Manipulation of Interrenal Cell Function in Developing Zebrafish Using Genetically Targeted Ablation and an Optogenetic Tool

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Zebrafish offer an opportunity to study conserved mechanisms underlying the ontogeny and physiology of the hypothalamic-pituitary-adrenal/interrenal axis. As the final effector of the hypothalamic-pituitary-adrenal/interrenal axis, glucocorticoids exert both rapid and long-term regulatory functions. To elucidate their specific effects in zebrafish, transgenic approaches are necessary to complement pharmacological studies. Here, we report a robust approach to specifically manipulate endogenous concentrations of cortisol by targeting heterologous proteins to interrenal cells using a promoter element of the steroidogenic acute regulatory protein. To test this approach, we first used this regulatory region to generate a transgenic line expressing the bacterial nitroreductase protein, which allows conditional targeted ablation of interrenal cells. We demonstrate that this line can be used to specifically ablate interrenal cells, drastically reducing both basal and stress-induced cortisol concentrations. Next, we coupled this regulatory region to an optogenetic actuator, *Beggiatoa* photoactivated adenylyl cyclase, to increase endogenous cortisol concentrations in a blue light-dependent manner. Thus, our approach allows specific manipulations of steroidogenic interrenal cell activity for studying the effects of both hypo- and hypercortisolemia in zebrafish. **(*Endocrinology* 156: 3394–3401, 2015)**

The interrenal organ of teleost fishes, which is equivalent to the adrenal gland in tetrapods, is composed of 2 cell types from different embryological origins: aminergic chromaffin cells and steroidogenic interrenal cells (1, 2). Glucocorticoids (GCs) are produced in the steroidogenic interrenal cells. As final effectors of the stress axis, GCs help to reestablish and maintain normal bodily functions (3, 4). Furthermore, GCs elicit ontogenic transitions during development (5, 6).

To date, studies of GC effects on zebrafish development and physiology have mostly used pharmacological and conventional genetic approaches. Cortisol analogues, such as dexamethasone, GC receptor antagonists, such as mifepristone, and GC receptor-mutant fish have been used to study effects of altered cortisol concentrations on zebrafish development and behavior (7–9). Also, optogenetics has been used to enhance the activity of the pituitary-adrenal axis, thereby causing transient and chronic hypercortisolemia (10). However, transgenic approaches directly targeting interrenal cells have been lacking.

In this study, we report a robust approach for manipulating endogenous concentrations of cortisol in developing zebrafish by means of interrenal-specific transgenic lines. These lines use a promoter element of the steroidogenic acute regulatory (StAR) protein, a cholesterol transporter and a rate-limiting mediator in steroidogenesis (11). Our results show that this regulatory element can drive expression of heterologous proteins exclusively in larval steroidogenic in-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in USA

Received January 11, 2015. Accepted June 25, 2015.
First Published Online July 1, 2015

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Abbreviations: bPAC, photoactivated adenylyl cyclase; dpf, days postfertilization; GC, glucocorticoid; GFP, green fluorescent protein; IHC, immunohistochemistry; ISH, in situ hybridization; MTZ, metronidazole; nfsB, *E. coli* nitroreductase; NTR, nitroreductase; POMC, proopiomelanocortin; PTU, phenylthiourea; StAR, steroidogenic acute regulatory; TH, tyrosine hydroxylase.

terrenal cells and that transgenic lines expressing the bacterial nitroreductase (NTR) enzyme or the *Beggiatoa* photoactivated adenylyl cyclase (bPAC) (12, 13) under the control of this StAR promoter can be used to reduce or enhance endogenous cortisol concentrations, respectively.

Materials and Methods

Fish maintenance

Zebrafish were bred and maintained according to standard methods on a 12-hour light, 12-hour dark cycle (14). For all experiments, the AB/TL zebrafish strain was used. To avoid unspecific activation of bPAC before the experiments were performed, *Tg(StAR:bPAC-2A-tdTomato)* embryos were raised in custom-made reflective containers covered by 550-nm long-pass filters (Thorlabs). All experimental procedures were performed according to the guidelines of the German animal welfare law and approved by the local government.

