Overnutrition induces β-cell differentiation through prolonged activation of β-cells in zebrafish larvae

Mingyu Li, Lisette A. Maddison, Patrick Page-McCaw, and Wenbiao Chen

Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee

Submitted 17 December 2013; accepted in final form 21 January 2014

Li M, Maddison LA, Page-McCaw P, Chen W. Overnutrition induces β-cell differentiation through prolonged activation of β-cells in zebrafish larvae. Am J Physiol Endocrinol Metab 306: E799–E807, 2014. First published January 28, 2014; doi:10.1152/ajpendo.00686.2013.—Insulin from islet β-cells maintains glucose homeostasis by stimulating peripheral tissues to remove glucose from circulation. Persistent elevation of insulin demand increases β-cell number through self-replication or differentiation (neogenesis) as part of a compensatory response. However, it is not well understood how a persistent increase in insulin demand is detected. We have previously demonstrated that a persistent increase in insulin demand by overnutrition induces compensatory β-cell differentiation in zebrafish. Here, we use a series of pharmacological and genetic analyses to show that prolonged stimulation of existing β-cells is necessary and sufficient for this compensatory response. In the absence of feeding, tonic, but not intermittent, pharmacological activation of β-cell secretion was sufficient to induce β-cell differentiation. Conversely, drugs that block β-cell secretion, including an ATP-sensitive potassium (KATP) channel agonist and an L-type Ca2+ channel blocker, suppressed overnutrition-induced β-cell differentiation. Genetic experiments specifically targeting β-cells confirm existing β-cells as the overnutrition sensor. First, inducible expression of a constitutively active KATP channel in β-cells suppressed the overnutrition effect. Second, inducible expression of a dominant-negative KATP mutant induced β-cell differentiation independent of nutrients. Third, sensitizing β-cell metabolism by transgenic expression of a hyperactive glucokinase potentiated differentiation. Finally, ablation of the existing β-cells abolished the differentiation response. Taken together, these data establish that overnutrition induces β-cell differentiation in larval zebrafish through prolonged activation of β-cells. These findings demonstrate an essential role for existing β-cells in sensing overnutrition and compensating for their own insufficiency by recruiting additional β-cells.

Compensatory increases in β-cell mass in response to increased insulin demand, for example, due to insulin resistance, have been documented in humans and rodents. In humans, evidence from postmortem analyses of β-cell mass has shown a positive correlation between β-cell mass and body mass index in nonobese patients. Furthermore, nonobese obese individuals have significantly more β-cells than diabetic obese individuals, suggesting a correlation between a failure of compensatory increase in β-cell number and development of diabetes (6, 15, 45). In rodents, both genetic and diet-induced insulin resistance increase β-cell mass (18, 49). β-Cell mass is markedly increased in genetically obese mice (both ob/ob and db/db) and rats (Zucker fa/fa) than control littermates (3, 14, 43). Similarly, mice with genetically induced insulin resistance also have a significant growth in β-cells (11, 41). Together these results suggest that compensatory increases in β-cell mass can be critical to maintain glucose homeostasis.

Compensatory β-cell genesis may arise from self-replication of existing β-cells, differentiation from stem/precursor cells, and/or transdifferentiation from other cell types (5). Although replication of existing β-cells is a major contributor to compensatory expansion, at least in rodents (4, 52), differentiation may also be an important source, especially during overnutrition (5, 22, 43). Where β-cell replication occurs, it is commonly thought that β-cells sense higher concentrations of glucose, insulin, GLP-1, or free fatty acids in the blood and reenter the cell cycle cell autonomously (1, 9). However, noncell autonomous mechanisms are also involved in inducing compensatory β-cell replication. For example, β-cell replication induced by insulin resistance resulting from either high-fat diet, genetic obesity, or liver-specific ablation of insulin receptor involves circulating factors (10, 12) and requires activation of Erk1/2 in liver and an intact vagal nerve (19, 31). Recently, a liver-derived factor, β-trophin, has been identified as a β-cell mitogen induced by insulin resistance (55).

