Original Article

MOLECULAR METABOLISM

Role of nutrients and mTOR signaling in the regulation of pancreatic progenitors development

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ABSTRACT

Objective: Poor fetal nutrition increases the risk of type 2 diabetes in the offspring at least in part by reduced embryonic β-cell growth and impaired function. However, it is not entirely clear how fetal nutrients and growth factors impact β-cells during development to alter glucose homeostasis and metabolism later in life. The current experiments aimed to test the impact of fetal nutrients and growth factors on endocrine development and how these signals acting on mTOR signaling regulate β-cell mass and glucose homeostasis.

Method: Pancreatic rudiments in culture were used to study the role of glucose, growth factors, and amino acids on β-cell development. The number and proliferation of pancreatic and endocrine progenitor were assessed in the presence or absence of rapamycin. The impact of mTOR signaling *in vivo* on pancreas development and glucose homeostasis was assessed in models deficient for mTOR or Raptor in Pdx1 expressing pancreatic progenitors.

Results: We found that amino acid concentrations, and leucine in particular, enhance the number of pancreatic and endocrine progenitors and are essential for growth factor induced proliferation. Rapamycin, an mTORC1 complex inhibitor, reduced the number and proliferation of pancreatic and endocrine progenitors. Mice lacking mTOR in pancreatic progenitors exhibited hyperglycemia in neonates, hypoinsulinemia and pancreatic agenesis/hypoplasia with pancreas rudiments containing ductal structures lacking differentiated acinar and endocrine cells. In addition, loss of mTORC1 by deletion of raptor in pancreatic progenitors reduced pancreas size with reduced number of β-cells.

Conclusion: Together, these results suggest that amino acids concentrations and in particular leucine modulates growth responses of pancreatic and endocrine progenitors and that mTOR signaling is critical for these responses. Inactivation of mTOR and raptor in pancreatic progenitors suggested that alterations in some of the components of this pathway during development could be a cause of pancreatic agenesis/hypoplasia and hyperglycemia.

Keywords Nutrients; mTOR; Rapamycin; Pancreas; Development; Islets; β-cells

1. INTRODUCTION

It has been established that both genetic and environmental factors contribute to type 2 diabetes (T2D) incidence [1]. The fetal nutrient environment plays a major role in the increased susceptibility to diabetes later in life (programming). The thrifty phenotype hypothesis proposes that poor fetal and infant growth increases the risk of type 2 diabetes and metabolic syndrome [2–4]. How poor fetal environment increases the susceptibility to diabetes is not completely clear, but nutrient availability is essential for proper cell growth, and survival and nutritional insults early in life are believed to have a deleterious effect on the developmental program of the pancreas and ultimately impair islet function [5]. Understanding the molecular mechanisms by which nutrient availability alters early pancreatic progenitors could lead to develop novel strategies that ultimately result in lowering the lifetime risk of type 2 diabetes in the offspring exposed to an adverse nutrient environment during pregnancy.

Published evidence demonstrates that the size of the mouse pancreas is constrained by an intrinsic program established early during development, one that is essentially not subject to growth compensation [6]. Specifically, final β-cell mass and pancreas size is limited by the size of the Pdx1 progenitor cell pool. This finding, together with the epidemiologic evidence supporting the "thrifty hypothesis" [7], suggests that autonomous growth constraints established during pancreatic development may lie beneath the normal regulative capacity of β-cells. Therefore, alterations in Pdx1 pancreatic progenitor proliferation and differentiation into endocrine cells can have major implications in the regulation of glucose homeostasis later in life. Ngn3

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Abbreviations: T2D, type 2 diabetes; mTOR, mammalian target of Rapamycin; TORC1, TORC2, target of Rapamycin complex 1, 2; AA, amino acids

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is a key transcription factor necessary for endocrine specification later during pancreatic organogenesis [8]. Ngn3-deficient mice fail to develop islets and die of diabetes early [9]. The current published evidence suggests that the proliferation rate of pancreatic and endocrine progenitors is subject to regulation, and that the proliferative rate of these cells could have major implications for glucose homeostasis. Mammalian cells have the ability to sense the nutritional status and to respond by triggering either anabolic pathways under nutrients abundance, or catabolic pathways under starvation and stress conditions [10,11]. The mechanisms by which these nutrition-related effects operate include signaling pathways that respond to changes in energy status. The mammalian target of Rapamycin (mTOR) integrates signals from both nutrients and growth factors. mTOR is a conserved serine/threonine protein kinase [12,13] that forms two structurally conserved, but functionally distinct complexes named (TORC1 and TORC2) [10,14–16]. These complexes phosphorylate and regulate different sets of substrates [17]. mTORC1 integrates inputs from various stimuli (growth factors, stress, energy status, oxygen, and amino acids) to control fundamental pathways implicated in cellular growth and metabolism. Amino acids, particularly leucine and arginine, are potent activators of mTORC1 [18,19]. Leucine supplementation during pregnancy increased fetal body weight, triggered fetal hyperglycemia and hypoinsulinemia, and decreased islet mass. Using an *in vitro* culture model for pancreatic explants during development, this study demonstrated that leucine resulted in decreasing the number of endocrine progenitors and β-cell mass, and this was associated with changes in HIF1α and the mTORC1 pathway [20].

In the current experiments, we determined the effect of different nutrients and growth factors on the control of embryonic pancreatic growth and development. In addition, we identified an important role of mTOR signaling on these processes, first by pharmacological inhibition of the pathway in pancreatic embryonic rudiment cultures and then in mice with conditional deletion of mTOR and Raptor in Pdx1 progenitors. Our studies highlighted the importance of leucine in the control of Pdx1 and Ngn3 progenitor proliferation. We also showed direct evidence implicating mTORC1 in the proliferation of both Pdx1 and Ngn3 progenitors. Our *in vivo* studies allowed us to demonstrate the importance of mTOR signaling for the pancreatic growth and the differentiation program of Pdx1 progenitors during early pancreatic organogenesis.

2. MATERIALS & METHODS

2.1. Animal generation

Mice carrying a floxed allele of mTOR were provided by S. Kozma and G. Thomas (Institut d'Investigació Biomèdica de Bellvitge, Barcelona, Spain). Animals carrying a floxed allele of Raptor were obtained from M. Hall and have been described [21]. Conditional deletion of mTOR or Raptor in the pancreas was achieved by crossing these mice to the Late Pdx1-Cre line (*L-Pdx1-Cre*) [22]. The CAG-YFP reporter transgenic animals were purchased from The Jackson laboratory (Stock number 011107; Bar Harbor, ME). Throughout the study, mice containing *L-Pdx1-Cre* transgene were used as controls for all the experiments. Mice were maintained on a mixed C57Bl6 background, and all the experiments were performed using control littermates. Genotyping of all mice was performed by PCR. All experiments were in accordance to the University of Michigan Institutional Animal Care and Use Committee approved protocols.

2.2. Pancreatic bud explants culture *in vitro* and calorie restricted studies *in vivo*

Pregnant CD1 females were purchased from Charles River Laboratories (Wilmington, MA). The morning of the vaginal plug discovery was designated as embryonic day 0.5 (E 0.5) Pancreatic rudiments were then dissected from E13.0 embryos. Pancreatic buds were placed in culture in collagen (Corning, Bedford, MA) as previously described [23,24]. Regular RPMI 1640 (Lonza, Walkersvile, MD) or amino acids depleted RPMI media (US Biological, Swampscott, MA). Leucine (Sigma, St-Louis, MO) was used at a low and high concentration (0.05 and 0.4 mM), and IGF1 (JRH Biosciences, Lenexa, KS, USA) was used at 5 μg/mL. Rapamycin (Sigma, St-Louis, MO) was used at 1 μg/mL. For control conditions, equivalent concentrations of DMSO (Sigma, St. Louis, MO) were added to the media (Table in Figure 1). Because of their respective half-life, the different factors were added every day whereas the media was replaced every other day. Cultures were maintained at 37 °C in a humidified atmosphere of 95% O_2/5% CO_2. For calorie-restricted studies, 8-week-old CD1 females were purchased from Charles River Laboratories and housed in 12:12-h light–dark cycle with *ad libitum* access to standard rodent chow (Lab Diet, St. Louis, MO) and water. After a week of adaptation to our facility, female mice were mated with a male. The presence of a vaginal plug was designated gestational day 0.5. The next day, random mice were either continued on the *ad libitum* feeding or restricted by 60% of the daily chow intake of the control group. At E13.0, the female mice were euthanized and the pancreatic buds were harvested from the fetuses.