Plasmid construction

A 2-kb DNA region upstream of the start codon of the zebrafish *star* gene (accession number NM_131663) was PCR amplified using the primers 5′-TGTATTGGAGGAACGAGTCTAAAAC-3′ and 5′-ATGTGAGCACACTGAGTAAAGTTGAATC-3′. The PCR product was cloned into a modified version of the zebrafish enhancer detection plasmid (pZED) vector (15, 16). The resulting plasmid (pT2–2kbStAR:GFP) is a Tol2-based vector consisting of the 2-kb regulatory region driving green fluorescent protein (GFP), flanked by hypersensitivity site 4 (HS4) insulators to reduce ectopic expression (15). As a transgenic marker, the plasmid contains a cassette expressing red fluorescent protein under the control of the *cmlc2* promoter. To create the pT2–2kbStAR:nfsB-GFP plasmid, we cloned the *Escherichia coli* NTR gene (*nfsB*) N-terminally fused to GFP. To create the plasmid p2kbStAR:bPAC-2A-tdTomato, we replaced the proopiomelanocortin (POMC) promoter of the pPOMC:bPAC-2A-tdTomato plasmid as reported (10) with the 2-kb StAR promoter fragment. The integrity of all of the cloned fragments was confirmed by sequencing.

Generation of transgenic lines

Recombinant plasmids were injected into 1-cell stage wild-type embryos at 10 ng/μL in the presence of 10-ng/μL Tol2 transposase mRNA and 0.05% phenol red. The progenies of injected fish were maintained in E2 medium + 0.2mM N-phenylthiourea (PTU) (P7629; Sigma-Aldrich) to prevent pigmentation and screened for heart red fluorescent protein expression and general GFP expression at 2 days postfertilization (dpf) using a Leica MZ6 fluorescence microscope. At least 3 founders were obtained per construct. The following stable transgenic lines were established: *Tg(2kbStAR:GFP)hd17*, *Tg(2kbStAR:nfsB-GFP)hd18*, and *Tg(2kbStAR:bPAC-2A-tdTomato)hd19*.

Immunohistochemistry (IHC) and fluorescent in situ hybridization (ISH)

IHC was performed as described (17) using the following antibodies: chicken anti-GFP (1:500; Abcam), rabbit antityrosine hydroxylase (TH) (1:250) (18), goat antichicken Alexa Fluor 488, and goat antirabbit Alexa Fluor 647 (1:1000; Invitrogen) (see Table 1). Fluorescent ISH was performed as described (17), using a *star* in situ probe (19). Larvae were imaged in 80% glycerol in PBS using a Nikon 20× objective on a Leica SP5 confocal microscope as described (17). Subsequent image processing and evaluation were performed as described (17).

Conditional targeted cell ablation

Conditional targeted cell ablation was performed as reported (20) using a solution of 10mM metronidazole (MTZ) (M1547; Sigma-Aldrich) in E2 medium + PTU. GFP-positive embryos were selected at 2 or 3 dpf and subsequently transferred to either E2 + PTU media or E2 + PTU + MTZ in 35-mm petri dishes. Embryos were maintained in the dark at 28°C for 48 hours, exchanging media after 24 hours. At 4 or 5 dpf, embryos were transferred to E2 + PTU media for 24 hours under 12-hour light, 12-hour dark conditions and fixed in 4% paraformaldehyde for ISH/IHC. To examine the stress response, we used a pool of 30 embryos as an experimental unit. MTZ treatment was performed as above but without PTU.

Light stimulation

A custom-made LED ring was placed at a fixed distance above a single petri dish, allowing homogeneous illumination of the samples. LEDs were controlled using custom-made drivers, pulse generators and a transistor-transistor logic control box (USB-IO box; Noldus). Four-day postfertilization larvae were exposed for 180 seconds to blue light (2V). The light pulse consisted of 100-millisecond flashes delivered at 5 Hz. Light power was measured using a hand-held light power meter (Newport). Samples were collected 2 minutes after the offset of light stimulation. After the larvae were immobilized in ice water, the media were removed, and the samples were frozen in an ethanol/dry ice bath; samples were stored at −20°C until cortisol extraction was performed. The cortisol ELISA assay was performed as reported (21).

Table 1. Antibody Table

Peptide/Protein Target	Name of Antibody	Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody	Species Raised in; Monoclonal or Polyclonal	Dilution Used
GFP	Anti-GFP	Abcam, ab13970	Chicken, polyclonal	1:500
TH	Anti-TH	Soojin Ryu	Rabbit, polyclonal	1:250
Chicken Ig-Fc	Alexa Fluor 488	Invitrogen, A11039	Goat, polyclonal	1:1000
Rabbit Ig-Fc	Alexa Fluor 647	Invitrogen, A21245	Goat, polyclonal	1:1000

Stressor treatment and cortisol assay

We developed a mechanosensory stress protocol that used a magnetic stirrer in a dish placed on a magnetic plate, thereby generating unpredictable perturbation of the medium. Petri dishes (inner diameter: 3.5 cm) containing 30 larvae and a plastic-covered stirrer (magnetic stir bar micro-polytetrafluoroethylene 6 × 3 mm; Fisher Scientific) were placed on a magnetic stir plate (Variomag Poly 15 stirrer plate; Thermo Scientific). The mechanosensory stress protocol was performed for 3 minutes at 330 rpm. Samples were collected 10 minutes after the onset of stimulation. Larvae were immobilized using ice-cold water, frozen in an ethanol/dry ice bath and stored at −20°C. Stimulation and sample collection were carried out between 11 AM and 2 PM. The cortisol ELISA assay was performed as reported (21).