Regardless of the mechanism or mechanisms by which β-cell mass increases, much less is known about how a persistently increased demand for insulin is sensed in the organism to trigger the compensatory β-cell differentiation. This is partly because of the lack of appropriate models to address the molecular mechanisms. Several groups have demonstrated that nutrient infusion in rats induces β-cell differentiation (5, 22, 42, 43, 53), whereas others have demonstrated that β-cell replication occurs in similar experimental paradigms (4, 50). The mixed occurrence of differentiation and replication in the rodent models, together with the low genetic tractability and the inaccessibility of the islet tissue of the rat model, makes it difficult to elucidate the molecular mechanisms of compensatory differentiation. To circumvent some of this experimental difficulty, we have developed a model of compensatory β-cell differentiation in the genetically tractable zebrafish (35). In this

Address for reprint requests and other correspondence: W. Chen, Dept. of Molecular Physiology and Biophysics, Vanderbilt Univ. School of Medicine, Light Hall, Rm. 711, 2215 Garland Ave., Nashville, TN 37232 (e-mail: wenbiao.chen@vanderbilt.edu).

Diabetes; nutrient sensing; zebrafish
model, the number of β-cells increases by 30% from 32 to more than 42 after 8-h culture in nutrient-containing medium by a single mechanism without replication or transdifferentiation. The new β-cells arise from endocrine precursor cells expressing mnx1 or nkt2.2 (35). The genetic and anatomical tractability of the zebrafish should facilitate molecular events underlying compensatory differentiation. This study focuses on determining the cellular and molecular mechanism by which insufficient insulin secretory capacity is sensed. Using a series of pharmacological and genetic analyses, we show that prolonged activation of the existing β-cells is necessary and sufficient for overnutrition-induced differentiation.

MATERIALS AND METHODS

Zebrafish strains and maintenance. Zebrafish were raised in an Aquatic-Habitats system on a 14:10-h light-dark cycle. Embryos were obtained from natural crossing and raised according to standard methods; animals were staged by hours postfertilization (hpf) and days postfertilization (dpf) (25). Tg(−1.2ins:HG6mCherry) was used to mark β-cells, and β-cells were counted as described (35). All procedures have been approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Establishment and identification of transgenic lines. New transgenic lines were generated using the Tol2 transposon system (51). For constitutive expression of human GCKV91L (23) in β-cells, a transgenic construct consisting of two engineered genes carried by the Tol2 transposon vector was made. Tg(crysa:tagRFP) marks the lens (referred to as lens red, LR) of transgenic fish while Tg(−1.2ins:GCKV91LR) directs β-cell expression of the mutant protein using a 1.2-kb insulin promoter (see Fig. 5A). This vector was designated as Tg(−1.2ins:GCKV91LR). For inducible expression of a truncated human BID (52), mouse Kir6.2CA-GFP (27), and mouse Kir6.2CA-GFP (27) in β-cells, each transgenic construct consists of three engineered genes: Tg(−1.2ins:rtTA-EcR) to drive a tetracycline- and ecdysone-dependent transcription activator in β-cells (26); either Tg(TRE:BiD), Tg(TRE:Kir6.2CA-GFP), or Tg(TRE:Kir6.2CA-GFP) to express the effector proteins; and Tg(crysa:tagRFP). For simplicity, rtTA-EcR and TRE were collectively designated as TE-ON. Hence, these transgenes were named Tg(−1.2ins:rtTAEcR-LR), Tg(−1.2ins:Kir6.2CA-GFPTE-ON-LR), and Tg(−1.2ins:Kir6.2CA-GFPTE-ON-LR), respectively. Individual founders with tagRFP expression in the lens at 4 dpf were raised to maturity and confirmed by PCR. Initial analyses were performed in at least two independent lines for each transgene, and similar results were obtained. All results reported here were from F1 or F2 fish of these lines.

