Glucagon receptor inactivation leads to α -cell hyperplasia in zebrafish

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Abstract

Glucagon antagonism is a potential treatment for diabetes. One potential side effect is α -cell hyperplasia, which has been noted in several approaches to antagonize glucagon action. To investigate the molecular mechanism of the α -cell hyperplasia and to identify the responsible factor, we created a zebrafish model in which glucagon receptor (gcgr) signaling has been interrupted. The genetically and chemically tractable zebrafish, which provides a robust discovery platform, has two *gcgr* genes (*gcgra* and *gcgrb*) in its genome. Sequence, phylogenetic, and synteny analyses suggest that these are co-orthologs of the human *GCGR*. Similar to its mammalian counterparts, *gcgra* and *gcgrb* are mainly expressed in the liver. We inactivated the zebrafish *gcgra* and *gcgrb* using transcription activator-like effector nuclease (TALEN) first individually and then both genes, and assessed the number of α -cells using an α -cell reporter line, *Tg(gcga:GFP)*. Compared to WT fish at 7 days postfertilization, there were more α -cells in *gcgra*−/−, *gcgrb*−/−, and *gcgra*−/−;*gcgrb*−/− fish and there was an increased rate of α -cell proliferation in the *gcgra*−/−;*gcgrb*−/− fish. Glucagon levels were higher but free glucose levels were lower in *gcgra*−/−, *gcgrb*−/−, and *gcgra*−/−;*gcgrb*−/− fish, similar to *Gcgr*−/− mice. These results indicate that the compensatory α -cell hyperplasia in response to interruption of glucagon signaling is conserved in zebrafish. The robust α -cell hyperplasia in *gcgra*−/−;*gcgrb*−/− larvae provides a platform to screen for chemical and genetic suppressors, and ultimately to identify the stimulus of α -cell hyperplasia and its signaling mechanism.

Key Words

- cell growth control
- glucagon
- mutations
- fish
- whole animal physiology

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Introduction

Glucagon is a peptide hormone secreted by pancreatic α -cells. It is the main counter-regulatory hormone of insulin. Glucagon acts primarily on liver through glucagon receptor (GCGR) to regulate hepatic glucose production (Mayo *et al.* 2003). The GCGR is a class B G-protein-coupled receptor that couples to Gs (G protein subunits) and activates adenylate cyclase to increase

intracellular levels of cAMP upon ligand binding (Brubaker & Drucker 2002). The cAMP subsequently activates protein kinase A, leading to increased glycogenolysis and gluconeogenesis and decreased glycogen synthesis (Jiang & Zhang 2003). GCGR is primarily expressed in the liver, but is also detected in other tissues, including β -cells, heart, intestinal smooth muscle, kidney,

brain, and adipose tissue (Hansen *et al.* 1995, Ali & Drucker 2009, Kedeas *et al.* 2009). Gcgr expression in hepatocytes is upregulated by glucose and factors known to raise intracellular cAMP levels (Abrahamsen *et al.* 1995).

Glucagon raises the blood glucose and excess glucagon action is seen in both types 1 and 2 diabetes (Lund *et al.* 2014). Knockout of Gcgr in mice results in mild hypoglycemia and decreased adiposity, and prevents diabetes induced either by streptozotocin or by a high fat diet (Gelling *et al.* 2003, Conarello *et al.* 2007, Lee *et al.* 2011), supporting glucagon antagonism as a potential treatment for both types of diabetes. However, as compensation to loss of glucagon action, *Gcgr*^{−/−} mice also exhibit α - and δ -cell hyperplasia, hyperglucagonemia (Gelling *et al.* 2003), and may develop pancreatic endocrine tumors (Gelling *et al.* 2003, Yu *et al.* 2011). Similarly, loss of GCGR function in humans is associated with hyperglucagonemia, α -cell hyperplasia, and endocrine tumor (Zhou *et al.* 2009). These undesirable consequences of glucagon antagonism must be mitigated if anti-glucagon treatment is to be useful and safe. A key question about the compensatory α -cell hyperplasia is the responsible mechanism. Liver-specific inactivation of Gcgr or its downstream transducer G_s alpha subunit (G α) in mice results in similar phenotype, suggesting the signal originates in the liver (Chen *et al.* 2005, Longuet *et al.* 2013). Furthermore, α -cell hyperplasia occurs in islets transplanted beneath the renal capsule, indicating the responsible factor circulates (Longuet *et al.* 2013). However, neither the factor that stimulates α -cell hyperplasia nor its signaling mechanism is known.

