, heart cells and neuromast cells appeared to be especially responsive to oestrogen. We show for the first time oestrogen responses (via GFP expression) in the muscle (somite and cranial) in live fish.

A key finding for the ERE-TG fish is that the tissue-specific expression patterns during development not only suggests a role for oestrogen receptors during development, but also indicates that there may be differences in tissue toxicities for different life stages. Importantly, our ERE-TG fish were able to identify different chemicals which are classified as environmental oestrogens can have different target tissues, and thus our system opens up new avenues for developing a better understanding of how different environmental oestrogens work in the body.

Life stage differences in responses to oestrogens and differences on health effects for different developmental stages, most likely relate to the expression patterns of the different ER subtypes (at least in part). In spite of the fact that there are three ER subtypes in fish, their different roles in developmental processes have not been well established. Our ERE-TG system in zebrafish larvae enables the ER subtype effect pathways to be investigated relatively easily using ER MO. We show this to good effect in the studies that have investigated how the oestrogenic responses within the different tissues were mediated (for exposure to EE2). Interestingly we show that normal development relating to the body trunk and hatching time depends on the ER signalling pathways. Our ER MO work has also shown that not all oestrogen signalling responses (as detected by GFP expression) occurred via ER α, β1 and β2 injection. Thus, some or these responses may include oestrogen receptor related receptors (ER α, β and γ) as is known to occur in mammals (Yang *et al*., 1996).

7.4 ERE-TG fish (immature and mature)

As a preliminary experiment, immature and mature transgenic zebrafish were examined for their responses to EE2 because the levels of oestrogens differ during different life stages and there may be life stage specific effects for oestrogen exposures. This work however, was much more restricted compared with the studies on larvae. This is because the skin thickness and pigmentation prevent responses studies (and visualisation of those responses) in the intact animal and in real time. The fish have to be killed to study the GFP responses to oestrogen. In this work however we were able to show responses to EE2 down to 5 ng/L in the liver and gonad, and 10 ngEE2/L in the muscle in both immature and mature transgenic zebrafish. This would suggest a different (higher) response threshold compared with in the larvae. This is not necessarily the case however as the analyses were conducted with conventional fluorescence microscopy to detect GFP expression, rather than confocal that was applied in some of the work on the larval stages detailed above.

7.5 Some final thoughts on the limitations/problems in applying the ERE-TG fish and some future prospects

Although this thesis work has been highly successful in developing ERE-TG (and has taken preliminary steps to apply them) for detecting responses to environmental oestrogens there are a number of inherent difficulties/limitations to the system, some of which can be overcome with further investment. One aspect is the need for careful planning and investment for maintaining the required number of ERE-TG fish for future studies. Separate lines of the ERE-TG zebrafish will need to be bred and subsequent generations tested on a regular basis through routine screening of their responses to oestrogens (tissue profiles and response sensitivity etc.). This is vital to ensure consistency in responses across generations.

A major limitation is the inability to observe GFP expression in the internal organs in the both immature and mature transgenic zebrafish using fluorescent microscopy. Pigmentation in the skin prevents this. As a consequence, unlike for the larval stages, the effects analysis cannot be conducted in live fish in immature/adult fish and this imposed further limits of possible experiments (repeat exposure analyses etc.). One possibility to circumvent this issue, as mentioned in chapter 6, is to develop the ERE-TG system in a pigment free line of zebrafish (e.g. caspers or absolute).

It is also the case that given female fish, both immature fish and as adults, contain varying (but significant) levels of circulating oestrogen, that studies on ERE-TG fish as immature/adult fish are likely only practicable in males. Separating immature zebrafish by sex is a very difficult proposition (it can easily be in adults). This would be made easier with the availability of a sex specific probe but this has not yet been forthcoming for this species.

The larval stages of the ERE-TG zebrafish however, offer huge potential for studying many aspects of oestrogen function and effects in fish (and other vertebrates). The fact that they are highly sensitive, show tissue related response specificities, that the signals (for the most part) operate through the different ERs, are able to conduct MO work to knock down effects through specific receptors and possess to the ability to visualise effects in live animals, in real time,

opens up this model for arguably a greater number of research questions than any other model system presently developed for oestrogens. The ERE-TG larval stages also offer the potential for development for high throughput screening of oestrogens. Some of the key areas of research for which the ERE TG zebrafish could usefully be applied include:

1.) Enhancing our fundamental understanding of the role of oestrogen, the mechanisms of oestrogen signalling and the specific functions of cells in organisms and how they are influenced by oestrogens (through over expression studies and ER MOs)

2.) Developing a more comprehensive understanding on life long oestrogen exposure effects. The fact that it is possible to visualise responsive tissue to oestrogen exposure during early life offers the possibility to track subsequent effects in those specific tissues in later life.

3. Advancing our understanding on mixture effects. The ERE-TG embryos/larvae could be very usefully applied to study how oestrogenic chemicals interact within the body to affect different body tissues, what the effects of sequential exposures are and how real world mixtures (e.g. oestrogenic STW effluents) affect developmental process and fish health.

CHAPTER 8

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