Feeding and cooing treatment. For glucose feeding, d-glucose (Sigma-Aldrich) was dissolved in Milli-Q water at 200 mmol/l and used at a working concentration of 10 or 20 mmol/l. For egg yolk feeding, chicken eggs were obtained from local grocery stores, and the yolk was separated and diluted to 5% by volume with 0.3× Danieau solution as described (35). All drugs were made in 1,000 mol/l stock solution and stored in light-protected Eppendorf tubes at −20°C; compound A (30 mmol/l; EMD Millipore), gibencamidyl (20 mmol/l; Sigma-Aldrich), and diazoxide (0.3 mol/l; Sigma-Aldrich) in DMSO and verapamil (10 mmol/l; Enzo) in water.

For induction of transgene expression, larvae were treated with doxycycline hyclate (100 mmol/l in ethanol stored in the dark at −20°C, 2,000×) and tebufenozide (50 mmol/l in DMSO at −20°C, 2,000×; Sigma-Aldrich) for 48 h (from 3 to 5 dpf) before feeding.

RNA extraction and RT-PCR. Total RNA was extracted from 10 zebrafish embryos using Trizol Reagents (Invitrogen) and digested by the RQI RNase-Free DNase (Promega) to remove any genomic DNA contamination. First-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega) with oligo(dT)18 as first-strand primers following the manufacturer’s instructions. PCR primers used were as follows: β-actin, 5′-CTTGCGGTATC-CACCAGAC-3′ and GGCCCATACAGACGGCAGG; human glucokinase (hGCK), 5′-GACAGAGGAGGACCTGGAAG-3′ and 5′-CCGGGGTGGCAGAGCTCT-3′; and mKir6.2, 5′-TGGCAGTACCACTCCT-3′ and 5′-TGGTAGCGTCCTGTTTC-3′. For β-actin, PCR was under the following conditions: 94°C for 3 min, then 28 cycles of 30 s at 95°C, 30 s at 58°C, 30 s at 72°C, and final extension at 72°C for 5 min. For hGCK and mKir6.2, 35 cycles of PCR with an annealing temperature of 60°C were used.

β-Cell ablation. Stable F1 Tg(−1.2ins:htBid+/-ON-LR) transgenic fish were crossed to homozygous Tg(−1.2ins:H2BmCherry) transgenic fish. Embryos were sorted based on the red lens fluorescence at 3 dpf and then induced as described above for 48 h, refreshing the media every 24 h. Animals were allowed to recover in drug-free media for 40 h before overnutrition treatment.

The larvae were then fixed in 4% paraformaldehyde and imaged using a Zeiss LSM710 confocal microscope.

Free glucose assay. Free glucose was determined by a glucose assay kit (BioVision). A pool of 10 larvae was homogenized in 100 µl of sample buffer, cleared by centrifugation, and stored at −80°C. Free glucose in the equivalent of one larva (10 µl of homogenate) was determined according to the manufacturer’s instructions. Fluorescence excitation (535 nm; emission, 590 nm) was measured using a SpectraMax M5 Microplate Reader (Molecular Devices). At least three pools of each sample were measured.

Immunofluorescence and 5-ethyl-2-deoxyuridine staining. The larval zebrafish of Tg(−1.2ins:H2BmCherry) were stained using proliferating cell nuclear antigen (PCNA, 1:2,000; Sigma-Aldrich P8825) using standard techniques. To identify proliferating β-cells, 5 dpf embryos were incubated with 100 µmol/l 5-ethyl-2-deoxyuridine (EdU) for 24 h labeling. EdU was detected using the Click-IT Edu Alexa Fluor 488 Imaging Kit (C10337; Invitrogen) according to published protocols (35). All images were collected using a Zeiss LSM510 or Zeiss LSM710 (Carl Zeiss).

Statistics. Data are means ± SE. Data were analyzed by one-way ANOVA followed by Fisher post hoc test or t-test (SPSS, Chicago, IL). Significance was accepted at P < 0.05.

