

# Nutrient Excess Stimulates $\beta$ -Cell Neogenesis in Zebrafish

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Persistent nutrient excess results in a compensatory increase in the  $\beta$ -cell number in mammals. It is unknown whether this response occurs in nonmammalian vertebrates, including zebrafish, a model for genetics and chemical genetics. We investigated the response of zebrafish  $\beta$ -cells to nutrient excess and the underlying mechanisms by culturing transgenic zebrafish larvae in solutions of different nutrient composition. The number of  $\beta$ -cells rapidly increases after persistent, but not intermittent, exposure to glucose or a lipid-rich diet. The response to glucose, but not the lipid-rich diet, required mammalian target of rapamycin activity. In contrast, inhibition of insulin/IGF-1 signaling in  $\beta$ -cells blocked the response to the lipid-rich diet, but not to glucose. Lineage tracing and marker expression analyses indicated that the new  $\beta$ -cells were not from self-replication but arose through differentiation of postmitotic precursor cells. On the basis of transgenic markers, we identified two groups of newly formed  $\beta$ -cells: one with *nkx2.2* promoter activity and the other with *mnx1* promoter activity. Thus, nutrient excess in zebrafish induces a rapid increase in  $\beta$ -cells through differentiation of two subpopulations of postmitotic precursor cells. This occurs through different mechanisms depending on the nutrient type and likely involves paracrine signaling between the differentiated  $\beta$ -cells and the precursor cells. *Diabetes* 61:2517–2524, 2012

**T**he insulin-producing  $\beta$ -cells of the pancreas are critical to maintain glucose homeostasis. Under increased metabolic demand, two compensatory  $\beta$ -cell responses occur: increased production and secretion of insulin and increased  $\beta$ -cell mass (1). Proliferation of  $\beta$ -cells has been considered by many to be the primary mechanism to increase postnatal  $\beta$ -cell mass in both mice and humans (2,3). However,  $\beta$ -cell neogenesis through differentiation of precursor cells or transdifferentiation from another pancreatic cell type may be equally important and occurs in both mice and humans under certain circumstances (4,5). There is considerable debate about the relative contribution of these two pathways under different physiological conditions and developmental stages (6,7).

A chronic excess in nutrients requires an increase in production of insulin in order to maintain metabolic control. Obesity, a result of prolonged nutrient excess or overnutrition, also leads to increased  $\beta$ -cell mass in rodents (8) and humans (9–11). Overnutrition likely increases glucose, amino acids, and lipids, and  $\beta$ -cells are sensitive to all of

these factors (12–14). Glucose has been implicated as a factor driving the increase in  $\beta$ -cells, supported by genetic evidence in mice in which diet-induced  $\beta$ -cell hyperplasia is compromised with haploinsufficiency of glucokinase (8). Furthermore, in a mouse  $\beta$ -cell regeneration model, glucose has been demonstrated to regulate  $\beta$ -cell proliferation in a metabolism-dependent manner (15). Nutrient excess not only will activate metabolic processes (13), but also nutrient-sensing pathways, including the mammalian target of rapamycin (mTOR) signaling pathway (16). Signaling through mTOR has been shown to regulate  $\beta$ -cell proliferation and  $\beta$ -cell mass (17). Insulin signaling has also been implicated in regulating  $\beta$ -cell hyperplasia (18). Furthermore, mice with haploinsufficiency for insulin receptor substrate 2 (8) or with  $\beta$ -cell-specific deletion of insulin receptor (19) have impaired compensatory  $\beta$ -cell hyperplasia. Although both glucose and insulin have been implicated as important factors regulating the compensatory  $\beta$ -cell response, levels of both fluctuate in normal physiology without an increase in  $\beta$ -cells, suggesting that other factors that remain to be determined are involved in regulating compensatory  $\beta$ -cell hyperplasia.

Zebrafish have been used extensively as a model to investigate many vertebrate biological processes, including  $\beta$ -cell development and regeneration (20–23). Zebrafish  $\beta$ -cells are similar to those in mammals both in development and function. Although the zebrafish has been used primarily as a model of developmental biology and innate behavior, it has increasingly been used to model human diseases (24) and is well poised to investigate  $\beta$ -cell physiology. With the exception of *ngn3*, all of the orthologs of genes important for mammalian  $\beta$ -cell development have been identified in zebrafish and have been shown to be functionally conserved (25). As in mammals, zebrafish  $\beta$ -cells play a central role in maintaining glucose homeostasis in adults (21,26). At larval stages, a single islet is present, containing ~30  $\beta$ -cells at 5 days postfertilization (dpf) and 60  $\beta$ -cells at 14 dpf (22,27). Starting in late larval stages, secondary islets are also present (28,29). We have found that zebrafish exhibit a rapid increase in the number of  $\beta$ -cells in states of overnutrition induced by glucose or a lipid-rich diet. Interestingly, mTOR signaling is involved only in the  $\beta$ -cell response to glucose and not to a lipid-rich diet. In contrast, inhibition of insulin/IGF-1 signaling in  $\beta$ -cells blocks the response to the lipid-rich diet, but not to glucose. Furthermore, this increase is not through  $\beta$ -cell proliferation but via differentiation of precursor cells.

## RESEARCH DESIGN AND METHODS

**Zebrafish strains and maintenance.** Adult zebrafish were raised in Aquatic Habitats systems on a 14/10-h light/dark cycle. Embryos were collected and raised in 0.3 $\times$  Danieau solution at 28.5°C in an incubator with lights on a 14/10-h light/dark cycle. The transgenic lines used for this study were *Tg(-1.2ins:H2BmCherry)*, *Tg(-1.2ins:tagRFP)*, *Tg(-1.2ins:EGFP)* (23), *Tg(-5.1mnx1:tagRFP)*, *Tg(-3.0hb9:GFP)* (23), *Tg(sst2:memRFP)* (23), *Tg(nkx2.2:mEGFP)<sup>VU17</sup>* (23), *Tg(gcga:EGFP)*, and *Tg(ptf1a:EGFP)* (23). We also

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generated a transgenic line with three elements separated by a 2A peptide, a membrane targeted TagRFP, nuclear mCherry, and nitroreductase and referred to as (*-1.2ins:mR-nC-NTR*).

