S6K1 controls pancreatic β cell size independently of intrauterine growth restriction

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Type 2 diabetes mellitus (T2DM) is a worldwide health problem that is characterized by insulin resistance and the eventual loss of β cell function. As recent studies have shown that loss of ribosomal protein (RP) S6 kinase 1 (S6K1) increases systemic insulin sensitivity, S6K1 inhibitors are being pursued as potential agents for improving insulin resistance. Here we found that S6K1 deficiency in mice also leads to decreased β cell growth, intrauterine growth restriction (IUGR), and impaired placental development. IUGR is a common complication of human pregnancy that limits the supply of oxygen and nutrients to the developing fetus, leading to diminished embryonic β cell growth and the onset of T2DM later in life. However, restoration of placental development and the rescue of IUGR by tetraploid embryo complementation did not restore β cell size or insulin levels in S6K1+/− embryos, suggesting that loss of S6K1 leads to an intrinsic β cell lesion. Consistent with this hypothesis, reexpression of S6K1 in β cells of S6K1−/− mice restored embryonic β cell size, insulin levels, glucose tolerance, and RPS6 phosphorylation, without rescuing IUGR. Together, these data suggest that a nutrient-mediated reduction in intrinsic β cell S6K1 signaling, rather than IUGR, during fetal development may underlie reduced β cell growth and eventual development of T2DM later in life.

Introduction

The common hallmark of frank type 2 diabetes mellitus (T2DM) is insulin resistance, which is initially compensated for by an increase in β cell mass and insulin production before eventually yielding to β cell failure (1). The number of diabetics worldwide is presently 347 million (2), with WHO projecting that diabetes will become the 7th leading cause of death by 2030 (3), underscoring the need for novel therapies (4). Ribosomal protein (RP) S6 kinase 1 (S6K1), a downstream effector of the mTOR Complex 1 (mTORC1) signaling pathway (5), has emerged as a potential drug target in the treatment of T2DM (6–8). In earlier studies, we demonstrated that mice deficient for S6K1 are resistant to high-fat diet–induced (HFD-induced) obesity due to increased lipolysis (9) and a lesion in adipogenesis, which we subsequently traced to an impairment in the ability of stem cells to commit to the adipocytic lineage (10). Consistent with a reduction in adiposity, as compared with WT mice, S6K1−/− mice maintained on a HFD remain insulin sensitive, despite increased glycemia (9). Increased insulin sensitivity may also result from the reduced circulating insulin levels in S6K1+/− mice, as well as the loss of a negative feedback loop mediated by S6K1 site-specific phosphorylation to elements of the insulin receptor pathway, particularly insulin receptor substrates 1/2 (IRS1/2) (5, 9). In the latter case, phosphorylation of IRS1/2 disrupts its interactions with the insulin receptor and the class 1 PI3K (11, 12), which is hypothesized to suppress glucose uptake in muscle and adipose tissue (5, 9). Consistent with these findings, liver-specific deletion of S6K1 has been recently shown to protect against HFD-induced hepatic steatosis and systemic whole-body insulin resistance, the latter being associated with reduced insulin levels and loss of the negative feedback loop in muscle and fat (13).

Despite the finding that depletion or loss of S6K1 leads to an increase in insulin sensitivity, there is a concern about the potential efficacy of S6K1 inhibitors for the treatment of T2DM. As noted above, this stems from the fact that S6K1-deficient mice are hypoinsulinemic, a phenotype which we found was not associated with the transcription, synthesis, degradation, or intrinsic secretion of insulin, but with diminished β cell size (9, 14). It is known that a decrease in β cell size has a proportionally larger negative effect on insulin secretion independent of secretory potential (15). Consistent with a role for S6K1 in this response, subsequent stud-
ies showed that targeted \( \beta \) cell expression of a constitutively active S6K1 cDNA leads to an increase in both \( \beta \) cell size and insulin secretion (16). However, at birth, S6K1–/– mice are also reduced in body size (17), a phenotype that defines intrauterine growth restriction (IUGR). IUGR is a risk factor for T2DM in adult life and is associated with reduced \( \beta \) cell function (18). IUGR affects over 5% of pregnancies, with the number of incidences progressively increasing over the past decade (19). IUGR is largely attributed to an insufficient oxygen and nutrient supply by the placenta, such that developing tissues and organs of the embryo fail to grow to their normal size (20). IUGR fetuses have reduced circulating insulin levels, impaired \( \beta \) cell function, and in more severe cases, reduced \( \beta \) cell mass (18). The impairment to \( \beta \) cell function is thought to be responsible for the increased insulin sensitivity observed in small gestational–age fetuses and newborns (21). The fetal oxygen and nutrient supply is normally actively transported from the mother to the placental trophoblasts, which develop during the early stages of pregnancy and are the first cells of the fertilized egg to differentiate. mTORC1, an upstream kinase required for S6K1 activation, is critical for the trophoblast’s development and function (22). Of note, S6K1 activity is markedly reduced in human placental tissues associated with restricted fetal growth (23). These findings and the observation that acute inhibition of S6K1 by rapamycin in \( \beta \) cells has no effect on glucose-stimulated insulin secretion (24) are consistent with IUGR, rather than an intrinsic \( \beta \) cell function, being responsible for the reduced body mass, hypoinsulinemia, and the \( \beta \) cell size deficit displayed by S6K1 deficient mice at birth (14, 17).