The zebrafish has emerged as a discovery platform for understanding molecular mechanisms of vertebrate biology. Its small size and transparent larvae make zebrafish an extremely useful for genetic and chemical modifier screens to discover critical components of a biological process (Lieschke & Currie 2007). Many signaling pathways and transcription factors important for mammalian pancreatic α - and β -cell development are conserved in zebrafish (Biemar *et al.* 2001, Field *et al.* 2003, Hesselson *et al.* 2011, Maddison & Chen 2012). Here we investigated the biology of the glucagon system in zebrafish in an effort to determine if the zebrafish might serve as a discovery platform for signals and mechanisms that regulate α -cell mass. We found that zebrafish have two *gcgr* genes, *gcgra* and *gcgrb*. We functionally characterized their gene products, and found that α -cells hyperplasia occurs in *gcgra*- and/or *gcgrb*-deficient zebrafish larvae.

Materials and methods

Zebrafish lines and maintenance

Zebrafish (*Danio rerio*) were raised in an Aquatic-Habitats system on a 14 h:10 h darkness cycle at 28 °C. Embryos were obtained by natural cross and kept in embryo rearing solution and staged according to standard methods (Kimmel *et al.* 1995). In this study, *Tg(gcga:GFP)* (Zecchin *et al.* 2007) was used to mark α -cells. All procedures have been approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Transcription activator-like effector nuclease-mediated mutagenesis of *gcgra* and *gcgrb*

The candidate transcription activator-like effector nuclease (TALEN) target sequences of *gcgra* and *gcgrb* were identified online using TALEN Targeter (<https://tale-nt.cac.cornell.edu/>). The target sequences for the *gcgra* TALEN pair are 5'-GCCCTGCCCAACTACAGT-3' (left) and 5'-GTATCTGCCCTGGCACAAGG-3' (right). The target sequences for the *gcgrb* TALEN pair are 5'-CTTCTGGGAAATCTCTGAAG-3' (left) and 5'-TGGAGGATCTACACCAATG-3' (right). The TALENs were assembled using the 'Golden Gate TALEN Assembly' Kit (Cermak *et al.* 2011). TALEN expression vectors were linearized and used as the template for capped mRNA synthesis using T3 mMessage mMachine Transcription Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Equal amounts of capped mRNAs were mixed and co-injected into one-cell stage zebrafish embryos at the dose of 200 pg/embryo. T7 Endonuclease I (NEB, Ipswich, MA, USA) digestion was used to evaluate the efficiency of the TALENs. Briefly, a ~400 bp fragment was amplified from genomic DNA isolated from injected-embryos at 24 h postfertilization, and purified product was digested and cleaved products resolved from intact products by agarose gel electrophoresis.

A *gcgra* allele with 22 bp deletion, designated as *gcgra*^{vu600} and a *gcgrb* allele with 1 bp deletion, designated as *gcgrb*^{vu601}, were selected for additional analysis. For *gcgra*^{vu600} carriers, PCR products amplified using *gcgra*-Mu22-F1 and *gcgra*-TN2-GTR following gel electrophoresis with 4% NuSieve GTG agarose (Lonza, Rockland, ME, USA) to distinguish the WT (180 bp) and mutant (158 bp). For *gcgrb*^{vu601} carriers, PCR products amplified using *gcgrb*-TN2-GTF and *gcgrb*-TN2-GTR were digested with PstI. PCR product from carriers could not be digested.

Cloning and sequence analysis

A putative *gcgra* gene was identified in Ensembl (<http://www.ensembl.org>; Ensembl ID: ENSDART00000156788). A pair of primers, gcgra-Long-F1 and gcgra-Long-R1 (Supplementary Table 1, see section on supplementary data given at the end of this article), was used to amplify the entire open reading frame (ORF) from zebrafish cDNA using Q5 High-Fidelity DNA Polymerase (NEB). To obtain the ORF of *gcgrb*, the cDNA sequence was first determined using 3'- and 5'-RACE based on the Ensembl ID: ENSDART00000021878. The entire ORF was subsequently cloned using primers gcgrb-Long-F1 and gcgrb-Long-R1.