2.3. Tissue preparation and immunofluorescence staining

After 72 h of culture, embryonic rudiments cultured in the presence of amino acids, rapamycin or growth factors were fixed in 3.7% Formalin in PBS, and then pre-embedded in Histogel (Thermo Scientific, Kalamazoo, MI) before paraffin processing and embedding. Newborn, P4 or adult pancreata from various genotypes were isolated and fixed in 3.7% Formalin in PBS. Formalin-fixed pancreatic tissues were embedded in paraffin using standard techniques. 5 or 8 μm sections were collected and analyzed for various staining. For detection of nuclear proteins, slides were subjected to antigen retrieval with citrate buffer and then permeabilized with 0.1% triton. The following primary antibodies were used overnight at the given dilution: Rat anti-E-cadherin (BD Biosciences, San Jose, CA, 1:100); rabbit anti-pS6 ribosomal protein (240/244) (Cell signaling, Davers, MA, 1:100); rabbit anti-Ki67 (Vector Laboratories, Burlingame CA, 1:200); goat anti-Pdx1 (Santa Cruz, 1:100); rabbit anti-Pdx1 (Millipore, Temecula, CA, 1:800); mouse anti-Ngn3 (Beta Cell Biology Consortium, 1:500); rabbit anti-Sox9 (Millipore, Temecula CA, 1:600); guinea pig anti-insulin (Dako, Denmark, 1:800); mouse anti-glucagon (Abcam, Cambridge, MA, 1:400); chicken anti-green fluorescent protein (EGFP and YFP) (Abcam, Cambridge, MA, 1:600); rabbit anti-amylase (Sigma, St Louis, MO, 1:300); rabbit anti-synaptophysin (Abcam, Cambridge, MA, 1:200). DBA (Dolichos Biflorus Agglutinin, Vector Laboratories, Burlingame CA, 1:100) staining was performed per the manufacturer's instructions. Sections were incubated with the appropriate secondary antibodies conjugated to FITC, AMCA, or Cy3 (Jackson Immunoresearch, West Grove, PA). When necessary, nuclei were counterstained with DAPI (Vector Laboratories, Burlingame CA).

Fluorescent images were acquired though Objective Imaging's Surveyor (V7.0.1, Objective Imaging Ltd, Cambridge, UK) using a Leica microscope DM6000B with a motorized stage (Leica Microsystems, Wetziar, Germany) interfaced with the OASIS-blue PCI controller and using and a Leica DFC360FX camera (Leica Microsystems, Wetziar, Germany). Embryos and organs pictures were acquired using a Leica D-LUX3 adapted to the light path of a Leica MZ6 microscope.

2.4. Morphometric analysis and cell counts

Entire pancreatic buds were sectioned at 5 μm thickness. For the *in vitro* studies, every other section of the bud was stained for

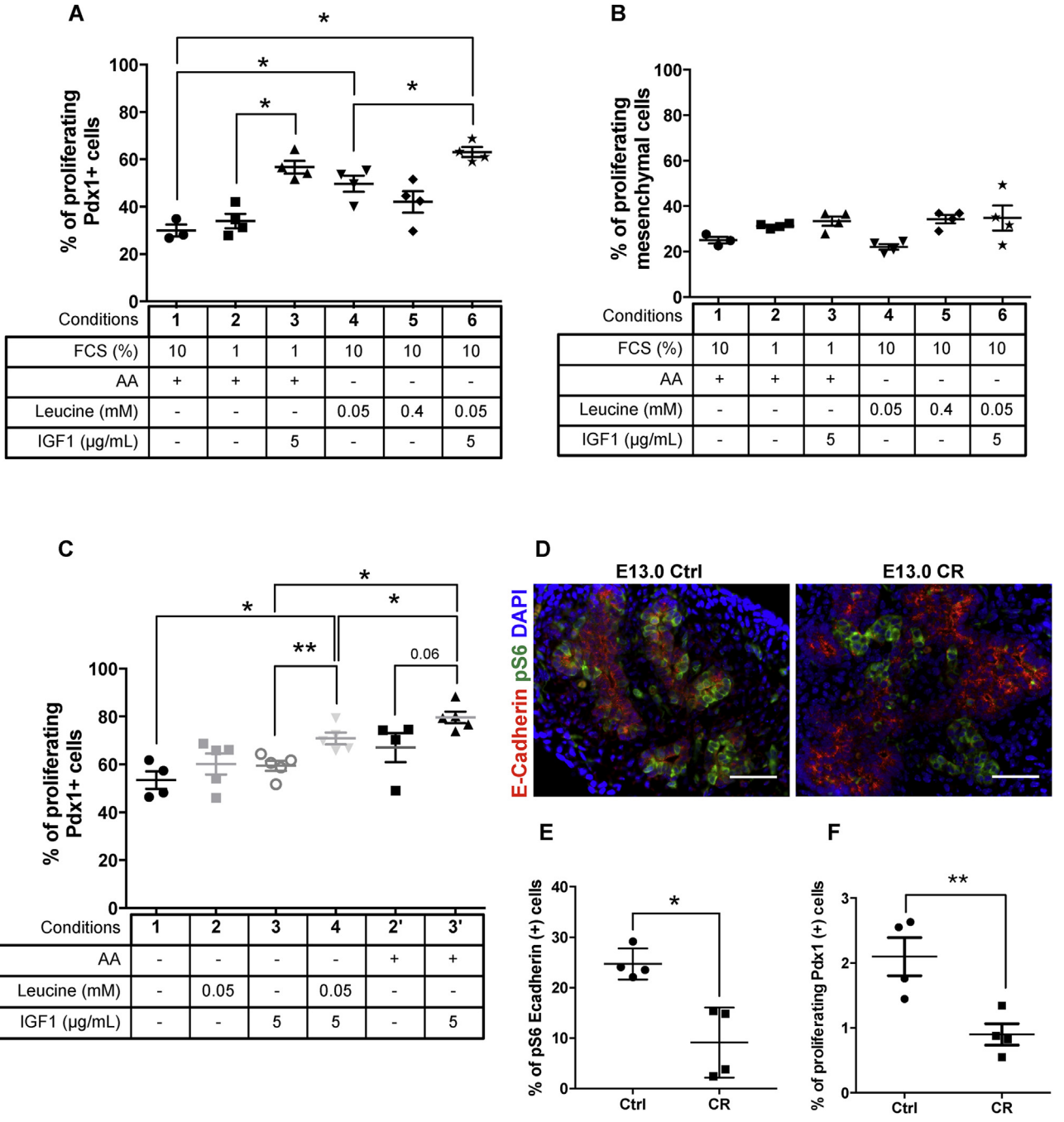

Figure 1: Effects of Amino acids and growth factors on pancreatic proliferation during development using embryonic pancreatic buds in culture. (A) Percentage of pancreatic progenitor proliferation in response to various treatments of growth factor (IGF1) and amino acids (leucine). (B) Percentage of proliferation within the mesenchyme of the embryonic buds in response to IGF1 and leucine. (C) Percentage of proliferation of Pdx-1 positive cells in serum starved pancreatic buds and in the presence of IGF1 and leucine. (D) Immunofluorescence staining for phospho-S6 (235/236) (green) and E-cadherin (red) in E13 pancreatic buds in which the mothers were subjected to normal diet (E13.0 Ctrl) or 60% calorie restricted diet (E13.0 CR) during pregnancy. (E) Percentage of pS6-positive cells within the E-Cadherin-positive epithelium. (F) Percentage of proliferating progenitor cells in response to these conditions was quantified. Data are presented as mean ± SEM. *p ≤ 0.05, **p ≤ 0.005. Nuclei were counterstained with DAPI. Scale bars, 50 µm.

Pdx-1 and Ki67 analysis. Alternate sections were stained for Ngn3 and Ki67 analysis. Different parameters (total number, Ki67, Pdx1, and Ngn3) were counted using ImageJ software version 1.50 g [25] and the Image-Based Tool for Counting Nuclei (ITCN) plug-in version 1.6 from the Center for Bio-Image Informatics (http://www.bioimage.ucsb.edu/automatic-nuclei-counter-plug-in-for-imagej) after contrast enhancement using the Enhance Local Contrast (CLAHE) http://imagej.net/Enhance_Local_Contrast_(CLAHE) tool for ImageJ, "Contrast limited adaptive histogram equalization", *Graphics gems IV*, Academic Press Professional, Inc., pp. 474–485). Mesenchyme parameters were calculated by subtracting the total number of Pdx1 positive cells from the total number of cells (based on a DAPI counterstain) in the rudiment.

For analysis of β-cell fraction, areas of acinar and β-cells were quantified in insulin- and amylase-stained sections. The analysis was performed in five independent sections (5 μm thick), separated by at least 200 μm using the Image-Pro Plus Software version 6.3.1 (Media Cybernetics, Inc, Bethesda, MD). Because of the inherent imprecisions over the measurements of the pancreas weight at this stage, the percentage area of β-cells over acinar cells was reported here instead of an absolute β-cell mass. Cell proliferation was quantified using sections co-stained for Insulin, Ki67 and DAPI nuclear counterstain. This analysis included 800 to 3000 cells per animal.

2.5. Metabolic studies

Blood samples were collected from the tail vein after an overnight fast. Glucose was measured on whole blood using AccuChek Aviva (Roche, South San Francisco, CA, USA). Glucose tolerance test was performed by intraperitoneally injecting (2 mg/g) of glucose on overnight fasting animals as previously described [26]. Animal weight and fed glucoses were measured at the given time point.

2.6. Statistical analysis

All values are expressed as mean ± standard errors. Statistical analyses were conducted using unpaired, non-parametric Mann—Whitney tests (*u* test) using Prism (version 6.0c, GraphPad Software, La Jolla, CA). Results were considered significant with a *p*-value ≤ 0.05.