**Feeding, nutrient, and compound treatment.** All feeding experiments used 0.3× Danieau solution as the medium. For Hatchfry encapsulon (Argent Laboratories) feeding, a small amount was placed on the surface of the solution. For egg yolk feeding, chicken eggs were obtained from local grocery stores, the yolk separated, and diluted to 5% by volume. The solution was shaken vigorously to ensure suspension. For purified amino acid treatment, L-amino acids were obtained (Sigma-Aldrich), dissolved in 1 M HCl at 50 mg/mL, diluted to appropriate concentration based on data from the U.S. Department of Agriculture nutrient database, and the pH adjusted to 7.4. Intralipid (Sigma-Aldrich) was diluted to 1.33% by volume. Rapamycin (Sigma-Aldrich) was dissolved in DMSO at 10 mmol/L and used at a working concentration of 2.5 μmol/L. To ablate β-cells, transgenic larvae were incubated in 10 mmol/L metronidazole (Sigma-Aldrich) for 24 h. After the appropriate exposure interval, larvae were killed in 3-amino-benzoic acid ethyl ester (Sigma-Aldrich) and fixed in 4% paraformaldehyde overnight at 4°C.

**Counting of β-cells.** After fixation, larvae were washed with 1× PBS plus 0.1% Tween-20 (PBST) and flat mounted in Aqua-Mount (Richard-Allan Scientific) with larvae oriented with their right side facing the coverslip. The larvae were flattened sufficiently to disrupt the islet slightly but allow better resolution of individual nuclei. All slides were coded to avoid bias. The β-cell number was determined by manually counting positive nuclei using a Zeiss AxioVert 100 under a 20× DIC lens or using a Zeiss AxioImager under a 40× lens (Carl Zeiss).

**Immunofluorescence and proliferation analysis.** Where necessary, after fixation, larvae were dehydrated in methanol and stored at -20°C. Larvae were rehydrated, permeabilized in acetone for 30 min at -20°C, and washed in PBST. Nonspecific binding was blocked using PBST plus 1.0% DMSO, 1% BSA, and 5% normal goat serum. Primary and secondary antibodies were diluted in PBST plus 1.0% DMSO, 1% BSA, and 2% normal goat serum. Antibodies and dilutions used were insulin (A0564; DakoCytomation), 1:1000, green fluorescent protein (GFP; ab6556; Abcam), 1:500, and appropriate Alexa Fluor secondary antibodies (Invitrogen), 1:2500. For proliferation analysis using the Click-iT EdU Alexa Fluor 488 Imaging Kit (C10337; Invitrogen), 2 nL of 100 μmol/L 5-ethynyl-2-deoxyuridine (EdU) was injected into the pericardial region of larvae. After recovery, larvae were treated for 8 h with appropriate stimuli, fixed in 4% paraformaldehyde, and staining performed (according to Ref. 23). All images were collected using a Zeiss LSM510 or Zeiss LSM710 (Carl Zeiss).

**Oil Red O staining.** After fixation, samples were washed in PBS and stained with filtered 0.3% Oil Red O in 60% 2-propanol for 2 h. Larvae were washed in PBS before imaging.

**Transferase-mediated dUTP nick-end labeling assay.** The Click-iT TUNEL imaging assay kit (Invitrogen) was used with the following modifications. Larvae were permeabilized with 20 μg/mL proteinase K in PBST for 45 min followed by refixation in 4% paraformaldehyde with 0.2% Tween-20. Larvae were then treated with 1× PBS with 1.0% DMSO and 0.5% Triton X-100. Manufacturer instructions were followed for the terminal deoxynucleotidyl-transferase-labeling reaction and detection.