Statistical analysis

All data are shown as bars or single measurement points, mean and SEM. We used a random experimental design and ANOVA for multiple group comparisons followed by Bonferroni's post hoc tests. Two- and one-sample Student's *t* tests (against a value of 1) were used for volume and fold change comparisons. Analyses were carried out using MS Excel (Microsoft) and Prism 5 (GraphPad Software, Inc).

Results

Identification of a *cis*-regulatory region of the *star* gene with specific activity in steroidogenic interrenal cells

To target heterologous proteins specifically to steroidogenic interrenal cells, we focused on the gene coding for the StAR protein, one of the earliest markers expressed in the embryonic interrenal tissue in zebrafish (22) and generated a reporter construct carrying a 2-kb fragment of its promoter (Figure 1). We identified a stable transgenic line with robust and discrete GFP expression in a cluster of cells dorsorostral to the yolk sac. To visualize steroidogenic and chromaffin cells, we examined cell identity using ISH for the *star* gene with IHC for TH and GFP. At 6–14 dpf, GFP-expressing cells colocalized with *star* expression in *Tg(2kbStAR:GFP)* larvae but not with intermingled TH-expressing cells (Figure 2 and Supplemental Figure 1). GFP-positive cells were not detected in other tissues. From these results, we concluded that the 2-kb promoter element is sufficient to recapitulate endogenous expression of the *star* gene in interrenal cells.

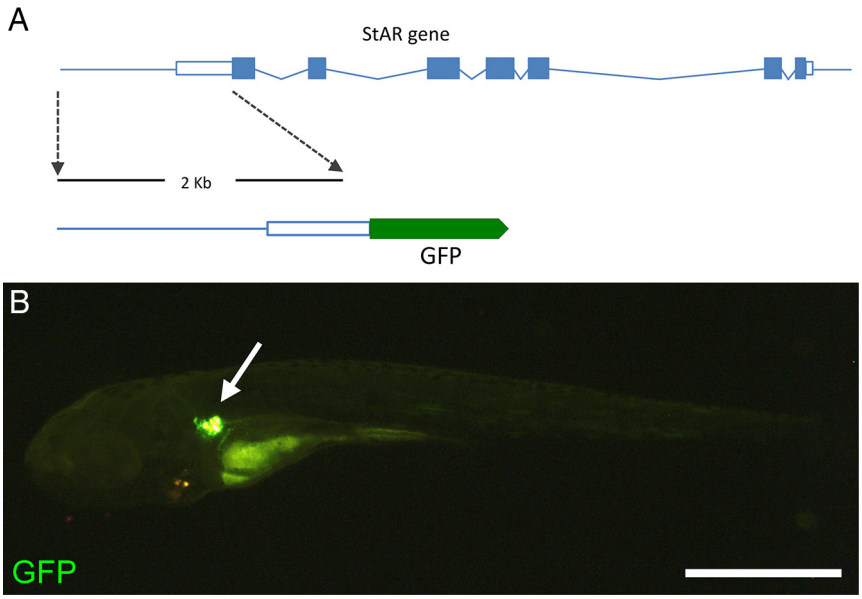

Figure 1. A *cis*-regulatory region of the *star* gene restricts the expression of a heterologous protein to the interrenal region. A, Genomic structure of the *star* gene in zebrafish. Filled boxes indicate exons. The 2-kb region tested is indicated. B, Live image of a larva showing GFP expression localized in the dorsovisceral region (arrow) above the yolk sac (autofluorescence). Scale bar, 500 μm.