3. RESULTS

3.1. Amino acids and growth factors regulate pancreatic progenitor proliferation during development

To assess the importance of mTOR activators during pancreatic development, we assessed the effects of growth factors and amino acids (AA) using E13.0 embryonic pancreatic rudiments placed in a collagen gel culture for three days [23]. At this stage, the anlage is still mostly undifferentiated, and the effects of nutrients on pancreas development can be assessed after a few days in culture. Pdx1-expressing progenitors exhibited a high degree of proliferation (~35%) at the end of a 3-day culture in complete (control) medium (RPMI containing 11 mM Glucose, 10% FCS and AA; Figure 1A, condition 1). Serum starvation in complete culture medium (containing amino acids) had no effect on the proliferation of the Pdx1 progenitors (Figure 1A, condition 1 *vs.* 2). IGF1 treatment induced Pdx1^{+} cell proliferation in low serum conditions containing amino acids (Figure 1A, condition 3). Compared to control conditions, culture of embryonic pancreas on AA-depleted medium supplemented with a low concentration of leucine (0.05 mM) and 10% FCS was sufficient to induced Pdx1 progenitor proliferation, suggesting that concentration of the other amino acids in standard RMPI may have inhibitory effects on Pdx1+ cell proliferation (Figure 1A, Conditions 1 *vs.* 4). No additional effect on proliferation could be observed when leucine concentrations in amino acid-free media were restored to the levels in standard RPMI (Figure 1A, conditions 4 *vs.* 5). Low levels of leucine in amino acid depleted medium were sufficient to induce Pdx1+ progenitor proliferation by IGF1 (Figure 1A, condition 4 *vs.* 6). Proliferation within the mesenchyme of the embryonic buds was not altered by most treatments, suggesting that epithelial progenitors are more sensitive than mesenchymal cells to changes in AA and growth factors (Figure 1B). The role of AA on IGFI-mediated induction of Pdx1+ progenitor proliferation was next examined in rudiment cultures. IGF1 induced Pdx1+ progenitor proliferation in RPMI with serum and full AA concentration (Figure 1C, compare conditions 1 and 3′) but not in the absence of AA (Figure 1C, compare conditions 1, 3 and 3′) (was only observed in the presence of full AA concentration (Figure 1C, compare conditions 3 to 3′). The effect of IGF1 on Pdx1+ progenitor proliferation observed in the presence of AA-free RPMI was supplemented with low levels of leucine (Figure 1C, compare conditions 1, 4 and 3′). Interestingly, IGF1 induction of proliferation was higher in RPMI with serum and full AA concentration when compared with AA-free RPMI was supplemented with low levels of leucine (Figure 1C, compare conditions 4 and 3′).

To assess the role of the nutrient abundance *in vivo*, we studied the impact of 60% calorie restriction (CR) on pancreatic progenitors proliferation during embryogenesis. Immunostaining for E-cadherin was used to outline the epithelial cells. E13.0 pancreatic rudiments displayed no discernable morphological differences between control and calorie-restricted mice (Figure 1D). The fraction of Phospho-S6-positive cells in the epithelium was significantly reduced in calorie-restricted rudiments compared to controls (Figure 1E), implying that this pathway was perturbed in calorie-restricted pancreata (Figure 1D). A significant reduction in proliferation of Pdx1+ cells was observed in calorie-restricted embryos compared to control littermates (Figure 1F).

3.2. Inhibition of mTORC1 by Rapamycin decreases the proliferation of the Pdx1+ population and pancreatic mesenchyme

Given the effect of AA on the regulation of pancreas progenitor proliferation and the lower phospho-S6 staining in response to caloric restriction, we tested the role of mTOR signaling on pancreas development by pharmacologic inhibition using rapamycin. Rapamycin treatment completely eliminated the phospho-S6 staining in E13.0 embryonic rudiments in culture for 3 days (Figure 2B). In contrast, stimulation of mTOR signaling by IGF1 treatment of embryonic rudiments for three days resulted in a more diffuse staining for phospho-S6 (Figure 2C). Rapamycin treatment reduced S6 phosphorylation in pancreatic rudiments cultured on normal conditions or after IGF1 treatment (Figure 2D). At the end of 3 days of culture, rapamycin treatment reduced the size of rudiment (Figure 2B F, F′). In contrast, IGFI treatment led to an increase in rudiment size, and this effect was not inhibited by rapamycin (Figure 2G, G′, H, H′, I). Rapamycin treatment reduced both the number and proliferation of the Pdx1+ progenitors (Figure 2F, F′ I, J) when compared to DMSO control conditions (Figure 2E, E′ I, J). The number and proliferation of Pdx1+ cells was significantly increased in response to IGFI (Figure 2G, G′, I, J). Surprisingly, when rudiments were treated with both IGF1 and Rapamycin, the number of Pdx1 cells still was increased, indicating that Rapamycin was not sufficient to prevent the increase in proliferation triggered by IGF1 (Figure 2H, H′, I, J). During early organogenesis, the surrounding mesenchyme secretes growth factors that control both proliferation and differentiation of the pancreatic epithelium. Thus, the effect of IGF1 or Rapamycin treatment on the mesenchyme was also determined. Rapamycin treatment led to a significant reduction in the proliferation and number of mesenchymal cells (Figure 2K). Interestingly, IGFI treatment was able only to induce mesenchymal cell proliferation in the presence of rapamycin, indicating that rapamycin treatment was amplifying growth signals in the mesenchyme.

3.3. Rapamycin treatment reduces the number and proliferation of endocrine progenitors

To further evaluate the effect of mTORC1 inhibition on endocrine progenitors in the developing anlage, we assessed the Ngn3+ population at the end of three-day culture (Figure 3A—D). When compared to control, rapamycin treatment led to a significant reduction in the total number and proliferation of Ngn3+ cells (Figure 3E,F). The size of the pancreatic rudiment and number of endocrine progenitor