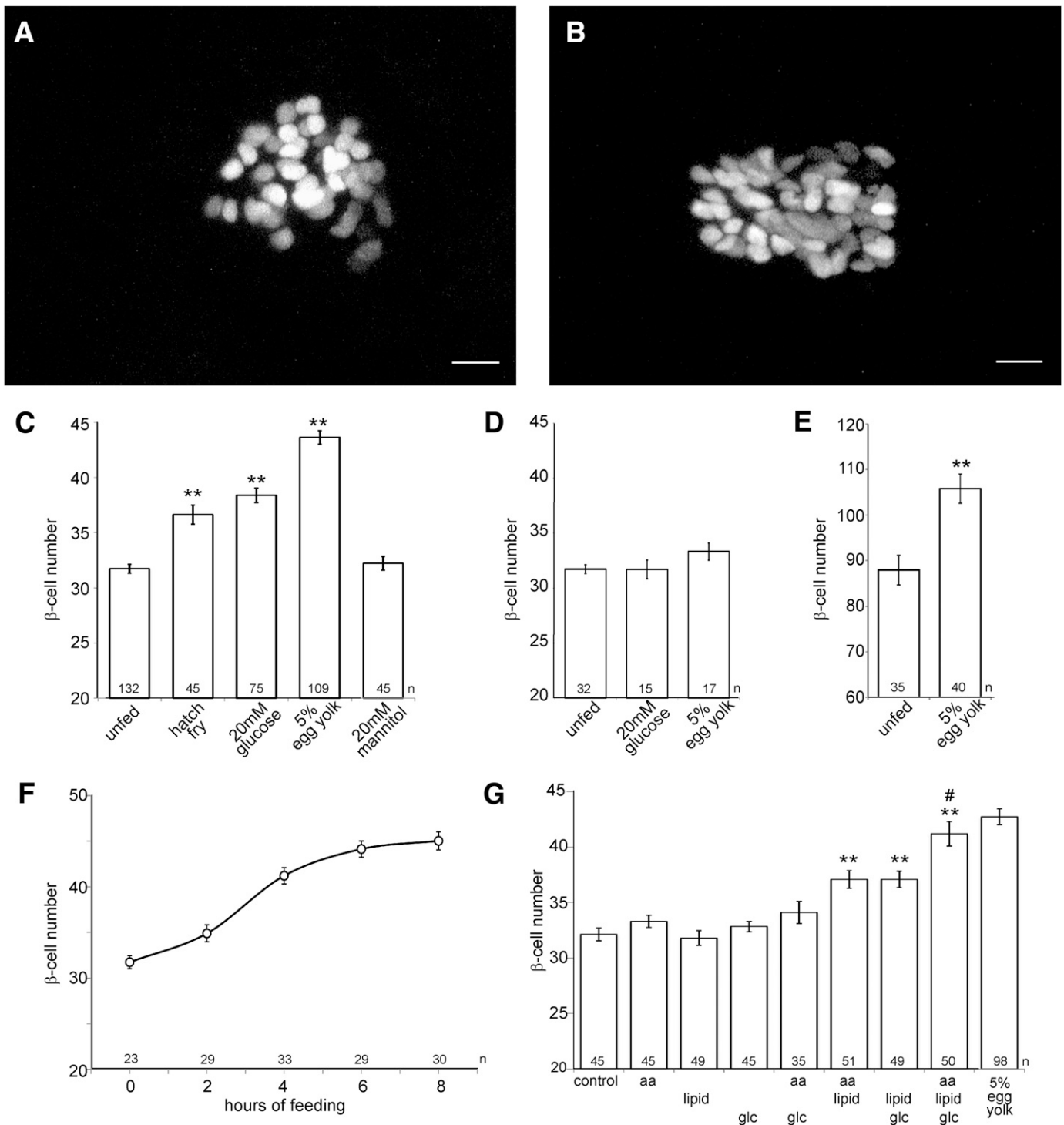
## RESULTS

**Overnutrition increases β-cells.** After 5 dpf, zebrafish have nearly exhausted the maternally provided nutrient store and begin feeding. At this time period, the second wave of endocrine differentiation from the ventral pancreas bud has begun (22,28,30). To facilitate quantification of β-cell number, we developed a transgenic line using a 1.2-kb fragment of the insulin promoter/5′-untranslated region to drive expression of a nuclear mCherry protein in the β-cells (*Tg:-1.2ins:H2B-mCherry*). We confirmed that the mCherry expression in the transgenic line labels all of the insulin-expressing cells (Fig. 3C and D). At 5 dpf, we found  $32.7 \pm 5.4$  (mean  $\pm$  SD) β-cells in the principal islet of unfed larvae (Fig. 1A), consistent with other reports (22). Larvae were fed ad libitum hatchfry encapsulon, a zebrafish larval diet, which consists of 50% protein and 12% lipid, or incubated in lipid-rich 5% chicken egg yolk solution, which consists of 26.5% lipid and 15.8% protein. When fed for 8 h, the number of β-cells increased to  $36.7 \pm 6.3$  with the larval diet (Fig. 1C) and  $43.1 \pm 7.1$  (Fig. 1B and C) with egg yolk. This was specific to the β-cells because the number of α-cells was similar in unfed larvae ( $17.9 \pm 4.6$

cells;  $n = 54$ ) and egg yolk-fed larvae ( $18.7 \pm 3.9$  cells;  $n = 56$ ) as determined using the *Tg(gcga:EGFP)* transgenic line (Fig. 4A and B). We also observed that the size of the β-cells is significantly larger in the egg yolk-fed larvae using the (*Tg:-1.2ins:tagRFP*) transgenic line, in which tagRFP expression is throughout the cell (data not shown). However, there is not a difference in the overall growth of unfed larvae or those fed for 8 h with the larval diet or egg yolk solution. Fish at 21 dpf had a similar β-cell response to 8-h incubation in egg yolk (Fig. 1E). A detailed time-course analysis of the response to egg yolk (Fig. 1F) revealed that the increase in β-cell number was evident after 4 h of feeding (ANOVA,  $P < 0.001$ , Tukey honest significant difference [HSD]), and the full effect was seen after 6 h of feeding. After incubation, larvae were found to have substantial egg yolk in the digestive tract (Fig. 3B and F), increased lipid in the liver (Fig. 3G), and chylomicrons in blood vessels (Fig. 3H). To determine whether the addition of β-cells is induced simply by the introduction of nutrients, larvae were treated with three rounds of 1-h incubation in the egg yolk solution followed by 2 h in yolk-free media. At the end of 9 h, the number of β-cells was not different from unfed larvae (Fig. 1D), suggesting that persistent feeding during the 8-h exposure period is required for the response. These data support that β-cell expansion in zebrafish larvae is induced by overfeeding.

We next investigated whether the β-cell expansion observed in larvae fed with egg yolk was due to a particular macronutrient type or growth factors and other components that may be present in the egg yolk. There are three major macronutrient classes in an egg yolk: amino acids, lipids, and glucose at a low concentration (1.5 mmol/L). To mimic these nutrient classes, we used defined solutions of purified amino acids, intralipid, and glucose at equivalent concentrations to that found in egg yolk based on U.S. Department of Agriculture data. Larvae were incubated for 8 h either with each individual nutrient class or in combinations (Fig. 1G). There was no change in the number of β-cells with the individual types of nutrients. The combination of amino acids and glucose together also had no significant effect. Intralipid in combination with amino acids or glucose significantly increased the number of β-cells over that of controls (ANOVA,  $P < 0.001$ , Tukey HSD), albeit not to the extent induced by egg yolk. However, all three nutrient classes together increased the number of β-cells to a similar degree as the egg yolk. The data strongly suggest that the β-cell addition induced by chicken egg yolk is due to a combination of the three macronutrient classes. The data further demonstrate that β-cell expansion is due to nutrient load rather than stimulation by growth factors in the chicken egg yolk.

