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ARTICLE

Exposure of mouse embryonic pancreas to metformin enhances the number of pancreatic progenitors

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Abstract
Aims/hypothesis Developing beta cells are vulnerable to nutrient environmental signals. Early developmental processes that alter the number of pancreatic progenitors can determine the number of beta cells present at birth. Metformin, the most widely used oral agent for treating diabetes, alters intracellular energy status in part by increasing AMP-activated protein kinase (AMPK) signalling. This study examined the effect of metformin on developing pancreas and beta cells.
Methods Pancreatic rudiments from CD-1 mice at embryonic day 13.0 (E13.0) were cultured with metformin, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, an AMPK activator) or vehicle control in vitro. In another set of studies, pregnant C57BL/6 mice were treated with metformin throughout gestation. Embryonic (E14.0) and neonatal pancreases were then analysed for their morphometry.
Results In vitro metformin treatment led to an increase in the proliferation and number of pancreatic duodenal homeobox 1-positive ($PDX1^+$) progenitors. These results were reproduced by in vitro culture of embryonic pancreas rudiments with AICAR, suggesting that AMPK activation was involved. Similarly, metformin administration to pregnant dams induced an increase in both $PDX1^+$ and neurogenin 3-positive progenitors in the embryonic pancreas at E14.0 and these changes resulted in an increased beta cell fraction in neonates.
Conclusions/interpretation These results indicate that exposure to metformin during gestation modulates the early steps of beta cell development (prior to E14.0) towards an increase in the number of pancreatic and endocrine progenitors. These changes ultimately result in a higher beta cell fraction at birth. These findings are of clinical importance given that metformin is currently used for the treatment of gestational diabetes.

Keywords AICAR · AMPK · Developmental programming · Metformin · mTOR · Pancreas development

Abbreviations

ACC	Acetyl-CoA carboxylase
AICAR	5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AMPK	AMP-activated protein kinase
mTOR	Mammalian target of rapamycin
mTORC1	mTOR complex 1
NGN3	Neurogenin 3
PDX1	Pancreatic duodenal homeobox 1

Introduction

Type 2 diabetes is one of the most prevalent conditions affecting human health today. It is understood that both genetic and environmental factors contribute to type 2 diabetes risk [1], and one important environmental factor is maternal nutrition during pregnancy [2]. Developing beta cells have been shown to be critically sensitive to nutrient status [3–6]. Experimental models of metabolic stress during pancreatic development

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show permanent impairments in offspring beta cell mass and function [7–10]. This phenomenon, termed beta cell programming, is also seen in observational studies in humans [11].

Pancreas development begins at embryonic day (E)8.5 within a region of the endoderm [12]. Pancreatic duodenal homeobox 1-positive ($PDX1^+$) cells represent a population of progenitor cells for all mature pancreatic cells [13]. These undifferentiated precursor cells can be specified towards the endocrine lineage by the expression of neurogenin 3 (NGN3) [14]. After the expression of a cascade of transcription factors, these cells differentiate into the five endocrine cell types: alpha cells (glucagon), beta cells (insulin), delta cells (somatostatin), PP cells (pancreatic polypeptide) and epsilon cells (ghrelin) (reviewed in [13, 15]).

The mechanisms by which nutrition-related changes influence beta cell development are unclear, but signalling pathways that respond to changes in energy status are prime candidates. Mammalian target of rapamycin (mTOR) is a nutrient sensor that has been shown to be important for beta cell mass and function in rodent models [16, 17]. The role of mTOR complex 1 (mTORC1) signalling in the regulation of mature beta cell mass and proliferation has been established [18, 19]. However, an understanding of the role of this pathway in the developing beta cell is only indirect [20]. Metformin, the most widely used oral glucose-lowering agent, has been demonstrated to decrease mTORC1 activity through various mechanisms including AMP-activated kinase (AMPK) induction [21]. Metformin also acts on nutrient signalling pathways via AMPK-independent mechanisms [22, 23]. Metformin is being studied for use during pregnancy in polycystic ovary syndrome and gestational diabetes [24, 25]. However, the implications for alterations in pancreatic embryonic development induced by metformin have not been characterised. We sought to directly examine the impact of metformin on pancreatic development using both an in vitro and an in vivo approach to assess the resultant alterations in the embryonic and neonatal pancreas.

Methods

Pancreatic bud culture in vitro Pancreatic rudiments were dissected from E13.0 embryos (the morning of vaginal plug was E0.5) of CD-1 dams purchased from Charles River (Wilmington, MA, USA) according to the University of Michigan School of Medicine-approved protocols. Pancreatic rudiments were cultured, as described previously [26, 27], for 72 h in DMSO with or without 2 mmol/l metformin or 1 mmol/l 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) (Sigma-Aldrich, St Louis, MO, USA). After culture, embryonic rudiments were fixed in 3.7% formalin in PBS and pre-embedded in Histogel (Thermo Scientific, Kalamazoo, MI, USA) for paraffin embedding.

In vivo metformin programming mouse model Eight-week-old virgin C57Bl6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and adapted to control diet (D02041001B; Research Diets, New Brunswick, NJ, USA) for 3 weeks. Upon vaginal plug detection female mice were given unadulterated water or water containing 5 mg/ml metformin (Sigma-Aldrich). Water was changed weekly until mice were killed. Blood glucose levels were measured using an AlphaTRAK blood glucose meter (Abbott Laboratories, Abbott Park, IL, USA).