RESULTS

Inhibition of membrane depolarization of nutrient-sensing cells suppresses overnutrition-induced β-cell differentiation. Hypothalamic neurons and pancreatic β-cells are two major postigestive nutrient sensors. In both cell types, nutrients inhibit the ATP-sensitive potassium (KATP) channels, resulting in membrane depolarization and Ca2+ influx through the voltage-sensitive L-type Ca2+ channel (40). To test whether nutrient inhibition of KATP channel is necessary for the overnutrition-induced β-cell differentiation, we used diazoxide, a KATP channel opener, to inhibit nutrient-induced membrane depolarization. As shown previously (35), sustained exposure of 6-day-old larvae to overnutrition results in a 25–30% increase in β-cell number with chicken egg yolk (Fig. 1, A and C) and an 20–25% increase in β-cell number with 20 mM glucose (Fig. 1, B and D). Diazoxide significantly attenuated the increase in β-cells induced by either egg yolk or glucose treatment (Fig. 1, A and B). Larvae treated with diazoxide had a significant increase of free glucose, indicating insulin secretion was suppressed as expected (Fig. 1E). These data show that the inactivation of KATP channels that occurs during nutrient exposure is necessary for overnutrition-induced β-cell differentiation. Similarly, verapamil, an inhibitor of L-type Ca2+ channels that open after membrane depolarization, also
significantly decreased β-cell differentiation induced by either 5% egg yolk or 20 mM glucose (Fig. 1, C and D). As expected, verapamil increased total free glucose levels (Fig. 1F). These data demonstrate that inhibition of K<sub>ATP</sub> channels or activation of L-type Ca<sup>2+</sup> channels is necessary for overnutrition-induced β-cell differentiation.

**Prolonged pharmacological activation of the K<sub>ATP</sub> channel and glucokinase induces β-cell differentiation.** Next, we tested whether inactivation of the K<sub>ATP</sub> channel, as would normally occur in the presence of nutrients, is sufficient to induce β-cell differentiation. When 6-day-old larvae were treated for 8 h with glibenclamide, an inhibitor of K<sub>ATP</sub> channels, β-cell number increased by about 20% even in the absence of nutrients (Fig. 2A). The new β-cells likely arise from differentiation of endocrine precursor cells, similar to those induced by overnutrition (35), since no increase of replicating β-cells was detected by EdU labeling or PCNA staining (Fig. 2C and data not shown). The scarcity of EdU incorporation was not due to technical issues, since it was readily detected in previously described Tg(CdK<sup>αb2</sup>C) larvae (33). Because only sustained, not intermittent, feeding results in β-cell differentiation (35), we hypothesized that intermittent glibenclamide treatment should not induce differentiation of new β-cells. Indeed, intermittent exposure to glibenclamide was insufficient to induce β-cell differentiation, demonstrating that prolonged pharmacological inhibition of K<sub>ATP</sub> channel is sufficient to elicit β-cell differentiation (Fig. 2A). These results demonstrate that prolonged K<sub>ATP</sub> channel inhibition is sufficient to drive β-cell differentiation in the absence of nutrients.