The central role of pancreatic β-cells is to regulate blood glucose levels, and both adult and larval zebrafish have been shown to be glucose-responsive (26,31). To determine if glucose stimulates an increase in β-cells, larvae were incubated in a solution of 20 mmol/L glucose. After 8 h of exposure, the number of β-cells increased to  $38.4 \pm 5.7$  (ANOVA,  $P < 0.001$ , Tukey HSD) (Fig. 1C). Although a significant increase over the unfed control, the degree of increase is not as large as in the lipid-rich egg yolk. The glucose-treated larvae did not exhibit abnormalities in morphology or behavior, nor did they have an increase in apoptosis (data not shown). Overt glucotoxicity has also not been observed in rats when the blood glucose was kept  $>20$  mmol/L for 4 days (32). Incubation



**FIG. 1. Nutrient excess increases the  $\beta$ -cell number.** *A* and *B*: Images of  $\beta$ -cells expressing a nuclear mCherry protein in 5-dpf *Tg(-1.2ins:H2B-mCherry)* larvae. Approximately 30  $\beta$ -cells are observed in unfed larvae (*A*), and this number rapidly increases after 8 h of culture in chicken egg yolk (*B*). Scale bars, 10  $\mu$ m. *C*: Effects of overnutrition on  $\beta$ -cell numbers in 6-dpf larvae. Mannitol changes the osmolarity similar to glucose. Bars indicate mean with SE (\*\*ANOVA versus unfed,  $P < 0.001$ , Tukey HSD). *D*: Effects of incubating larvae in 20 mmol/L glucose or 5% chicken egg yolk in an intermittent or meal-type schedule. *E*: Quantification of  $\beta$ -cells in the principal islet of 21-day larvae either in control conditions or in 5% chicken egg yolk for 8 h. A significant increase ( $t$  test,  $P < 0.001$ ) is observed between the two groups. *F*: Time course of  $\beta$ -cell number increase within the first 8 h of culturing in 5% chicken egg yolk. The number of  $\beta$ -cells was determined at 2-h intervals. *G*: Deconvolution of egg yolk effects on  $\beta$ -cell number.  $n$  indicates the number of individual larvae in each sample group. aa, amino acids; control, no treatment; glc, 1.5 mmol/L glucose; lipid, intralipid (\*\*ANOVA versus control,  $P < 0.001$ , Tukey HSD; #not significant from 5% egg yolk).

of larvae in 20 mmol/L mannitol for 8 h did not change the number of  $\beta$ -cells (Fig. 1*C*), suggesting the induction of  $\beta$ -cells was not a result of changes in osmolarity. Similar to the results with the egg yolk, intermittent incubation

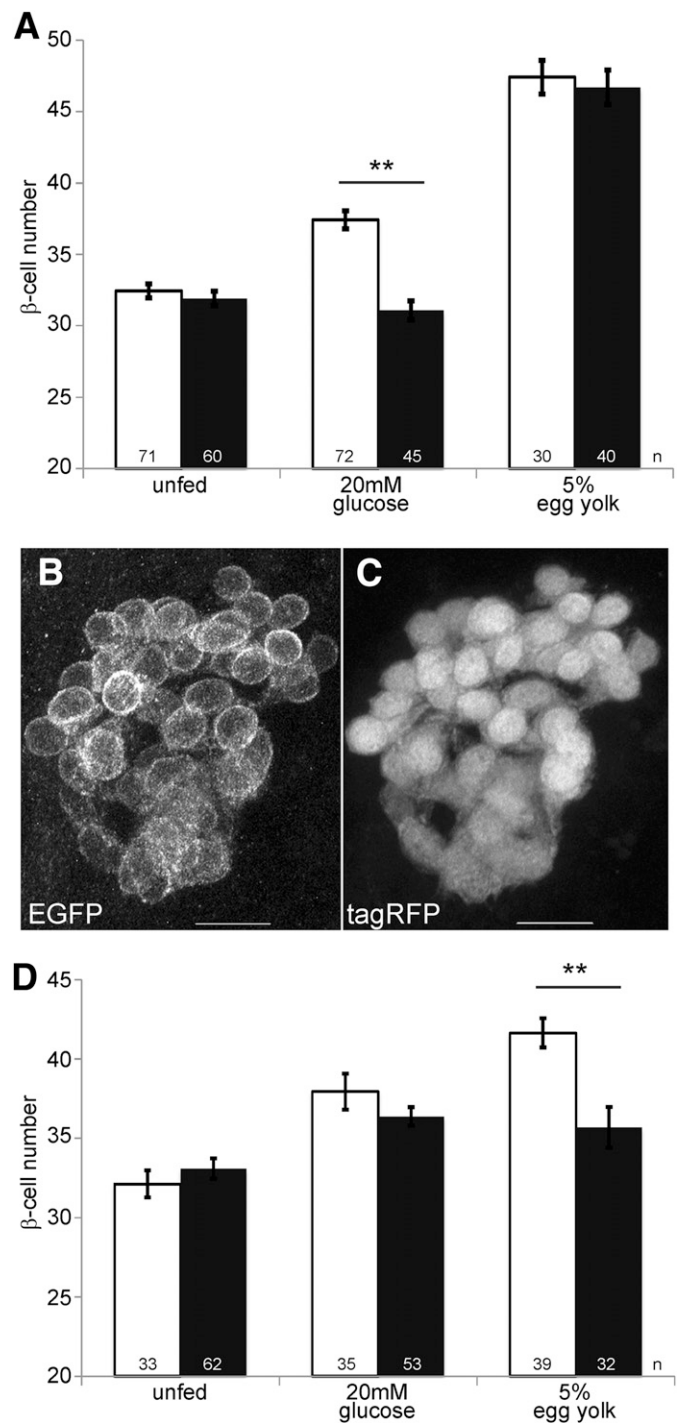
in 20 mmol/L glucose was insufficient to increase the  $\beta$ -cell number (Fig. 1*D*). Taken together, continuous presence of nutrients is necessary to induce additional  $\beta$ -cells.

**mTOR activity is required only for glucose-induced  $\beta$ -cell addition.** To discern the role of mTOR, a major nutrient sensor, in the overnutrition-induced  $\beta$ -cell expansion, we used rapamycin to inhibit TORC1. Zebrafish larvae are very amenable to this type of pharmacological approach. Using rapamycin in conjunction with egg yolk or 20 mmol/L glucose for 8 h, we found that mTOR activity was only necessary for glucose stimulated  $\beta$ -cell expansion (Fig. 2A). In larvae cultured in 20 mmol/L glucose, rapamycin treatment abolished the  $\beta$ -cell expansion (*t* test,  $P < 0.001$ ). In contrast, rapamycin had no effect on the  $\beta$ -cell expansion induced by egg yolk (Fig. 2A). We did not observe a difference in apoptosis between the treatment groups (data not shown). These data suggest that different signaling pathways are activated to induce addition of  $\beta$ -cells depending on the nutrient context.