Metformin quantification in mouse plasma Metformin was quantified using HPLC with UV detection. Three hundred microlitres of calibrators, controls and samples were mixed with 30 μl of 10 μg/ml phenformin (internal standard) and 1.0 ml methanol. Samples were vortexed and centrifuged at 3200 *g* for 10 min. Supernatant fractions were dried to residue in glass tubes, re-dissolved in 200 μl of mobile phase (35% acetonitrile, 65% 40 mmol/l KH_2PO_4, pH 4.0) and filtered using a microfilterfuge tube. A 100 μl volume was injected into the HPLC–UV system at room temperature with a flow rate of 1.0 ml/min and 234 nm wavelength of absorbance. The ratio of the peak area of metformin to the internal standard was compared against a linear regression of ratios of calibrators at concentrations of 0, 62.5, 125, 250, 1000, 2000 and 4000 ng/ml. The HPLC–UV system consisted of a Dionex Omnipac PCX 500 column (4.6×250 mm) (Thermo Fisher Scientific, Sunnyvale, CA, USA) and a Waters 2487 UV detector, 717 autosampler and 515 HPLC pump (Waters Corporation, Milford, MA, USA).

Morphometric analysis and immunostaining Pancreatic rudiments were dissected from C57Bl6 mouse embryos at E14.0. Embryonic rudiments were fixed in 3.7% formalin in PBS then pre-embedded in Histogel (Thermo Scientific) for paraffin embedding. Newborn mouse pancreases, harvested on postnatal day 1 (P1), were fixed in 3.7% formalin in PBS for 6 h before embedding.

The entire pancreatic bud and neonatal pancreases were sectioned at 5 μm thickness. For the in vitro study every other section of the bud was stained for PDX1 and KI-67 (8–16 sections counted per bud). Alternate sections were stained for NGN3 and KI-67. For the in vivo studies at E14.0, four sections were taken from each quartile of the organ. For the neonatal studies, five sections were taken at equal intervals [28]. Sections were deparaffinised, rehydrated and incubated overnight at 4°C with primary antibodies as previously described [29]. Specific primary antibodies used were insulin (guinea pig; Dako, Glostrup, Denmark), KI-67 (rabbit; Vector Laboratories, Burlingame, CA, USA), E-cadherin (mouse;

BD Biosciences, San Jose, CA, USA), PDX1 (rabbit; Millipore, Temecula, CA, USA), NGN3 (mouse; Beta Cell Biology Consortium, www.betacell.org), phospho-S6 (rabbit, Ser240; Cell Signaling, Danvers, MA, USA) and these were followed by secondary antibodies conjugated to FITC, aminomethyl coumarin acetate (AMCA) or Cy3 (Jackson Immunoresearch, West Grove, PA, USA). TUNEL staining was performed using the ApopTag kit (Millipore, Billerica, MA, USA). Images were acquired using a Leica DM5500B fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

For neonates, the areas of the pancreatic section and of insulin-positive tissue were assessed in five independent sections. For proliferation analysis at E14.0, KI-67-positive (KI-67$^+$) nuclei were hand-counted from four sections. The total number of nuclei was counted using ImageJ64 software (imagej.nih.gov/ij/) [30]. In neonates (n=4 or 5 per group), a total of 1000–3000 beta cells were manually counted from three sections. The number of mesenchymal cells was calculated by subtracting the total number of PDX1$^+$ cells from the total number of cells staining positive for DAPI (DAPI$^+$). For determination of cell size at E14.0 the area of each E-cadherin-positive (E-cadherin$^+$) cell was directly measured in 25 cells per bud from pancreatic sections using the magic wand tool in Photoshop CS4 (Adobe, San Jose, CA, USA) to assess the area of the cytoplasm delimited by the E-cadherin staining.

Immunoblotting Immunoblotting was performed as previously described [20, 31]. Briefly, whole embryonic pancreases at E13.0 pooled from three dams were cultured in collagen for 3 days, then removed from collagen gel and immediately suspended in RIPA lysis buffer and sonicated. Thirty micrograms of protein lysates were used for the western blots using phospho-S6 (p-S6, Ser 240) and phosphorylated acetyl-CoA carboxylase (p-ACC) (Cell Signaling) antibodies. Mouse Cyclophilin B (Fisher Scientific, Pittsburgh, PA, USA) was used as a loading control.

Statistical analysis Statistical significance was assessed by the Mann–Whitney test (U test) or t test, where appropriate, using GraphPad Prism (version 6.0c; GraphPad Software, La Jolla, CA, USA). Results were considered significant with a p value<0.05.