Glucokinase (GCK) controls the glucose sensitivity of a subset of cells, including β-cells and hepatocytes as well as neurons and glia of the hypothalamus (21). Compound A (CpdA) is an allosteric activator of GCK that increases the sensitivity of GCK-expressing cells to circulating glucose (13, 37). We have previously shown that culturing larvae in 20 mM glucose inside of the bars. Groups labeled with different letters are significantly different from each other (P < 0.05).
tain nutrient-sensing cells is necessary and sufficient to induce β-cell differentiation. We hypothesized that β-cells might be a critical component of the sensor for overnutrition. To determine whether β-cells act as a component of overnutrition sensor, we genetically manipulated K<sub>ATP</sub> channels in the β-cells. The transgenic line Tg(1.2ins:Kir6.2<sup>DN</sup>-GFP<sup>TE-ON</sup>; LR) allows inducible expression of a dominant-negative form of Kir6.2, Kir6.2(AAA)-GFP (28), in β-cells (Fig. 3A). Expression of Kir6.2<sup>DN</sup>-GFP should maintain the channel in the closed state, depolarizing the cells independent of intracellular ATP production (28). Transgenic and control larvae were exposed to doxycycline and tebufenozide for 48 h from 3 to 5 dpf to induce Kir6.2<sup>DN</sup>-GFP. Transgene expression was confirmed by RT-PCR analysis at 6 dpf (Fig. 3B). Expression of Kir6.2<sup>DN</sup> is expected to result in constitutive secretion of insulin, resulting in lowered baseline glucose levels (27). Indeed, the transgene was shown to be functional by the decrease in free glucose levels measured in larvae at 6 dpf (Fig. 3C). There were significantly more β-cells in unfed Kir6.2<sup>DN</sup>-GFP-expressing larvae than sibling control larvae at 6 dpf (Fig. 3D). The additional β-cells were not derived from β-cell replication, since no increase of EdU-labeled β-cells was detected, similar to those induced by overnutrition (Fig. 3E). Interestingly, overnutrition did not further increase the number of β-cells in Tg(1.2ins:Kir6.2<sup>DN</sup>-GFP<sup>TE-ON</sup>; LR) larvae (Fig. 3D), either because the pool of available undifferentiated cells is fully induced or because the K<sub>ATP</sub> channels in transgenic β-cells are no longer ATP-sensitive. The results demonstrate that genetically induced constitutive depolarization of β-cells is sufficient to induce differentiation.

**Genetic activation of K<sub>ATP</sub> channel in β-cells suppresses overnutrition-induced β-cell differentiation.** To determine whether depolarization of β-cells is necessary for overnutrition-induced β-cell differentiation, we generated a transgenic zebrabfish Tg(1.2ins:Kir6.2<sup>CA</sup>-GFP<sup>TE-ON</sup>; LR) that inducibly and β-cell specifically expresses Kir6.2<sub>ΔN2-30/K185Q-GFP</sub>, a constitutively active form of Kir6.2 (Kir6.2<sup>CA</sup>-GFP) (27) (Fig. 4A). Kir6.2<sup>CA</sup>-GFP locks K<sub>ATP</sub> in the open state, preventing β-cell depolarization. Transgenic expression of Kir6.2<sup>CA</sup> in β-cells in mice results in profound neonatal diabetes, since the β-cell can no longer respond to nutritional cues by secreting insulin (27). After 48 h of induction starting at 3 dpf, Tg(1.2ins:Kir6.2<sup>CA</sup>-GFP<sup>TE-ON</sup>; LR) larvae showed robust Kir6.2<sup>CA</sup> expression as measured by RT-PCR at 6 dpf (Fig. 4B) and had significantly increased free glucose at 6 dpf (Fig. 4C). When cultured for an additional 8 h with or without 5% egg yolk at 6 dpf, induction of Kir6.2<sup>CA</sup>-GFP expression significantly reduced compensatory β-cell differentiation (Fig. 4D). Similarly, induction of Kir6.2<sup>CA</sup>-GFP expression significantly reduced compensatory β-cell differentiation induced by 20 mM glucose (Fig. 4D). Expression of Kir6.2<sup>CA</sup>-GFP had no effect on basal β-cell number compared with sibling controls (Fig. 4D). These data indicate that depolarization of existing β-cells resulting from ATP-dependent inhibition of K<sub>ATP</sub> channels is necessary for overnutrition-induced β-cell differentiation.