Phylogenetic analysis was performed by the neighbor-joining method using the MEGA4 Software (The Biodesign Institute, Tempe, AZ, USA) and full-length amino acid sequences. Bootstrap analyses were run on 1000 replicates. The genomic structure of zebrafish *gcgra* and *gcgrb* was determined by the Blat program (<http://genome.ucsc.edu/cgi-bin/hgBlat>) using the cloned full-length cDNA sequence as query to search zebrafish Assembly Zv9 (July 2010). Synteny analysis was carried out based on zebrafish Assembly Zv9 (http://www.ensembl.org/Danio_reio/Info/Index) and human Build GRCh38 (http://www.ensembl.org/Homo_sapiens/index.html), and from zebrafish and human synteny map respectively.

RNA extraction, RT-PCR, and quantitative real-time RT-PCR

Total RNA was isolated from adult zebrafish and embryos using TRIzol reagent (Invitrogen Life Technologies) and digested by the RQ1 RNase-Free DNase (Promega) to remove any genomic DNA contamination. First strand cDNA was synthesized using M-MLV (Promega) with oligo(dT)₁₆ as primer. RT-PCR was carried out using GoTaq Flexi DNA Polymerase (Promega) at MyCycler Thermal Cycler (Bio-Rad). The primers are listed in Supplementary Table 1. β -actin was used as an internal control. RT-PCR products were fractionated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed using ChemiDoc XRS Gel Documentation System (Bio-Rad). Quantitative real-time RT-PCR (qRT-PCR) was performed in a CFX96 system (Bio-Rad). After a 3-min incubation at 95 °C, the amplification was performed as follows: 95 °C, 10 s; 60 °C, 30 s for 40 cycles. Each assay for an unknown sample was performed in triplicate. mRNA levels were calculated using $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001) and presented as relative (fold) levels normalized to the level of β -actin.

cAMP accumulation

Zebrafish glucagon, Gcga (HSEGTFSNDYSKYLETR-RAQDFVQWLMNA) and Gcgb (HSEGTFSNDYSKYLETR-RAQDFVQWLMNS) peptides were synthesized by GENEWIZ (Cambridge, MA, USA). Mouse glucagon was from Sigma-Aldrich (Sigma G2044). ORF of zebrafish *gcgra* and *gcgrb* were subcloned in the pcDNA 3.1+ at KpnI and EcoRI sites. Mouse *Gcgr* ORF was subcloned in the pcDNA 3.1+ at EcoRI and XhoI sites. HEK293T cells are co-transfected with CRE-luciferase reporter plasmid and pcDNA3.1, pcDNA3.1-*gcgra*, pcDNA3.1-*gcgrb*, and pcDNA3.1-*Gcgr* using Dilute LipoD393 (SigmaGen Laboratories, Gaithersburg, MD, USA). Forty-eight hours after transfection, cells were washed and dissociated, then evenly seeded into each well of a 96-well plate. Different final concentrations of glucagon peptides were added to each well with forskolin as the control. After a 4 h incubation at 37 °C, ONE-Glo Luciferase mixture was added to each well. After a 5 min incubation at room temperature, the signal in the plate was read using SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Nonlinear regression analysis was performed to calculate cAMP concentrations using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

Free glucose assay

Free glucose of zebrafish larvae was determined using the Amplex Red Glucose/Glucose Oxidase Assay Kit (Life Technologies). A pool of ten larvae was homogenized in 100 μ l of sample buffer. The homogenate was cleared by centrifugation. According to the manufacturer's instructions, 10 μ l of supernatant (equivalent of one larva) was measured. The reaction was incubated for 30 min at room temperature and fluorescence was measured at Ex/Em = 535/590 nm in a SpectraMax M5 Microplate Reader (Molecular Devices). At least three ten-fish pools of each genotype were measured.

Whole mount *in situ* hybridization and whole mount immunofluorescence

Digoxigenin-labeled antisense and sense RNA probes were synthesized *in vitro* using the linearized plasmid as a template. Hybridization was carried out as described previously (Ni *et al.* 2012). The larval zebrafish of *Tg(gcga:GFP)* were stained using monoclonal anti-glucagon antibody (Sigma G2654) using standard techniques as described previously (Li *et al.* 2014).

Proliferation analysis

Proliferation was analyzed using the Click-iT EdU Alexa Fluor 594 Imaging Kit (C10339; Invitrogen). To identify proliferating α -cell, embryos were incubated with 1 mmol/l 5-ethynyl-2-deoxyuridine (EdU) for 24 h starting at 4 days postfertilization (dpf). EdU was detected according to published protocols (Li *et al.* 2013). All images were collected using a Zeiss LSM710 (Carl Zeiss, Jena, Germany).