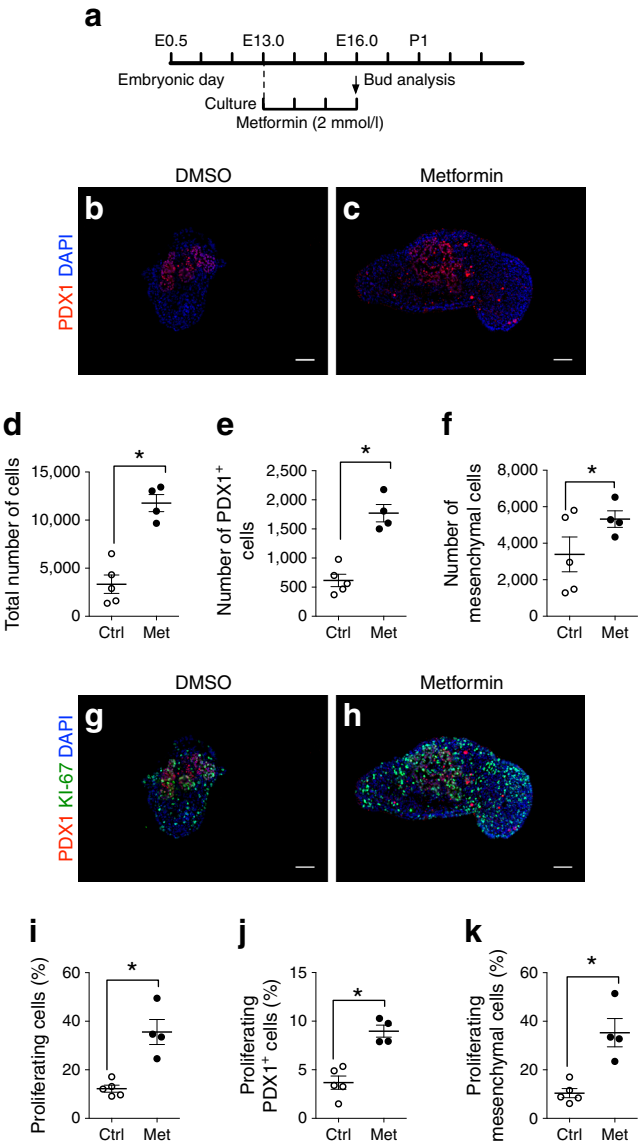

Fig. 1 Metformin increases the number and proliferation of pancreatic progenitors in vitro. (**a**) Schematic representation of in vitro experiments. (**b**, **c**) E13.0 buds cultured for 72 h with DMSO (**b**) or metformin (**c**) and stained for PDX1 (red) and a nuclear marker, DAPI (blue). (**d**–**f**) Quantification of the total cell number (**d**) and number of PDX1$^+$ cells (**e**) and mesenchymal cells (**f**). (**g**, **h**) E13.0 pancreatic buds exposed to DMSO (**g**) or metformin (**h**) and stained for PDX1 (red), KI-67 (green) and DAPI (blue). (**i**–**k**) Overall proliferation rates at the end of the culture (**i**), of PDX1 cells (**j**) and mesenchymal cells (**k**). *p<0.05 for the indicated comparison; scale bars, 50 μm. Ctrl, control (DMSO); Met, Metformin

Results

Metformin increases the number of pancreatic progenitors in vitro To assess the effect of metformin during embryonic development, E13.0 pancreatic rudiments were cultured with metformin (2 mol/l) or vehicle (DMSO) for 72 h (Fig. 1a). Buds exposed to metformin were strikingly larger than control buds (Fig. 1b, c). The total number of cells counted throughout the bud was higher in the metformin-exposed group (Fig. 1d). To determine the cause of the size difference, we examined the number of PDX1$^+$ progenitors and found it to be increased in the metformin-exposed buds (Fig. 1e). Exposure to metformin also increased the number of mesenchymal cells (Fig. 1f).

We next assessed the contribution of proliferation to the increased cell populations observed. Metformin-exposed pancreatic buds displayed an increased proliferation rate compared with controls (Fig. 1g–i). The metformin-exposed rudiments also showed an increase in PDX1$^+$ (Fig. 1j) and mesenchymal cell proliferation (Fig. 1k). These studies suggest that metformin increases the size of the pancreatic bud by increasing the number and proliferation of mesenchymal and epithelial cells.

Metformin decreases the number of endocrine progenitors in vitro We then examined the effect of metformin on the number and proliferation of the endocrine progenitor NGN3 cells. The fraction of NGN3-positive (NGN3$^+$) cells was decreased in the metformin-exposed rudiments compared with controls (Fig. 2a–c). Proliferation of the NGN3$^+$ progenitors at the end of the culture, however, was unchanged (Fig. 2d–f). This suggests that the increase in proliferation of PDX1$^+$ progenitors delays the differentiation to NGN3$^+$ cells.