**Targeted expression of hyperactive GCK enhances glucose-induced β-cell differentiation.** To further verify that prolonged activation of β-cells is necessary and sufficient to induce β-cell differentiation, we expressed a human GCK mutant (GCK<sup>V91L</sup>) that has a >8.5-fold higher affinity for glucose than wild-type GCK (23) in β-cells (Fig. 5, A and B). The glucose affinity of GCK in β-cells determines the threshold of glucose-triggered K<sub>ATP</sub> depolarization. At 6 dpf, unfed Tg(1.2ins:GCK<sup>V91L</sup>; LR) larvae had an unchanged β-cell number compared with nontransgenic larvae (Fig. 5C). However, when cultured in subthreshold doses of 10 mM glucose, GCK<sup>V91L</sup>-expressing animals had significantly more β-cells than sibling controls (Fig. 5C). Again, no increase of EdU-positive β-cells was detected, consistent with these cells arising from differentiation and not β-cell replication (Fig. 3E). These results are similar to what we observed using CpdA to activate GCK (Fig. 2B). Taken together, these data demonstrate that persistent activation of glucose metabolism in β-cells drives β-cell differentiation.
Overnutrition-induced β-cell differentiation requires β-cells. To confirm the hypothesis that β-cells are required for sensing insufficient insulin secretory capacity, we generated a transgenic line that can be used to specifically ablate β-cells. In this line, Tg(−1.2ins:htBidTE-ON; LR), the proapoptotic protein tBid (32), is expressed under the control of the tetracycline-and edcsyne-inducible system (26) (Fig. 6A). Expression of tBid was induced for 48 h starting at 72 hpf and followed by 48-h recovery (2). Most of the remaining β-cells have lower levels of mCherry signal (Fig. 6B), suggesting that they are likely immature, either newly regenerated as in the in MTZ-treated Tg(ins:CFP-NTR)c0692 larvae (2) or had low insulin promoter activity that allowed them to survive the ablation. The larvae with β-cell ablation had increased free glucose compared with controls, even 48 h after recovery, further suggesting insufficient β-cell function (Fig. 6C). To determine whether overnutrition-induced β-cell differentiation was impaired in the β-cell-ablated larvae, ablated and control larvae were treated with 5% egg yolk for 8 h after the 40 h of recovery. At the end of the experiment (7 dpf), the control animals had an increased β-cell count by ~20–30% (Fig. 6D), consistent with our previous findings (35); however, the number of β-cells did not increase in ablated animals (Fig. 6D). It is unlikely that overnutrition-induced new β-cells were killed since the tBID inducers doxycycline and tebufenozide had been washed out for 40 h before overnutrition treatment. Whereas a complete ablation of the β-cells has not proven possible in our hands or others (2), these data demonstrate that more than eight mature, functional β-cells are necessary for compensatory β-cell differentiation upon overnutrition.

DISCUSSION

Whereas it is well established that mammals have the ability to change β-cell number and synthetic capacity in response to changes in insulin demand, the sensing mechanisms of the mismatch between demand and capacity that drive these changes remain incompletely understood. Defects in the ability
to increase β-cell number to compensate for increased demand for insulin may account for the susceptibility of type 2 diabetes. We have found that, similar to compensatory differentiation of β-cells in rodents under conditions of nutrient infusion, overnutrition induces compensatory β-cell differentiation in zebrafish larvae (35). In zebrafish larvae, this compensation is rapid, occurring in <24 h; easily produced by soaking the fish in nutrients; and robust, facilitating mechanistic analysis. We show here that the compensatory response requires prolonged activation of existing β-cells, suggesting that β-cells are the sensor of overnutrition and function non-cell autonomously to promote differentiation of endocrine precursor cells.

Our conclusion that overnutrition-induced β-cell differentiation requires glucosensing cells is based on several lines of evidence. First, suppression of β-cell depolarization by activating KATP either pharmacologically or genetically in the β-cells inhibits the induction (Figs. 1 and 4). Second, activation of β-cells by inhibiting KATP either pharmacologically or genetically in the β-cell is sufficient for induction of β-cell genesis in the absence of nutrients (Figs. 2 and 3). Third, modulation of β-cell metabolism by manipulation of GCK activity or β-cell exocytosis by manipulation of L-type Ca2+ channel activity also lead to similar results (Figs. 1, 2, and 5). Fourth, intermittent inhibition of KATP, as for meal-style feeding, fails to elicit the induction, demonstrating the importance of prolonged activation of the sensor (Fig. 2A). Last, after genetic ablation of β-cells, there is a failure to activate overnutrition-induced compensatory β-cell genesis (Fig. 6).