Counting of α -cells

After fixation in 4% paraformaldehyde overnight at 4 °C, larvae were washed with 1× PBS plus 0.1% Tween-20 (PBST) and flat mounted in Aqua-Mount (Richard-Allan Scientific, Kalamazoo, MI, USA) with their right side facing the coverslip. The larvae were flattened just to disrupt the islet slightly to allow better resolution of α -cell. The α -cells were counted according to the GFP signal using a Zeiss AxioImager under a 40× lens or using confocal projections taken by Zeiss LSM710 under a 40× lens (Carl Zeiss).

Whole fish glucagon content measurement

Glucagon content in zebrafish was measured by RIA in the Vanderbilt Hormone Assay Core similar to mouse whole pancreata measurements (Longuet *et al.* 2013). The two zebrafish peptides share 76% identity with mammalian glucagon. Ten zebrafish larvae (7 dpf) were collected in 0.11 N HCl in water and homogenized with a Kontes motorized homogenizer and pestle. The samples were solubilized for 72 h at 4 °C while rotating. Cleared extracts were stored at −80 °C after centrifugation until use. We used a glucagon RIA Kit (Glucagon KGND1, Siemens Healthcare Diagnostics, Los Angeles, CA, USA) according to the manufacturer's protocol with modifications to increase the sensitivity. Specifically, the glucagon antibody was diluted twofold and the glucagon ¹²⁵I diluted to 5000 c.p.m./tube. The antibody detected the two zebrafish peptides with equal affinity, albeit with a small reduction (right shift) compared to the mammalian peptide (data not shown). There was no difference in the slope of the binding curve between mammalian, zebrafish a, or zebrafish b glucagon ($P > 0.05$).

Statistical analysis

Data are means and s.e.m. Data were analyzed by one-way ANOVA followed by Newman–Keuls *post hoc* test or *t*-test (SPSS). Significance was accepted at $P < 0.05$.

Results

Cloning and characterization of two *gcgr* genes from zebrafish

Two putative *gcgr* genes were found in Ensembl zebrafish genome assembly (http://www.ensembl.org/Danio_rerio/Info/Index). Subsequently, primers were designed to amplify both ORFs. The ORF of *gcgra* and *gcgrb* were 1563 and 1527 bp and encoding proteins of 520 and 508 amino acids respectively. Structural analysis revealed that both zebrafish *gcgra* and *gcgrb* had a structure similar to their mammalian counterparts, which contained an extra-cellular domain, seven transmembrane domains, and an intracellular domain.

We next generated a phylogenetic tree using the neighbor-joining method. As shown in Fig. 1A, zebrafish Gcgr forms a cluster with known GCGRs with a high bootstrap support value and well separated from GIP receptors, GLP1 receptors and GLP2 receptors. Synteny analysis revealed that zebrafish *gcgra* is located on chromosome 3, *gcgrb* is located on chromosome 1, while the human *GCGR* locus is on chromosome 17. To explore the possible synteny relationship between zebrafish *gcgr* and human *GCGR*, we compared genes surrounding the *GCGR* loci. Several neighboring genes of *GCGR*, namely *BAIAP2*, *AATK*, *SLC25A10*, *FN3KRP*, *TBCD*, *ZNF750*, *B3GNTL1*, and *METRNL*, also had their orthologs near zebrafish *gcgra* or *gcgrb* (Fig. 1B). This conserved synteny relationship provides further evidence that *gcgra* and *gcgrb* are both orthologous to human *GCGR*.

Expression patterns of *gcgra* and *gcgrb* mRNA

RT-PCR detected *gcgra* and *gcgrb* mRNA in all stages examined, including 1, 2, 3, 5, 7 dpf, and 3 weeks of age (Fig. 2A). In adult tissues, *gcgra* mRNA was most abundant in liver, followed by brain, muscle, and heart, but was undetectable in intestines. Similarly, *gcgrb* mRNA was most abundant in liver, but was also expressed in intestines at a level similar to brain and was undetectable in muscle and heart. Therefore, *gcgra* and *gcgrb* have distinctive expression in muscle, heart, and intestines (Fig. 2B).