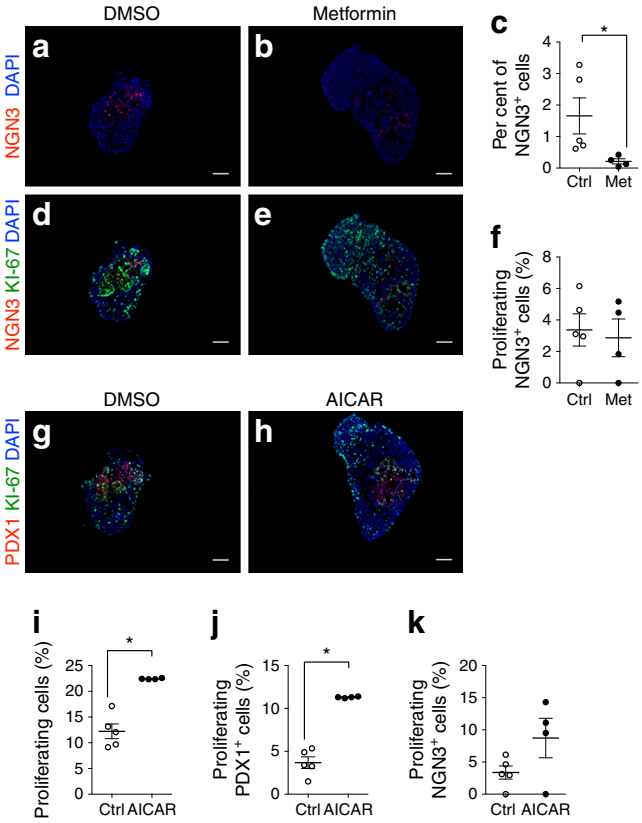

Fig. 2 Metformin decreases endocrine progenitors in vitro and AICAR increases PDX1$^+$ progenitor proliferation in vitro. (**a**, **b**) E13.0 control (DMSO-treated) (**a**) or metformin-treated (**b**) buds stained for NGN3 (red) and DAPI (blue). (**c**) The fraction of NGN3$^+$ cells. (**d**, **e**) Staining for NGN3 (red) and KI-67 (green) in the control (**d**) and metformin group (**e**). (**f**) The proliferation of NGN3$^+$ cells. (**g**, **h**) E13.0 control buds (**g**) or buds exposed to AICAR (**h**) stained for PDX1 (red), KI-67 (green) and DAPI (blue). (**i**–**k**) Proliferation rates at the end of the culture (**i**), of PDX1$^+$ (**j**) and NGN3$^+$ cells (**k**). *$p<0.05$ for the indicated comparison; scale bars, 50 μm. Ctrl, control; Met, metformin

AICAR increases the bud size and rate of proliferation in vitro Metformin regulates multiple intracellular processes by inducing changes in signalling pathways including AMPK and mitochondrial function. To understand the mechanisms responsible for the effect of metformin on pancreas development, we assessed the contribution of AMPK by culturing pancreatic buds with AICAR, an AMPK activator. Buds exposed to AICAR were larger than those exposed to DMSO control (Fig. 2g, h), similar to the findings with metformin. The overall proliferation rate of bud cells exposed to AICAR was increased compared with controls (Fig. 2i). PDX1$^+$ cell proliferation was also increased, as seen with metformin (Fig. 2j). Thus, treatment of embryonic pancreas rudiments with an AMPK activator had a similar effect on bud size, PDX1$^+$ cell number and proliferation to that of treatment with metformin. Also similar to the results with metformin there was no change in the proliferation rate of the NGN3$^+$ cells (Fig. 2k).

Metformin exposure in utero does not alter birth variables The previous studies showed that metformin had a significant effect on the growth of the embryonic pancreas in vitro. We next sought to determine whether metformin administration in vivo recapitulated these changes. Metformin was given to pregnant dams in drinking water at a dose that had been demonstrated to yield a blood level in the human therapeutic range (equivalent to 0.03 mmol/l) [32, 33]. Pregnant dams were exposed to metformin throughout gestation, from E0.5 (day of the vaginal plug) to delivery of the pups (Fig. 3a). The weight gain of metformin-treated dams was equal to that of the control-treated dams and the two groups displayed a similar change in blood glucose at G14.0 (Fig. 3b, c). There was no difference in litter size between the two groups (Fig. 3d). Plasma analysis revealed that metformin levels were in the upper therapeutic range in dams at delivery, and at the lower therapeutic limit in neonates (Fig. 3e).

Gestational metformin exposure increases the number of pancreatic progenitors in vivo Given the augmentation in pancreatic bud size and proliferation of pancreatic progenitors seen in vitro, we then looked for alterations in pancreatic development at E14.0. E14.0 was selected as a time when there is a high number of undifferentiated PDX1$^+$ cells and when NGN3$^+$ cells begin appearing in increasing numbers, just before the secondary transition for endocrine differentiation [34]. Metformin was administered to pregnant dams from E0.5 to E14.0, at which point embryonic pancreases were harvested. Upon microscopic examination of the E14.0 embryonic pancreases, metformin-exposed buds were again noted to be larger than controls (Fig. 4a, b). The total number of cells across the bud was significantly higher in the metformin-exposed pancreases (Fig. 4c). Assessment of the total number of PDX1$^+$ cells showed this to be significantly increased in the