Given the importance of S6K1 as a potential therapeutic target in insulin resistance (8) and recent data suggesting that fetal \( \beta \) cell impairment, rather than being a consequence, is instead a cause of IUGR (25), we set out to trace the basis of the reduced \( \beta \) cell size and hypoinsulinemia observed in adult S6K1–/– mice. To address this question, we first determined \( \beta \) cell size and insulin content in S6K1–/– mouse embryos. Next, we asked which stage of S6K1–/– mouse embryonic development was affected and whether the developmental effects could be accounted for by reduced \( \beta \) cell size. Finally, employing 2 distinct genetic paradigms, we determined whether the effects on \( \beta \) cell size and hypoinsulinemia observed in adult S6K1–/– mice were due to IUGR or were intrinsic to \( \beta \) cells.

**Results**

\( \beta \) Cells from S6K1–/– embryos exhibit reduced cell size and insulin content. As limited intrauterine nutrient availability has been associated with impaired pancreatic \( \beta \) cell development (26, 27), we asked whether \( \beta \) cells are affected in S6K1–/– mice during embryogenesis. Examination of H&E-stained pancreata at E16.5 revealed clusters of endocrine cells in the pancreatic interstitium (Figure 1A). In S6K1–/– embryos, we observed a higher density of nuclei in the endocrine cell clusters, as compared with WT embryos (Figure 1A). The same \( \beta \) cell phenotype was observed in S6K1–/– mice obtained either from heterozygous or homozygous parental crosses. Analysis of tissue sections of \( \beta \) cells, immunostained for the membrane-associated GLUT2 transporter, by IF staining; (C) cleaved caspase-3 (red), insulin (green) by IF staining and DAPI (blue) staining; and (D) insulin (red) and glucagon (green), by IF staining. (E) \( \beta \) cell number in embryonic pancreata (n = 3 per genotype). (F) \( \beta \) cell size in embryonic pancreata from 45–52 determinations in WT embryos and 29–44 determinations in S6K1–/– embryos (n = 3 per genotype; \( \beta \) cell surface: WT, 95.60 ± 2.69 \( \mu \)m\(^2\); S6K1–/–, 69.54 ± 1.5 \( \mu \)m\(^2\)). (G) Reduced insulin content, normalized for body weight, in embryonic pancreata (n = 6–9 per genotype). Scale bars: 25 \( \mu \)m. In E–G, values are given as mean ± SEM. *P < 0.05, **P < 0.01 vs. WT, unpaired t test.

S6K1–/– mice during embryogenesis. Examination of H&E-stained pancreata at E16.5 revealed clusters of endocrine cells in the pancreatic interstitium (Figure 1A). In S6K1–/– embryos, we observed a higher density of nuclei in the endocrine cell clusters, as compared with WT embryos (Figure 1A). The same \( \beta \) cell phenotype was observed in S6K1–/– mice obtained either from heterozygous or homozygous parental crosses. Analysis of tissue sections of \( \beta \) cells, immunostained for the membrane-associated GLUT2 transporter, confirmed that S6K1–/– \( \beta \) cells were smaller than those from WT embryos (Figure 1B). Given that S6K1 deficiency affects apoptosis (28, 29), we examined apoptotic \( \beta \) cells by staining for cleaved caspase-3 and insulin, together with DAPI, to observe nuclei. The result revealed significant levels of cleaved caspase-3 staining in S6K1–/– \( \beta \) cells (WT 0%, S6K1–/– 17%, P < 0.008) and surrounding
tissue, whereas none was detected in WT tissue sections (Figure 1C). Morphometric analysis of pancreatic tissue sections contained for insulin and glucagon (Figure 1D) revealed a 25% and a 27% reduction in β cell number and size, respectively, in S6K1−/− versus WT embryos (Figure 1, E and F). However, there was no significant difference in the size or number of glucagon-positive α cells, suggesting a selective effect on β cells (Figure 1, E and F). The decrease in β cell size was consistent with a 50% reduction in the insulin content of S6K1−/− versus WT embryos, normalized to embryonic body weight (Figure 1G). The reduction in insulin content and β cell size during embryonic development persists throughout adult life, suggesting that the phenotype observed in adult S6K1−/− mice arises from impaired embryonic pancreatic β cell growth.

S6K1−/− mice are developmentally retarded and display IUGR. To determine if the reduction in β cell number and size observed in S6K1−/− embryos was associated with IUGR, we examined embryonic body weights from E11.5–E18.5. The results show that S6K1−/− embryos displayed IUGR as early as E12.5, with their weight reaching only 80% of that of WT embryos (Figure 2A). Previously, we demonstrated that flies deficient for the Drosophila orthologue of S6K1, dS6K, were not only severely reduced in size, but were strongly delayed in development (30). To establish whether IUGR was associated with a developmental delay in S6K1−/− embryos, we analyzed cartilage formation and bone ossification during development. We found that IUGR was accompanied by a developmental delay — as measured at E16.5 by reduced staining for cartilage and bone, with alcian blue and alizarin red, respectively — as well as by deferred chondrocyte hypertrophy in the cervical vertebrae (Figure 2B). By E17, the differences in bone ossification and bone growth were less striking but not resolved (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI77030DS1). As with the β cell growth, the effects on skeletal development were equivalent in S6K1−/− mice obtained either from maternal heterozygous or null S6K1 crosses. To determine if the delay in development was elicited at an earlier stage, we analyzed whole embryos by scanning electron microscopy at
E9.5. We found that S6K1−/− embryos had a reduced number of somites and a delay in otic pit closure, as well as the appearance of limb buds, indicating an approximate half-day delay in development (Figure 2C). Finally, we assessed preimplantation blastocysts to determine whether IUGR observed in S6K1−/− embryos reflected an intrinsic defect in embryonic development. This analysis revealed no visible anomalies in the S6K1−/− preimplantation blastocysts, with 60% of the eggs of both genotypes developing to the blastocyst stage and maturing by E3.5 (Figure 2D). Although a deficit in blastocysts cannot be excluded in contributing to S6K1−/− embryonic phenotype, the growth retardation arises at the time of, or immediately following, trophoblast differentiation and intra-uterine implantation, strongly suggesting that it appears as a function of impaired extraembryonic tissue development.

