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A living biosensor model to dynamically trace glucocorticoid transcriptional activity during development and adult life in zebrafish

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ABSTRACT

Glucocorticoids (GCs) modulate many cellular processes through the binding of the glucocorticoid receptor (GR) to specific responsive elements located upstream of the transcription starting site or within an intron of GC target genes. Here we describe a transgenic fish line harboring a construct with nine GC-responsive elements (GREs) upstream of a reporter (EGFP) coding sequence. Transgenic fish exhibit strong fluorescence in many known GC-responsive organs. Moreover, its enhanced sensitivity allowed the discovery of novel GC-responsive tissue compartments, such as fin, eyes, and otic vesicles. Long-term persistence of transgene expression is seen during adult stages in several organs. Pharmacological and genetic analysis demonstrates that the transgenic line is highly responsive to drug administration and molecular manipulation. Moreover, reporter expression is sensitively and dynamically modulated by the photoperiod, thus proving that these fish are an *in vivo* valuable platform to explore GC responsiveness to both endogenous and exogenous stimuli.

1. Introduction

Glucocorticoids (GCs) are essential steroid hormones secreted by the adrenal cortex and the interrenal tissue of the head kidney in mammals and teleost fish, respectively, through a regulatory feedback loop under the control of the hypothalamic–pituitary–adrenal/interrenal (HPA/I) axis.

Cortisol is the main circulating GC both in teleosts and most mammals, including humans, while corticosterone is the major GC in rodents, amphibians, reptiles and birds (Bury and Sturm, 2007). GCs regulate many physiological processes, including intermediary metabolism, immune system, behavior and stress response (Sapolsky et al., 2000; Gross and Cidlowski, 2008). In mammals, GCs are also crucial for embryogenesis and development (Nesan and Vijayan, 2013). In the zebrafish, *Danio rerio*, unfertilized eggs and embryos during early stages of development have been shown to contain both cortisol and glucocorticoid receptor (*gr*) mRNAs (Alsop and Vijayan, 2008; Pikulkaew et al., 2010). The latter has been previously postulated to be essential for embryonic development, since its morpholino-mediated knockdown triggers several developmental defects, altered mesoderm patterning and limited survival of the embryos (Pikulkaew et al., 2011; Nesan et al., 2012).

The activation of the GC signaling pathway mainly depends on the binding to the cognate GC receptor, GR, a member of the nuclear receptor family of ligand-activated transcription factors, that is expressed in most tissues where it regulates tissue-specific sets of genes (Gross and Cidlowski, 2008; Chrousos and Kino, 2009). In the absence of ligand, GR is confined in the cytosol as part of a multiprotein complex that includes heat shock protein 70 (HSP70) and HSP90 (Rose et al., 2010). After GC binding, GR translocates into the nucleus, where it directly binds to GC responsive elements (GREs) in the promoter region of target genes or indirectly by means of protein–protein interactions with other DNA-binding proteins (Schoneveld et al., 2004; Rose et al., 2010).

An adult viable mutant zebrafish strain, named s357gr−/− has been recently identified (Ziv et al., 2013). In this mutant line, DNA binding activity of the receptor has been abolished by a single base-pair substitution in the DNA-binding domain leading to the replacement of an Arginine with a Cysteine (R443C). Gr-s357 mutants are viable, but show behavioral abnormalities, such as elevated startle response (Griffith et al., 2012) as well as a hyper-activated HPA axis (Ziv et al., 2013). Viability of larvae and

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adult s357 homozygous mutants apparently contrasts with *gr*-MO knockdown results (Pikulkaew et al., 2011; Nesan et al., 2012). However, during early development, Gr-s357 homozygous mutants are supplied with GR protein and gr mRNA of maternal origin.

The GRE is shared by activated homodimerized receptors for GCs, mineralcorticoids, progesterone and androgens (Adler et al., 1992; Merkulov and Merkulova, 2009). It is composed of two imperfect palindromic, hexameric half-sites separated by a 3-nucleotide hinge (GGTACAnnnTGTTCT). A GR monomer binds first to the 3′-half-site, the most conserved one, followed by a second monomer that binds to the 5′-half-site, resulting in a DNA-bound GR dimer (Schoneveld et al., 2004). Alternatively, GR can work as a monomer bound only to the 3′-half-site (Merkulov and Merkulova, 2009).

To study GC activity a transgenic zebrafish line (GRE:Luc), in which four GRE tandem repeats drive luciferase reporter gene expression, has been recently developed (Weger et al., 2012). However, the GRE:Luc reporter gene allows less spatial resolution than that obtained by the green fluorescent protein (GFP) *in vivo* imaging (Hoffman, 2008). The advantage of using fluorescent proteins has been already shown in stable transgenic zebrafish lines, where the expression of reporter proteins is driven by responsive elements for different intracellular signaling pathways (Schwend et al., 2010; Laux et al., 2011; Gorelick and Halpern, 2011; Moro et al., 2012).

Hence, we here report the generation and validation of a stable transgenic zebrafish line in which Enhanced-GFP (EGFP) expression is driven by nine GRE tandem repeats. This line shows, in the absence of exogenous GCs, strong EGFP fluorescence starting with an ubiquitous pattern at early somitogenesis, and becoming mostly localized in brain and trunk muscles by 24 h post-fertilization (hpf). By 2–3 days post-fertilization (dpf), the fluorescence is detectable in well-known GC targets, such as liver, pancreas and intestine, and in new unpredicted tissues such as the cristae and lateral canals of the otic vesicles, scattered dermal mesenchymal-like cells and presumptive Kolmer-Agdur (KA″) interneurons, thus revealing novel GC targets.

