1   **Interruption of glucagon signaling augments islet non-alpha cell proliferation in** SLC7A2-

2   **and mTOR-dependent manners**

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ABSTRACT

Objective: Dysregulated glucagon secretion and inadequate functional beta cell mass are hallmark features of diabetes. While glucagon receptor (GCGR) antagonism ameliorates hyperglycemia and elicits beta cell regeneration in pre-clinical models of diabetes, it also promotes alpha and delta cell hyperplasia. We sought to investigate the mechanism by which loss of glucagon action impacts pancreatic islet non-alpha cells, and the relevance of these observations in a human islet context.

Methods: We used zebrafish, rodents, and transplanted human islets comprising six different models of interrupted glucagon signaling to examine their impact on delta and beta cell proliferation and mass. We also used models with global deficiency of the cationic amino acid transporter, SLC7A2, and mTORC1 inhibition via rapamycin, to determine whether amino acid-dependent nutrient sensing was required for islet non-alpha cell growth.

Results: Inhibition of glucagon signaling stimulated delta cell proliferation in mouse and transplanted human islets, and in mouse islets. This was rapamycin-sensitive and required SLC7A2. Likewise, *gcgr* deficiency augmented beta cell proliferation via SLC7A2- and mTORC1-dependent mechanisms in zebrafish and promoted cell cycle engagement in rodent beta cells but was insufficient to drive a significant increase in beta cell mass in mice.

Conclusion: Our findings demonstrate that interruption of glucagon signaling augments islet non-alpha cell proliferation in zebrafish, rodents, and transplanted human islets in a manner requiring SLC7A2 and mTORC1 activation. An increase in delta cell mass may be leveraged for future beta cell regeneration therapies relying upon delta cell reprogramming.

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1. INTRODUCTION

Diabetes is a multifactorial disease, but insufficient insulin secretion due to inadequate functional beta cell mass and dysregulated, often increased, glucagon secretion are fundamental to essentially all forms of diabetes and contribute to hyperglycemia.[1-6] The utility of glucagon receptor (GCGR) antagonism as a means of treating diabetic hyperglycemia has been intensively examined because it markedly improves glycemic control in diabetic rodents, non-human primates, and humans.[7-10] More recently, studies have shown that inducible elimination of glucagon action by administration of a GCGR monoclonal antibody (GCGR-Ab) enhances beta cell survival, regeneration, and function in pre-clinical models of type 1 (T1D) and type 2 (T2D) diabetes.[11-16]

However, inhibition of glucagon action elicits adverse effects as well, including increased serum and liver lipid levels, increased blood pressure, and robust pancreatic islet alpha cell hyperplasia.[10] We and others[17-21] have shown that the response of islet alpha cells to GCGR antagonism is caused by disruption of the liver-alpha cell axis, which results in hyperaminoacidemia, hyperglucagonemia, and rapamycin-sensitive alpha cell proliferation through a mechanism also involving induction of a glutamine transporter, *Slc38a5*, in alpha cells.[19,20] Recently, we extended these observations by showing that the alpha cell-enriched cationic amino acid transporter, *Slc7a2*, is required for high amino acid-stimulated mTOR activation, *Slc38a5* induction, alpha cell proliferation, and islet hormone secretion[22], underscoring a prominent role for amino acids in the regulation of alpha cell function and phenotypes.[23]

Interestingly, pancreatic islet non-alpha cells, namely delta and beta cells, have also been shown to be impacted by GCGR antagonism.[17,24-26] For example, constitutive ablation of the *Gcgr* gene was associated with 2- and 3-fold increases in pancreatic delta cell number and

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79   somatostatin content, respectively, and increased postnatal beta cell proliferation and

80   mass.[17,24,25] Furthermore, some[11,26] but not all[18-20,27] studies have shown that GCGR-Ab

81   treatment increases pancreatic delta and beta cell numbers in mice and cynomolgus monkeys,

82   respectively. However, the mechanism by which constitutive or inducible elimination of glucagon

83   action elicits changes in islet non-alpha cells under insulin-sufficient conditions, and the

84   relevance of these observations in a human islet context, are unknown.

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86   Here, we used several complementary approaches to show that interruption of glucagon

87   signaling augments islet non-alpha cell proliferation in SLC7A2- and mTORC1-dependent

88   manners. We found that constitutive and inducible elimination of glucagon action stimulated

89   delta cell proliferation and mass expansion in mouse and transplanted human islets, and that in

90   mouse islets, this required mTORC1 activation and the amino acid transporter, SLC7A2.

91   Likewise, we found that *gcgr* deficiency increased beta cell number in a SLC7A2- and

92   rapamycin-sensitive manner in zebrafish and promoted cell cycle engagement in rodent beta

93   cells but was insufficient to drive a significant increase in beta cell mass in mice. Our findings

94   highlight key differences in the regulation of beta versus delta and alpha cell proliferation and

95   reveal a new mechanism linking inhibition of glucagon signaling to expansion of islet delta cell

96   mass that may be leveraged for future beta cell regeneration therapies via delta cell

97   reprogramming.[28,29]

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101 ## 2. MATERIALS AND METHODS

102 ### 2.1. Mouse studies

103 All studies were performed at Vanderbilt University Medical Center and conducted in

104 accordance with protocols and guidelines approved by the Vanderbilt University Institutional

105 Animal Care and Use Committee. Mice were provided ad libitum access to standard rodent

106 chow and water and housed under a 12-hour light/12-hour dark cycle. The following mice were

107 obtained from The Jackson Laboratory: $Gcg^{-/-}$ (NOD.Cg-Gcg^{em1Dvs} $Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/DvsJ,

108 strain #: 029819)[30], C57BL/6J (strain #000664), NSG (NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ, strain #:

109 005557)[31], and $Slc7a2^{-/-}$ (B6.129S7-$Slc7a2^{tm1Clm}$/LellJ, strain #: 022767).[32] $Gcgr^{-/-}$ and $Gcgr^{Hep-/-}$

110 mice were generated as described previously.[17] Wildtype (+/+) and knockout (-/-) mice obtained

111 from heterozygous crosses were used for all experiments. For inducible elimination of glucagon

112 action, mice were treated once per week for up to 8 weeks with control (IgG or PBS) or 10mg/kg

113 of a humanized monoclonal antibody (10mg/kg) targeting the glucagon receptor (GCGR-Ab;

114 "Ab-4" and REMD 2.59)[33] via intraperitoneal (i.p.) injection.