**Impaired placental development in S6K1−/− mice.** As nutritional restriction is associated with impaired trophoblast development and mTORC1 appears to play a critical role in placental function (22, 31), we questioned whether the absence of S6K1 could affect placental development. The placenta is composed of 3 distinct extraembryonic tissues largely derived from the fetus: an outer trophoblast giant-cell sheath, a middle spongiotrophoblast layer, and the innermost labyrinth (32). The labyrinth contains cells of both trophoblast and mesodermal origin that undergo branching morphogenesis to produce the large surface area required for the placental exchange of nutrients and gases (32). Morphological analysis revealed that S6K1−/− placentas weighed 12%–16% less than those of WT mice from E11.5–E13.5 (Figure 3A), whereas, the S6K1−/− embryos weighed 25%–36% less than those of WT mice during this same stage of development (Figure 2A). It is well documented that a reduction in placental size can directly impact the size of the embryo, due the placenta’s ability to transfer nutrients from the mother to the fetus (33). These observations suggested that the negative effect of the loss of S6K1 on embryonic growth may be attributed to impaired placental development (see below). Histological examination of placentas from S6K1−/−, S6K1+/−, and WT embryos showed normal morphology of the giant cells that form a layer between the maternal decidual tissue and the embryonic labyrinth (Supplemental Figure 2, A–C). However, compared with WT and S6K−/− placentas, the spongiotrophoblast layer of S6K1−/− placentas was reduced in size, as assessed by H&E staining (Figure 3, B and C, and Supplemental Figure 2, A–C, G, and H). An intact spongiotrophoblast layer is required for normal development of the labyrinth, which secretes vascular endothelial growth factor to induce normal blood vessel function (32). Moreover, H&E staining revealed that the S6K1−/− placentas appeared to have a slightly larger–sized labyrinth layer but displayed a sharp reduction in embryonic and maternal blood vessel association, with the fetal blood vessels highly dilated (Figure 3D). This result was confirmed by immunostaining of platelet/endothelial cell adhesion molecule-1 (PECAM-1) (Figure 3E and Supplemental Figure 2, D–F), again indicating a decreased vascular capacity of the labyrinth layer in S6K1−/− placentas. The decrease in blood vessel intermingling suggested restricted nutrient and oxygen exchange between the mother and the developing embryo, which could directly impact the size of the fetus (33). In addition, S6K1−/− placentas displayed improperly positioned, trophoblast-specific, Tpbbp-expressing cells in the labyrinth region at E19.5 (Supplemental Figure 2, I and J). The fact that the S6K1−/− placental phenotypes observed were alike, regardless of the parental genotype, is consistent with the trophoblast playing a dominant role in the development of the placenta (33). Taken together, these results suggest that impaired placental development, as depicted by the defects in the spongiotrophoblast and labyrinthine layers, may be responsible for IUGR of S6K1−/− mice.

**Rescue of IUGR, but not reduced β cell size, by tetraploid WT placentas.** Given the importance of the placenta in pancreatic β cell growth and development (27, 34), we reasoned that the placental
to the embryo proper (32). To initiate these studies, we derived 4 independent S6K1−/− ES cell lines (see Methods), and were able to obtain fetuses from one of them. Using this line, we obtained E13.5 S6K1−/− tetraploid rescue (S6K1−/− TR) embryos, which developed to a size larger than S6K1−/− embryos and even slightly larger than WT embryos. Both were cultured in vitro and transplanted in WT surrogate mothers (Figure 4, A–D). The larger-sized tetraploid embryo is consistent with previous studies, showing that tetraploid embryo complementation leads to increased embryonic weight, a phenotype termed hybrid vigor (37). The genotype of each tissue was confirmed by Southern blot analysis (Figure 4E). These results support the hypothesis that it is the failure of the S6K1−/− trophoblasts to differentiate properly, which leads to impaired placental development, ultimately triggering IUGR. Such a phenotype has been described in S6K1−/− mice was the cause of IUGR, resulting in impaired β cell function. To test this hypothesis, we took advantage of tetraploid embryo complementation (35), such that S6K1−/− embryonic stem cells (ES cells) were aggregated with tetraploid 2-cell-stage WT embryos. This allows one to supply a WT placenta to ES cells during embryonic development (35). The extent to which tetraploid embryo complementation can salvage IUGR is underscored by the complete rescue of a homozygous mouse derived from lethal trophoblastic mutant (36). The ensuing embryos were then transplanted into foster CD1 mothers (35). In this model, WT tetraploid cells supply the extraembryonic placental trophoblasts and yolk sac endodermic cells but are excluded from the embryo, owing to their increased ploidy. In contrast, the diploid ES cells cannot develop into trophoblast tissues and are confined to the embryo proper (32). To initiate these studies, we derived 4 independent S6K1−/− ES cell lines (see Methods), and were able to obtain fetuses from one of them. Using this line, we obtained E13.5 S6K1−/− tetraploid rescue (S6K1−/− TR) embryos, which developed to a size larger than S6K1−/− embryos and even slightly larger than WT embryos. Both were cultured in vitro and transplanted in WT surrogate mothers (Figure 4, A–D). The larger-sized tetraploid embryo is consistent with previous studies, showing that tetraploid embryo complementation leads to increased embryonic weight, a phenotype termed hybrid vigor (37). The genotype of each tissue was confirmed by Southern blot analysis (Figure 4E). These results support the hypothesis that it is the failure of the S6K1−/− trophoblasts to differentiate properly, which leads to impaired placental development, ultimately triggering IUGR. Such a phenotype has been described in S6K1−/−
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Results indicate that the IUGR displayed by vessels (Figure 4B), as well as normal weights (Figure 4D). These displayed typical WT structures with proper intermingling of blood cell number in pancreas. A total of 18 sec-β-glucagon (green), by IF staining. (E) cleaved caspase-3 (red), insulin (green), and DAPI (blue), by IF; and (F) insulin (red) and &C by H&E staining; (G) cleaved S6K1–/– transgenic expression was obtained S6K1–/– cell number, but surprisingly not β cell size, suggesting an independent role for S6K1 in β cell growth.