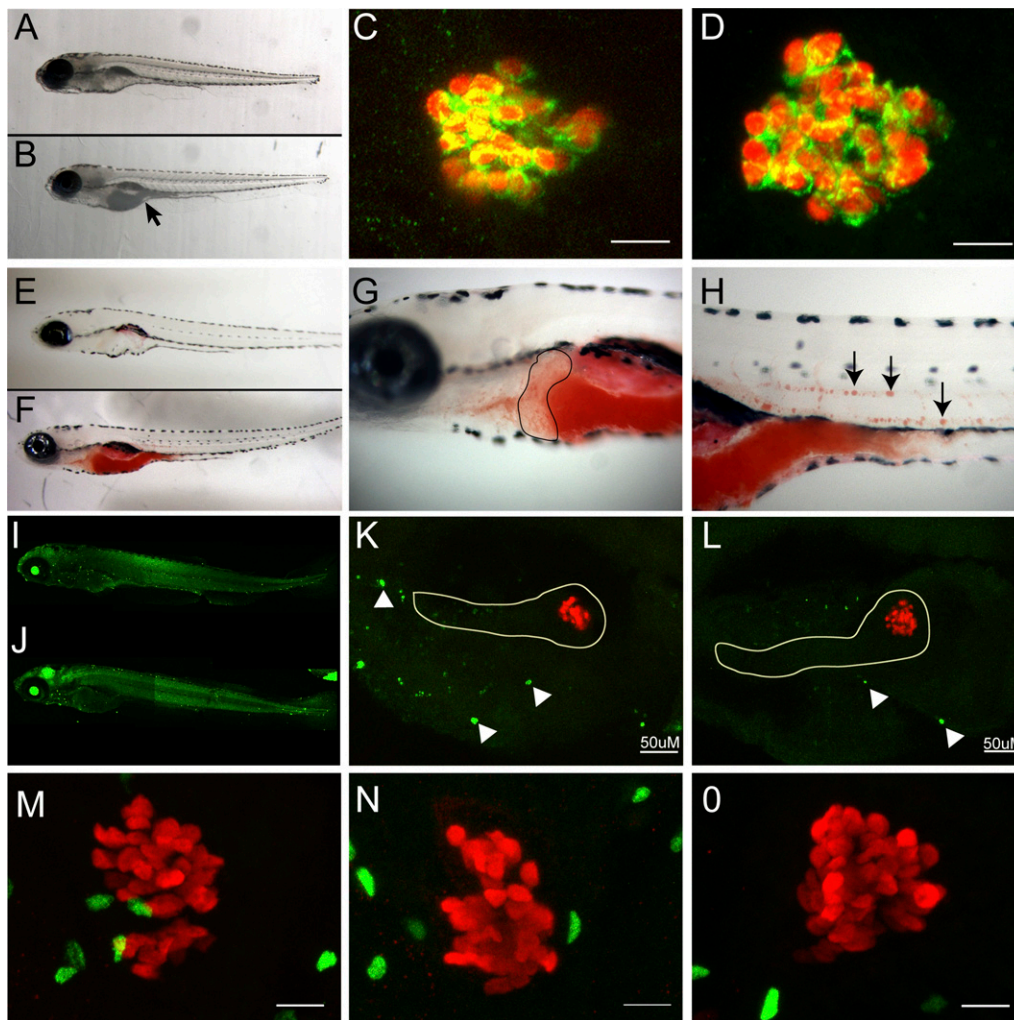
**Insulin/IGF-1 signaling in differentiated  $\beta$ -cells is necessary to induce compensatory  $\beta$ -cell expansion.** Autocrine/paracrine insulin/IGF-1 signaling has been implicated in postnatal  $\beta$ -cell expansion and high-fat diet-induced compensatory hyperplasia in mice (19). To determine if insulin/IGF-1 signaling in  $\beta$ -cells has a role in regulating compensatory  $\beta$ -cell expansion, we made use of a dominant-negative IGF-1R (dnIGF-1R) (33,34). In mice, dnIGF-1R forms hybrid receptors between insulin receptor and IGF-1R and interferes with signaling (35). We developed a transgenic zebrafish that express the dnIGF-1R in  $\beta$ -cells under the control of the insulin promoter. We confirmed that dnIGF-1R was expressed exclusively in the pancreatic  $\beta$ -cells (Fig. 2B and C). In unfed larvae, we did not observe any abnormalities in the number and morphology of the  $\beta$ -cells. However, when larvae are overfed egg yolk for 8 h,  $\beta$ -cell number was significantly reduced compared with controls (Fig. 2D) (*t* test,  $P < 0.001$ ), but the response to 20 mmol/L glucose was not statistically different than control larvae. This suggests that, similar to the mouse, autocrine/paracrine insulin/IGF-1 signaling is necessary in regulating the compensatory response to high-fat diet.

**Origin of the new  $\beta$ -cells.** We sought to determine the origin of the new  $\beta$ -cells induced by overnutrition. New  $\beta$ -cells could form through three distinct mechanisms:  $\beta$ -cell self-replication, transdifferentiation from other pancreatic cells, or neogenesis from precursor/progenitor cells. To determine whether the new  $\beta$ -cells result from  $\beta$ -cell replication, EdU was used to label replicating cells during the  $\beta$ -cell expansion period. In unfed larvae ( $n = 34$ ), we observed many proliferating cells that are readily labeled with EdU. However, we rarely detected insulin-positive, EdU-positive  $\beta$ -cells (Fig. 3M). Strikingly, in larvae with overnutrition ( $n = 40$ ), we observed no additional EdU-positive  $\beta$ -cells (Fig. 3N and O). EdU-positive cells are occasionally observed within the islet, but these cells do not colocalize with insulin. Similar results were obtained with 20 mmol/L glucose (Fig. 3N) or egg yolk (Fig. 3O) as the stimulus. The lack of EdU-positive  $\beta$ -cells suggests the new  $\beta$ -cells are not from self-replication but arise potentially through transdifferentiation from non- $\beta$ -cells or neogenesis from post-mitotic precursor cells.