Efficient conditional steroidogenic interrenal cell ablation

To ablate steroidogenic interrenal cells, we used the NTR-MTZ method, which is suitable for noninvasively ablating targeted cells (20). First, we generated stable transgenic lines expressing the *E. coli nfsB* gene, which encodes the NTR protein, and fused it to GFP under the control of the 2-kb regulatory region of the *star* promoter. We then incubated transgenic *Tg(2kbStAR:nfsB-GFP)* larvae in E2 medium with or without 10mM MTZ. 12 hours of MTZ treatment did not lead to ablation (n = 100) and 24 hours of MTZ treatment only induced ablation in 14/92 of the animals (data not shown). In contrast, after 48 hours of MTZ incubation, we observed a dramatic decrease in GFP expression in many larvae preincubated with MTZ; all GFP-expressing cells were ablated in most larvae (49/93 animals) (data not shown). These cells did not recover up to 14 dpf (Supplemental Figure 2). Several studies have shown a functional interdependence of adrenocortical steroidogenic cells and adrenomedullary chromaffin cells (23, 24). To evaluate the effect of steroidogenic interrenal cell ablation on chromaffin cells, we performed ISH for the *star* gene combined with IHC for TH and GFP. At 4 and 5 dpf, we observed ablation of *star*-expressing cells but not of TH-expressing cells (Figure 3).

Conditional steroidogenic interrenal cell ablation causes hypocortisolemia

Next, we asked whether *Tg(2kbStAR:nfsB-GFP)* larvae show any decrease in whole-body

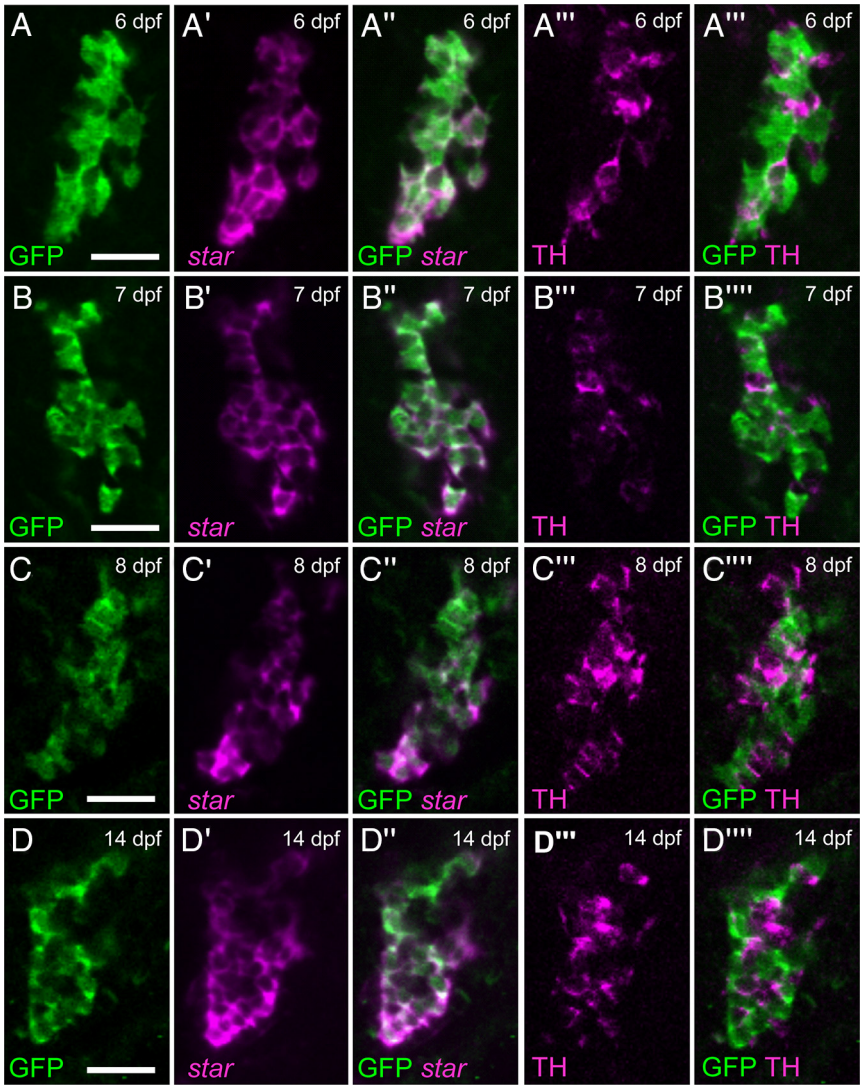

Figure 2. Expression of GFP in *Tg(2kbStARp:GFP)* is specific to *star*-positive interrenal cells. Single planes of confocal stacks show GFP (IHC staining) colocalizing with *star* (ISH staining), but not with TH (IHC staining), in 6-dpf (A–A'''', n = 6), 7-dpf (B–B'''', n = 11), 8-dpf (C–C'''', n = 10), and 14-dpf (D–D'''', n = 9) larvae. Scale bars, 25 μm.