S6K1 affect intrinsically embryonic β cell size. Given the ability of tetraploid embryos to completely rescue placental development of ES cells of a lethal trophoblast mutant (36), it would imply that loss-of-S6K1 effects on embryonic β cell size are independent of IUGR. If this is the case, it raises the possibility that S6K1 controls this response in a cell-autonomous manner. We have previously demonstrated that Drosophila ds6k plays an intrinsic role in controlling cell size in specific tissues, including the wing and the eye (30, 39), which we also found later to be the case in mouse embryonic fibroblasts (MEFs) (40). Moreover, others have shown that targeted β cell expression of a constitutively active S6K1 cDNA leads to an increase in β cell size (16). To test this possibility, we first generated a transgenic mouse line expressing a myc-epitope–tagged S6K1 cDNA driven by the rat insulin II promoter (RIP, RIP-S6K1) (41). By breeding WT Tg(RIP-S6K1) with S6K1–/– mice, we obtained S6K1–/– Tg(RIP-S6K1) mice. Although we could not detect the myc-epitope tag by immunofluorescence, we could detect S6K1, which was coexpressed with insulin in S6K1–/– Tg(RIP-S6K1) β cells of E16.5 embryos, but not in those of S6K1–/– E16.5 embryos (Supplemental Figure 3). Reexpression of the RIP-S6K1 transgene in β cells did not rescue fetal weight (Supplemental Figure 4), whereas, in striking contrast to S6K1–/– β cells of E16.5 embryos, β cells of E16.5 S6K1–/– Tg(RIP-S6K1) embryos were equivalent in size to those derived from E16.5 WT Tg(RIP-S6K1) embryos, as assessed by either H&E or GLUT2 staining (Figure 5, A and B, respectively). Moreover, even though staining with cleaved caspase-3 revealed increased apoptosis in β cells of S6K1–/– Tg(RIP-S6K1) versus WT Tg(RIP-S6K1) embryos, apoptosis occurred mainly outside of embryonic β cells (Figure 5C). Morphometric analysis of insulin and glucagon containing of tissue sections (Figure 5D) showed that the RIP-S6K1 transgene was sufficient to rescue β cell number and size in E16.5 S6K1–/– embryos (Figure 5, E and F, respectively). The rescue of β cell number and size by RIP-S6K1 transgenic expression was paralleled by a restoration in β cell insulin content (Figure 5G).

in mouse KOs of other protein kinases (38). Consistent with the rescue being dependent on proper placental development, the placentas from E13.5 S6K1–/– TR embryos, unlike the S6K1–/– placenta, displayed typical WT structures with proper intermingling of blood vessels (Figure 4B), as well as normal weights (Figure 4D). These results indicate that the IUGR displayed by S6K1–/– embryos results from impaired placental development. To determine the impact of a WT placenta on S6K1–/– β cell development, we analyzed S6K1–/– TR embryos at E16.5. Unexpectedly, the results of H&E staining showed that the nuclei were still densely packed in the pancreas from S6K1–/– TR embryos, as compared with those from WT embryos (Figure 4F). These findings are consistent with the apparently smaller β cell size revealed by GLUT2 transporter immunostaining (Figure 4G). Morphometric analysis of tissue sections costained with insulin and glucagon (Figure 4H) confirmed that the WT tetraploid placenta did not rescue the reduction in β cell size (Figure 4I). The cell number also appeared to be slightly reduced in S6K1–/– TR versus WT embryos, though the difference was not significant (Figure 4J). Compatible with the reduction in β cell size, insulin content was still strongly reduced in S6K1–/– TR versus WT embryos (Figure 4K). Thus, the apparent reversion of the IUGR deficit in the tetraploid rescue, as determined by the restoration of a normal placenta phenotype (Figure 4B), does not appear to rescue the β cell size deficit (Figure 4, F–I). The placenta derived from WT trophoblasts also appears to rescue S6K1–/– TR β cell number, but surprisingly not β cell size, suggesting an independent role for S6K1 in β cell growth.