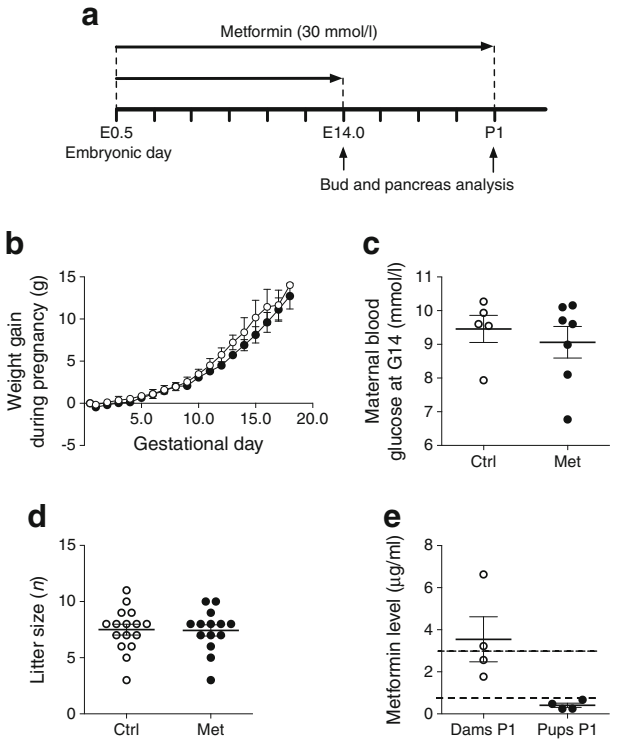

Fig. 3 Characteristics of dams and offspring after gestational exposure to metformin. (**a**) Schematic representation of in vivo experiments. (**b**) Gestational weight gain, from baseline, in control (open circles) or metformin-treated (black circles) dams (n=5). (**c**, **d**) Maternal blood glucose at G14.0 (**c**) and litter size (**d**). (**e**) Metformin level in pregnant dams and offspring. The dashed lines indicates the therapeutic window of metformin. Ctrl, control; Met, metformin

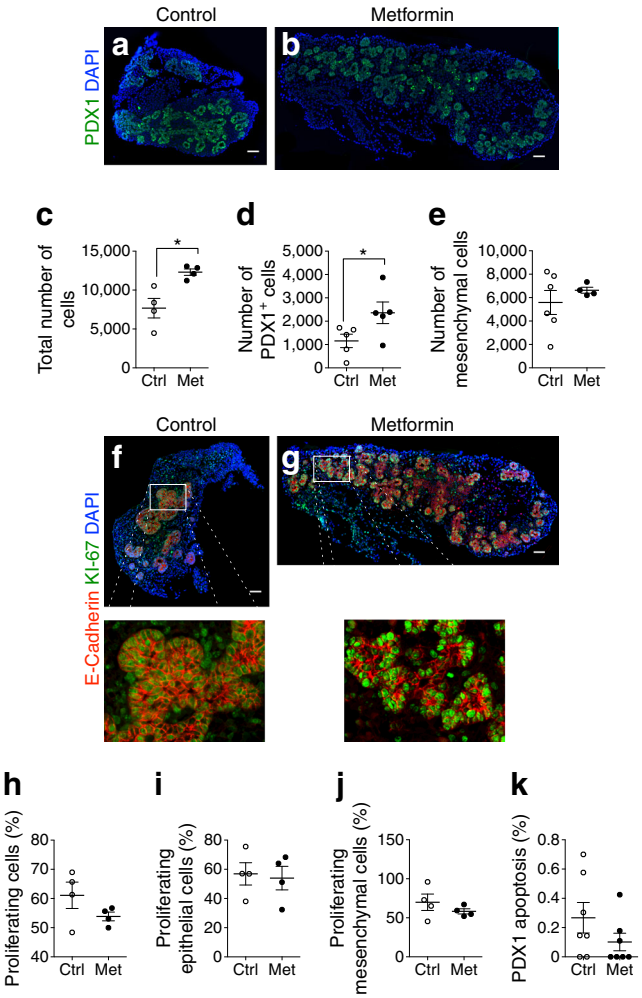

Fig. 4 Metformin increases bud size and number of pancreatic progenitors in vivo. (**a**, **b**) E14.0 embryonic pancreas, unexposed (**a**) or exposed to metformin (**b**), was stained for PDX1 (green) and DAPI (blue). (**c**–**e**) Results for: total number of bud cells (**c**); number of PDX1$^+$ cells (**d**) and number of mesenchymal cells (**e**). (**f**, **g**) E14.0 control (**f**) and metformin-exposed buds (**g**) stained for E-cadherin (red), KI-67 (green) and DAPI (blue), with higher-magnification views also shown. (**h**–**j**) Proliferation rates for: total bud cells (**h**); epithelial cells (**i**) and mesenchymal cells (**j**). (**k**) Apoptosis rate for PDX1$^+$ cells as measured by TUNEL. *p<0.05 for the indicated comparison; scale bars, 50 μm. Ctrl, control; Met, metformin

metformin-exposed group (Fig. 4d). The total number of mesenchymal cells, however, was not altered by exposure to metformin (Fig. 4e).

The rate of proliferation was then quantified in the different compartments of the embryonic pancreas. Overall, the percentage of proliferating cells in the embryonic rudiment was the same in both conditions (Fig. 4f–h) and there was no significant difference in the proliferation rates of the epithelial (E-cadherin$^+$ cells, Fig. 4i) or mesenchymal cells between the groups (Fig. 4j). We also assessed the contribution of apoptosis during development to the changes induced by metformin but found no difference in apoptosis of the PDX1$^+$ progenitors (Fig. 4k). Thus, exposure of the developing pancreas to metformin in vivo does not alter the rate of proliferation or apoptosis when assessed at E14.0.