cortisol concentrations after interrenal cell ablation. We compared cortisol values from 6-dpf *Tg(2kbStAR:nfsB-GFP)* and *Tg(2kbStAR:GFP)* larvae preincubated in E2 medium with or without 10mM MTZ (Figure 4) and found that the values from the 2 groups differed in terms of both basal and stress-induced concentrations (one-way ANOVA, $F_{9,114} = 129.3$, $P < .0001$, followed by Bonferroni's post hoc tests) (Figure 4B). *Tg(2kbStAR:GFP)* larvae had basal concentrations similar to those of untreated wild-type larvae, irrespective of MTZ treatment. The same occurred with *Tg(2kbStAR:nfsB-GFP)* larvae that had not been preincubated in MTZ (Bonferroni's post hoc tests, $P < .001$). In contrast, basal cortisol was reduced in *Tg(2kbStAR:nfsB-GFP)* larvae preincubated in MTZ (Bonferroni's post hoc tests, $P < .001$). Upon exposure to a mechanosensory stressor, untreated wild-type, *Tg(2kbStAR:GFP)*, and *Tg(2kbStAR:nfsB-GFP)* larvae had similar cortisol concentrations. However, MTZ incubation completely abolished the stressor-mediated rise in cortisol in *Tg(2kbStAR:nfsB-GFP)* larvae (Bonferroni's post hoc tests, $P < .001$). MTZ-incubated *Tg(2kbStAR:GFP)* larvae exhibited a stressor-mediated rise in cortisol, but to a lesser degree than without MTZ, revealing MTZ effects that are independent of NTR expression. Therefore, we examined the effect of MTZ alone on interrenal cells in larvae that do not express NTR. In such larvae, interrenal cells were present (Supplemental Figure 3), but MTZ treatment altered the overall volumes of interrenal *star* clusters as well as that of the rostral pituitary *pomc* (Supplemental Figure 4). Nevertheless, despite this effect of MTZ alone, a complete hypocortisolic state is only achieved in *Tg(2kbStAR:nfsB-GFP)* larvae, which exhibited only 0.38 ± 0.06 and 0.27 ± 0.03 of the basal and stressor-mediated cortisol concentrations found in *Tg(2kbStAR:GFP)* larvae, respectively (1-sample *t* tests against 1, nonstressed: $t_{(19)} = 10.3$, $P < .0001$ and stressed: $t_{(7)} = 27.0$, $P < .0001$) (Figure 4C).

Optogenetic manipulation of steroidogenic interrenal cell function causes hypercortisolemia

After binding to its receptor on adrenocortical cells, ACTH elicits cAMP production and Ca^{2+} influx, which stimulates the synthesis and release of GC (25). We reasoned that a transient stimulation of cAMP concentrations in steroidogenic interrenal cells may produce hypercortisolemia. We have previously shown that a sudden change in illumination can be a stressful event for zebrafish larvae and that placing an optogenetic actuator in the pituitary corticotroph cells enhances this stress response (10). Using a similar approach targeted to interrenal cells, we generated a transgenic line expressing bPAC, shown to increase cAMP production upon blue light, under the control of the 2-kb regulatory region of the StAR promoter.