We also did not observe an increase of apoptosis in the pancreas of larvae with overnutrition (Fig. 3L). Transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells were rare in the pancreas of 6 dpf unfed larvae (Fig. 3K) and overfed larvae, although TUNEL-positive cells in the intestine could be identified in both. This suggests that the  $\beta$ -cell increase was not triggered



**FIG. 2.** Nutrient-specific action of mTOR and insulin/IGF-1 signaling. **A:** Effect of mTOR inhibition. Larvae (6 dpf) were incubated in 0.3× Danieau solution plus 0.1% DMSO or 2.5  $\mu$ mol/L rapamycin in 0.1% DMSO either alone or with 20 mmol/L glucose or 5% egg yolk. The number of  $\beta$ -cells determined after 8 h of treatment is shown. Bars indicate mean  $\beta$ -cell number with SE.  $^{***}P < 0.001$  (*t* test). **B** and **C:** Expression of dnIGF-1R-EGFP in  $\beta$ -cells of *Tg(-1.2ins:dnIGF1R-EGFP)*; *Tg(-1.2ins:tagRFP)* larvae. EGFP (**B**) is localized to the membrane of all the tagRFP-positive  $\beta$ -cells (**C**). **D:** Response of *Tg(-1.2ins:dnIGF1R-EGFP)* larvae to overnutrition. The  $\beta$ -cell number was determined in *Tg(-1.2ins:H2BmCherry)* or *Tg(-1.2ins:dnIGF1R-EGFP)* larvae either unfed or after 8 h of treatment with 20 mmol/L glucose or 5% egg yolk. Graphed are means and SE. *n* indicates the number of individual larvae in each sample group.  $^{***}P < 0.01$  (*t* test).



**FIG. 3.** Overnutrition-induced new  $\beta$ -cells are not newly divided. Larvae cultured in egg yolk (*B*) have distended intestine (arrow) full of yolk particles, which is absent in control (*A*) larvae. All  $\beta$ -cells in the *Tg(-1.2ins:H2BmCherry)* larvae are fluorescently labeled. Insulin expression (green) overlaps completely with mCherry (red) in both unfed (*C*) and egg yolk-overfed (*D*) larvae. *E-H*: Oil Red O-stained larvae. Control larvae have little stored lipid at 6 dpf (*E*). Egg yolk-fed larvae have substantial lipid-containing egg yolk particles in the intestine (*F*). Egg yolk-fed larvae have increased lipid accumulated in the liver, outlined in black (*G*), and overt lipid droplets in the blood vessels (arrows) (*H*). *I-L*: TUNEL labeling of larvae. Apoptotic cells are present in both control (*I*) and larvae fed for 8 h with chicken egg yolk (*J*). These images were acquired with serial confocal projections and stitched together to show the entire larvae. In the abdomen of 6-dpf unfed larvae (*K*) or larvae fed for 8 h with chicken egg yolk (*L*), few apoptotic cells are present (arrowheads). The region of the pancreas is outlined in white for reference. Cell proliferation in the islet region within 8 h in control (*M*), 20 mmol/L glucose-treated (*N*), and egg yolk-overfed (*O*) larvae showing  $\beta$ -cells (red) rarely incorporated EdU (green). All images are confocal projections, and scale bars indicate 10  $\mu\text{m}$  except *I* and *J*, in which the scale bars indicate 50  $\mu\text{m}$ . (A high-quality digital representation of this figure is available in the online issue.)

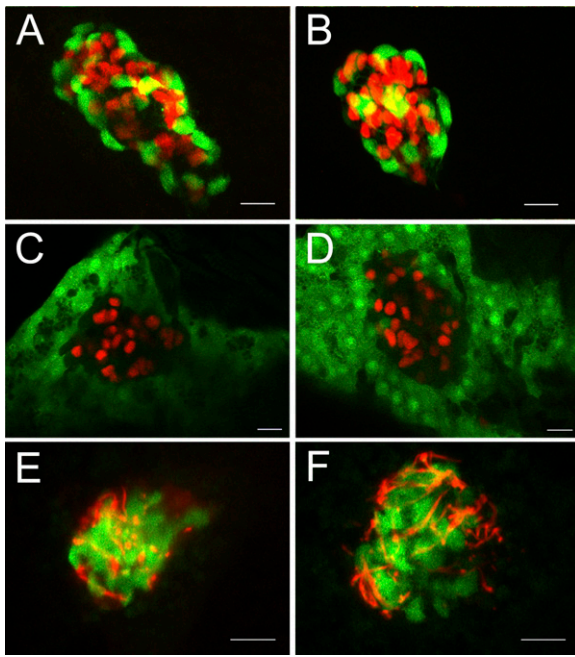
by apoptosis in the pancreas or islet. Apoptotic cells throughout the larvae were more prevalent in unfed larvae (Fig. 3*I*) than larvae with overnutrition (Fig. 3*J*).

To determine the cell of origin of the new  $\beta$ -cells, we used an array of transgenic lines that highlight cell types implicated as a potential source of  $\beta$ -cells. Given the addition of  $\beta$ -cells is a rapid process, and fluorescent proteins are fairly stable in this time frame, we reasoned that new  $\beta$ -cells should retain the fluorescent marker present in the previous state. We first examined transdifferentiation from  $\alpha$ -cells using *Tg(gcga:EGFP)* transgenic larvae, which labeled all  $\alpha$ -cells (data not shown). Although we did observe endocrine cells that coexpress the markers for glucagon and insulin, there is no change in the number of these cells between unfed ( $2.3 \pm 1.4$ ;  $n = 18$ ) (Fig. 4*A*) and larvae that were incubated for 8 h in egg yolk ( $1.6 \pm 1.3$ ;  $n = 22$ ) (Fig. 4*B*). We did not observe transdifferentiation of acinar cells into  $\beta$ -cells using *Tg(ptf1a:EGFP)* (Fig. 4*C* and *D*), as

no cells were found to coexpress the two markers. We also excluded transdifferentiation from  $\delta$ -cells using *Tg(sst2:memRFP)* (Fig. 4*E* and *F*), as no cells that coexpress the two markers were identified in either unfed larvae or those overfed with egg yolk. The number of  $\delta$ -cells was difficult to quantify due to morphology and the membrane localization of RFP. These data suggest that new  $\beta$ -cells are not derived from transdifferentiation of differentiated pancreatic endocrine or exocrine cells.