Functional characterization of zebrafish Gcgra and Gcgrb

There are two glucagon genes in zebrafish, *gcga* and *gcgb* (Argenton *et al.* 1999, Cruz *et al.* 2010). We synthesized Gcga and Gcgb peptides and determined their dose–response curve in HEK293T cells expressing Gcgra,

Figure 1

Identification of zebrafish *gcgra* and *gcgrb*. (A) Phylogenetic analysis of glucagon receptors. Full-length amino acid sequences of various receptors for glucagon-like peptides were analyzed using the neighbor-joining method. Posterior probability values (1000 runs) for branches of the tree are given on nodes. GIPR, glucose-dependent insulinotropic polypeptide receptor; GLP1R, glucagon-like peptide 1 receptor; GLP2R, glucagon-like peptide 2 receptor. The prefixes used are: h, human; m, mouse; r, rat; c, chicken; x, *Xenopus laevis*; zf, zebrafish; gf, goldfish; lf, lungfish. *Predicted sequences. (B) Conserved synteny of *gcgra* and *gcgrb* to human GCGR. Zebrafish *gcgra* and *gcgrb* are located on chromosome 3 and chromosome 1 respectively. They share several syntenic genes of human GCGR on chromosome 17.

Gcgrb, and mouse GCGR using the CRE-luc reporter system. As shown in Fig. 2, zebrafish Gcga and Gcgb stimulated cAMP accumulation in cells expressing zebrafish Gcgra with EC₅₀ value of 4.16 and 14.6 nM respectively. Mouse GCG had a much higher (1.96 μM) EC₅₀ on zebrafish Gcgra (Fig. 2C and Table 1). The mouse and two zebrafish glucagon peptides activated zebrafish Gcgrb at similar EC₅₀ values (Gcga, 27.95 nM; Gcgb, 14.88 nM; and GCG, 40.46 nM; Fig. 2D and Table 1). However, Gcga and Gcgb only weakly activated mouse

GCGR, contrasting to an EC₅₀ value of 8.33 nM for mouse GCG. These data suggested that the two zebrafish GCGRs have similar characteristics in responding to zebrafish glucagons (Gcga and Gcgb), but only Gcgrb responds to mouse glucagon. Zebrafish glucagons have a limited activity on the murine GCGR.

Generation of *gcgra* and *gcgrb* mutations using TALEN

To investigate the physiological function of Gcgra and Gcgrb, we generated loss-of-function alleles of *gcgra* and *gcgrb* using TALEN-mediated mutagenesis. The TALENs targeted the third exon of *gcgra* and second exon of *gcgrb* respectively. From the TALEN mRNA-injected founders, we obtained one germline mutation in *gcgra* with a 22 bp deletion (*gcgra*^{vu600}), and one germline mutation in *gcgrb* with 1 bp deletion (*gcgrb*^{vu601}), both resulting in reading-frame shift and premature stop codon (Fig. 3A, B, C, D, E, F, G and H). QRT-PCR analysis showed that *gcgra* mRNA levels were not changed in the *gcgrb*^{vu601}/*gcgrb*^{vu601} mutants, but were decreased in *gcgra*^{vu600}/*gcgra*^{vu600} (0.65 ± 0.02) and *gcgra*^{vu600}/*gcgrb*^{vu601}/*gcgrb*^{vu601} (0.55 ± 0.02). The levels of *gcgrb* mRNA also were not changed in the *gcgra*^{vu600}/*gcgrb*^{vu601} mutants, but were decreased in *gcgrb*^{vu601}/*gcgrb*^{vu601} (0.67 ± 0.05) and *gcgra*^{vu600}/*gcgrb*^{vu601}/*gcgrb*^{vu601} (0.56 ± 0.16) (Fig. 3I). The results suggest that there is no compensatory regulation of the two glucagon genes. The reduction of the mutant mRNA levels is likely due to nonsense-mediated decay of mRNA.

Disruption of zebrafish *gcgra* and *gcgrb* causes pancreatic α -cell hyperplasia in larvae

To determine the role of GCGRs in the regulation of pancreatic α -cell mass, we crossed the *gcgra*^{vu600} and *gcgrb*^{vu601} into *Tg(gcga:GFP)* fish whose α -cells are labeled with GFP (Zecchin *et al.* 2007), allowing cell number determination. We confirmed by immunofluorescence that all GFP positive cells in the islet of the transgenic fish express glucagon (Supplementary Fig. 1). We then determined the number of α -cells in WT and mutants at 7 dpf. Compared to *Tg(gcga:GFP)* with WT *gcgr* genes (20.63 ± 0.53; mean ± S.E.M.), there was an increase of α -cell number in the principal islet of *gcgra*^{vu600}/*gcgrb*^{vu601}; *Tg(gcga:GFP)* (27.73 ± 1.50; *P* < 0.01) and *gcgrb*^{vu601}/*gcgrb*^{vu601}; *Tg(gcga:GFP)* (23.64 ± 0.54; *P* < 0.01) (Fig. 4A and B). There, increase was even greater in *gcgra*^{vu600}/*gcgrb*^{vu601}; *Tg(gcga:GFP)* (32.89 ± 0.84; *P* < 0.001; Fig. 4A and B). To determine