These results strongly implicate the β-cells as the primary sensor mediating compensatory β-cell genesis and that the molecular components of this sensing apparatus are shared with the glucose-stimulated insulin secretion system. Other glucosensing cells are known to modulate β-cell number and express these same molecular components. For instance, hypothalamic neurons and glia express these components and are implicated in β-cell mass regulation (8, 30, 34). Our study, however, demonstrates that the β-cells emit a sufficient signal for induction of the compensatory β-cell genesis, since sustained depolarization of the β-cells through the Kir6.2DN was sufficient for the induction. Likewise, β-cell-specific expression of a hypersensitive GCK that triggers membrane depolarization at lower glucose threshold confers the compensatory response at a suboptimal level of glucose. Furthermore, β-cell ablation and β-cell-specific expression of the Kir6.2CA inhibit overnutrition-driven β-cell genesis. Although other sensors may be involved, these data suggest that β-cell is the primary sensor in our assay.

The identity of the neogenic signals released by β-cells after prolonged activation is of great interest for future research. Although insulin may be an integral part of the neogenic signal, the signal likely consists of other factors. This is because that pulsatile activation of β-cell secretion by either intermittent feeding (35) or intermittent inhibition of KATP (this study) fails to induce β-cell differentiation and differentiation occurs only when β-cell activation is sustained. One possible explanation is that only persistently high insulin levels drive β-cell differentiation. A precedent for differential effects of transient and prolonged insulin exposure comes from insulin’s known mitogenic effects (20). Prolonged insulin exposure initiates signal transduction events that are distinct from transient incubation in cultured hepatocytes (29). However, the time scale in our in vivo experiments (2 vs. 8 h) is very
different from each other (Fig. 6). Groups labeled with different letters are significantly different from each other (P < 0.05).

Fig. 5. β-Cell expression of a hyperactive GCK potentiates glucose-induced β-cell differentiation. A: schematic representation of the Tg([1.2ins:GCKV91L, LR]) transgene used to express a hyperactive human GCK mutant (GCKV91L) in β-cells. B: RT-PCR analysis of nontransgenic wild-type larvae or Tg([1.2ins:GCKV91L, LR]) transgenic larvae with (RT+) or without (RT−) reverse transcriptase confirmed expression of GCKV91L. C: the number of β-cells was significantly increased in Tg([1.2ins:GCKV91L, LR]); Tg([1.2ins:H2BmCherry]) transgenic larvae incubated in subthreshold 10 mM glucose compared with nontransgenic larvae. All values are means ± SE; n are shown inside of the bars. Groups labeled with different letters are significantly different from each other (P < 0.05).