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116 For transplantation experiments, mouse islets were isolated by intraductal infusion of

117 collagenase P, separated via histopaque gradient, and cultured overnight in Roswell Park

118 Memorial Institute complete medium (RPMI; 5.6 mmol/L glucose with 10% FBS) before

119 transplantation. Islets were isolated from 13-15-week-old $Slc7a2^{+/+}$ and $Slc7a2^{-/-}$ mice and

120 transplanted beneath the renal capsule of contralateral kidneys in 16-18-week-old syngeneic

121 $Slc7a2^{+/+}$ recipients. Following 2 weeks of engraftment, $Slc7a2^{+/+}$ recipient mice were injected

122 i.p. with control IgG or GCGR-Ab (10 mg/kg) once per week for 2 weeks, after which the kidneys

123 were harvested for tissue embedding and graft analysis as described.[22] In the $Gcgr$ mouse line,

124 islets isolated from 14-week-old $Gcgr^{+/+}$ mice were transplanted beneath the renal capsule of

125 14-week-old syngeneic $Gcgr^{Flox}$ or $Gcgr^{Hep-/-}$ recipients as described.[17] Human islet transplants

126 were performed exactly as described.[19] Human islets were obtained from the Integrated Islet

127   Distribution Program (https://iidp.coh.org/) or the Human Pancreas Analysis Program

128   (https://hpap.pmacs.upenn.edu).[34,35] Individual donor characteristics may be found in

129   Supplemental Table 1 and Dean et al.[19]

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2.2. Zebrafish studies

132   We used previously described *gcgra/b* double-knock[19] out (abbreviated *gcgr$^{-/-}$* here) and *slc7a2$^{-/-}$*

133   zebrafish lines.[22] Proliferating beta cells were identified by incubating zebrafish embryos with 1

134   mmol/l 5-ethynyl-2-deoxyuridine (EdU) at four days post-fertilization (dpf) and chasing them for

135   24 hours. EdU was detected as described previously.[36] Beta cell number was measured by

136   counting *Tg(ins:H2B-mcherry)* labeled beta cells in the islet of five dpf zebrafish as described.[36]

137   In the rapamycin experiments, starting at 3 dpf zebrafish were treated with a concentration of

138   200 nM rapamycin. Treatment continued for 3 days and then beta cell number was determined

139   at 6 dpf in *gcgr -/-* and control fish.

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2.3. Immunofluorescence staining and image analysis

142   Tissue preparation and sectioning were performed as described previously.[19,22] Tissue sections

143   were stained for Ki67, a marker of cell proliferation (Abcam, ab15580), phosphorylated

144   ribosomal protein S6 (pS6$^{240/244}$), a marker of mTOR activation (Cell Signaling, #2215), and

145   insulin (Dako, A0564), somatostatin (Santa Cruz, sc-7819), or glucagon (LSBio, LS-C202759) to

146   mark beta, delta, and alpha cells, respectively. Alpha cells in *Gcg$^{-/-}$* sections were identified by

147   pro-glucagon staining (Cell Signaling, #8233). Whole pancreatic sections and islet grafts were

148   imaged using a Scanscope FL System (Aperio Technologies) and an Olympus FV3000 laser

149   scanning confocal microscope and analyzed using Halo image analysis software (Indica Labs).

150   Colocalization of Ki67 with DAPI in insulin+ and somatostatin+ cells was determined by manual

151   counting and a CytoNuclear FL v1.4 algorithm (Indica Labs). Percent beta and delta cell

152   proliferation was quantified from at least 1500 beta and up to 1200 delta cells per animal (and at

153   least 450 beta and 400 delta cells per donor) by dividing the number of Ki67+/insulin+ or

154   Ki67+/somatostatin+ cells by the total number of insulin+ or somatostatin+ cells, respectively.

155   Beta and delta cell mass was determined using an area classifier in Halo and calculated as

156   described.[37] Briefly, the fractional areas for insulin and somatostatin from 5-7 pancreatic

157   sections of differing tissue depths were multiplied by the pancreas weight to obtain an estimate

158   of total beta and delta cell mass.

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160   **2.4. Statistical Analyses**

161   All data are presented as mean $\pm$ SEM. Comparisons between 2 groups were analyzed using

162   unpaired two-tailed t tests. Comparisons between more than 2 groups were determined by one-

163   way or two-way ANOVA with Fisher's LSD or Tukey's post-hoc tests. $P < 0.05$ denotes

164   statistical significance. Analyses were performed using Prism 9 software.

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3. RESULTS

3.1. Loss of glucagon action augments delta cell proliferation and mass expansion in mouse and transplanted human islets. To determine how interruption of glucagon signaling impacts pancreatic islet non-alpha cells, we first measured delta cell proliferation and mass in two different mouse models: one with constitutive global glucagon deficiency (*Gcg*$^{-/-}$) and another with inducible elimination of glucagon action via treatment with a GCGR-Ab. We found that delta cell proliferation, quantified as the percentage of Ki67-positive delta cells, was increased by 4.7- and 6.2-fold in *Gcg*$^{-/-}$ and GCGR-Ab treated mice, respectively, compared with controls (**Fig 1A-C**). This resulted in a ~ 3-fold increase in delta cell mass compared to controls (**Fig 1D-F**). Consistent with earlier reports[24,25], we also observed a distinctive shift in the distribution of somatostatin-positive cells from the mantle to the core of the islet in both *Gcg*$^{-/-}$ and GCGR-Ab treated mice (**Fig 1A and 1D**). To evaluate the translational relevance of these findings, we measured delta cell proliferation in human islets transplanted into immunocompromised recipient mice (i.e., NSG) treated with IgG (control) or GCGR-Ab and found a 3.5-fold increase in the percentage of Ki67-positive delta cells after 4 weeks of treatment (**Fig 1G-I**). Our findings suggest that interruption of glucagon signaling promotes islet delta cell mass expansion by stimulating delta cell proliferation in mouse and transplanted human islets.