Gestational metformin exposure increases the number of endocrine progenitors in vivo We next examined the effect of metformin on the endocrine precursors and found that the total number of NGN3$^+$ endocrine progenitors across the bud was significantly increased in the metformin-exposed group compared with the control group (Fig. 5a–c). NGN3$^+$ cells did not display a higher proliferation rate at E14.0 when the mother had received metformin (Fig. 5d–f).

Gestational metformin exposure alters intracellular signals downstream of mTORC1 To find out whether the changes observed in the developing pancreases could be due to altered mTOR signalling, we assessed the expression of phosphorylated ribosomal protein S6 (p-S6) at E14.0 on embryonic tissue sections from metformin-exposed animals. While this approach is not quantitative, pancreases from the metformin-exposed embryos displayed increased staining intensity for p-S6 (Fig. 6a, b). The average cell area of the epithelial (E-cadherin$^+$) cells was also increased (Fig. 6c–e), as would

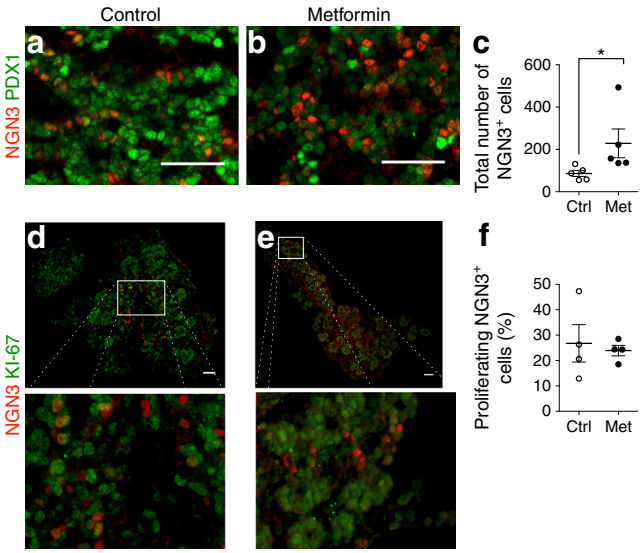

Fig. 5 Metformin increases the number of endocrine progenitors in vivo. (**a**, **b**) E14.0 control buds (**a**) or metformin-exposed buds (**b**) were stained for PDX1 (green) and NGN3 (red). (**c**) Total number of NGN3$^+$ cells. (**d**, **e**) Staining for NGN3 (red) and KI-67 (green) in control (**d**) and metformin-exposed pancreases (**e**) at low power and high power. (**f**) Proliferation of NGN3$^+$ cells. *p<0.05 for indicated comparison; scale bars, 50 μm. Ctrl, control; Met, metformin

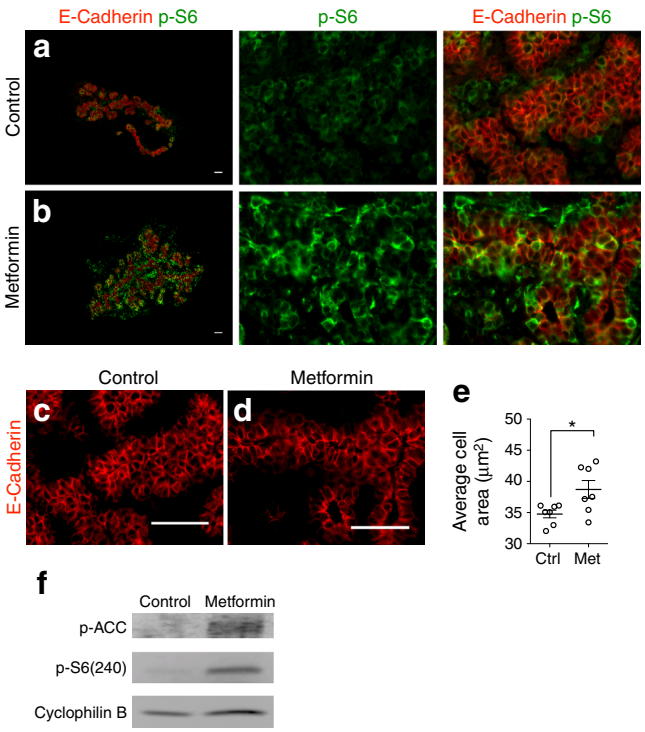

Fig. 6 Metformin exposure increases mTORC1 signalling. (**a**, **b**) E14.0 control (**a**) or metformin buds (**b**) stained for E-cadherin (red) and p-S6 (green). (**c**, **d**) High-power images from control (**c**) and metformin-exposed buds (**d**) stained for E-cadherin (red). (**e**) Results were quantified for average cell area. (**f**) Representative immunoblotting for p-ACC and p-S6 from E13.0 pancreatic buds cultured with or without metformin for 72 h. *p<0.05 for indicated comparison; scale bars, 50 μm. Ctrl, control; Met, metformin

be expected from the increased S6 activity. Finally embryonic pancreases were dissected at E13.0 and cultured with or without metformin for 72 h. At the end of the experiments, western blots indicated that there was an increase in p-ACC (known target of metformin, used as a control) and p-S6 serine 240 in the metformin-treated rudiments (Fig. 6f).