This transgenic line (named ia20Tg following the Zebrafish Model Organism Database nomenclature) with enhanced sensitivity and spatial resolution represents a promising readout model to investigate the physiological functions of GC signaling *in vivo* during zebrafish development and adult life. Moreover, it may allow to study the circadian rhythm and modulation of neuronal and behavioral responses during feeding and stress as well as to detect compounds able to influence glucocorticoid-dependent responsiveness in pharmacological, toxicological and environmental research.

2. Materials and methods

2.1. Animals maintenance and handling

Zebrafish (*D. rerio*) were raised, staged and maintained according to standard protocols (Kimmel et al., 1995; Westerfield, 1995). Fish are kept in a 14 h light/10 h dark light cycle with light turning on at 8.00 am and off at 10.00 pm. For screening after 48 hpf and *in vivo* imaging, embryos and larvae were anesthetized with 0.04% tricaine (Westerfield, 1995). Analysis of light-dependent modulation of transgene reporter expression was performed in 5 dpf larvae starting from 2 h before light onset and collecting samples at 2 h interval for 28 h. The transgenic line Tg(12×Gli-HSV.Ul23:nlsmCherry)ia10 was used to localize the floor plate cells (Corallo et al., 2013). All live animals procedures were approved by the institutional ethics committee for animal testing (C.E.A.S.A.).

2.2. Generation of Tg(9×GCRE-HSV.Ul23:EGFP)ia20 reporter plasmid

To prepare the GRE reporter plasmid, we placed in tandem nine consensus GREs (TGTACAggaTGTTCT, with uppercase letters representing the GRE from the rat *tyrosine aminotransferase* promoter) (Grange et al., 1991). Briefly, we annealed and PCR amplified two phosphorylated oligonucleotides (5′-GTA GCT GAA CAT CCT GTA CAG GAT GTT CTA GC-3′ and 5′-GTA GCT AGA ACA TCC TGT ACA GCT CGA CGT AGC TAG AAC ATC CTG TAC A-3′; consensus GRE sequence is underlined), under the following reaction conditions: enzyme activation (Iproof High Fidelity PCR kit, Biorad, Milan, Italy) at 95 °C for 30 s followed by 40 cycles of denaturation (95 °C for 30 s), annealing (40 °C for 5 s) and extension (72 °C for 20 s). Reaction products were gel purified (Wizard® SV Gel and PCR Clean-Up System, Promega, Milan, Italy), ligated to one another using T4 DNA ligase (Promega) and cloned into pGEM-T Easy plasmid (Iproof High Fidelity PCR kit) pGEM®-T Easy Vector System, Promega). Nine GRE tandem repeats were PCR amplified (from a positive clone using two specific oligonucleotides (pGEM-GRE-F: 5′- *CCC***AAGCTT***GGG*TTCGATTGGATG-3′ with *Hind*III restriction site in bold letters and pGEM-GRE-R: 5′-*CCG***CTCGAG***CGG*TAGTGATTTAGC-3′ with *Xho*I restriction site in bold), purified (Wizard® SV Gel and PCR Clean-Up System, Promega), digested with *Hind*III and *Xho*I (Promega), gel purified, and ligated into the *Hind*III/*Bam*HI sites of the p5E-MCS vector from the Tol2 kit (Kwan et al., 2007) together with the *thymidine kinase* promoter (*tk*), retrieved by *Sal*I/*Bam*HI double digestion from PCR-blunt II-TOPO-*tk* (Moro et al., 2009).

Ligated 9×GRE-*tk* products were confirmed by sequencing. The resulting plasmid (p5E-9×GCRE-HSV.Ul23) was a 5′-entry clone suitable for the Gateway system. This clone, along with two Multisite Gateway-compatible entry vectors from the Tol2 kit (Kwan et al., 2007), a middle entry vector carrying the *egfp* open reading frame named pME-EGFP and a 3′-entry vector carrying a SV40 polyA tail from pCS2+(p3E-polyA), were incubated in the presence of the LR Clonase II Plus Enzyme mix (Invitrogen) and the destination vector pDestTol2pA2 as previously described (Kwan et al., 2007). The resulting destination plasmid contained a GRE-dependent EGFP reporter construct flanked by the minimal Tol2 transposon elements and was named Tg(9×GCRE-HSV.Ul23:EGFP) reporter plasmid. Reporter plasmid DNA (25–50 pg) was co-injected along with 25–50 pg of *in vitro* transcribed Tol2 *transposase* mRNA (Kawakami et al., 2004) into wild type (WT) 1-cell stage embryos.

2.3. Imaging

For confocal microscopy, transgenic embryos, larvae and adult tissues were embedded in 0.8% low-melting agarose and placed on a Petri capsule filled with fish water. The Nikon C2 confocal system was used to record images. WMISH-stained embryos were mounted in 87% glycerol in PBT or cleared and mounted in 2:1 benzyl benzoate/benzyl alcohol, observed under a Leica DMR microscope, and photographed with a Leica DC500 digital camera.

2.4. Drug treatments and microinjection of morpholinos (MOs)

Zebrafish transgenic embryos were incubated with different chemicals, all purchased from Sigma-Aldrich (Milan, Italy). All the chemicals were dissolved in ethanol to prepare stock solutions. Drug stocks were directly diluted 1:1000 in fish water (50×: 25 g Instant Ocean, 39.25 g $CaSO_4$, and 5 g $NaHCO_3$ for 1 l) to reach the desired final concentrations. Each treatment was performed in triplicate with 15 embryos per replica.

MO (Gene Tools, Philomath, OR) treatment was performed with gr^{ATG}MO (MO2-nr3c1), an antisense non-overlapping MO against

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