Figure 5. Pancreatic β cell–specific expression of S6K1 restores diminished β cell size and insulin content in S6K1–/– embryos. (A–D) Pancreas sections from WT Tg(RIP-S6K1), S6K1–/–, and S6K1–/– Tg(RIP-S6K1) embryos at E16.5: (A) histology by H&E staining; (B) GLUT2 by immunofluorescence (IF) staining; (C) cleaved caspase-3 (red), insulin (green), and DAPI (blue), by IF; and (D) insulin (red) and glucagon (green), by IF staining. (E) β cell number in pancreas. A total of 18 sections from the middle of the pancreas was analyzed (n = 3 for per genotype from 3 independent crosses). (F) β cell size, obtained from 29–51 determinations (n = 3 for per genotype). (G) Insulin content from embryonic pancreata (n = 3–10 for per genotype). Scale bars: 25 μm. Values in E–G are given as mean ± SEM. *P < 0.05, **P < 0.01 vs. other genotypes, ANOVA.

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These studies strongly support the hypothesis that the reduction in β cell number and size explains the reduction in insulin content in S6K1−/− embryos (Figures 1 and 5).

**RIP-S6K1 transgene expression improves glucose tolerance.** Western blot analysis of adult 3- to 5-month-old mice revealed that the expression of the Myc-epitope-tagged S6K1 was maintained in both WT Tg(RIP-S6K1) and S6K1−/− Tg(RIP-S6K1) mice (Figure 6A). Following birth, reexpression of the RIP-S6K1 transgene in S6K1−/− mice did not rescue the reduction in body size, nor were there any obvious alterations in the body composition in terms of either fat mass or lean mass, when compared with S6K1−/− mice (Figure 6, B–D). Likewise, RIP-S6K1 transgene expression had little effect on whole-body energy expenditure (EE) when compared with S6K1−/− mice (Figure 6E). In contrast to these observations, RIP-S6K1 transgene expression in β cells restored circulating insulin in S6K1−/− mice to levels similar to those observed in either WT Tg(RIP-S6K1) or WT mice (Figure 6F). Previously, we had shown that S6K1−/− mice are mildly glucose intolerant, which we attributed to their hypoinsulinemia (14). However, despite hypoinsulinemia, such mice maintain glucose homeostasis and insulin sensitivity (9), apparently due to both reduced insulin levels and adiposity, as well as the loss of the negative feedback loop from S6K1 to IRS1 in muscle and adipose (14). Consistent with these findings, RIP-S6K1 transgene expression in S6K1−/− mice not only rescued the mild glucose intolerance, but dramatically enhanced this response over that of WT RIP-S6K1 or WT mice (Figure 6G). It should be noted that the expression of S6K1 in β cells, but not its levels of expression (Figure 6A), correlated with the restoration of normal circulating levels of insulin (9), apparently due to both reduced insulin levels and adiposity, as well as the loss of the negative feedback loop from S6K1 to IRS1 in muscle and adipose (14). Consistent with these findings, RIP-S6K1 transgene expression in S6K1−/− mice not only rescued the mild glucose intolerance, but dramatically enhanced this response over that of WT RIP-S6K1 or WT mice (Figure 6G). It should be noted that the expression of S6K1 in β cells, but not its levels of expression (Figure 6A), correlated with the restoration of normal circulating levels of insulin (9), apparently due to both reduced insulin levels and adiposity, as well as the loss of the negative feedback loop from S6K1 to IRS1 in muscle and adipose (14). Consistent with these findings, RIP-S6K1 transgene expression in S6K1−/− mice not only rescued the mild glucose intolerance, but dramatically enhanced this response over that of WT RIP-S6K1 or WT mice (Figure 6G). It should be noted that the expression of S6K1 in β cells, but not its levels of expression (Figure 6A), correlated with the restoration of normal circulating levels of insulin. Thus, the increase in glucose tolerance displayed by S6K1−/− Tg(RIP-S6K1) mice, as compared with S6K1−/− mice, appears to be due to restoration of normal circulating levels of insulin.

**RIP-S6K1 transgene expression restores β cell size and S6 phosphor-ylation.** Consistent with the restoration of normal insulin levels, H&E and insulin staining of adult islets showed a clear increase in β cell size of S6K1−/− Tg(RIP-S6K1) mice (Figure 7, A and B), which was confirmed by morphometric analysis of insulin-stained cells.
This finding led to the suggestion that the size reduction observed in islets from S6K1–/– mice was due to IUGR or were mediated by an intrinsic β cell defect. The results demonstrate that S6K1 deficiency leads to IUGR, but independently affects β cell size, in a cell-autonomous manner. Although it is generally accepted that a deficiency in the placental nutrient supply leads to IUGR and subsequent lesions in β cell development (18), others have recently reported in an ovine model of IUGR that the placental nutrient supply leads to IUGR and subsequent lesions in β cell development (18), others have recently reported in an ovine model of IUGR that following midgestation, the stage at which IUGR is diagnosed in humans, there is already a decrease in β cell proliferation and fetal insulin levels (25). From these observations, they hypothesize that impaired β cell development and decreased insulin levels may be a cause rather than an effect of IUGR (25). Nevertheless, the RPS6 knockin mice, harboring serine to alanine substitutions in the 5′ RPS6 phosphorylation sites (45) are substituted by alanines, that mice harboring a knockin of 40S RPS6 cDNA, where the 5′ RPS6 phosphorylation sites (45) are substituted by alanines, may be mediated, at least in part, by decreased insulin secretion by β cells (43).

