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⁻/⁻ fish and there was 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

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,

Glucagon raises the blood glucose and excess glucagon action is seen in both types 1 and 2 diabetes (Lund et al. 2013). Furthermore, treatment for both types of diabetes. However, as compensation to loss of glucagon action, Gcgr−/− mice also exhibit α- and β-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 Gsα subunit (Gsα) 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(gcgra: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′-GCCCTGCCCAACACTACAGT-3′ (left) and 5′-GTATCTGCCCTGACACAAGG-3′ (right). The target sequences for the gcgrb TALEN pair are 5′-CTCTGGAATCTCTGAAG-3′ (left) and 5′-TGGAGGATCTACACAATG-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 RNAs 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<sup>vua600</sup> and a gcgrb allele with 1 bp deletion, designated as gcgrb<sup>d603</sup>, were selected for additional analysis. For gcgra<sup>vua600</sup> carriers, PCR products amplified using gcgra-Mu22-F1 and gcgrb-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<sup>d603</sup> 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: ENSDART0000021878. 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 replications. 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_rerio/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−ΔΔCt method (Livak & Schmittgen 2001) and presented as relative (fold) levels normalized to the level of β-actin.

cAMP accumulation

Zebrafish glucagon, Gcga (HSEGTFSDYKYLETR-RAQDFVQWLMA) and Gcgb (HSEGTFSDYKYLETR-RAQDFVQWLMS) 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 (SignaGen 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 Amplesx Red Glucose/Glucose Oxidase Assay Kit (Life Technologies). A pool of ten larvae was homogenized in 100 μl of sample buffer. The homogenate was cleared by centrifugation. According to the manufacturer’s instructions, 10 μl 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-ethyl-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 Axiosmager 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 KGN1, 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 extracellular 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, FN3KR, TBCC, ZNF750, B3GNT1L1, and METRN, 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, gcgra and gcgrb (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,
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\textsuperscript{vu600}), and one germline mutation in gcgrb with 1 bp deletion (gcgrb\textsuperscript{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\textsuperscript{vu601}/gcgrb\textsuperscript{vu601} mutants, but were decreased in gcgra\textsuperscript{vu600}/gcgra\textsuperscript{vu600} (0.65 \pm 0.02) and gcgra\textsuperscript{vu600}/gcgrb\textsuperscript{vu600}/gcgrb\textsuperscript{vu601} (0.55 \pm 0.02). The levels of gcgrb mRNA also were not changed in the gcgra\textsuperscript{vu600}/gcgrb\textsuperscript{vu600} mutants, but were decreased in gcgrb\textsuperscript{vu601}/gcgrb\textsuperscript{vu601} mutants (0.67 \pm 0.05) and gcgrb\textsuperscript{vu600}/gcgrb\textsuperscript{vu600}/gcgrb\textsuperscript{vu601}/gcgrb\textsuperscript{vu601} mutants (0.56 \pm 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 a-cell hyperplasia in larvae

To determine the role of GCGRs in the regulation of pancreatic a-cell mass, we crossed the gcgra\textsuperscript{vu600} and gcgrb\textsuperscript{vu601} into Tg(gcgra:GFP) fish whose a-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 a-cells in WT and mutants at 7 dpf. Compared to Tg(gcgra:GFP) with WT gcgr genes (20.63 \pm 0.53; mean \pm S.E.M.), there was an increase of a-cell number in the principal islet of gcgra\textsuperscript{vu600}/gcgrb\textsuperscript{vu600}/Tg(gcgra:GFP) (27.73 \pm 1.50; P < 0.01) and gcgrb\textsuperscript{vu601}/gcgrb\textsuperscript{vu601}/Tg(gcgra:GFP) (23.64 \pm 0.54; P < 0.01) (Fig. 4A and B). There, increase was even greater in gcgra\textsuperscript{vu600}/gcgrb\textsuperscript{vu600}/gcgrb\textsuperscript{vu601}/gcgrb\textsuperscript{vu601}/Tg(gcgra:GFP) fish (32.89 \pm 0.84; P < 0.001; Fig. 4A and B). To determine...
These data suggest that disruption of zebrafish Gcgra 4.161 and Gcgrb 2.798 mRNA, when the difference occurs, we followed α-cell number from 3 to 8 dpf in Tg(gcga:GFP) and gcgra\textsuperscript{v600}/gcgrb\textsuperscript{v601}; gcgrb\textsuperscript{v601}/gcgrb\textsuperscript{v601};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\textsuperscript{v600}/ gcgrb\textsuperscript{v601}/gcgrb\textsuperscript{v601};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\textsuperscript{v600}/gcgrb\textsuperscript{v601};Tg(gcga:GFP) (4.56±0.99; P<0.05; Fig. 5). The number of EdU positive α-cells trended to be increased in both gcgra\textsuperscript{v600};gcgrb\textsuperscript{v601};Tg(gcga:GFP) (3.11±0.45) and gcgrb\textsuperscript{v601};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,
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\textsuperscript{vu600} gcgrb\textsuperscript{vu601} and gcgra\textsuperscript{vu600} gcgrb\textsuperscript{vu601} larvae, but not in gcgrb\textsuperscript{vu601} gcgrb\textsuperscript{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\textsuperscript{K} (Supplementary Fig. 2), suggesting increased Glp1 in the islet of the double mutants ( Supplementary Fig. 2).

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\textsuperscript{vu600} gcgrb\textsuperscript{vu601}, gcgrb\textsuperscript{vu601} gcgrb\textsuperscript{vu601}, and gcgrb\textsuperscript{vu600} gcgrb\textsuperscript{vu601} gcgrb\textsuperscript{vu601} mutants (Fig. 5E).

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±S.E.M., *P<0.05 and ***P<0.001 by one-way ANOVA.

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 \(\alpha\)-cell hyperplasia. However, the molecular explanation for the \(\alpha\)-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 \(\alpha\)-cell hyperplasia occurs in zebrafish, like in rodents and humans, when GCGR signaling is interrupted.
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 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), gcgra−/−; gcgrb−/− 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), gcgra−/−; gcgrb−/−; Tg(gcga:GFP), gcgra−/−; gcgrb−/−; Tg(gcga:GFP), gcgra−/−; gcgrb−/− from 3 to 8 dpf, n=8–16. The values shown are means±S.E.M., **P<0.01 and ***P<0.001 by t-test.

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±S.E.M., *P<0.05 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.

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 ± S.E.M. from three independent experiments. *P<0.05, **P<0.01, and ***P<0.001 by one-way ANOVA.

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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