Gestational metformin exposure leads to an increase in neonatal beta cell fraction Both in vivo and in vitro experiments indicated that the developing pancreatic rudiment grows larger under metformin treatment. The in vitro experiments also led us to hypothesise that acute metformin treatment could delay the differentiation of the endocrine progenitors while significantly increasing the number of undifferentiated PDX1$^+$ progenitors. Given these findings, we sought to determine the extent to which these changes might result in altered beta cell fraction at birth.

Offspring of mothers characterised above were killed on the morning of birth (P1). Metformin-exposed dams delivered pups with no difference in birth length or weight compared with pups from control dams (Fig. 7a, b). The blood glucose was significantly decreased in metformin-exposed neonates (Fig. 7c).

The pancreases of offspring were analysed to establish the beta cell fraction and beta cell and acinar proliferation rates. Metformin exposure throughout gestation led to a significant increase in the beta cell fraction at birth in metformin vs control pups (Fig. 7d–f) but there was no increase in the overall size of the pancreas estimated by the total cross-sectional area of the representative images (Fig. 7g). Assessment of beta cell and acinar proliferation rates at birth also indicated no differences between groups (Fig. 7h, i). A summary of the findings in each set of experiments is presented in Table 1.

Discussion

The studies presented here provide surprising evidence of a previously unreported effect of metformin—modulation of the developmental programme of the pancreas. The studies using an in vitro culture system demonstrate that direct treatment of embryonic pancreas with metformin enhances the proliferation and number of PDX1$^+$ and mesenchymal cells. In the in vivo model, maternal exposure to metformin also induced the number of PDX1$^+$ and NGN3$^+$ cells at E14.0. Continuing the treatment throughout gestation led to an increase in the beta cell fraction at birth. These findings are summarised in Table 1 and suggest that a commonly used glucose-lowering agent alters the developmental programme of the embryonic pancreas in favour of increasing beta cell

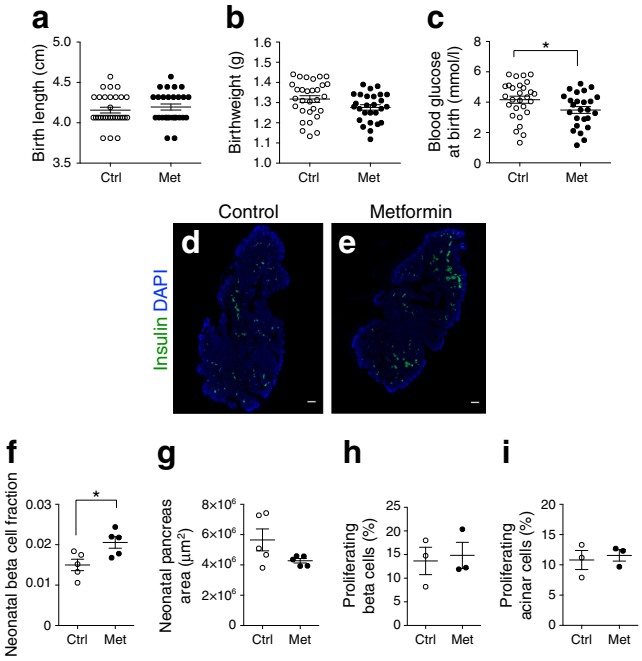

Fig. 7 Metformin exposure in utero increases beta cell fraction at birth. (**a**–**c**) Body length (**a**), body weight (**b**) and blood glucose (**c**) of control or metformin-treated offspring on their day of birth. (**d**, **e**) Staining of neonatal pancreases for insulin (green) and DAPI (blue) for control (**d**) and metformin-exposed neonates (**e**). (**f**–**i**) Beta cell fraction (**f**), neonatal pancreas area (**g**), beta cell proliferation (**h**) and acinar proliferation (**i**) were quantified. *$p<0.05$ for indicated comparison; scale bars, 50 μm. Ctrl, control; Met, metformin

fraction at birth, which may then confer protection from type 2 diabetes in adulthood. These observations are clinically relevant because of the ongoing studies of metformin for use in pregnancy [25] and suggest that metformin could have beneficial effects on beta cell mass in humans.

In the in vitro culture system exposure of the developing pancreatic buds to a high dose of metformin increased the pool of PDX1$^+$ progenitors and augmented PDX1$^+$ cell proliferation. The associated decrease in NGN3$^+$ cells may result from a delay in differentiation following the proliferation of the PDX1$^+$ progenitors, as cell cycle exit is generally linked with differentiation [35]. It is possible that a longer incubation could have had an effect on the NGN3$^+$ cell number, as pancreatic development does not occur at a typical pace in culture. Interestingly, a positive effect of metformin on the number of NGN3$^+$ cells was observed in vivo at E14.0. The discrepancy of these findings could reflect a longer exposure to metformin, since the developing pancreas in the in vivo model was exposed to metformin from the very early stages of pancreatic development (E9.5). Alternatively, it is possible that the differences could reflect the different concentrations of metformin used in vivo and in vitro.