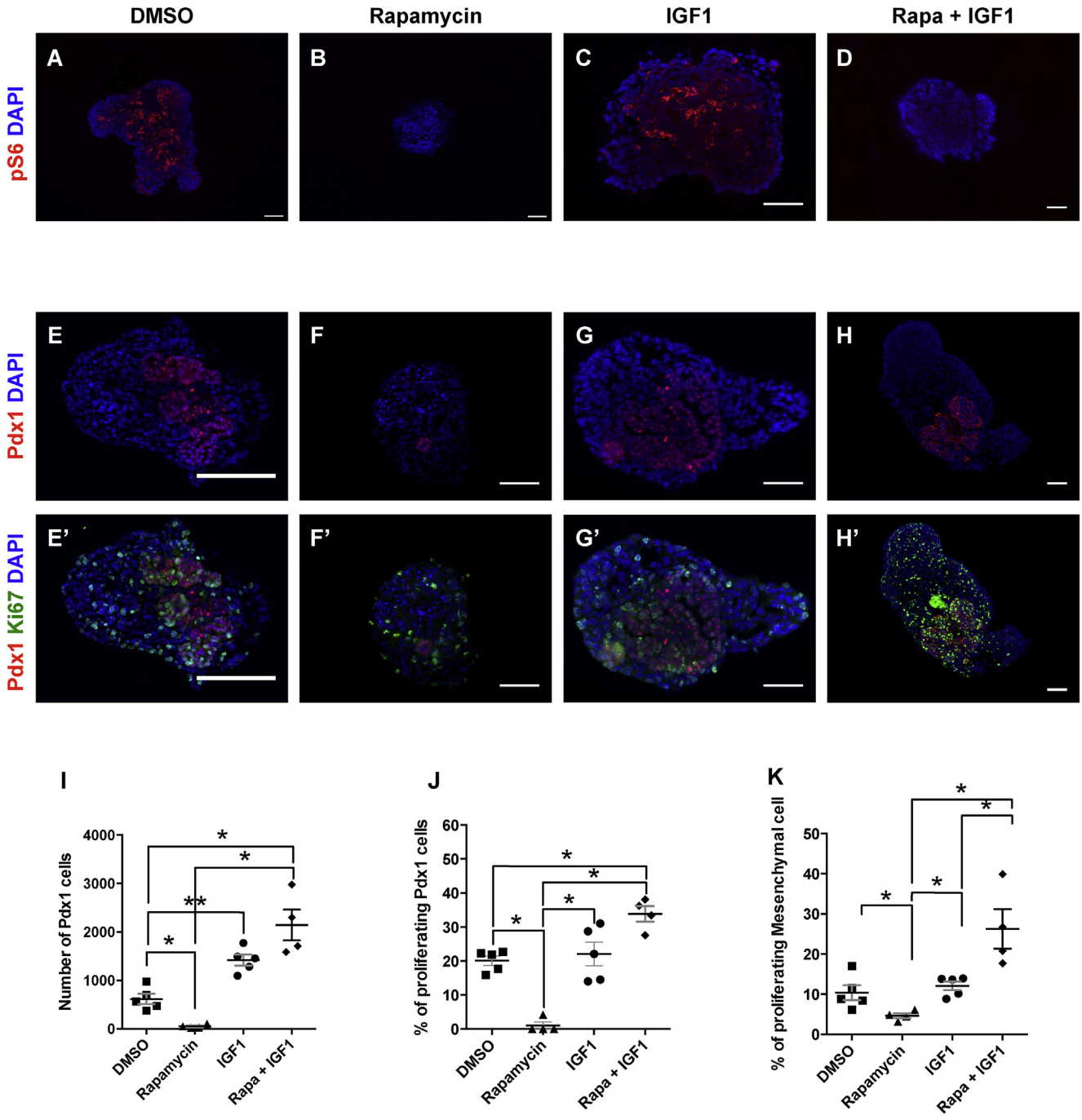

Figure 2: Inhibition of mTORC1 by Rapamycin decreases the proliferation of the Pdx1+ population. (A–D) Immunofluorescence staining for phospho-S6 (235/236) (red) of E13 pancreatic buds cultured for 3 days with either DMSO (A), rapamycin (B), IGF1 (C), or rapamycin and IGF1 (D). Nuclei were counterstained with DAPI. (E–H) Immunofluorescence staining for Ki67 (green), Pdx-1 (red) of E13 pancreatic buds cultured for 3 days with either DMSO (E), rapamycin (F), IGF1 (G), or rapamycin and IGF1 (H). Nuclei were counterstained with DAPI. (I–K) Quantification of different parameters from E13 pancreatic buds. (I) Number of progenitors cells represented by Pdx1 positive cells. (J) Percentage of proliferating Pdx1 positive cells. The percentage of proliferating mesenchymal cells is plotted in (K). Data are presented as mean ± SEM. *$p \leq 0.05$ Scale bars, 50 μm.

population was increased in the presence of IGF1, and this effect was partially attenuated by rapamycin treatment (Figure 3E, note a difference in magnification). In contrast, IGF1 treatment alone or in combination with rapamycin had no effect on Ngn3+ cell proliferation (Figure 3F).

3.4. Deletion of mTOR in Pdx1 progenitors results in pancreatic agenesis

We then sought to determine the role of mTOR *in vivo* by conditionally inactivating mTOR after E12.5 of pancreas development using the Pdx1-Cre^{late} strain (*L-Pdx1-Cre; mTOR$^{f/f}$* or *mTOR^{PANCKO}*). Efficiency of

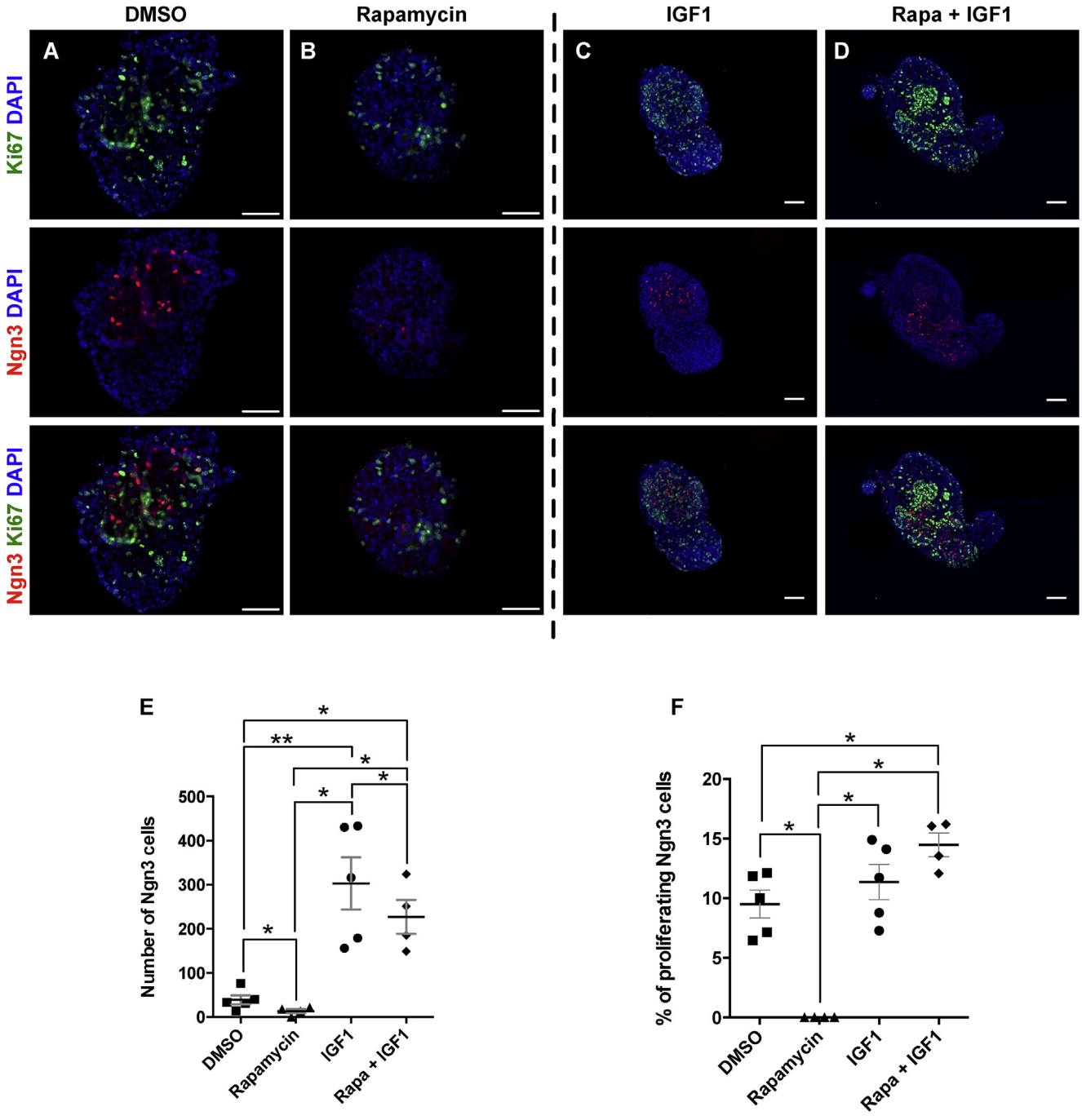

Figure 3: Inhibition of mTORC1 by rapamycin reduced endocrine progenitor proliferation. (A–D) Immunofluorescence staining for Ki67 (green) (top panel) and Ngn3 (red) (middle panel) of E13 pancreatic buds cultured for 3 days with either DMSO (A), rapamycin (B), IGF1 (C), or rapamycin and IGF1 (D). Nuclei were counterstained with DAPI and the merged pictures are shown in the lower panel. Scale bars, 50 μm. Quantification of different parameters from E13 pancreatic buds cultured in various conditions. (E) Total number of Ngn3 positive cells. (F) Percentage of proliferating endocrine progenitors. Data are presented as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.005$. Rudiment sections differ in size depending on the conditions and are not presented at the same magnification. Magnification shown for A–B is higher than that of C–D, but all scale bars are equal, at 50 μm.

the *L-Pdx1-Cre* was demonstrated by the expression of the YFP reporter in the majority of exocrine and β-cells at 1 month of age (Supp Figure 1). Some mosaicism in the efficiency of Cre-mediated recombination was observed (Supp Figure 1C, C′). In order to trace cells in which mTOR was deleted, we studied $mTOR^{PANCKO}$ animals in a CAG-YFP reporter background. $mTOR^{PANCKO}$ newborn mice appeared to be in poor general health, and about 75% failed to survive after 4 days. $mTOR^{PANCKO}$ animals displayed growth retardation, dehydration, and died by 4 days of life (Figure 4A). Pancreas dissection of mice at P4 or newborn revealed a significant reduction in pancreatic size and a fraction of these mice exhibited pancreas agenesis/hypoplasia (Figure 4C,F). Glucose levels in $mTOR^{PANCKO}$ sick newborn mice were significantly lower suggesting poor feeding (Figure 4G). These results indicate that mTOR is essential for the development of the pancreas.