However, we did not detect expression of S6K1 in hypothalamus in S6K1–/– mice (Figure 2) long before the onset of β cell growth defects at E16.5, when insulin-producing β cells of mice start forming. Moreover, when S6K1 is reintro-duced in the β cells of S6K1–/– mice, there is no impact on IUGR, despite the correction in β cell size and insulin levels (Figures 5–7).

Discussion

Given the potential role of S6K1 inhibitors in the treatment of T2DM, we set out to determine whether the effects on β cell size and hypoinsulinemia observed in S6K1–/– mice were due to IUGR or were mediated by an intrinsic β cell defect. The results demonstrate that S6K1 deficiency leads to IUGR, but independently affects β cell size, in a cell-autonomous manner. Although it is generally accepted that a deficiency in the placental nutrient supply leads to IUGR and subsequent lesions in β cell development (18), others have recently reported in an ovine model of IUGR that following midgestation, the stage at which IUGR is diagnosed in humans, there is already a decrease in β cell proliferation and fetal insulin levels (25). From these observations, they hypothesize that impaired β cell development and decreased insulin levels may be a cause rather than an effect of IUGR (25). Nevertheless, the RPS6 knockin mice, harboring serine to alanine substitutions in the 5′ RPS6 phosphorylation sites (45) are substituted by alanines, may be mediated, at least in part, by decreased insulin secretion by β cells (43).
It should be noted that in the mouse, deletion of Mtor — the S6K1 T389 kinase — abrogates development at E5.5, as it is required for trophoblast differentiation, outgrowth, and invasion of the uterine stroma during placentation development (22, 31, 47). Deletion of S6K1 may have less damaging developmental effects because it represents only one of the multiple downstream targets of mTOR.

Taken together, these findings support the possibility that nutrient deprivation during early development may use independent routes to affect IUGR and impair β cell function.

mTORC1 plays a key role in the activation of S6K1 by providing a docking site for phosphoinositide-dependent protein kinase (PKD1), the T229 S6K1-activation loop kinase (48). Previous studies have shown that mice or embryos lacking PKD1 exhibit both reduced β cell number and size (49, 50). PKD1 also serves as the S508 PKB-activation loop kinase. As PKB mediates the canonical PI3K signaling pathway to mTORC1, this suggests that PKD1 plays a key role in 2 distinct pathways leading to S6K1 activation: one through nutrients and the other through hormone-mediated PKB activation. The effect on cell number, but not cell size, observed in PKD1-deficient β cells is largely rescued by crossing the PKD1-deficient mice with mice haploinsufficient for FoxO1, whose activity is negatively regulated by PKB phosphorylation (49). In line with this finding, mice lacking rictor, an essential component of the mTORC2 required for PKB-S473 phosphorylation, exhibit reduced β cell number and increased FoxO1 activity (51). These observations led to the hypothesis that the effects of PKD1 deficiency on cell number are regulated through suppression of PKB, whereas those on cell size are regulated by S6K1 (49). Consistent with the effects of S6K1 on β cell size, TSC1/TSC2 and Rheb (52) — upstream effectors of mTORC1/S6K1 — have been implicated in this response, and ectopic expression of a constitutively active S6K1 transgene in β cells results in increased β cell size (16). Genetic studies in mice suggest that the size reduction observed in the β cells of S6K1−/− mice is mediated by a decrease in RPS6 phosphorylation (46), which has been implicated in the recruitment of mRNAs to the cap-binding complex translation initiation complex (53). Nevertheless, it should be noted that the effects of the loss of RPS6 phosphorylation on β cell size are based on a whole-animal replacement with the multiple-alanine site RPS6 mutant, such that the effects on β cell size may not be cell autonomous. At this stage, it would be informative to determine whether the effects of S6K1 on β cell size are mediated through RPS6 by generating a β cell–specific knockin of the RPS6 alanine allele.

Earlier studies from this laboratory showed that S6K1-deficient mice exhibit mild glucose intolerance, which was attributed to reduced β cell size, leading to decreased glucose-stimulated insulin secretion (14). From the studies described here, the reduction in β cell size does not appear to be mediated through either PKB or ERK, but most likely through reduced RPS6 phosphorylation (Figure 7E). We initially attributed the mild effect on glucose intolerance to the fact that S6K1-deficient mice were still able to increase circulating insulin levels in answer to a glucose challenge, albeit less robustly (5). However, our later studies revealed that the ability of S6K1-deficient mice to maintain glucose homeostasis could also be explained, in part, by increased insulin sensitivity (9, 14). This effect was particularly evident when mice were challenged with an HFD (9, 14). WT mice became insulin resistant, whereas S6K1-deficient mice remained insulin sensitive, as they did on a normal chow diet (9, 14). Here we show that reexpression of S6K1 in the β cells of S6K1−/− Tg(RIP-S6K1) mice dramatically enhances glucose tolerance over that of WT mice (Figure 6G) by restoring normal plasma insulin levels (Figure 6F) under conditions where reduced adiposity (Figure 6C) and increased insulin sensitivity displayed by S6K1−/− mice are maintained (Figure 6H). The observation that rapamycin does not have an inhibitory effect on acute insulin secretion (54) but can rapidly increase insulin sensitivity in obese rats (55) initially raised the possibility of employing the drug as an insulin sensitizer. However, chronic treatment of posttransplant patients with rapamycin has been associated with impaired glucose tolerance and T2DM (56), due in part to β cell toxicity (57). Unlike in the case of S6K1 deletion, we observe a similar phenotype in mice where we have conditionally deleted the Mtor gene in β cells (J. Park, S.H. Um, and S.C. Kozma, unpublished observations). Moreover, others have provided evidence that insulin resistance caused by chronic treatment of mice with rapamycin is due to disruption of mTORC2 and not inhibition of mTORC1 (58). Thus the more deleterious effects of rapamycin could be attributed to the inhibition of multiple mTORC1 substrates, in addition to S6K1 (57), and its ability to inhibit mTORC2 (58). This argument is consistent with preliminary studies showing that in cell culture, compared with deletion of S6K1, rapamycin treatment leads to a more profound and lasting inhibition of the negative feedback loop to IRS1/2, as measured by insulin stimulation of PKB (K. Masuda and G. Thomas, unpublished observation). These findings, and the mild glucose intolerance displayed by S6K1−/− mice, raise the possibility that a selective inhibitor of S6K1 may have a clinical window of efficacy as an insulin sensitizer, beyond that of mTOR1 and mTORC2 inhibition.