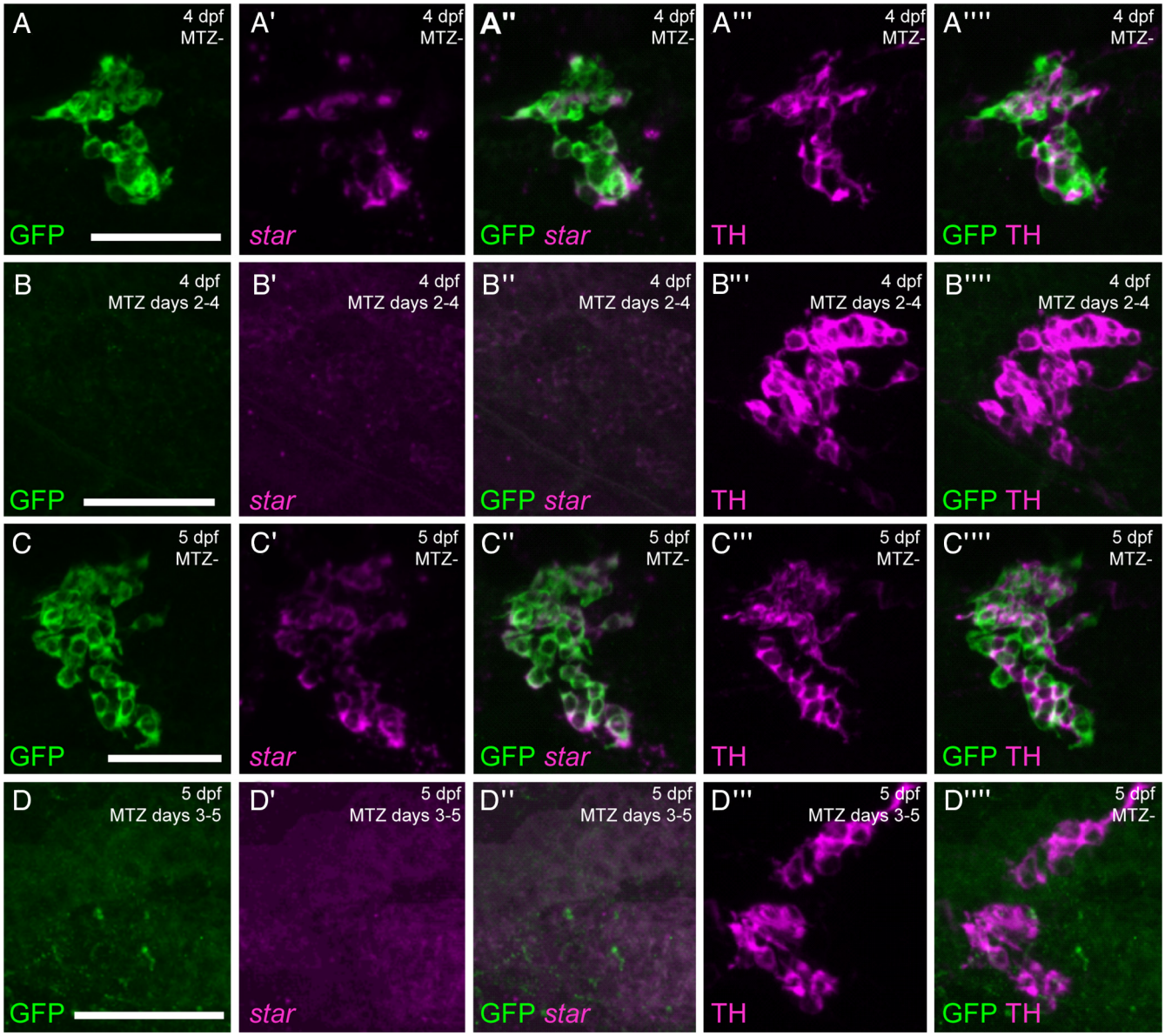

Figure 3. Ablation using MTZ eliminates *star*-positive interrenal cells in *Tg(2kbStARp:nfsb-GFP)* larvae. Maximum intensity projections of confocal stacks show GFP (IHC staining) colocalizing with *star* (ISH staining), but not with TH (IHC staining), in 4-dpf (A–A'''', n = 25) and 5-dpf (C–C'''', n = 36) larvae. These cells are eliminated after treatment with MTZ between 2 and 4 dpf (B–B'''', 12/26 animals) or between 3 and 5 dpf (D–D'''', 14/21 animals). Scale bars, 50 μm.

Tg(2kbStAR:bPAC-2A-tdTomato) larvae showed restricted tdTomato expression in the interrenal gland, similar to *Tg(2kbStAR:GFP)* and *Tg(2kbStAR:nfsB-GFP)* animals (Figure 4E). The progenies of *Tg(2kbStAR:bPAC-2A-tdTomato)* larvae were then sorted into those expressing the transgene (bPAC+) or not (bPAC−) under a dissecting microscope, based on the signal of fused tdTomato fluorescence. Upon blue light illumination, bPAC+ and bPAC− 4-dpf larvae had different cortisol profiles (one-way ANOVA, $F_{5,39} = 63.0$, $P < .0001$, followed by Bonferroni's post hoc tests) (Figure 4F). Wild-type and bPAC− larvae had similar basal and light-stimulated cortisol concentrations, whereas bPAC+ larvae had similar basal but much higher light-stimulated cortisol than both such groups (Bonferroni's post hoc tests, $P < .001$). As a result, bPAC+ larvae had the same basal concentration of cortisol (fold change, 1.0 ± 0.1) but significantly higher cortisol concentration upon stress (fold change, 1.8 ± 0.05) as compared with their negative siblings (1-sample *t* tests against 1, nonstimulated: $t_{(5)} = 0.02$, $P = .98$ and stimulated: $t_{(6)} = 17.5$, $P < .0001$; 2-tailed *t* test, $t_{(11)} = 7.6$, $P < .0001$) (Figure 4G).