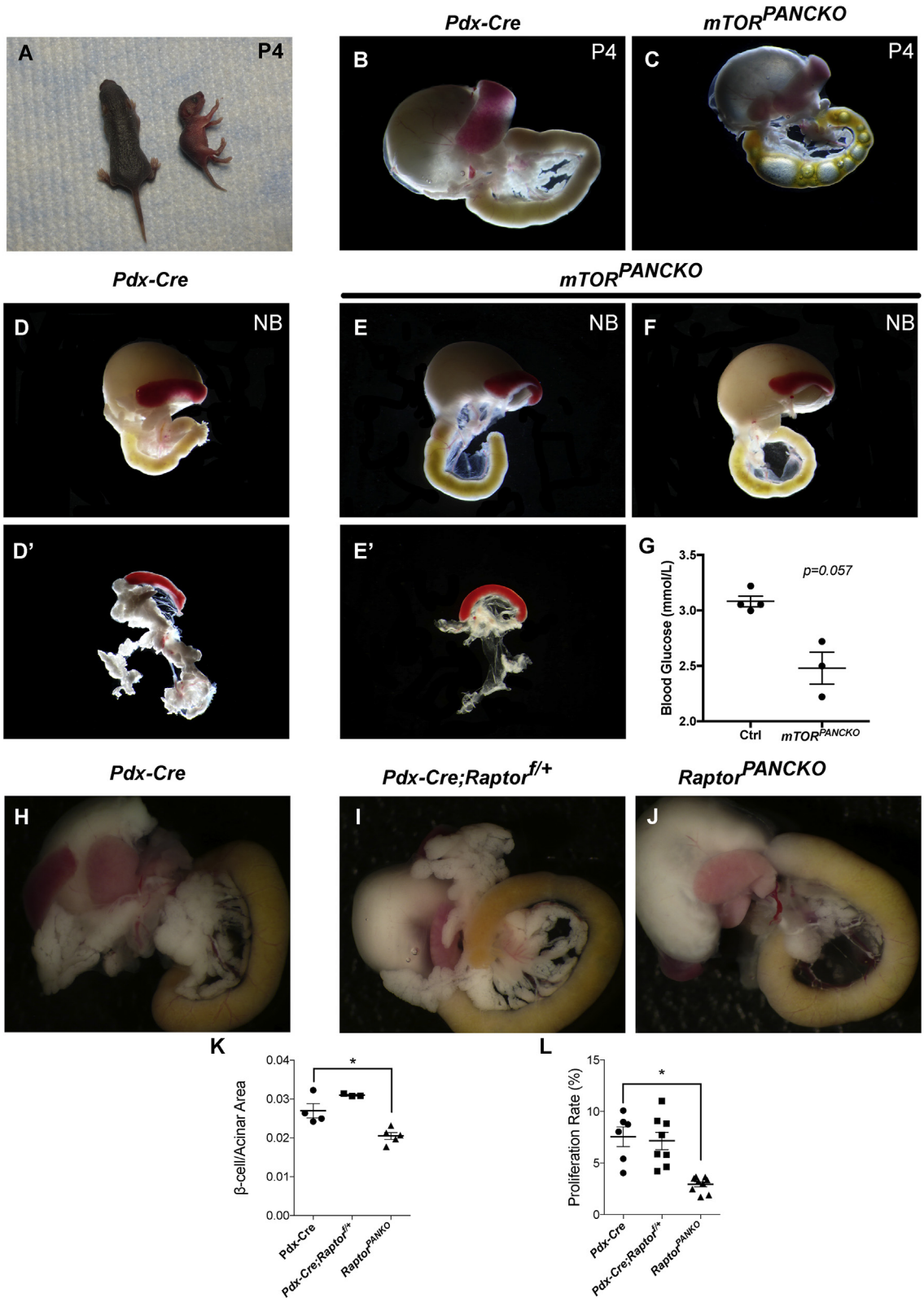

Figure 4: Conditional deletion of mTOR and Raptor in pancreatic progenitors reduced animal and pancreas size. (A) Photographs of animals 4 days after birth. Gastric tracts of post-natal day 4 animals from control (B) and *mTOR* ^{PANCKO}*; YFP* (C). (D—F) Gastric tracts of newborn animals from control (D) and *mTOR* ^{PANCKO}*; YFP* (E, F). (D′, E′) enlarged photographs of the pancreatic tissue from (D, E) (G) Random blood glucose measured at the time of dissection in control and mTOR deleted neonates. (H—J) Photographs of newborn pancreas from control and *Raptor* ^{PANCKO}. (K) β-cell/acinar area and proliferation rate in (L).

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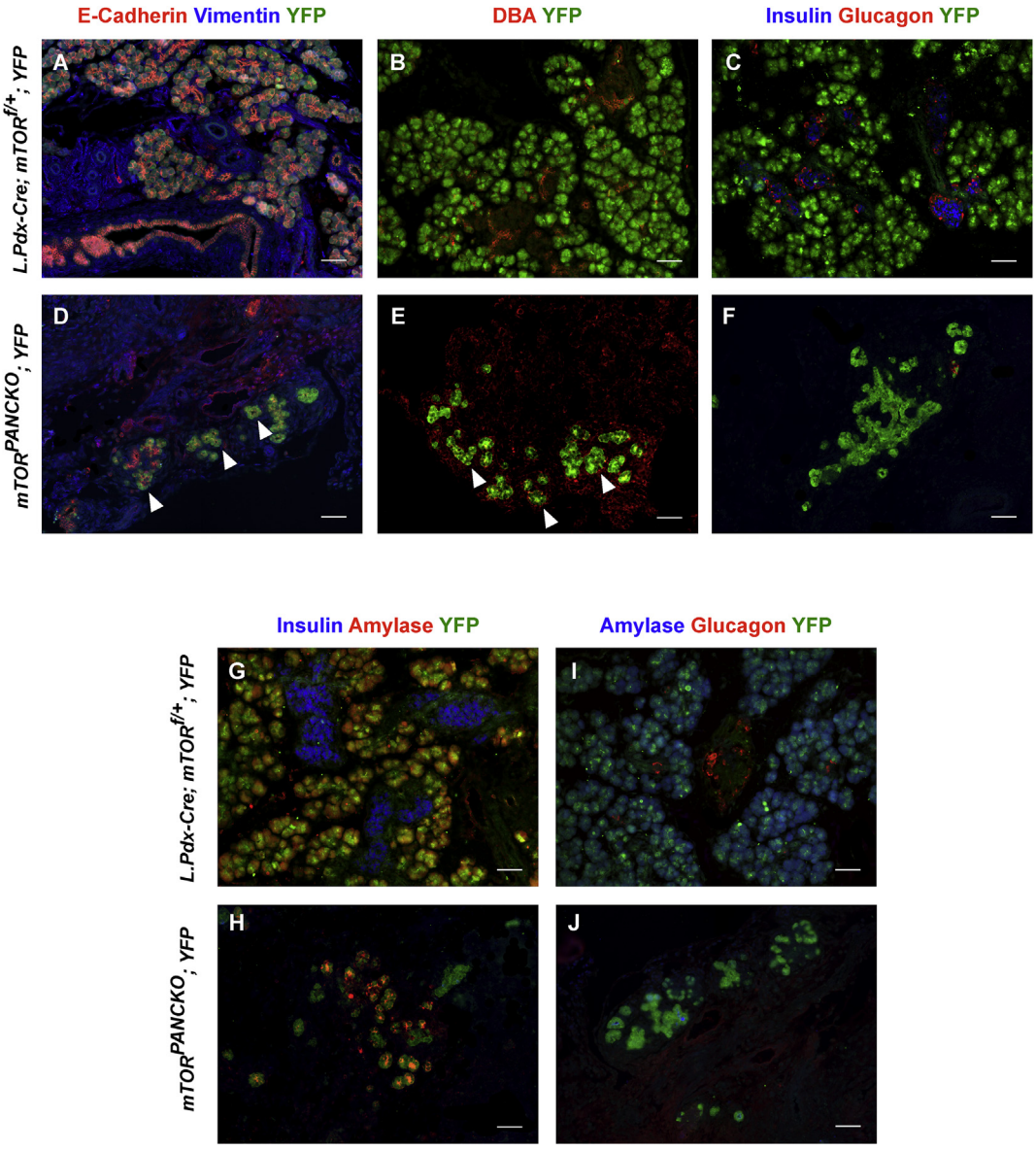

Figure 5: mTOR is key to the differentiation of the endocrine and acinar tissues in neonates. (A, D) Immunofluorescence staining for vimentin (blue), E-cadherin (red), and YFP (green) of newborn pancreata. (B, E) staining for DBA (red) and YFP (green). (C, F) Immunofluorescence for insulin (blue), glucagon (red) and YFP (green). Newborn pancreata from *L.Pdx-Cre; mTOR* $^{f/+;}$ *YFP* (A—C) and *mTOR* $^{PANCKO;}$ YFP (D—F). (G, H) Immunofluorescence staining for insulin (blue), amylase (red) and YFP (green). (I, J) staining for amylase (blue), glucagon (red), and YFP (green). Neonatal pancreata from *L.Pdx-Cre; mTOR*$^{f/+;}$ *YFP* (G, I) and *mTOR* $^{PANCKO;}$ YFP (H, J). Scale bars, 50 μm.

3.5. Deletion of Raptor in Pdx1 progenitors reduces pancreas size at birth

Since mTOR is a major component of the mTORC1 complex, and rapamycin treatment *in vitro* had major effects on pancreas development *in vitro*, we then assessed the extent to which mTOR regulates pancreas development through formation of the mTORC1 complex. To do this, we developed mice with conditional deletion of raptor in pancreas progenitors. No changes in body weight in neonates were observed in *Raptor*^{PANCKO} (data not shown). Raptor deficiency in pancreatic progenitors resulted in reduced pancreas size (Figure 4H—J). However, the reduction in pancreas size resulted in a decrease in β-cell/acinar ratio and β-cell proliferation in *Raptor*^{PANCKO} mice (Figure 4K,L). Deletion of one Raptor allele was not sufficient to alter pancreas size, β-cell/acinar ratio or β-cell proliferation (Figure 4K,L).