Figure 2

Expression patterns of *gcgra* and *gcgrb* and pharmacological profiles of their gene products. (A) RT-PCR analysis *gcgra* and *gcgrb* mRNA at different stages of development. The upper panel is *gcgra* mRNA, the middle panel is *gcgrb* mRNA, and lower panel is β -actin mRNA. The developmental stages are shown at the top as days or weeks post fertilization. nc, negative control. (B) RT-PCR analysis *gcgra* and *gcgrb* mRNA in different tissues in adult fish. Total RNA was isolated from liver (Li), intestine (In), muscle (Mu), heart (He), and brain (Br) from adult zebrafish. nc, negative control.

(C, D and E) Dose-response curves of zebrafish and mouse glucagon receptors (GCGRs) to zebrafish and mouse glucagon peptides as measured by cAMP-dependent CRE-luciferase expression. HEK293T cells were co-transfected with pCRE-luc and expression vector pcDNA3.1(+) containing zebrafish Gcga (C), zebrafish Gcgrb (D), and mouse GCGR (E). The cells were exposed to different concentrations of zebrafish glucagon-a (Gcga), glucagon-b (Gcgb), and mouse glucagon (mGCG) peptides 48 h after transient transfection. Each data point is the mean of triplicates.

when the difference occurs, we followed α -cell number from 3 to 8 dpf in *Tg(gcga:GFP)* and *gcgra^{vu600}/gcgra^{vu600};gcgrb^{vu601}/gcgrb^{vu601};Tg(gcga:GFP)* fish. The increase of α -cell number was observed as early as 4 dpf (19.38 ± 0.65 vs 25.00 ± 0.96 , $P < 0.001$; Fig. 4C). The largest single-day increase of α -cells occurred from 4 to 5 dpf in *gcgra^{vu600}/gcgra^{vu600};gcgrb^{vu601}/gcgrb^{vu601};Tg(gcga:GFP)* fish (Fig. 4C). These data suggest that disruption of zebrafish *gcgra* and *gcgrb* causes supernumerary α -cells in zebrafish.

To determine whether α -cell replication contributed to the supernumerary α -cells in zebrafish *gcgr* mutants, we incubated 4 dpf larvae with EdU for 24 h to label replicating cells. Compared to *Tg(gcga:GFP)* (1.71 ± 0.56), there were a significant increase of EdU positive α -cells

in *gcgra^{vu600}/gcgra^{vu600};gcgrb^{vu601}/gcgrb^{vu601};Tg(gcga:GFP)* (4.56 ± 0.99 ; $P < 0.05$; Fig. 5). The number of EdU positive α -cells tended to be increased in both *gcgra^{vu600}/gcgra^{vu600};Tg(gcga:GFP)* (3.11 ± 0.45) and *gcgrb^{vu601}/gcgrb^{vu601};Tg(gcga:GFP)* (2.75 ± 0.45) fish, although the difference was not statistically significant (Fig. 5). These data suggest that the supernumerary α -cells are partially due to replication.

Mild hypoglycemia and hyperglucagonemia in *gcgr*-deficient zebrafish

To assess whether supernumerary α -cells in *gcgr*-deficient zebrafish contributes to hyperglucagonemia,

Table 1 EC50 of glucagons to glucagon receptors (GCGRs) as measured by cAMP-mediated luciferase activity. EC50 values represent glucagon concentrations that elicit 50% of the maximum response to GCGRs. EC50 (upper panel in each cell) and 95% CIs (lower panel in each cell) were shown. Each data point was the average of triplicates

Receptor	Gcga	Gcgb	mGCG
Gcgra	4.161×10^{-9} (1.125×10^{-9} – 1.539×10^{-8})	1.461×10^{-8} (5.827×10^{-9} – 3.664×10^{-8})	1.962×10^{-6} (8.530×10^{-7} – 4.513×10^{-6})
Gcgrb	2.798×10^{-8} (1.354×10^{-8} – 5.779×10^{-8})	1.488×10^{-8} (8.770×10^{-9} – 2.524×10^{-8})	4.046×10^{-8} (1.562×10^{-8} – 1.048×10^{-7})
mGCGR	~ 0.03148	~ 0.02404	8.329×10^{-9} (5.481×10^{-9} – 1.266×10^{-8})