Discussion

We present a transgenic approach for manipulating steroidogenic interrenal cells in larval zebrafish. Using this

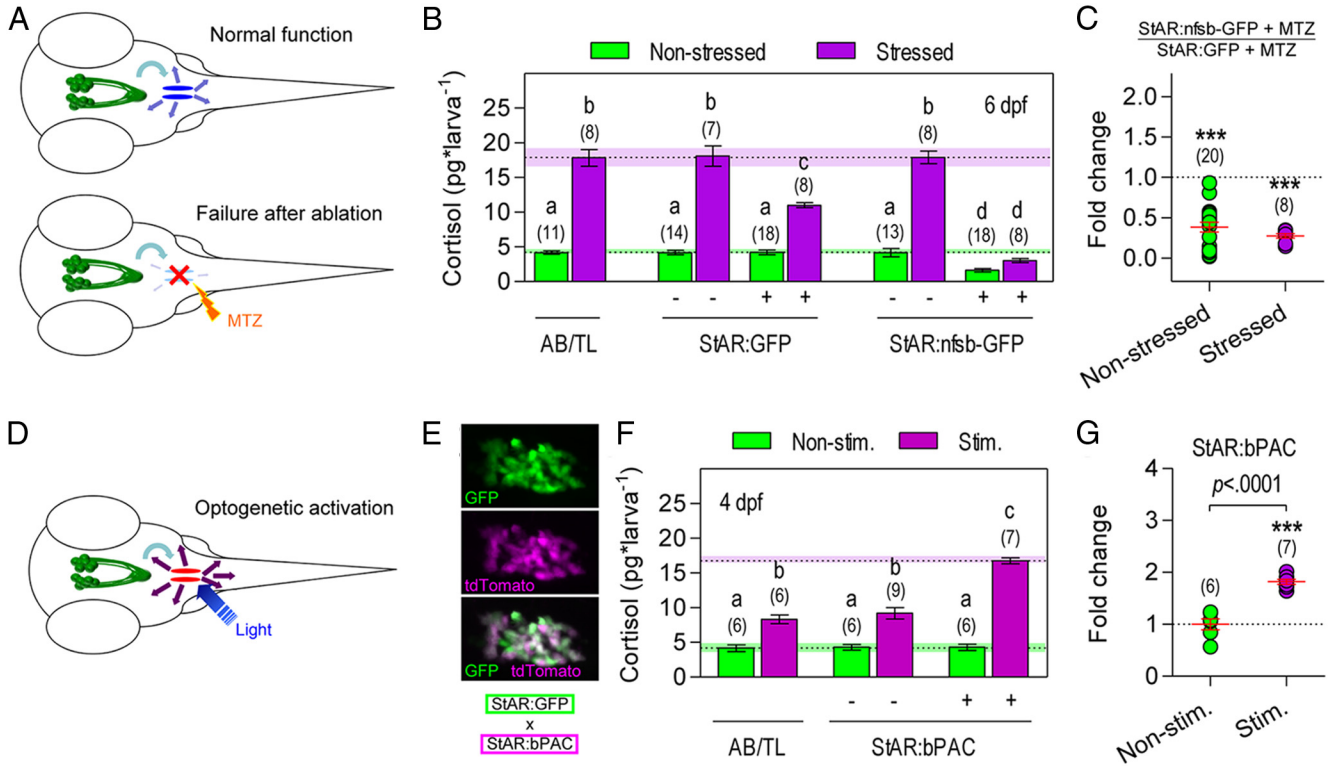

Figure 4. Cortisol concentrations are altered after manipulation of interrenal-specific transgenic lines. A, Schematic illustration of interrenal ablation in MTZ-treated *Tg(2kbStARp:nfsb-GFP)* larvae. The hypothalamo-pituitary complex (green) triggers the release of ACTH (curved arrow), which acts on interrenal cells (blue) to induce the release of cortisol (purple arrows). Ablation eliminates the steroidogenic interrenal cells, thereby reducing cortisol output. B, Basal (green) and stress-induced (purple) cortisol concentrations in wild-type, *Tg(2kbStARp:GFP)*, and *Tg(2kbStARp:nfsb-GFP)* larvae, either untreated (−) or treated (+) with MTZ. Note that basal cortisol concentrations are reduced and upon stress induction, cortisol concentrations fail to increase in MTZ-treated *Tg(2kbStARp:nfsb-GFP)* larvae. C, Fold change of basal (green) and stress-induced (purple) cortisol concentrations in MTZ-treated *Tg(2kbStARp:nfsb-GFP)* larvae relative to those of *Tg(2kbStARp:GFP)* larvae. D, Schematic illustration of optogenetic interrenal activation in *Tg(2kbStARp:bPAC-2A-tdTomato)* larvae. Illumination with blue light activates the interrenal cells, thereby increasing cortisol output. E, Specificity of the *Tg(2kbStARp:bPAC-2A-tdTomato)* line shown by colocalization of GFP and tdTomato in a double-transgenic 4-dpf larva expressing GFP and bPAC-2A-tdTomato under the control of the 2-kb *star* promoter. F, Nonstimulated (green) and light-stimulated (purple) cortisol concentrations in wild-type, bPAC-negative (−), and bPAC-positive (+) 4-dpf progenies of the *Tg(2kbStARp:bPAC-2A-tdTomato)* line. Note that cortisol concentrations are increased in bPAC-positive light-stimulated animals. G, Fold change of nonstimulated (green) and light-stimulated (purple) cortisol concentrations in bPAC-positive larvae relative to their negative siblings.

approach, we could either reduce or increase endogenous concentrations of cortisol. To achieve this, we first identified and characterized a *cis*-regulatory region of the *star* gene with specific activity in steroidogenic interrenal cells. The StAR protein is expressed in steroidogenic tissues, such as the gonads, the brain (neurosteroids), and the interrenal organ (26). We did not observe expression outside the interrenal tissue in any of the transgenic lines until 14 dpf, but we cannot exclude promoter activity in other tissues in adult animals. This regulatory element thus offers an excellent tool to express heterologous proteins specifically in larval steroidogenic interrenal cells, allowing careful manipulations of steroidogenic cell function.