3.6. mTOR is essential for differentiation of the endocrine and acinar compartments

In order to further characterize the impact of mTOR deletion in pancreatic progenitors, pancreatic histology of *mTOR*^{PANCKO} mice was then analyzed at birth. Deletion of one copy of mTOR in Pdx1 progenitors had no effect on animal size or animal survival (data not shown). Examination of the neonatal pancreas from *L-Pdx1-Cre; mTOR* $^{f/+;}$ *YFP* mice showed normal pancreas architecture for this stage of development with mosaic YFP expression, islets, acinar cells, and significant ductal structures (Figure 5A—C, G, I). Therefore, heterozygote animals (*L-Pdx1-Cre; mTOR* $^{f/+}$; *YFP*) were used as controls for our histological analysis. Control mice displayed normal differentiation with conserved acinar, ductal, and islet architecture (Figure 5A—C, G, I). The YFP reporter was also expressed in all

pancreatic populations, as shown by YFP, E-cadherin, amylase, glucagon insulin, and DBA staining (Figure 5A–C,G,I). In the *mTOR*^{PANCKO}*; YFP* animals, neonatal pancreas exhibited YFP+ cells organized into epithelial structures that also stained positive for E-cadherin (Figure 5D, see arrowheads) and also expressed the ductal marker DBA (Figure 5E, see arrowheads). Most of the remaining tissue was of mesenchymal nature and expressed vimentin (Figure 5D). Further characterization of the neonatal pancreata from *mTOR*^{PANCKO} mice indicated that β- and α-cells were totally absent in these animals (Figure 5F,H,J) as opposed to the control littermates (Figure 5C,G,I). Very few YFP-negative α-cells were observed indicating that these cells escaped Cre-mediated recombination (data not shown). The differentiated acinar tissue assessed by amylase staining was also significantly reduced in *mTOR*^{PANCKO}*; YFP* animals (Figure 5H,J).

In the normal developing pancreas, Pdx1 expression is progressively restricted to β-cells, and most of the β-cells are positive for Pdx1 as observed in neonates from control *L-Pdx1-Cre; mTOR* $^{f/+}$; YFP mice (Figure 6A). In contrast, the number of Pdx1+ in the pancreatic epithelium was extremely rare in *mTOR*^{PANCKO} neonates (6 cells out of 3,300 YFP+ epithelial cells—Fig. 6B). Interestingly, the YFP+ structures observed in the *mTOR*^{PANCKO} were also negative for Pdx-1 expression. However, when analyzing the expression of other transcription factors critical for pancreas development, we observed staining for GATA 4 and 6 as well as Sox9 expression in ductal structures from *mTOR*^{PANCKO} neonates (Figure 6C–H, see enlarged pictures for the *mTOR*^{PANCKO} animals). Interestingly, in the *mTOR* ^{PANCKO}, Sox9 and GATA6 were expressed diffusely over the YFP+ ductal structures whereas GATA4 expression was mainly observed at the tips of the ductal structures.

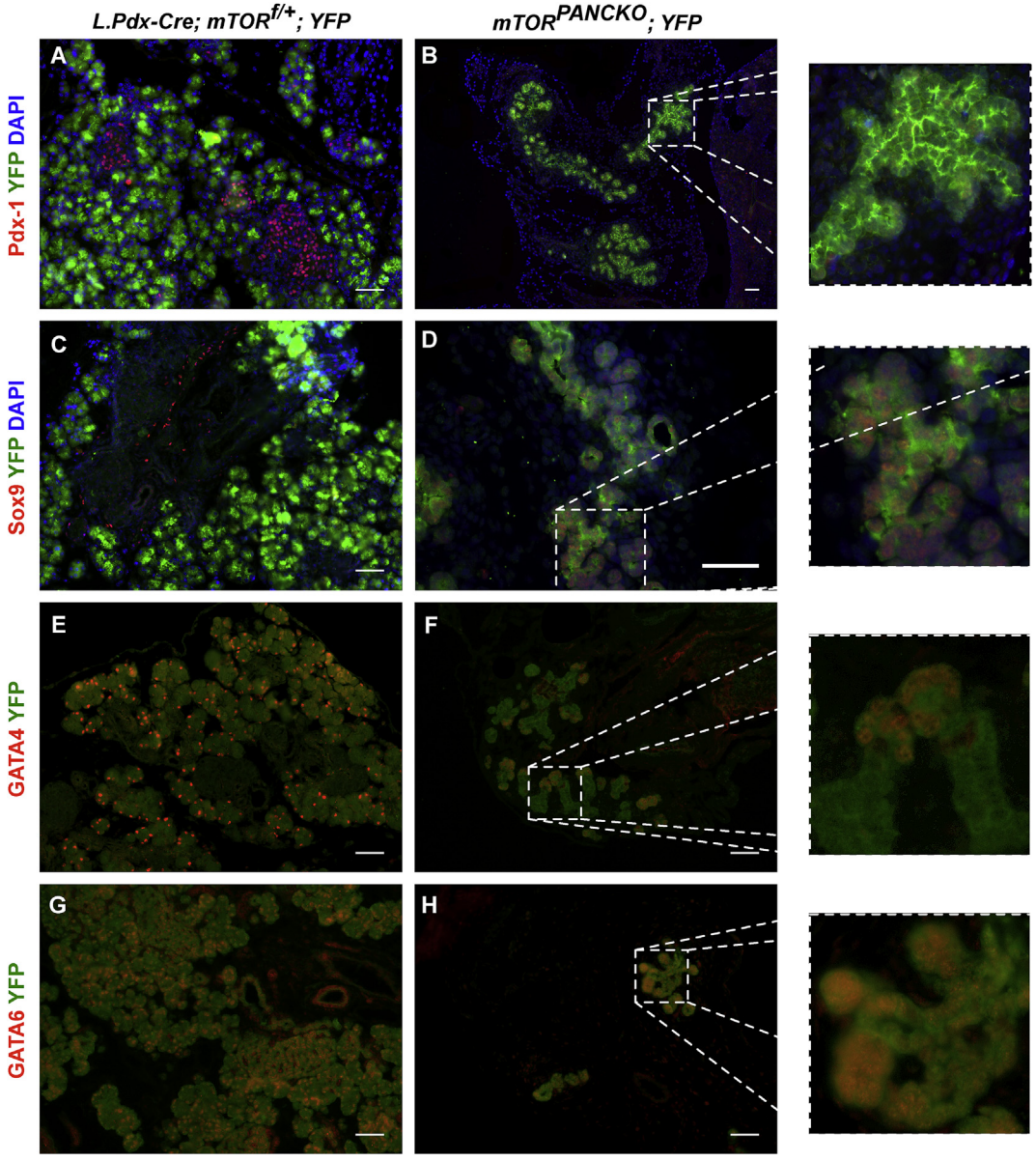

Figure 6: mTOR is required for the expression of Pdx-1 but dispensable for the expression of other transcription factors. (A, B) Immunofluorescence for Pdx-1 (red) and YFP (green). Nuclei were counterstained with DAPI. (C, D) Immunostaining for Sox9 (red), and YFP (green). (E, F) Immunofluorescence for Gata4 (red) and YFP (green). (G, H) Staining for GATA 6 (red) and YFP (green). (A–D) Nuclei were counterstained with DAPI. Neonatal pancreata from *L.Pdx-Cre; mTOR* $^{f/+;}$ *YFP* are shown in panels (A, C, E, G) and *mTOR* ^{PANCKO}; YFP in panels (B, D, F, H). Scale bars, 50 μm.

3.7. Characterization of the pancreas from four day old *mTOR*^{PANCKO} mice

At postnatal day 4, pancreata from control animals showed expression of the YFP reporter within the acinar, ducts and endocrine tissue (Figure 7A—E). *mTOR*^{PANCKO} mice exhibited a variable penetrance of the phenotype. A quarter of the *mTOR*^{PANCKO} mice had a less severe phenotype with a reduced pancreas size but exhibited normal acinar and endocrine cells (Figure 7A′—E′). The majority of mice presented a more severe phenotype with the majority of the animals exhibiting a very small pancreas with total absence of both insulin and glucagon cells (Figure 7A″—E″). The number of YFP-positive cells was considerably reduced in the few *mTOR*^{PANCKO} mice that survive beyond this stage (Figure 7A′—E′; Figure 7A″—E″). Similar to the neonatal pancreas, few YFP+ cells were organized into monolayers of undifferentiated epithelial cells that were surrounded by mesenchymal tissue that stained positive for E-Cadherin and DBA (Figure 7D″, E″). Some *mTOR*^{PANCKO} mice exhibited a less severe phenotype with some areas of the pancreas that were less affected by mTOR deletion. Histologic examination of these areas revealed normal amylase staining and conserved islet architecture with insulin cells surrounded by glucagon positive cells (Figure 7A'-D′). Importantly, the majority of the acinar, insulin and glucagon cells failed to express YFP, suggesting that most of these cells originated from progenitors that escaped recombination (YFP-negative areas; Figure 7A′—D′). While the total number of cells was reduced, Amylase staining in YFP- cells indicated that at acinar differentiation appeared unaffected by the deletion of mTOR at P4 (Figure 7A′, A″—B′, B″).