Figure 3

Generation of *gcgra*^{vu600} and *gcgrb*^{vu601}. (A, B, C, D, E and F) Schematic representations of TALEN targets and mutant alleles. Both *gcgra* (A) and *gcgrb* (D) consists of 13 exons (filled box). The TALEN pairs target exons 3 and 2 respectively. The sequences of the target regions are aligned to the selected alleles for *gcgra* (B) and *gcgrb* (E). The location of the truncation in the mutant gene product for *gcgra* (C) or *gcgrb* (F) are indicated with a

dotted line. (G and H) Genotyping of WT, heterozygous, and homozygous of *gcgra* (G) and *gcgrb* (H). (I) Real-time PCR analysis of *gcgra* and *gcgrb* mRNA levels. The results were from three independent experiments. All the values shown are means $\pm$ S.E.M., * $P < 0.05$ and *** $P < 0.001$ by one-way ANOVA.

we determined the mRNA and protein levels of glucagon at 7 dpf. Compared to WT controls, the levels of *gcga* and *gcgb* mRNA were both significantly increased in *gcgra*^{vu600}/*gcgra*^{vu600} and *gcgra*^{vu600}/*gcgrb*^{vu601}; *gcgrb*^{vu601}/*gcgrb*^{vu601} larvae, but not in *gcgrb*^{vu601}/*gcgrb*^{vu601} larvae (Fig. 6A and B). No change in insulin expression was detected (Fig. 6C). Expression analysis by *in situ* hybridization confirmed an increase of *gcga* and *gcgb* in the islet of the double mutants (Supplementary Fig. 2). The analysis also indicated elevated intestinal expression of the preproglucagon mRNA in the double mutants (Supplementary Fig. 2), suggesting increased Glp1 in the zebrafish mutants as in GCGR^{-/-} mice (Gelling *et al.* 2003). To determine whether the increase of *gaca* and *gcgb* mRNA in the islet resulted in more glucagon peptide, we measured the total glucagon content using RIA. Compared to total glucagon in the control group (3.23 ± 0.30 pg/fish), *gcgra*^{vu600}/*gcgra*^{vu600} (4.07 ± 0.16 pg, $P < 0.05$), and *gcgra*^{vu600}/*gcgrb*^{vu601}; *gcgrb*^{vu601}/*gcgrb*^{vu601} (5.83 ± 0.35 pg, $P < 0.001$) had more glucagon per fish (Fig. 6D). The increased glucagon content was consistent with an increased α -cell number in the mutants.

To assess the effect of GCGR deficiency on gluconeogenesis, we measured free glucose levels at 7 dpf. Compared to WT control, there was a significant decrease in *gcgra*^{vu600}/*gcgrb*^{vu601}, *gcgrb*^{vu601}/*gcgrb*^{vu601} and

gcgra^{vu600}/*gcgrb*^{vu601}; *gcgrb*^{vu601}/*gcgrb*^{vu601} mutants (Fig. 5E). To determine whether this is due to changes in the expression of gluconeogenic genes, we measured the expression of phosphoenolpyruvate carboxykinase (soluble) (*pck1*), *pck2* (mitochondrial), glucose-6-phosphatase (*g6pca.1*) mRNA by qRT-PCR (Fig. 6F, G and H). Compared to WT control, there was a significant reduction of *pck1*, *pck2*, and *g6pca.1* mRNA levels in *gcgra*^{vu600}/*gcgrb*^{vu601}; *gcgrb*^{vu601}/*gcgrb*^{vu601} larvae (Fig. 6F, G and H). However, no significant change in *pck1* and *g6pca.1* expression was found in *gcgra*^{vu600}/*gcgra*^{vu600} fish.

Discussion

Antagonism of glucagon action is a potential therapeutic approach in type 2 diabetes. To advance glucagon antagonism as a therapy requires a thorough understanding of its compensatory responses to α -cell hyperplasia. However, the molecular explanation for the α -cell hyperplasia is unknown. In an effort to identify the responsible factor or factors, we have used the genetic tractability of zebrafish to determine if the underlying compensatory mechanism is conserved. In this study, we characterize the GCGRs of zebrafish and demonstrate that α -cell hyperplasia occurs in zebrafish, like in rodents and humans, when GCGR signaling is interrupted.