3.2. SLC7A2 and mTORC1 activation are required for delta cell proliferation in response to interrupted glucagon signaling. To gain insight into the mechanism(s) that may be driving delta cell proliferation in *Gcg*$^{-/-}$ and GCGR-Ab treated mice, we first measured delta cell proliferation in mice with constitutive global inactivation of the cationic amino acid transporter, SLC7A2. We and others[18-20] have shown that GCGR-Ab-induced hyperaminoacidemia, especially arginine and glutamine, triggers pancreatic islet alpha cell proliferation in an mTOR-dependent manner, and that this response requires SLC7A2.[22] Here, we found that SLC7A2 is

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194   also required, at least in part, for delta cell proliferation in response to GCGR-Ab treatment

195   since the percentage of Ki67-positive delta cells was abrogated, albeit incompletely, in *Slc7a2*

196   knockout (-/-) mice compared with wild-type (+/+) controls (**Fig 2A-B**). To delineate the

197   intracellular signaling pathway involved, we immunostained for phosphorylated (p) S6 protein, a

198   downstream target of mTOR kinase, and observed an 18-fold increase in the percentage of

199   pS6-positive delta cells in islets of $Gcg^{-/-}$ compared with $Gcg^{+/+}$ mice, indicative of mTOR

200   activation (**Fig 2C-D**). To determine whether mTOR signaling was required for delta cell

201   proliferation, we co-treated mice with GCGR-Ab and rapamycin (RAPA), an mTOR inhibitor, and

202   found that RAPA abolished GCGR-Ab-induced delta cell proliferation in C57BL6 mice (**Fig 2E-**

203   **F**). These data indicate that loss of glucagon action stimulates RAPA-sensitive delta cell

204   proliferation through a mechanism requiring, at least in part, the amino acid transporter

205   SLC7A2.

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207   **3.3. Loss of glucagon receptor function stimulates beta cell proliferation in a species-**

208   **specific manner.** Since the response of islet delta cells to interrupted glucagon signaling

209   resembled that of alpha cells shown previously[17-20,22,24,25,36,38] we wanted to determine if this

210   mechanism was likewise conserved in beta cells under insulin-sufficient conditions. First, we

211   examined a zebrafish model with constitutive global deficiency of both forms of the $gcgr$[36]

212   ($gcgra/b^{-/-}$, abbreviated $gcgr^{-/-}$) and found that beta cell proliferation and number were both

213   increased in $gcgr^{-/-}$ zebrafish compared to WT ($gcgr^{+/+}$) controls (**Fig 3A-B**). Furthermore, this

214   response was significantly blunted in $slc7a2^{-/-};gcgr^{-/-}$ double mutants, or in $gcgr^{-/-}$ zebrafish

215   treated with RAPA (**Fig 3C-D**). These data indicate that the mechanism for GCGR deficiency-

216   induced delta and alpha cell mass expansion is conserved in zebrafish beta cells.

217   To examine the response of beta cells in a mammalian context, we treated adult

218   C57BL6 mice with IgG or GCGR-Ab for 8 weeks and observed a 4.1-fold increase in the

219   percentage of Ki67 positive beta cells (**Fig 3E-F**). This measure of beta cell proliferation was

220   also increased in mice with constitutive global inactivation of glucagon signaling (**Fig S1A-B**), as

221   shown previously.[25] Furthermore, we detected an increase in beta cell proliferation in *Gcgr*^{+/+}

222   islets only when transplanted into liver-specific *Gcgr*^{-/-} (*Gcgr*[Hep-/-]) and not *Gcgr*[Flox] mice (**Fig**

223   **S1C-E**), supporting the premise that inhibition of hepatic glucagon action produces circulating

224   factors that augment islet cell proliferation.[15,18-20]

225   To determine whether the amino acid transporter SLC7A2 was required for beta cell

226   proliferation, we treated *Slc7a2*^{+/+} and *Slc7a2*^{-/-} mice with IgG or GCGR-Ab for 2 weeks and

227   found that the GCGR-Ab-induced increase in Ki67-positive beta cells (~2.8-fold) was abolished

228   in *Slc7a2*^{-/-} islets (**Fig 3G**), and in *Slc7a2*^{-/-}, but not *Slc7a2*^{+/+}, islets transplanted into *Slc7a2*^{+/+}

229   recipients (**Fig S2A-C**). These data suggest that SLC7A2 is required in an islet autonomous

230   manner for GCGR-Ab-induced beta cell proliferation. In contrast to the response of mouse delta

231   and zebrafish beta cells, however, we did not detect a coordinate increase in beta cell mass in

232   GCGR-Ab-treated mice (**Fig S2D**). The discordance between beta cell proliferation and mass

233   did not appear to be due to a detectable increase in beta cell death since we did not detect any

234   TUNEL positive beta cells in islets of GCGR-Ab treated mice (data not shown). These findings

235   imply that inducible elimination of glucagon action is sufficient to stimulate beta cell cycle entry,

236   but insufficient to promote cell cycle completion in healthy mice.

237   Lastly, we sought to evaluate the translational relevance of these observations by

238   measuring beta cell proliferation in transplanted human islets and likewise observed an increase

239   in the mean percentage of Ki67-positive beta cells following 4 weeks of GCGR-Ab treatment

240   (**Fig 3H-J**), though the magnitude of the response varied widely among donors and was lower

241   than that of human delta cells.