Fig. 6. Overnutrition-induced β-cell differentiation requires existing β-cells. A: schematic representation of the transgene Tg([1.2ins:htBidTE-ON, LR]) used to inducibly ablate insulin-expressing cells in the presence of doxycycline and tebufenozide. In the transgene, the proapoptotic gene truncated BID (tBID) is under the control of a TRE-based promoter that is activated by a TE-ON driven by the zebrafish insulin promoter. An α-crystalline-driven tagRFP was used to mark transgene carriers. B: Tg([1.2ins:H2BmCherry]) (Control) or double transgenic Tg([1.2ins:htBidTE-ON, LR]; Tg([1.2ins:H2BmCherry]) (Ablation) larvae were incubated for 48 h with doxycycline and tebufenozide or drug-free medium and allowed to recover for 40 h in drug-free medium. Islets were then imaged with equivalent imaging parameters for acellular debris (arrows) that remained 48 h after drug washout. The images are confocal projections, and scale bars indicate 10 μm. C: total free glucose level of 6 dpf Tg([1.2ins:H2BmCherry]) (tBid−) and Tg([1.2ins:htBidTE-ON, LR]; Tg([1.2ins:H2BmCherry]) (tBid+) larvae treated with or without doxycycline and tebufenozide. Significantly increased free glucose levels were observed in Dox− Tbf-induced Tg([1.2ins:htBidTE-ON, LR]; Tg([1.2ins:H2BmCherry]) larvae. D: Dox− and Tbf-treated 6 dpf Tg([1.2ins:H2BmCherry]) (−) and double-transgenic Tg([1.2ins:htBidTE-ON, LR]; Tg([1.2ins:H2BmCherry]) (−) and control double-transgenic Tg([1.2ins:htBidTE-ON, LR]; Tg([1.2ins:H2BmCherry]) (−) larvae were cultured in 5% egg yolk or nutrient-free medium for 8 h. Overnutrition did not significantly affect β-cell number following β-cell ablation in unfed and yolk-fed double-transgenic larvae, but control animals responded normally. All values are means ± SE; n are shown inside of the bars. Groups labeled with different letters are significantly different from each other (P < 0.05).
not embryonic stage, the larval stage zebrafish as the model organism. At this stage the animal is dependent for all its nutrient and metabolic needs on its ability to identify and consume food. It therefore likely possesses more mature physiology and metabolism than neonatal mammals. Consistent with this, its β-cell number is relatively stable in normal rearing conditions (17, 35). The large increase only occurs after continuous culture in nutrient solutions (35) or prolonged stimulation of β-cells (this study), consistent with a compensatory mechanism. Perhaps more important than the developmental stage of the animal is the unusual replication independent nature of the β-cell genesis observed in our system. The advantage of this system, over and above the advantage of a large and robust compensatory response that occurs within 8 h, lies in the reduction of the complexity of the experimental system. This reductionist model allowed unambiguous demonstration of the non-cell autonomous nature of the response as well as a functional dissection of the underlying cellular and molecular processes. These results provide models that must then be tested in the more complicated and generally used systems. Because zebrafish also generate β-cells through replication at later stages (36, 39), it will be of great interest to explore these systems to determine if the sensor system identified here functions universally in β-cell genesis or whether this is a model specific.

The relevance to β-cell physiology in adult mammals notwithstanding, it is worth noting that the β-cell secretion pathway has been shown to play a critical role in β-cell replication in adult mice. For example, acute administration (24 h or less) of a GK activator, a KATP inhibitor, or an L-type channel blocker promotes β-cell replication (44, 47). Although it is proposed that the hyperactivated β-cells replicate cells autonomously (9), existing data cannot rule out a paracrine mechanism. By manipulating membrane depolarization at a stage when β-cells are refractory to replication (33, 39), we revealed a cell nonautonomous role of hyperactivated β-cells in β-cell genesis. It is tantalizing to speculate that the same factor(s) may be secreted from hyperactivated β-cells in adult mammals and contribute to the ensuing β-cell replication.

ACKNOWLEDGMENTS

We thank Colin G. Nichols (Washington University) for providing the Kir6.2ON-GFP and Kir6.2CA-GFP constructs.

GRANTS

This work was supported by the Vanderbilt Diabetes Research and Training Centers and National Institutes of Health (NIH) Grant DK-088686 (W. Chen) and by the American Diabetes Association Grant 1-13-BS-027 (W. Chen). We used the core(s) of the Vanderbilt Diabetes Research and Training Center funded by Grant DK-02593 from NIH, and confocal imaging was performed in the VUMC Cell Imaging Shared Resource (supported by NIH Grants CA-68485, DK-2593, DK-56404, HD-15052, DK-59637, and EY-08126).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

Molecular Mechanism of Compensatory β-Cell Differentiation