Figure 4

Homozygous mutants of *gcgr* genes develop α -cell hyperplasia. (A) Representative images of the principal islet of *Tg(gcga:GFP)*, *gcgra*^{-/-}; *Tg(gcga:GFP)*, *gcgrb*^{-/-}; *Tg(gcga:GFP)*, *gcgra*^{-/-}; *gcgrb*^{-/-}; *Tg(gcga:GFP)* at 7 dpf. The images are confocal projections; scale bars indicate 10 μm. (B) Quantification of the α -cell number in different

genotypes of zebrafish at 7 dpf, $n=8-12$. * $P<0.05$ and *** $P<0.001$ by one-way ANOVA. (C) α -cell number in *Tg(gcga:GFP)* and *gcgra*^{-/-}; *gcgrb*^{-/-}; *Tg(gcga:GFP)* from 3 to 8 dpf, $n=8-16$. The values shown are means $\pm$ s.e.m., ** $P<0.01$ and *** $P<0.001$ by t-test.

We show that the zebrafish have two *gcgr* genes and that both are functional. This is likely due to the additional round of genome duplication in the teleost lineage (Venkatesh 2003). As with many other gene duplications, *gcgra* and *gcgrb* are likely preserved because of functional partitioning. While both are expressed in the liver and brain, *gcgra* is not expressed in the intestines whereas *gcgrb* is not expressed in the heart and muscle (Fig. 2B). Nevertheless, their protein products

respond similarly to both zebrafish glucagon peptides, Gcga and Gcgb, and couple to Gs to increase intracellular cAMP. An interesting pharmacological difference between the two receptors is that Gcgra is much less responsive to mammalian glucagon than Gcgrb. A surprising finding is that both zebrafish glucagon peptides fail to activate mouse GCGR (Fig. 2E). This is similar to goldfish glucagon, which fails to bind rat GCGR (Chow *et al.* 2004).

Figure 5

Increased α -cell proliferation in *gcgr*-deficient zebrafish larvae. (A) Representative images of different genotypes larvae by EdU staining. Arrows indicated the EdU (red) positive α -cell (green). The images are

confocal projections; scale bar indicates 10 μm. (B) Quantification of EdU labeled α -cells, $n=7-10$. All the values shown are means $\pm$ s.e.m., * $P<0.05$ by one-way ANOVA.

Figure 6

Defects in glucose metabolism in *gcgr*-deficient zebrafish at 7 dpf. (A, B and C) Real-time PCR analysis of *gcga* (A), *gcgb* (B), and insulin (C) mRNA in WT and mutant fish. (D) Total glucagon content in WT and mutant fish. (E) Total free glucose content in WT and mutant fish. (F, G and H) Quantitative RT-PCR analysis of the expression of key

gluconeogenic genes *pck1* (F), *pck2* (G), and *g6pca.1* (H). Expression of β -actin was used as an internal control for all real-time PCR experiments. All the values shown are means $\pm$ S.E.M. from three independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by one-way ANOVA.

We show that *gcgr*-deficient zebrafish display many phenotypes similar to *Gcgr*-deficient mice, having lower free glucose content and higher glucagon content, similar to the hypoglycemia and hyperglucagonemia seen in mice (Gelling *et al.* 2003, Vuguin *et al.* 2006). Importantly, *gcgr*-deficient zebrafish have a greater α -cell mass (Fig. 4) and the increase of α -cells is at least partially from α -cell replication (Fig. 5). Similar to our results, Ye *et al.* (2015) reported that knockdown of zebrafish *gcga* by morpholino resulted in supernumerary α -cells partially from proliferation of existing α -cells. These data indicate that the compensatory mechanism to glucagon deficiency or interruption of glucagon signaling is conserved in zebrafish. In *gcgr*-deficient mice, increased α -cell number is observed as early as day e15 (Vuguin *et al.* 2006). Similarly, the α -cell hyperplasia phenotype is very robust as early as 7 dpf in zebrafish, a stage that is amenable for large-scale genetic and small molecule screens. Therefore, GCGR-deficient zebrafish offer an opportunity to unravel the molecular mechanism of compensatory α -cell hyperplasia resulting from glucagon antagonism and to discover other signals that regulate α -cell mass.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-15-0284>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

W C is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and

the accuracy of the data analysis. W C, M L, E D D, and A C P designed the study. M L, E D D, L Z, and W E N performed the key experiments. W C, M L, D D, A C P, L Z, and W E N participated in the planning of the work and the interpretation of the results. M L and W C drafted the manuscript. W C, M L, D D, and A C P have participated in revising the paper.

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