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245 **4. DISCUSSION**

246   The mechanism linking inhibition of glucagon signaling to changes in pancreatic islet

247 delta and beta cells, and the relevance of these observations in a human islet context, are

248 unknown. In this study, we used zebrafish, rodents, and transplanted human islets comprising

249 six different models of altered glucagon signaling to show that inhibition of glucagon action

250 stimulated delta and beta cell proliferation via SLC7A2- and mTORC1-dependent mechanisms.

251 Constitutive global deletion of *Gcg*, or inducible elimination of glucagon signaling with a GCGR-

252 Ab, promoted RAPA-sensitive delta cell proliferation and mass expansion through a mechanism

253 requiring the cationic amino acid transporter, SLC7A2. Likewise, we identified an increase in

254 beta cell proliferation and mass in *gcgr*-deficient zebrafish that was abrogated upon deletion of

255 SLC7A2 or treatment with RAPA. While constitutive and inducible inhibition of glucagon

256 signaling also augmented the percentage of Ki67-positive beta cells in rodent islets, this

257 resulted in only a modest, non-significant increase in beta cell mass. Consistent with previous

258 reports in human alpha cells[19,20], we also showed that GCGR-Ab treatment stimulated human

259 delta, and to a lesser extent beta, cell proliferation in transplanted islets, highlighting the

260 translational relevance of our observations.

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262   We and others[18-20] showed previously that GCGR antagonism elicits

263 hyperaminoacidemia, which promotes mTOR-dependent alpha cell hyperplasia through a

264 mechanism involving induction of SLC38A5, a glutamine transporter, in alpha cells.[19,20] More

265 recently, we found that SLC7A2, an arginine transporter, is the most highly expressed amino

266 acid transporter in zebrafish, rodent, and human alpha cells and required for activation of

267 mTORC1 signaling, induction of SLC38A5, and stimulation of alpha cell proliferation in response

268 to GCGR antagonism.[22] Here, we extended these observations by demonstrating that SLC7A2

269 and mTORC1 activation are likewise required for islet non-alpha cell proliferation in response to

270 interrupted glucagon signaling. This was surprising since the expression of *Slc7a2* is much

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271   lower in delta and beta cells compared to alpha cells.[22] Because we used mice with constitutive

272   global deletion of SLC7A2, we cannot delineate the direct versus indirect requirement for this

273   transporter on delta and/or beta cell proliferation. Notwithstanding, we showed that GCGR-Ab

274   treatment augmented the percentage of Ki67-positive beta cells in transplanted donor islets

275   from *Slc7a2$^{+/+}$*, but not *Slc7a2$^{-/-}$*, mice supporting at least an islet-autonomous role for this

276   transporter in amino acid-regulated cell proliferation. However, the possibility cannot be

277   excluded that GCGR antagonism-dependent proliferative signal(s) emanating from SLC7A2-

278   enriched alpha cells act in a paracrine manner on neighboring delta and/or beta cells to promote

279   their proliferation. Future studies using conditional *Slc7a2* gene targeting approaches in islet

280   delta, beta, and/or alpha cells will be necessary to delineate its requirement in specific

281   endocrine cell subsets.

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283   We were also surprised to find that mTORC1 signaling was required for islet non-alpha

284   cell proliferation, since previous studies reported GCGR-Ab-mediated activation of S6 protein, a

285   downstream target of mTORC1, in only rare beta[20] or delta[19] cells, and the results are

286   conflicting. Here, we observed an 18-fold increase in the percentage of pS6-positive delta cells

287   in *Gcg$^{-/-}$* islets, indicative of heightened mTORC1 activity. It is possible that the pattern of S6

288   activation in islet cells differs between models of constitutive versus inducible elimination of

289   glucagon action. Nevertheless, we showed that pharmacologic inhibition of mTORC1 signaling

290   with RAPA abolished delta cell proliferation in GCGR-Ab-treated mice, and beta cell proliferation

291   in *gcgr* deficient zebrafish, supporting a central role for mTORC1-dependent nutrient sensing in

292   islet non-alpha cell proliferation in non-diabetic models.

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294   Our results agree with previous studies in *Gcgr$^{-/-}$* mice, which showed up to 3.5-fold

295   increases in islet delta cell number, mass, and pancreatic somatostatin content.[24,25] These

296   studies also identified a shift in the labeling pattern of somatostatin-positive cells from being

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297   restricted to the mantle zone in *Gcgr*^{+/+} islets to being scattered within the core as well in *Gcgr*^{-/-}

298   islets, much like alpha cells.[24,25] We confirmed and extended these observations in *Gcg*^{-/-} mice

299   by showing that the increase in delta cell mass was due, at least in part, to delta cell

300   proliferation as evidenced by a 4.7-fold increase in the percentage of Ki67-positive delta cells.

301   More recently, Gu et al.[26] showed that treatment of C57BL6 mice with a GCGR-Ab at a weekly

302   dose of 5 mg/kg for 4 weeks increased islet delta cell number by ~35% in association with

303   marginally significant (P=0.05) delta cell proliferation. Conversely, we showed that treatment of

304   C57BL6 mice with a GCGR-Ab at a weekly dose of 10 mg/kg for 8 weeks triggered a 6.2-fold

305   increase in delta cell proliferation and a 2.5-fold increase in delta cell mass. These

306   discrepancies are likely explained by differences in the dose and/or duration of GCGR-Ab

307   exposure, since Kim et al.[20] also found no change in pancreatic delta cell mass in C57BL6 mice

308   after 3 weeks of GCGR-Ab treatment at a weekly dose of only 3 mg/kg.