In the in vivo studies, delivery of a pharmacologically relevant dose of metformin to pregnant dams resulted in an increase in the number of PDX1$^+$ and NGN3$^+$ cells in the bud. The increase in the number of PDX1$^+$ cells was not accompanied by increased proliferation suggesting that metformin induces proliferation of the PDX1$^+$ progenitors prior to E14.0, as demonstrated in vitro. In addition, there was no evidence that apoptosis contributed to the metformin-induced changes in PDX1$^+$ progenitors. The increase in the number of both PDX1$^+$ and NGN3$^+$ cells is likely responsible for the increased beta cell fraction in neonates. This is consistent with previous evidence demonstrating that the initial pool of PDX1$^+$ and NGN3$^+$ progenitors are major determinants of the final beta cell number [36, 37]. Interestingly, the proliferation of PDX1$^+$ cells in vitro was in marked contrast to the lack of alteration in NGN3$^+$ cell proliferation. These intriguing findings suggest that PDX1$^+$ progenitors are more susceptible than NGN3$^+$ cells to proliferative signals induced by metformin. Another possibility may be that a rise in the proliferation of NGN3$^+$ cells occurs at a later time point than was examined in this study, as the peak of NGN3$^+$ cell proliferation typically occurs at E14.5 [38]. Finally, it is possible that fetal administration of metformin induces long-lasting consequences that could alter early postnatal beta cell remodelling and the responses of beta cells to diabetogenic conditions. This could be tested by assessing beta cell proliferation and apoptosis during the first 4 weeks of life and by exposing these mice to a high-fat diet.

The in vitro experiments demonstrated that metformin increased mesenchymal cell number and proliferation. These results are particularly interesting, as the mesenchyme has been shown to secrete growth factors and mitogenic signals

Table 1 A summary of the morphologic findings in each group of experiments

Stage of development	Epithelium size (PDX1$^+$ cells)	Epithelium proliferation (E-cadherin$^+$, KI-67$^+$)	No. of endocrine progenitors (NGN3$^+$)	Endocrine progenitor proliferation (NGN3$^+$, KI-67$^+$)	Beta cell fraction (insulin$^+$)	Beta cell proliferation (insulin$^+$, KI-67$^+$)
In vitro (E13.0)	↑	↑	↓	↔		
In vivo (E14.0)	↑		↑	↔		
In vivo neonatal					↑	↔

The groups examined were embryonic pancreas in vitro at E13.0, in vivo at E14.0 and neonatal pancreas in vivo. Variables examined were epithelium size and proliferation, and endocrine progenitor number and proliferation

that regulate proliferation and differentiation of PDX1$^+$ progenitors; this may have contributed to the effects seen in PDX1$^+$ cells [36, 39, 40]. Culturing the pancreatic epithelium with metformin could allow assessment of the direct role of metformin on PDX1$^+$ cell proliferation in the absence of mesenchyme.

Metformin has been shown to induce AMPK activity, but the role of AMPK signalling in the developing endocrine pancreas has not been evaluated in detail [41, 42]. Adult animals with AMPK α1 and α2 double knockdown have no change in beta cell mass when compared with controls [43]. However, no evaluation of embryonic stages or neonatal beta cell fraction was done in that model. Here we demonstrate that modulation of these signalling pathways by metformin during embryogenesis has a beneficial effect on endocrine pancreatic development. How activation by metformin induces proliferation during developmental stages is not completely understood, but it is possible that these effects could be mediated by inducing the secretion of mitogenic factors by the mesenchyme. Metformin has been shown to have a negative impact mTOR signalling through several pathways and most often demonstrates an anti-proliferative effect on cells [23, 44–48]. In these experiments we present surprising evidence suggesting that metformin increases mTORC1 signalling in the embryonic pancreas. This positive effect of metformin has been demonstrated recently in hypothalamic tissue and suggests that the responses to metformin could be tissue and developmental stage specific [49]. The effect on PDX1$^+$ cell proliferation induced by metformin was reproduced after treatment with AICAR in vitro, providing strong evidence that this is an AMPK-dependent effect. The ability of AICAR to induce cell proliferation has also been demonstrated previously in an embryonic cell line [50]. Whether the effects of metformin on the embryonic pancreas are AMPK- and mTOR-dependent may be clarified by further studies using genetic mouse models.

The results of these studies revealed that in vivo metformin exposure during pancreatic development leads to an increase in the pancreatic beta cell population at birth. It remains to be demonstrated that this change persists into adult life. These findings provide important clinical information because metformin may be used during pregnancy, depending on the outcome of ongoing clinical trials. The relevance of this study to the clinical use of metformin in pregnancy is currently unknown. One important difference is that administration of metformin throughout pregnancy, as in the studies described here, is not often used in pregnant women. However, a potentially relevant clinical scenario is one in which women are treated for polycystic ovary syndrome from the first trimester to delivery, as described in some studies [24, 51–55]. Future studies will be required to explore the effect of dosing and timing of metformin treatment in diabetic women. The findings described here also underscore the importance of possible programming phenomena resulting from altering cellular energy status in both the mother and the developing fetus. Further examination of these programming phenomena is warranted and this may provide a strategy aimed at enhancing beta cell mass at birth in populations at risk for type 2 diabetes later in life.

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Contribution statement BG designed experiments, generated and analysed data and wrote and approved the final manuscript. LE and CC-M designed experiments, generated and analysed data, assisted with manuscript preparation and approved the final version. EA helped with experimental design and manuscript preparation and approved the final version. MS, MB-R and DG performed experiments, edited the manuscript and approved the final version. EB-M conceived the study, designed the experiments, helped to prepare the manuscript and approved the final version. EB-M is the guarantor of this work.

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