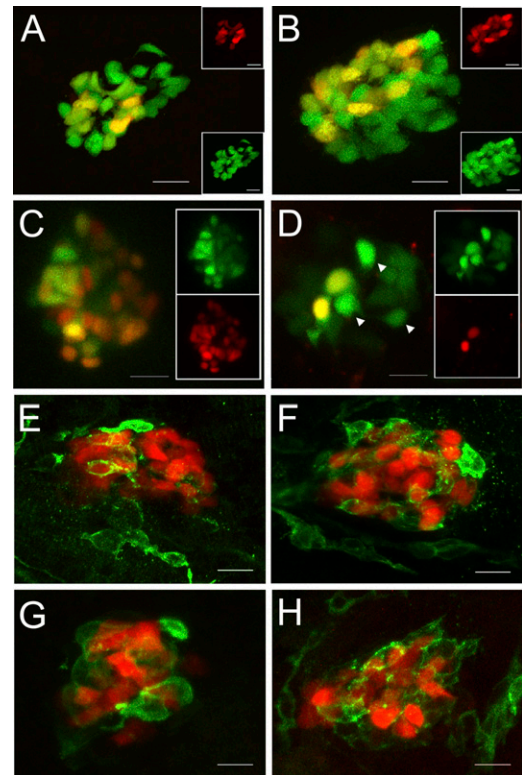
The newly differentiated  $\beta$ -cells arose via neogenesis from postmitotic endocrine precursor cells. Using *Tg(-5mnx1:tagRFP)*, we found that overnutrition increased the number of TagRFP-positive  $\beta$ -cells from  $17.1 \pm 2.4$  cells in the unfed larvae ( $n = 16$ ) (Fig. 5*A*) to  $23.3 \pm 5.3$  cells in larvae overfed egg yolk for 8 h (Fig. 5*B*) ( $n = 30$ ) (*t* test,  $P < 0.001$ ). Although highly expressed during early development (data not shown) (36,37), TagRFP was expressed only in a subset of the  $\beta$ -cells in the larval islet





**FIG. 4.** New  $\beta$ -cells do not transdifferentiate from  $\alpha$ ,  $\delta$ , or acinar cells. Confocal projections of insulin- and glucagon-expressing cells in double-transgenic *Tg(gcga:EGFP);Tg(-1.2ins:tagRFP)* larvae that were unfed (A) or overfed 5% egg yolk (B). C and D: Expression of *ptf1a* and *insulin* in *Tg(ptf1a:EGFP);Tg(-1.2ins:tagRFP)* larvae. No coexpressing cells are observed in unfed (C) or egg yolk-overfed (D) larvae. Each image is a single confocal slice. Confocal projections showing expression of somatostatin and insulin in *Tg(sst2:memRFP);Tg(-1.2ins:EGFP)* larvae that were unfed (E) or overfed with egg yolk (F). For each image: scale bars, 10  $\mu$ m. (A high-quality digital representation of this figure is available in the online issue.)

(Fig. 5A and B). The increase in TagRFP-positive cells likely highlights newly differentiated, immature  $\beta$ -cells. This was suggested by the observations that *mnx1* (*hb9*) promoter-directed marker expression preceded *insulin* promoter-directed marker expression during the time course of egg yolk feeding and that *mnx1* promoter-directed marker expression was absent in the secondary islets of adult fish (Supplementary Fig. 1). Furthermore, after nitroreductase-mediated  $\beta$ -cell ablation in *Tg(-3.0hb9:GFP);Tg(-1.2ins:mR-nC-NTR)* larvae, GFP-positive mCherry-negative cells appeared in the regenerating islets (Fig. 5D), whereas all GFP-expressing cells in untreated larvae are mCherry positive (Fig. 5C). These data strongly suggest that the increase of cells with *mnx1* promoter activity was due to newly differentiated  $\beta$ -cells rather than reactivation of the promoter in mature  $\beta$ -cells. However, the increase in TagRFP-positive cells, an average of six cells, did not fully account for the 10 new  $\beta$ -cells induced by overnutrition. We also observed an increase in enhanced GFP (EGFP) expression both in and surrounding the islet in overfed *Tg(nkx2.2:mEGFP)<sup>VUI7</sup>; Tg(ins:TagRFP)* larvae (Fig. 5F) compared with unfed larvae (Fig. 5E). There were significantly more cells that coexpress both EGFP (*nkx2.2*) and tagRFP (*insulin*) in egg yolk-overfed ( $8.06 \pm 2.93$  cells;  $n = 16$ ) larvae compared with unfed larvae ( $4.14 \pm 1.51$  cells;  $n = 14$ ) ( $t$  test;  $P < 0.001$ ). However, like the increase of TagRFP cells in *Tg(-5.1mnx1:TagRFP)*, the four new EGFP-positive cells in *Tg(nkx2.2:mEGFP)<sup>VUI7</sup>* do not account for all 10 of the new  $\beta$ -cells induced by overnutrition. This may indicate two separate pathways of  $\beta$ -cell differentiation. In mice, two parallel



**FIG. 5.** Differentiation of precursors in overnutrition-induced  $\beta$ -cell expansion. *Tg(-5.1mnx1:tagRFP);Tg(-1.2ins:EGFP)* larvae left unfed (A) or overfed with 5% egg yolk (B) showing an increased number of tagRFP-positive  $\beta$ -cells in the overfed larvae compared with unfed larvae. The insets show individual channels for each image. Images of the islets from *Tg(-3.0hb9:GFP);Tg(-1.2ins:mR-nC-NTR)* larvae prior to (C) and 24 h after (D) nitroreductase-mediated  $\beta$ -cell destruction. In untreated larvae, all GFP-positive cells are mCherry-positive  $\beta$ -cells. During regeneration, some GFP-positive cells in the islet are not  $\beta$ -cells (red) (arrowheads). *Tg(nkx2.2:mEGFP)<sup>VUI7</sup>;Tg(-1.2ins:tagRFP)* larvae left unfed (E) or overfed with egg yolk (F) showing increased EGFP expression in the islet of overfed larvae compared with unfed larvae. EGFP is detected by immunofluorescence. The islet region of *Tg(nkx2.2:mEGFP);Tg(-5.1mnx1:tagRFP)* larvae either unfed (G) or egg yolk-overfed (H), highlighting that EGFP- and tagRFP-expressing cells do not overlap. EGFP is detected by immunofluorescence. All images are confocal projections. Scale bars, 10  $\mu$ m for each image. (A high-quality digital representation of this figure is available in the online issue.)