3.8. Pancreas morphology and glucose homeostasis in four-week-old *mTOR*^{PANCKO} mice

We then examined the pancreas morphology and glucose homeostasis in the few mice that survive the first week of life. As expected, one-month-old *mTOR* $^{f/+}$; *YFP* pancreata lack YFP expression (Figure 8A,D). YFP was ubiquitously observed within the endocrine islet as well as in the acinar tissue of the control *L-Pdx1-Cre; mTOR* $^{f/+}$; *YFP* animals (Figure 8B,E), suggesting that loss of one allele of mTOR was dispensable for pancreas development and β-cell maintenance during the first few weeks of life. One month-old pancreata from *L-Pdx1-Cre; mTOR* $^{f/+}$; *YFP* and *mTOR*^{PANCKO}; *YFP* mice also exhibited normal endocrine, acinar, and ductal architectures (Figure 8C,F, arrowheads). In contrast, a very small population of YFP+ cells was detected within few islets and acinar cells in *mTOR*^{PANCKO}; *YFP* mice (Figure 8C,F, arrowheads). The remaining pancreatic tissue displayed normal cells with no evidence of recombination suggesting that cells that escaped recombination were sufficient to repopulate and allow normal pancreas formation. *mTOR*^{PANCKO}; *YFP* mice showed a slight decrease in body weight and random blood glucose at 1 month (Figure 8G,H). However, these abnormalities were transient and returned to normal by 3 months of age (Figure 8I,J). No change in glucose tolerance was observed in three-month-old *mTOR*^{PANCKO}; *YFP* animals (Figure 8K).

4. DISCUSSION

The current studies identified a role of mTOR during pancreas development and demonstrate that the pancreatic epithelium is sensitive to the concentration of amino acids. These experiments established that amino acids play a permissive role in growth factor induced proliferation during pancreas development. In addition, the use of genetic models lacking mTOR signaling confirmed the essential role of this pathway in pancreas development and further suggests that genetic abnormalities in this pathway could result in pancreatic agenesis/hypoplasia. Finally, these studies shed light into the function of circulating amino acids during fetal stages and suggest that these nutrients could have major implications in pancreas development as an important factor in fetal programming.

The first set of experiments aimed to assess the effect of different concentrations of amino acids and the cross talk with growth factor signaling on pancreatic and endocrine progenitors. An important observation of these studies is that proliferation of mesenchymal cells is less responsive to alterations in growth factor and amino acid treatments when compared to the pancreatic epithelium (Figure 1A,B). However, it is possible that signals from the mesenchyme stimulated by amino acids could indirectly regulate the proliferation of the pancreatic epithelium as previously described [27—31]. Assessment of the responses in the pancreatic epithelium demonstrated that serum starvation had no effect on Pdx1+ progenitors proliferation in embryonic rudiments in culture, implying that some of the components in RPMI medium are sufficient to maintain growth (Figure 1A). As expected, the growth factor, IGF1, led to increased proliferation of Pdx1+ precursors cells in low serum concentrations, indicating that serum wasn't required to potentiate IGF1 effects. Interestingly, low levels of leucine were sufficient to induce Pdx1+ progenitor proliferation by IGF1, suggesting that this amino acid could play permissive roles in modulating pancreatic progenitor proliferation during development (Figure 1C). Leucine has previously been shown to be the most potent AA for controlling mTOR signaling [32—34]. Surprisingly, RPMI containing low levels of leucine increased Pdx1+ precursor proliferation when compared to the complete medium, suggesting that AA concentration in RMPI could exert inhibitory effects on pancreatic progenitor proliferation. These findings are consistent with previous data showing that high concentrations of leucine (in complete RPMI media) repress β-cells differentiation in pancreatic bud cultures through the activation of the mTOR signaling pathway [20]. Together, the current evidence suggests that hyperstimulation of mTORC1 signaling by high concentrations of leucine or AA could inhibit proliferation by alterations on HIF-1α or activation of a negative feedback on IRS signaling as described [14].

In order to expand our *in vitro* observations to *in vivo* models, pregnant females were subjected to a calorie-restricted diet. Calorie restriction also reduced pancreatic precursor proliferation, and this was associated to a reduction in phospho-S6 staining, a downstream target of mTOR signaling pathway. Similarly, pharmacological inhibition of mTOR *in vitro* on embryonic rudiment by Rapamycin abolished p-S6 expression even in the presence of IGF1 (Figure 2A—D). mTOR inhibition by rapamycin treatment reduced proliferation of mesenchymal, Pdx1+ and Ngn3+ cells, suggesting that this pathway is critical for the expansion of the pool of pancreatic progenitors in this *in vitro* system (2J, K and 3F). Interestingly, IGF1 was able to induce mesenchymal cell, Pdx1+ and Ngn3+ progenitor proliferation in the presence of rapamycin indicating that the proliferative effect of IGF1 during development could be independent of mTORC1 signaling and perhaps involve the MAPK pathway as previously described [23]. An important outcome of IGF1 treatment was the increase in the number of Ngn3+ progenitors and this effect was independent of changes in proliferation (Figure 3E,F). The increased in proliferation and number of the pool of Pdx1+ progenitors that ultimately differentiated to Ngn3+ progenitors could explain this finding. However, our experimental paradigm was not designed to assess differentiation and it is possible that mTOR could also alter the differentiation program of the pancreas.

The *in vitro* results were further validated by *in vivo* conditional deletion of mTOR in pancreatic progenitors. This model confirmed a central role of mTOR signaling on the developmental program of the

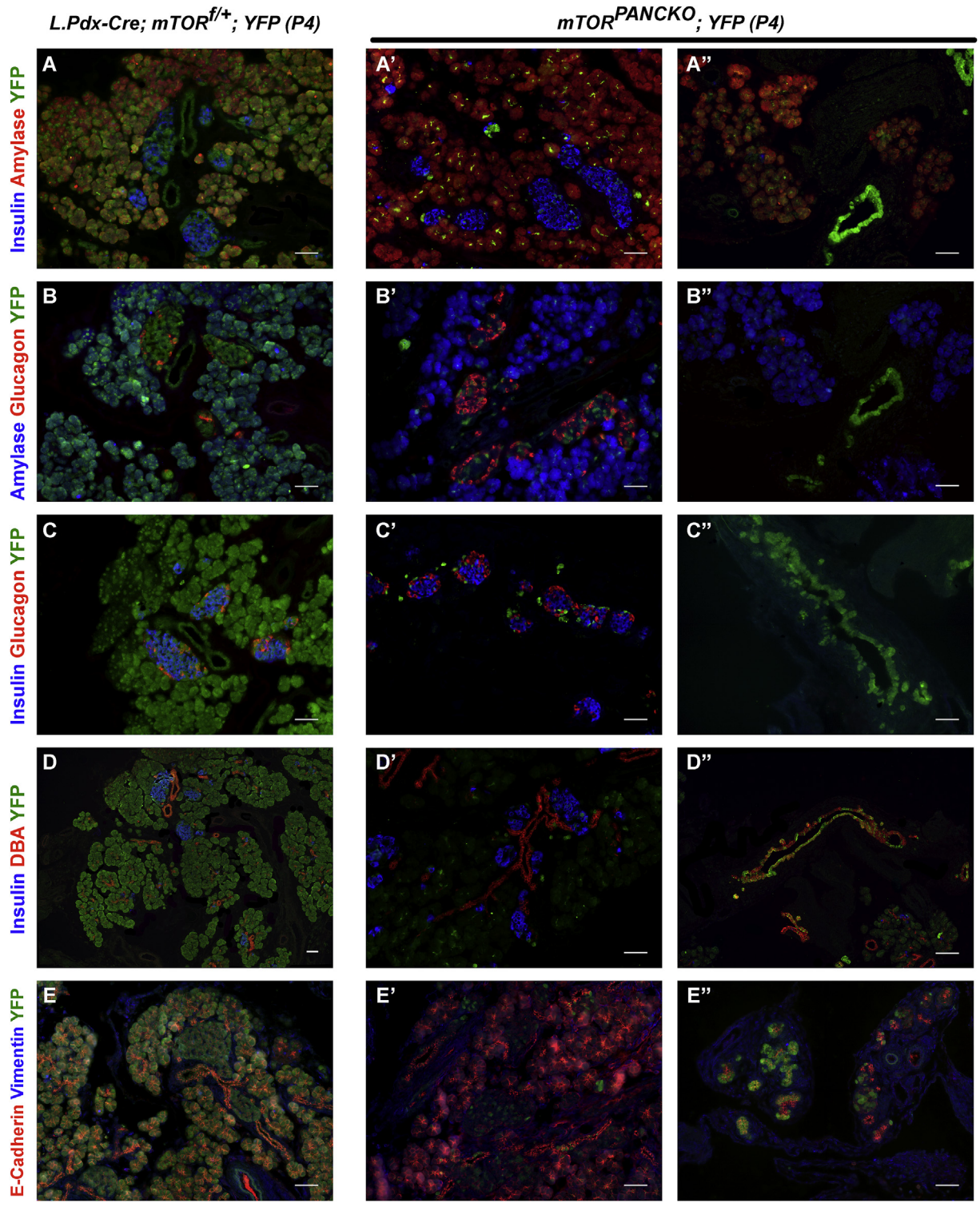

Figure 7: mTOR is required to the differentiation of the endocrine tissues after birth. (A, A′, A″) Immunofluorescence staining for insulin (blue), amylase (red) and YFP (green) of post-natal day 4 pancreata. (B, B′, B″) staining for amylase (blue), glucagon (red), and YFP (green). (C, C′, C″) staining for insulin (blue), glucagon (red), and YFP (green). (D, D′, D″) staining for insulin (blue), DBA (red), and YFP (green). (E, E′, E″) staining for vimentin (blue), E-cadherin (red), and YFP (green). Pancreata from *L.Pdx-Cre; mTOR* $^{f/+;}$ *YFP* are shown in panel (A–E). *mTOR* $^{PANCKO;}$ *YFP* pancreata with a mild phenotype are shown in panels A′–E′, while *mTOR* $^{PANCKO;}$ *YFP* pancreata with a more severe phenotype are shown in panels A″–E″. Scale bars, 50 μm.