**Methods**

**Mice.** S6K1−/− mice on C57BL/6 background (9) were obtained from heterozygous or homozygous parental crosses, as indicated for the different methods. Genotyping was performed on tail DNA — or DNA derived from dissected embryos and placenta, in the case of tetraploid embryo complementation studies — by Southern blot analysis or PCR. The PCR primers corresponded to: S6K1 forward, 5′-GTAAGGGAACCT-TAATGACCAC-3′; S6K1 reverse, 5′-TGTCCCATATATT-GCT-CAAGG-3′; neo reverse, 5′-GCCTTCTTGA CGA GTCTTCTGTGAG-3′.

**Histological analysis.** WT, S6K1−/−, and S6K1−/− mouse embryos and placenta were collected and fixed in 10% formalin overnight at 4°C. Fixed samples were embedded in paraffin, and 5-µm sections were cut and mounted on slides to generate a series of 6 slides with comparable adjacent sections. The average number of slides collected were 90-100 from pancreata per genotype using 3 heterozygous and 9 comparable adjacent sections. The average number of slides collected were 90-100 from pancreata per genotype using 3 heterozygous and 4 homozygous mouse matings. For morphological analyses, slides of each series were H&E stained. For immunofluorescence, sections of embryonic and adult pancreata were stained with rabbit anti-glucagon (1:50, Invitrogen), guinea pig anti-insulin (1:400, Millipore), and rabbit anti-Glut2 (1:200, provided by B. Thorens) antisera. Detection was performed using Alexa Fluor 488 and 594 (Invitrogen). Morphometric analyses and cell counting were done using ImagePro Plus software (Media Cybernetics). Insulin and glucagon staining was performed using 15–16 slides per pancreas, representing 1/6 of the organ. Forty-five to 52 slides were analyzed per genotype. To calculate the
mean size of individual α and β cells, a defined area was divided by the number of cell nuclei positive for either insulin or glucagon, as previously described (14). Apoptosis was determined in pancreatic sections stained for insulin (guinea pig anti-swine insulin, 1:800, Dako) and cleaved caspase-3 (rabbit anti-cleaved caspase-3, 1:50, Cell Signaling Technology) as described (52). Apoptotic cells were identified by co-staining for insulin and cleaved caspase-3. Three sections per pancreas were examined, and at least 50–100 insulin-stained cells were counted for each animal. IHC and ISH were performed using placental tissue sections from the middle of each placenta. For anti-PCEM-1 staining, the sections were incubated with 0.1% trypsin after rehydration for 12 minutes at 37°C, followed by 20 minutes incubation with 2N HCl. Sections were then incubated overnight at 4°C with rat monoclonal anti-PCEM-1 (1:50, Pharmingen). Biotinylated horse anti-rat (Vector Laboratories) was used as the secondary antibody, followed by incubation with the ABC reagent (Vector Laboratories), detection with DAB (Vector Laboratories), and nuclear counterstaining with hematoxylin. The endocrine mass was quantified using the following equation: β cell mass = pancreas weight (mg) × relative insulin surface (total islet area μm²/total pancreas area μm²) × 100.

ISH analysis. The trophoblast-specific protein α (Ttpb/-4311) gene was used as a molecular marker of spongiotrophoblast cells (59). Placenta sections of WT, S6K1+/–, and S6K1–/– embryos, from both heterozygous or homozygous matings, were rehydrated and permeabilized using 20 μg/ml proteinase K in 10 mM Tris pH8/20 mM EDTA for 15 minutes at room temperature, fixed in 4% paraformaldehyde/0.2% glutaraldehyde, and dehydrated in a graded methanol series. Anti-sense and sense riboprobes were generated by in vitro transcription using the DIG RNA Labeling Kit (Roche Diagnostics) following the manufacturer’s instruction. NBT (Nitroblue tetrazolium chloride) and BCIP (5-Bromo-4-chloro-3-indoyl-phosphate, 4-toluidine salt), both from Roche Diagnostics, were used as the substrate for visualization.