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310   The impact of interrupted glucagon signaling on beta cell proliferation and mass

311   expansion under insulin-sufficient conditions is inconclusive. Several studies[18-20,27,30], primarily in

312   rodent models of inducible elimination of glucagon action, have failed to detect an increase in

313   beta cell proliferation or mass. On the other hand, constitutive global or liver-specific ablation of

314   the *Gcgr* gene was associated with increased postnatal beta cell proliferation[25] and up to a 1.7-

315   fold increase in beta cell mass.[17,25] Furthermore, Xi et al.[11] found a ~20% increase in the

316   percentage of insulin-positive cells in islets of healthy cynomolgus monkeys treated with GCGR-

317   Ab at a weekly dose of 60 mg/kg for 13 weeks, but it is unknown if this was due to an increase

318   in beta cell proliferation. Here, we showed a significant increase in beta cell mass in *gcgr*-

319   deficient zebrafish, but not in GCGR-Ab treated mice, despite cell cycle engagement as

320   evidenced by 2- to 4-fold increases in the percentage of Ki67 positive beta cells. An uncoupling

321   of beta cell cycle entry from cell cycle completion was also observed by Furth-Lavi and

322   colleagues[39] in a diabetic rodent model of extreme beta cell-ablation, where some beta cells

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323   were capable of entering the cell cycle but failed to complete it under conditions of severe, but

324   not moderate, hyperglycemia. In pre-clinical models of T1D and T2D, however, GCGR

325   antagonism improves glycemia and readily promotes the regeneration of functional beta cell

326   mass through, for example, beta cell proliferation and alpha-to-beta-cell transdifferentiation.[11-16]

327   These studies suggest that the blood glucose level and/or magnitude of insulin deficiency may

328   be determinants of beta cell regenerative capacity, and by extension, beta cell mass expansion,

329   in diabetic rodent models. We posit that under healthy, insulin-sufficient conditions, rodent beta

330   cells, but not alpha or delta cells, require additional proliferative signals – or disinhibition of

331   repressive signals – for GCGR antagonism to promote cell cycle completion and an increase in

332   beta cell mass. Tight control over insulin production is necessary to protect against

333   hypoglycemia and may reflect physiologic autoregulation of beta cell mass in healthy animals.

334   Species-specific differences in the response of beta cells to GCGR loss likely reflect the

335   heightened plasticity, regenerative capacity and developmental stages of zebrafish versus

336   mammalian islet cells.[40-43] Future studies aimed at identifying the extra- and/or intra-cellular

337   effectors that couple beta cell cycle engagement with cell cycle completion in health and

338   diabetes will shed new light on unique mechanisms of mammalian beta cell regulation.

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340   These studies demonstrate a physiologic role for glucagon in the regulation of islet delta

341   and beta cell mass and expose notable differences in their proliferative capacity in healthy mice.

342   Future studies should address how amino acid-dependent nutrient sensing stimulates islet non-

343   alpha cell proliferation, and whether GCGR-Ab induces delta cell mass expansion in diabetic

344   models. By enhancing our understanding of the mechanism(s) linking inhibition of glucagon

345   action to expansion of islet cell mass, we may improve our ability to mitigate the negative side

346   effects of GCGR antagonism while leveraging its favorable effects, including the possibility for

347   beta cell regeneration therapies relying upon delta (and/or alpha) cell reprogramming in T1D

348   and T2D.[26,28,29]

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DECLARATION OF COMPETING INTERESTS

The authors declare no conflicts of interests.

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CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Katie C. Coate: Conceptualization, Formal analysis, Validation, Investigation, Visualization, Supervision, Project Administration, Funding acquisition, Writing – original draft, Writing – review and editing; **Chunhua Dai:** Conceptualization, Methodology, Formal analysis, Validation, Investigation, Visualization, Supervision, Project Administration, Writing – review and editing; **Ajay Singh:** Investigation, Validation, Formal analysis; **Jade Stanley:** Investigation, Validation, Formal analysis; **Brittney A. Covington:** Investigation, Validation, Formal analysis; **Amber Bradley:** Software, Investigation, Validation; **Favour Oladipupo:** Investigation, Validation, Formal analysis; **Yulong Gong:** Investigation, Validation, Formal analysis; **Scott Wisniewski:** Investigation, Validation, Formal analysis; **Erick Spears:** Resources, Methodology, Investigation; **Greg Poffengerber:** Methodology; **Alexandra Bustabad:** Investigation, Validation, Formal analysis; **Tyler Rodgers:** Investigation, Validation, Formal analysis; **Nandita Dey:** Investigation, Validation; **Leonard D. Shultz:** Resources, Writing – review and editing; **Dale L. Greiner:** Resources, Writing – review and editing; **Hai Yan:** Resources; **Alvin C. Powers:** Conceptualization, Resources, Writing – review and editing, Supervision, Project administration, Funding Acquisition; **Wenbiao Chen:** Conceptualization, Resources, Writing – review and editing, Supervision, Project administration, Funding Acquisition; **E. Danielle Dean:** Conceptualization, Methodology, Validation, Investigation, Formal Analysis, Visualization, Resources, Writing – review and editing, Supervision, Project administration, Funding Acquisition

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526   **FIGURE LEGENDS**

527   Figure 1. ***Loss of glucagon action augments delta cell proliferation and mass expansion***

528   ***in mouse and transplanted human islets.*** (**A**) Representative images of pancreatic islet and

529   delta cell proliferation in $Gcg^{+/+}$/$Gcg^{-/-}$ (upper row) and IgG/GCGR-Ab-treated C57BL6 (bottom

530   row) mice. Somatostatin (green), Ki67 (red), and DAPI (blue) are shown. White arrows indicate

531   Ki67+ somatostatin+ cells. (**B-C**) Quantification of pancreatic islet delta cell proliferation in (**B**)

532    $Gcg^{+/+}$ (black bar) and $Gcg^{-/-}$ (red bar) mice (n=1-2 females and 2-3 males per genotype) and (**C**)

533   control IgG (black bar) and GCGR-Ab-treated (blue bar) mice (all males, unpaired t test, ***p <

534   0.001 versus $Gcg^{+/+}$, **p < 0.01 versus IgG). (**D**) Representative images of pancreatic islet

535   hormones in $Gcg^{+/+}$/$Gcg^{-/-}$ (upper row) and IgG/GCGR-Ab-treated (bottom row) mice. Insulin

536   (green), somatostatin (red), and pro-glucagon ($Gcg^{+/+}$/$Gcg^{-/-}$; blue) or glucagon (IgG/GCGR-Ab;