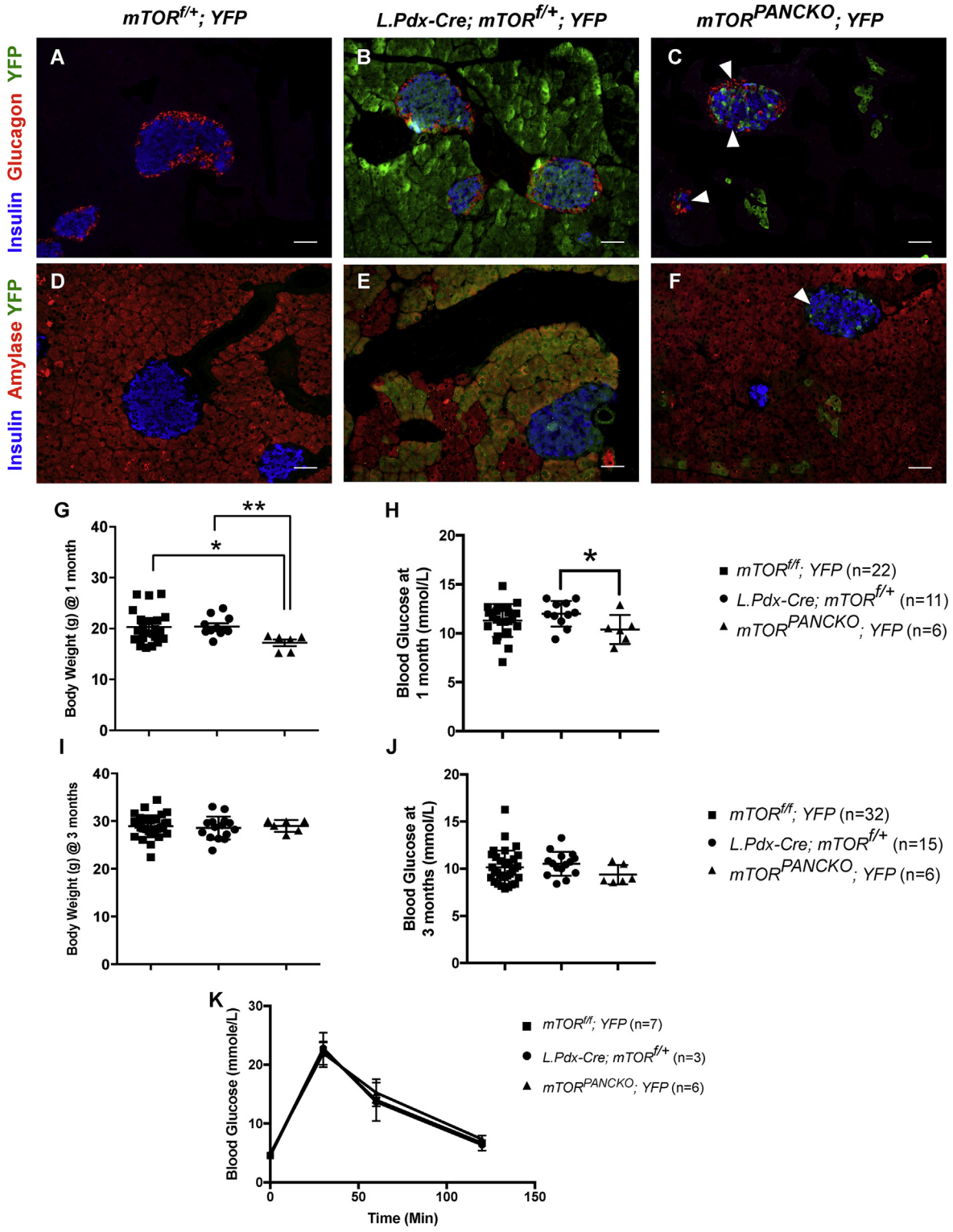

Figure 8: Adult L-Pdx1-Cre; mTOR $^{f/f}$ mice are metabolically normal due positive selection of cells that escaped recombination. Immunofluorescence staining for insulin (blue), glucagon (red), and YFP (green) of 1-month-old pancreata from controls (A), heterozygotes (B) and deleted animals (C). Immunofluorescence staining for insulin (blue), amylase (red), and YFP (green) of 1-month-old pancreata from control (D), heterozygotes (E), and deleted animals (F). (G, I) Body weight measured in control, heterozygotes, and deleted animals at 1 (G) and 3-months of age (I). (H, J) Random blood glucose levels measured in control, heterozygotes and deleted animals at 1 (H) and 3-months of age (J). (K) Intraperitoneal glucose tolerance tests were performed on control, heterozygotes and deleted animals at 3 months of age using (2 g/kg) of Dextrose after an overnight fast. Data are from males animals and presented as mean $\pm$ SEM. *p $\leq$ 0.05, **p $\leq$ 0.005. Scale bars, 50 μm.

pancreas. The macroscopic assessment of the pancreas from *mTOR*^{PANCKO}; *YFP* mice suggests that these mice exhibited pancreatic agenesis/hypoplasia. A fraction of these mice survive and exhibited normal glucose homeostasis and pancreas morphology. However, expression of the YFP reporter was absent in the pancreas of these mice, suggesting that the pancreatic progenitors that escape Cre-mediated recombination were sufficient to maintain normal pancreas development. These animals exhibited mild reductions in body weight and blood glucose at 1 month (Fig. 8G–H), but quickly catch up and are undistinguishable from control mice at 3 months of age (Fig. 8I–J). This phenotype is consistent with the mosaic expression of this mouse model as previously described [35,36]. In *mTOR*^{PANCKO}; *YFP* mice with a severe phenotype, the pancreatic rudiment in *mTOR*^{PANCKO} animals exhibited Pdx1-; YFP+ structures expressing Sox9, GATA-4, and -6 surrounded by pancreatic mesenchyme (Figures 5 and 6). No mature cells from the endocrine lineage were found. This phenotype suggests that pancreatic progenitors in which mTOR was deleted failed to progress through the maturation program and remained undifferentiated. This phenotype is similar to other mouse models of pancreatic agenesis/hypoplasia by deletion of Pdx1 and Gata4 and 6 [37,38]. The phenotype of pancreatic agenesis implies that genetic abnormalities in components that regulate this pathway could potentially cause pancreatic agenesis/hypoplasia in humans. Mutations in Pdx1, Gata4, and Gata6 have been associated to pancreatic agenesis, and we hypothesized that mTOR signaling could regulate these genes. Staining for Gata4 and Gata 6 was conserved in *mTOR*^{PANCKO}; YFP mice suggesting that expression of these transcription factors was conserved in cells lacking mTOR. Interestingly, Pdx1 staining was absent in ductal structures from *mTOR*^{PANCKO}; YFP mice suggesting that this could be a mechanism implicated in pancreas agenesis/hypoplasia in these mice. Further experiments could be designed to assess how mTOR signaling modulates Pdx1 expression. Finally, Raptor deficiency in pancreatic progenitors resulted in reduced pancreas size and reduced β-cell/acinar ratio and β-cell proliferation, suggesting that mTORC1 activity in the pancreatic epithelium plays a major role in pancreas development. We have not followed these mice beyond the neonatal stage, but Raptor deletion in β-cells results in severe diabetes by 3 months (unpublished data).

In summary, we have demonstrated a crucial role for mTOR in pancreas development and differentiation. The *in vitro* studies illustrated that mTOR plays a major part in the nutrient sensing pathway. This pathway is activated in the response to growth factor and specific amino acids and controls pancreatic progenitor proliferation. The *in vivo* data illustrated that the same processes control pancreatic development in models of calorie restriction. In the most extreme situations, complete inactivation of mTOR eventually leads to pancreatic agenesis.

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CONFLICT OF INTEREST

The authors have nothing to disclose.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.03.010.

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