Optogenetic tools provide hitherto unparalleled means for noninvasive manipulations of cellular activity. Zebrafish larvae are highly suitable for noninvasive optogenetics due to their genetic amenability and transparent body (27). Notably, they are highly sensitive to photic stimuli and react to sudden illumination changes with humoral and behavioral stress reactions (10). By targeting bPAC specifically to pituitary corticotroph cells, we have previously reported stressor-mediated hypercortisolemia presumably caused by enhanced ACTH release. Exposing larvae expressing bPAC specifically in steroidogenic interrenal cells to blue light also caused stressor-mediated hypercortisolemia. When combined, both approaches provide an opportunity for dissecting fast and long-term effects of the pituitary-adrenal system during development.

The NTR-MTZ system has successfully been applied in zebrafish to ablate different cell types, particularly in regeneration studies (28). Upon MTZ treatment, as expected, the number of steroidogenic interrenal cells appeared significantly reduced in *Tg(2kbStAR:nfsB-GFP)* larvae; these cells did not recover up to 14 dpf. The NTR-MTZ system therefore will be useful in examining the

effect of interrenal ablation on larval development and physiology, although regeneration of interrenal tissue at later stages has yet to be determined.

Interrenal ablation is expected to reduce endogenous cortisol concentrations. MTZ treatment confirmed this prediction, causing dramatically reduced basal and stressor-induced cortisol concentrations. However, we observed that MTZ treatment alone can also decrease stressor-induced cortisol concentrations, irrespective of NTR. Therefore, to further examine MTZ effects on cells within the hypothalamic-pituitary-interrenal axis, we measured preoptic corticotropin-releasing hormone (*crh*), pituitary *pomc*, and interrenal *star* expression in MTZ-treated larvae. MTZ increased the volume of the rostral *pomc* cluster, as compared with the caudal cluster, a phenomenon that is consistent with recent findings (29). MTZ also decreased the volume of the interrenal StAR cluster, whereas the number of StAR cells appeared unaffected. These effects may account for the reduced cortisol concentrations observed in NTR-negative larvae. Altogether, the results indicate that MTZ effects should be tested independently when employing the NTR-MTZ system.

The small size and complex organization of the adrenal homolog in teleosts impedes surgical manipulations. The possibility to noninvasively manipulate interrenal cell function using specific modulators of cell activity and viability provides an interesting opportunity for studying the roles of steroidogenic interrenal cells and GCs during early vertebrate development.

Acknowledgments

We thank Patrick Lichtner for the experimental assistance provided. We also thank Regina Singer, Gabi Shoeman, Angelika Schoell, and Christiane Brandel for technical assistance and expert fish care. The pZED vector was kindly provided by Dr. Jose Luis Gomez-Skarmetta.

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This work was supported by the Max Planck Society and the German Research Foundation Grant DFG-FOR1279.

Disclosure Summary: The authors have nothing to disclose.

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