Insulin content in blood and embryonic pancreata. Blood samples from adult mice issued from both heterozygous and homozygous matings were collected and centrifuged. Embryonic pancreas from each genotype obtained from both heterozygous and homozygous matings were placed into PBS and then sonicated 3 times for 10 seconds each time. Insulin concentration in the plasma or in pancreas extracts was measured using the ultrasensitive insulin ELISA kit (Crystal Chem Inc.) or by radioimmunoassay (Millipore), respectively.

Embryonic growth analysis. For embryo staging, the day of vaginal plug appearance was considered E0.5. Embryos were obtained from 4 heterozygous and 26–32 homozygous crosses for WT and S6K1–/– mice, respectively. For developmental analyses of growth, embryos and placenta were dissected from pregnant mice, patted dry, and weighed on a microbalance. For embryos obtained from heterozygous parental crosses, were flushed at the morula stage and incubated overnight in M16 medium. On the next day, blastocysts were transferred to 6-cm dishes coated with inactivated mouse fibroblasts and 5637 cells (ATCC HTB9) to facilitate ES cell development. A few days later, the inner cell masses (ICMs) were collected with a glass pipette, treated with trypsin (Invitrogen) for 10 minutes at RT, and further propagated on inactivated fibroblasts. The medium used throughout the process was KO-DMEM supplemented with pen/strep/glutamine, gentamycin (Invitrogen), β-mercaptoethanol (Sigma-Aldrich), LIF (Novartis), and 15% FBS (PAN-Biotech GmbH).

Aggregation of tetraploid WT embryos with diploid S6K1+/– ES cells. WT embryos were recovered at the 2-cell stage (E1.5). The blastomeres were fused by electrofusion (60), and the resulting tetraploid embryos were cultured for 48 hours at 37°C, 5% CO₂, and 100% humidity in M16 medium (60). Next, the zona pellucida was removed from the 4-cell-stage tetraploid embryos by Tyrode’s Acid treatment and further cultured in microdrops (60). Three independent S6K1+/– ES cell clones were brought to single-cell suspension by a 5-minute trypsin treatment (Invitrogen). The S6K1+/– ES cell suspensions were left to reaggregate into small clumps of 10–15 ES cells and placed in separate wells containing 2 tetraploid embryos, as described (60). The next day, blastocyst-stage embryos were harvested from the wells and implanted into the uteri of E2.5 pseudopregnant-recipient CD1 outbred female mice. This technique allows for the development of embryos in utero; however, it generates less than 5% living birth, and mice die within hours after birth (37). Therefore, analyses were performed at E13.5 and E16.5.

Generation of transgenic mice expressing S6K1 in pancreatic β cells. The RIP-S6K1 DNA construct consisted of a 720-bp fragment of RIP driving transcription of a myc-S6K1 cDNA. RIP-S6K1 DNA was injected into B6CF2 — second-generation (F2) obtained from the crosses between C57Bl/6J and BALB/C mice — fertilized eggs. Founders were identified by PCR analysis, and germline transmission was confirmed by Southern blot analysis. Genotyping was performed by PCR using primers derived from RIP and S6K1 cDNA sequences as follows: Forward, 5′-CAGGCGAACCCAGGACCC-3′; reverse 1, 5′-GCATGAGTTCCTC CGGCTTC AGG-3′; reverse 2, 5′-GTAAATGGCCC-AAAGCCATGGAG-3′. The transgenic mice were backcrossed for 6 generations on C57BL/6J background prior to breeding with S6K1+/– mice. Isolated islets were obtained from adult WT Tg(RIP-S6K1) and S6K1+/– Tg(RIP-S6K1) mice by collagenase P digestion (14). The lysates were analyzed by Western blotting with antibodies against S6K1 (Santa Cruz Biotechnology Inc.) and c-Myc (Upstate Biotechnology Inc.). Two of 15 S6K1+/– Tg(RIP-S6K1) mouse lines were positive for S6K1 expression, and one of them could be maintained for experimentation. The hypothalamus was dissected from 3–6 mice per established genotypes to assess S6K1 expression.

Metabolic phenotyping. Indirect calorimetry, locomotor activity, metabolic performance, and home-cage activity were studied by using a combined indirect calorimetry system (TSE Systems GmbH).
Volumes of oxygen consumed (VO₂, ml/kg/h) and carbon dioxide produced (VCO₂, ml/kg/h) were measured continuously and simultaneously every 45 minutes to determine the respiratory quotient (RQ: VCO₂/VO₂) and to calculate EE (kcal/kg/h, EE = 3.815×10⁻³ × VO₂ + 1.232×10⁻³ × V CO₂). During the 95 hours of measurements, the animals remained unconstrained except for a single filling of both water bottles and food hoppers. Prior to the experiment, all mice were allowed to acclimatize to the air-tight cages for 22 hours.

**Body composition analysis.** Whole-body composition (fat and lean mass) was measured using NMR technology (EchoMRI).

**Statistics.** Data are presented as mean ± SEM. The main and interactive effects were analyzed by ANOVA factorial or repeated measures, or by 1-way ANOVA. Differences between individual group means were analyzed by a post-hoc Bonferroni test or 2-tailed unpaired t test. Analyses were performed using GraphPad Prism software. Differences were considered to be statistically significant at P < 0.05.

**Study approval.** Animal care and study protocols were approved by the Institutional Animal Care and Use Committee of Sungkyunkwan University School of Medicine and the Animal care committee of the University of Cincinnati, and were in accordance with NIH guidelines.

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