537   blue) are shown. (**E-F**) Pancreatic islet delta cell mass in (**E**) $Gcg^{+/+}$ (black bar) and $Gcg^{-/-}$ (red

538   bar) mice (n=1-2 females and 3 males per genotype) and (**F**) control IgG (black bar) and GCGR-

539   Ab-treated (blue bar) mice (all males, unpaired t test, **p < 0.01 versus $Gcg^{+/+}$ or IgG). (**G**)

540   Schematic of approach for human islet subcapsular renal transplantation in NSG recipient mice

541   followed by control IgG or GCGR-Ab treatment. Created with BioRender.com (**H**)

542   Representative images of delta cell proliferation in human islet grafts after 4 weeks of control

543   IgG (upper row) or GCGR-Ab-treatment (bottom row). Grafts were immunostained for

544   somatostatin (green), Ki67 (red), and DAPI (blue). White dashed boxes indicate regions

545   selected for insets. (**I**) Quantification of delta cell proliferation in transplanted human islets in

546   control IgG (black circles) and GCGR-Ab-treated (orange circles) mice (n=3 donors [see

547   Supplemental Table 1], unpaired t test, **p < 0.01 versus IgG).

548

549   Figure 2. SLC7A2 ***and mTOR activation are required for delta cell proliferation in response***

550   ***to interrupted glucagon signaling.*** (**A**) Representative images of pancreatic islet delta cell

551   proliferation in $Slc7a2^{+/+}$ (upper row) and $Slc7a2^{-/-}$ (bottom row) IgG/GCGR-Ab-treated mice.

552   Somatostatin (green), Ki67 (red), and DAPI (blue) are shown. White arrows indicate Ki67+

553   somatostatin+ cells. (**B**) Quantification of pancreatic islet delta cell proliferation in $Slc7a2^{+/+}$

554   (black bars) and $Slc7a2^{-/-}$ (blue bars) IgG or GCGR-Ab-treated mice (n=4 females and 1-3

555   males per genotype, one-way ANOVA with Tukey's multiple comparisons test, ****$p < 0.0001$

556   versus $Slc7a2^{+/+}$ IgG, ***$p < 0.001$ versus $Slc7a2^{+/+}$ GCGR-Ab). (**C**) Representative images of

557   pancreatic islets in $Gcg^{+/+}$/$Gcg^{-/-}$ mice immunostained for somatostatin (green), phosphorylated

558   ribosomal protein S6 (pS6$^{240/244}$; red), and DAPI (blue). White arrows indicate pS6+

559   somatostatin+ cells. White dashed boxes indicate regions selected for insets. (**D**) Quantification

560   of the percentage of pS6+ somatostatin+ cells in $Gcg^{+/+}$ (black bar) and $Gcg^{-/-}$ (red bar)

561   pancreatic islets (n=1-2 females and 3-4 males per genotype, unpaired t test, ****$p < 0.0001$

562   versus $Gcg^{+/+}$). (**E**) Schematic of approach for IgG/GCGR-Ab (once weekly) and rapamycin

563   (RAPA; once daily) co-treatment in C57BL6 mice. Created with BioRender.com (**F**)

564   Quantification of pancreatic islet delta cell proliferation in mice co-treated with IgG (black bars)

565   or GCGR-Ab (blue and white bar) and PBS or RAPA (all males, one-way ANOVA with Tukey's

566   multiple comparisons test, ****$p < 0.0001$ versus IgG, ***$p < 0.001$ versus PBS/GCGR-Ab).

567

568   Figure 3. ***Loss of glucagon receptor function stimulates beta cell proliferation in a***

569   ***species-specific manner.*** (**A**) Beta cells stained for EdU to assess their proliferation in 5 dpf

570   (days post-fertilization) wild-type ($gcgr^{+/+}$, black bar) and $gcgra/b^{-/-}$ (abbreviated $gcgr^{-/-}$, green

571   bar) zebrafish (n=8 per group, unpaired t test, **$p < 0.01$ versus $gcgr^{+/+}$). (**B**) Beta cell number in

572   5 dpf $gcgr^{+/+}$ (black bar) and $gcgr^{-/-}$ (green bar) zebrafish (n=24-30 per group, unpaired t test,

573   ****$p < 0.0001$ versus $gcgr^{+/+}$). (**C**) Beta cell number after knockdown of $slc7a2$ (+/+, black bar; -

574   /-, green bar) in 5 dpf $gcgr^{-/-}$ zebrafish (n=8-15 per group, unpaired t test, ****$p < 0.0001$ versus

575    $slc7a2^{+/+}$). (**D**) Beta cell number in 6 dpf g$cgr^{+/+}$ (black bar) and $gcgr^{-/-}$ (green bar) zebrafish after

576   3 days of treatment with PBS or RAPA (n=6-7 per group, one-way ANOVA with Fisher's LSD,

577   ****$p < 0.0001$ versus PBS/$gcgr^{+/+}$, * $p < 0.05$ versus PBS/$gcgr^{-/-}$). (**E**) Representative images of

578   pancreatic islet beta cell proliferation in IgG/GCGR-Ab-treated C57BL6 mice. Insulin (green),

579   Ki67 (red), and DAPI (blue) are shown. White arrow indicates a Ki67+ insulin+ cell. (**F**)

580   Quantification of pancreatic islet beta cell proliferation in control IgG (black bar) and GCGR-Ab-

581   treated (blue bar) mice (all males, unpaired t test, **p < 0.01 versus IgG). (**G**) Quantification of

582   pancreatic islet beta cell proliferation in $Slc7a2^{+/+}$ (black bars) and $Slc7a2^{-/-}$ (blue bars) IgG or

583   GCGR-Ab-treated mice (n=2-5 females and 3-6 males per group, one way ANOVA with Tukey's

584   multiple comparisons test, **p < 0.0001 versus $Slc7a2^{+/+}$ IgG, ***p < 0.001 versus $Slc7a2^{+/+}$

585   GCGR-Ab). (**H**) Schematic of approach for human islet subcapsular renal transplantation in

586   NSG recipient mice followed by PBS or GCGR-Ab treatment. Created with BioRender.com (**I**)

587   Representative images of beta cell proliferation in human islet grafts after 4 weeks of PBS or

588   GCGR-Ab-treatment. Grafts were immunostained for insulin (green), Ki67 (red), and DAPI

589   (blue). Dashed yellow lines indicate kidney-graft boundary. (**J**) Quantification of beta cell

590   proliferation in transplanted human islets in PBS (black circles) or GCGR-Ab-treated (orange

591   circles) mice (n=2 female and 5 male donors [see Supplemental Table 1], unpaired t test, *p <

592   0.05 versus PBS).