pathways of  $\beta$ -cell differentiation have been suggested: one Pax6-dependent and the other Pax4-Mnx1(Hb9)-dependent (38,39). We have found that the  $\beta$ -cells with TagRFP expression and those with expression of EGFP are distinct populations in *Tg(-5.1mnx1:tagRFP);Tg(nkx2.2:mEGFP)<sup>VUI7</sup>* larvae in both unfed (Fig. 5G) and overnutrition (Fig. 5H) conditions. Because the *nkx2.2* promoter is active in both ductal cells and endocrine precursor cells (40), these new EGFP-positive  $\beta$ -cells could be from transdifferentiation of differentiated pancreatic ductal epithelial cells, differentiation of endocrine precursor cells, or both. More focused lineage tracing will be required to distinguish these possibilities. Taken together, these data support that a large fraction of the new  $\beta$ -cells are likely from differentiation of postmitotic endocrine precursor cells.

## DISCUSSION

The maintenance of an appropriate number of  $\beta$ -cells and adequate production of insulin is essential for glucose homeostasis. Although zebrafish have been used to study

the development of the pancreas and  $\beta$ -cells (20,22,23) and regeneration of  $\beta$ -cells in adult animals (21), how  $\beta$ -cells respond to an increased metabolic demand has not been determined. We have shown in this study that a rapid increase in  $\beta$ -cells occurs upon overfeeding and suggests that the zebrafish  $\beta$ -cells are indeed able to sense and respond to an increase of metabolic demand by 5 dpf. We did not observe an increase with meal-style feeding, suggesting that the response requires a persistently elevated metabolic demand and is not simply a normal response to nutrients. This is consistent with the compensatory hyperplasia of  $\beta$ -cells that occurs in the face of nutrient excess and obesity. In humans and mice, this is an essential mechanism to prevent, or at least delay, the development of diabetes (9–11). Because childhood overnutrition and obesity predicts type 2 diabetes later in life, our model may potentially be used to understand the underlying mechanism (41). Given that zebrafish are an excellent platform for genetic and chemical screens (41,42), this system can be adapted to identify genes and pathways that are involved in sensing and responding to nutrient excess.

Type 2 diabetes results when  $\beta$ -cells can no longer supply sufficient insulin to maintain glucose homeostasis, which may be a consequence of  $\beta$ -cell apoptosis,  $\beta$ -cell failure, or defective  $\beta$ -cell hyperplasia (1,43). A chronic excess in nutrients leads to obesity and is a major contributor to  $\beta$ -cell failure (44). Although we have not observed  $\beta$ -cell failure after 4 days of overnutrition (data not shown), a longer duration of overnutrition may be required. If  $\beta$ -cell failure does result with a longer period of overnutrition, the zebrafish system may also be used to identify genes and pathways leading to  $\beta$ -cell failure in conditions of nutrient excess.

We have observed differences in signaling pathways that are involved in the  $\beta$ -cell differentiation induced by glucose and egg yolk. Although the glucose effect requires mTOR but not autocrine/paracrine insulin/IGF signaling, the opposite is true for the egg yolk effect. Although we cannot pinpoint if mTOR activity in  $\beta$ -cells is necessary, the data from genetic suppression of insulin/IGF1 signaling indicate that differentiated  $\beta$ -cells are an important component in the compensatory response. The requirement of insulin signaling for  $\beta$ -cell expansion has also been observed in adult mice, in which  $\beta$ -cell replication seems to be the main mechanism (18,19), although in our model,  $\beta$ -cell replication does not occur. It is possible that in all systems, a common factor or factors is produced by  $\beta$ -cells under conditions of overnutrition. Whether this factor(s) results in  $\beta$ -cell proliferation or differentiation of precursor cells may depend on the replication competence of  $\beta$ -cells or the availability of precursor cells.

Because we do not observe  $\beta$ -cell proliferation in this model of nutrient excess, the population of cells that are recruited to become  $\beta$ -cells is of great interest. It is unlikely that the increase of  $\beta$ -cells is simply a phenomenon of increased insulin promoter activity in pre-existing  $\beta$ -cells because all of the insulin-positive cells are marked by our transgenic approach and the  $\beta$ -cell numbers observed in unfed larvae are consistent with other reports (22). It is conceivable that a postmitotic precursor cell pool resides within the islet and is poised to differentiate with appropriate cues. Identification of the precursor population may be challenging, however, because many of the genes used to highlight potential precursor populations in zebrafish, such as *pax6* and *neurod*, are also expressed in differentiated endocrine cell types. In rodent models, NGN3 expression is used as a marker of a precursor cells (45). However, *ngn3*

expression in the zebrafish pancreas has not been observed (46). The *mnx1* promoter-directed reporter gene expression in the *Tg(-5.0mnx1:tagRFP)* and *Tg(-3.0hb9:GFP)*-transgenic lines likely mark a subpopulation of differentiating precursor cells and immature  $\beta$ -cells. Interestingly, the activity of the *mnx1* promoter in the transgenic zebrafish contrasts to that of the endogenous *Mnx1* in mice in which it has been shown to be active in both developing and adult  $\beta$ -cells (47). Whether our observation is a consequence of the lack of additional regulatory elements in the promoter used or a fundamental difference between zebrafish and mammals remains to be resolved. Overall, our findings support overnutrition can induce new  $\beta$ -cells in zebrafish through induction of precursor differentiation into  $\beta$ -cells. Through a combination of genetics, pharmacology, and transgenesis, the zebrafish larvae is well poised to identify the mechanisms, signals, and cells involved in this response, which can lend insights into  $\beta$ -cell responses involved in conditions of nutrient excess, insulin resistance, and the development of diabetes.

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L.A.M. and W.C. designed the experiments. L.A.M. researched data. L.A.M. and W.C. wrote the manuscript. W.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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