593

594   Supplemental Figure 1. ***Genetic interruption of glucagon signaling stimulates beta cell***

595   ***proliferation in pancreatic and transplanted mouse islets.*** (**A-B**) Quantification of pancreatic

596   islet beta cell proliferation in (**A**) 6 week-old $Gcgr^{+/+}$ (black bar, all males) and $Gcgr^{-/-}$ (red striped

597   bar, all males) and (**B**) 8 week-old $Gcg^{+/+}$ (black bar) and $Gcg^{-/-}$ (red bar) mice (n=2-4 females

598   and 3 males per group, unpaired t test, ***p < 0.001 versus $Gcgr^{+/+}$, *p < 0.05 versus $Gcg^{+/+}$). (**C**)

599   Schematic of approach for subcapsular renal transplantation of $Gcgr^{+/+}$ (wild type, WT) donor

600   islets into control ($Gcgr^{Flox}$) or liver-specific $Gcgr$ knockout ($Gcgr^{Hep-/-}$) recipient mice. Created

601   with BioRender.com (**D**) Representative images of islet grafts from WT to Flox and WT to Hep$^{-/-}$

602   recipients after four weeks. Grafts are immunostained for insulin (green), Ki67 (red) and DAPI

603   (blue). White arrows indicate Ki67+ insulin+ cells. Dashed yellow lines indicate kidney-graft

22

604   boundary. (**E**) Quantification of beta cell proliferation in transplanted islets from WT to Flox

605   (black bar) and WT to Hep$^{-/-}$ (red striped bar) groups (n=4 males per group, unpaired t test, **p <

606   0.05 versus WT to Flox).

607

608   Supplemental Figure 2. SLC7A2-*dependent stimulated beta cell proliferation is islet*

609   *autonomous.* (**A**) Schematic of approach for subcapsular renal transplantation of *Slc7a2$^{+/+}$*

610   (wild type, WT) and *Slc7a2$^{-/-}$* (KO) donor islets into *Slc7a2$^{+/+}$* (WT) recipient mice followed by

611   control IgG or GCGR-Ab treatment. Created with BioRender.com (**B**) Representative images of

612   *Slc7a2$^{+/+}$* (upper row) and *Slc7a2$^{-/-}$* (bottom row) islet grafts from *Slc7a2$^{+/+}$* kidney capsules after

613   two weeks of IgG or GCGR-Ab treatment. Grafts are immunostained for insulin (green), Ki67

614   (red) and DAPI (blue). White arrows indicate Ki67+ insulin+ cells. Dashed yellow lines indicate

615   kidney-graft boundary. (**C**) Quantification of beta cell proliferation in transplanted islets from

616   *Slc7a2$^{+/+}$* and *Slc7a2$^{-/-}$* donors treated with IgG (black circles) or GCGR-Ab (blue circles; n=2

617   females and 2 males per treatment group, two-way ANOVA with Fisher's LSD test, **p < 0.01

618   versus IgG treated). (**D**) Quantification of pancreatic islet beta cell mass in *Slc7a2$^{+/+}$* (black bars)

619   and *Slc7a2$^{-/-}$* (blue bars) IgG or GCGR-Ab-treated mice (n=2-5 females and 3-6 males per

620   group).

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632 Supplemental Table 1: *Human Islet Donor Information.*

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Donor ID	Age	Ethnicity/Race	Sex	BMI (kg/m^2)	HbA1c (%)	Cause of Death	Islet Source
AELC213	10	Hispanic/Latino	F	25.4	N/A	Head Trauma/Blunt Injury	Other
AFEA331	45	Black	M	29.3	5.0	CVA/ stroke	IIDP
AIFV371	28	Hispanic/Latino	F	24.7	5.0	CVA/ stroke	HPAP
1	32	N/A	M	29.5	N/A	N/A	IIDP
2	47	N/A	M	22.3	N/A	N/A	IIDP
3	55	N/A	M	28.4	N/A	N/A	IIDP
4	43	N/A	M	29.6	N/A	N/A	IIDP
5	46	N/A	M	28.8	N/A	N/A	IIDP
6	41	N/A	F	31.1	N/A	N/A	IIDP
7	47	N/A	F	25.6	N/A	N/A	IIDP
8	52	N/A	M	33.2	N/A	N/A	IIDP

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Figure 1

A Somatostatin/Ki67/DAPI

$Gcg^{+/+}$ $Gcg^{-/-}$

IgG   GCGR-Ab

50 μm

D Insulin/Somatostatin/(Pro)Glucagon

$Gcg^{+/+}$ $Gcg^{-/-}$

IgG   GCGR-Ab

50 μm

B Ki67+ delta cells (%)

$Gcg^{+/+}$ $Gcg^{-/-}$ ***

C Ki67+ delta cells (%)

IgG   GCGR-Ab   **

E Delta cell mass (mg)

$Gcg^{+/+}$ $Gcg^{-/-}$ **

F Delta cell mass (mg)

IgG   GCGR-Ab   **

G Human Islets

NSG Recipient

IgG or GCGR-Ab

Analysis

0   2   4   6   weeks

H Somatostatin/Ki67/DAPI

IgG

GCGR-Ab

100 μm

I Ki67+ delta cells (%)

IgG   GCGR-Ab   **

Figure 2

A Somatostatin/Ki67/DAPI

Slc7a2⁺/⁺ : IgG, GCGR-Ab

Slc7a2⁻/⁻ : IgG, GCGR-Ab

50 μm

B

Ki67+ delta cells (%)

**** ***

Slc7a2 +/+ -/- +/+ -/-

IgG   GCGR-Ab

C Somatostatin/pS6(p240/p244)/DAPI

Gcg⁺/⁺   *Gcg*⁻/⁻

75 μm

D

pS6⁺ delta cells (%)

****

Gcg⁺/⁺   *Gcg*⁻/⁻

E

1x/week

IgG or GCGR-Ab ± RAPA (daily)

Analysis

0   2   4 weeks

C57BL6

F

Ki67+ delta cells (%)

**** ***

IgG GCGR-Ab IgG GCGR-Ab

PBS   RAPA

Figure 3

Supplemental Figure 1

A   6 week-old mice

Ki67+ beta cells (%)

***

- Gcgr$^{+/+}$
- Gcgr$^{-/-}$

B   8 week-old mice

Ki67+ beta cells (%)

*

- Gcg$^{+/+}$
- Gcg$^{-/-}$

C

Gcgr$^{+/+}$ (WT) Donor Islets

Analysis

Gcgr^{Flox} Recipient   Gcgr$^{Hep-/-}$ Recipient   4 weeks

D   Insulin / Ki67 / DAPI

WT to Flox   WT to Hep$^{-/-}$

E   4 week post tx

Ki67+ beta cells (%)

**

- WT to Flox
- WT to Hep$^{-/-}$
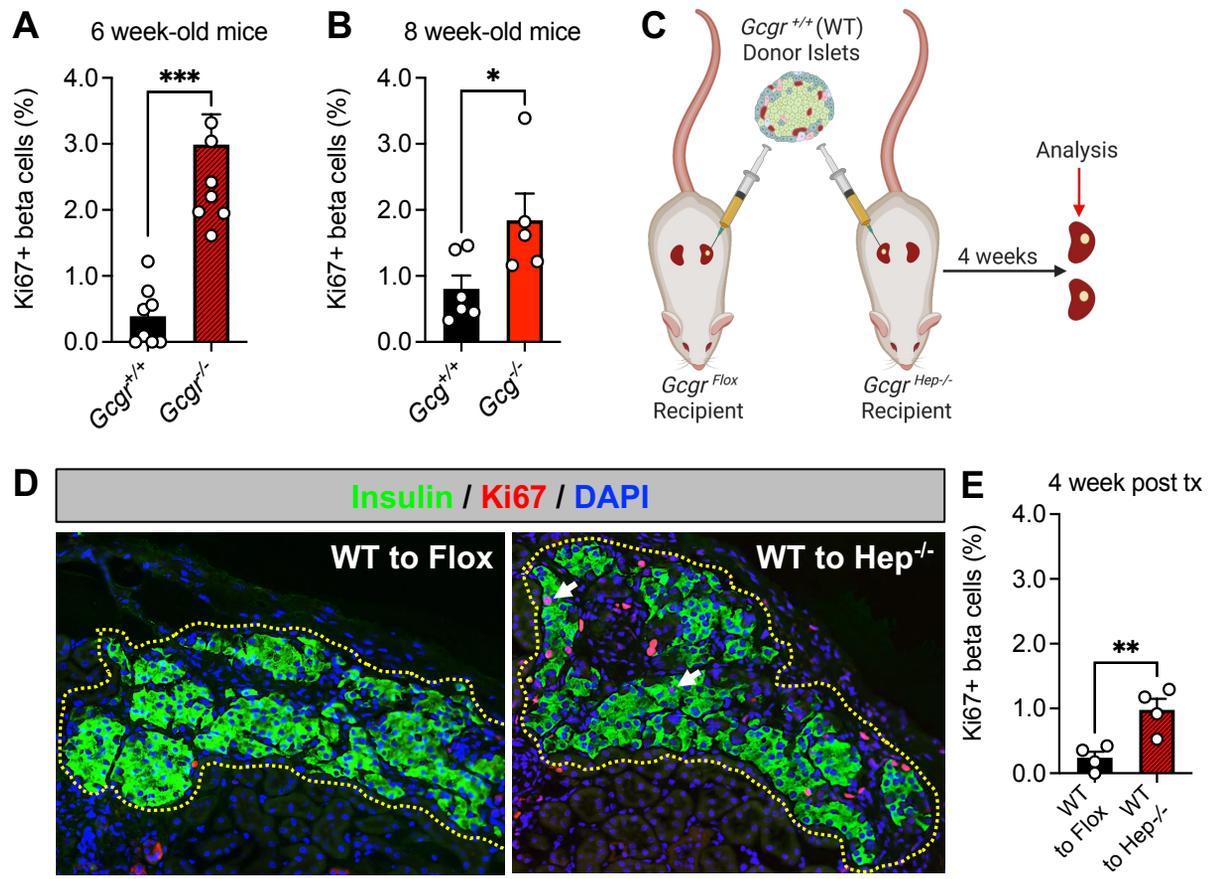
Supp Fig 2: Slc7a2 is required for Gcgr-Ab-induced beta cell proliferation in transplanted mouse islets

A

Slc7a2$^{+/+}$ (WT) Donor Islets

Slc7a2$^{-/-}$ (KO) Donor Islets

Analysis

IgG or GCGR Ab

0   2   4   weeks

Slc7a2$^{+/+}$ (WT) Recipient

B Insulin / Ki67 / DAPI

Slc7a2$^{+/+}$ IgG GCGR-Ab

Slc7a2$^{-/-}$ IgG GCGR-Ab

100 µm

C

Ki67$^+$ beta cells (%)

● IgG
● GCGR-Ab

**

Slc7a2 +/+ -/-

D

Beta cell mass (mg)

Slc7a2 +/+ -/- +/+ -/-

IgG   GCGR-Ab