Functional roles of fructose

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During the periimplantation period of pregnancy, pig blastocysts undergo morphological changes and differentiation requiring secretion and transport of nutrients (histotroph) into the uterine lumen. Of these nutrients, glucose is converted to fructose, an isomer of glucose, by conceptus trophectoderm. Although glucose is an energy source for proliferation and growth of mammalian cells, the role of fructose in uterine histotroph is unclear although it is the most abundant hexose sugar in fetal blood and fluids of ungulate mammals (e.g., cows, sheep, and pigs). In this study, we used porcine trophectoderm cells to determine that fructose increased cell proliferation, as did glucose. Western blot analyses of porcine trophectoderm cell extracts revealed that fructose increased the abundance of phosphorylated-RPS6K, -EIF4EBP1, and -RPS6 over basal levels within 30 min, and those levels remained elevated to 120 min. Phosphorylation of both RPS6K and EIF4EBP1 proteins in response to fructose was inhibited by inhibitors of both PI3K and MTOR. Further, when we investigated the inhibition of glutamine-fructose-6-phosphate transaminase 1 (GFPT1) by azaserine (an inhibitor of GFPT1) and GFPT1 siRNA, we found that MTOR-RPS6K and MTOR-EIF4EBP1 signaling in response to fructose is mediated via GFPT1 activation and the hexosamine pathway. We further demonstrated that fructose stimulates the production of hyaluronic acid via GFPT1 and the hexosamine biosynthesis pathway. Collectively, these results demonstrate critical roles for fructose that are mediated via the hexosamine biosynthesis pathway to stimulate MTOR cell signaling, proliferation of porcine trophectoderm cells, and synthesis of hyaluronic acid, a significant glycosaminoglycan in the pregnant uterus.

trophoblast | glycosaminoglycans

Domestic livestock species are invaluable animal models used extensively in research relevant to human and veterinary reproductive medicine as well as basic biological and biomedical sciences (1, 2). In particular, the ewe is an established animal model for studies of intrauterine growth restriction during fetal life that is a predisposition for the adult onset of metabolic disease (3). Ewes have a protracted periimplantation period of pregnancy, a relatively short period of gestation, and are well suited for metabolic studies to assess temporal and cell-specific changes in gene expression in response to various hormones, growth factors, and nutrients that are relevant to human and veterinary medicine and basic aspects of reproductive biology. Although fructose clearly is the major hexose sugar in fetal blood and fetal fluids of ungulate species such as sheep and pigs, it is largely ignored with respect to function because it is not metabolized via the glycolytic pathway or the Krebs cycle as an energy source. Here we provide evidence that fructose affects multiple metabolic pathways critical to pregnancy and metabolism in multiple organ systems.

Fructose is the most abundant hexose sugar in fetal fluids of ungulate mammals (4–6). Fructose also is present in human fetal fluids (7), but it is a relatively minor sugar compared with glucose (8, 9). Fructose also is a minor sugar in fetal blood and fetal fluids of dog, cat, guinea pig, rabbit, rat, and ferret (9). In general, high levels of fructose are found in fetal blood and fetal fluids of mammals having epitheliochorial and synepitheliochorial placentas (9) that are used extensively as animal models for intrauterine growth (1, 2, 10). The placentas of these mammals contain little or no glycogen, i.e., less than 0.5% of that in the fetus. In contrast, mammals with endotheliochorial and hemochorial placentas (11) have fetuses in which glucose is converted to glucose-6-PO_4 that enters either the pentose phosphate pathway or the glycolytic pathway to meet the metabolic demands of the rapidly developing conceptus.

Studies of pregnant ewes have revealed that (*i*) injection of glucose into ewes results in a rapid increase in glucose followed by a protracted increase in fructose in fetal blood; (*ii*) injection of glucose into the umbilical vein of the fetus increases glucose in maternal blood and hyperfructosemia in the fetus, indicating that glucose can move from conceptus vasculature to maternal blood, whereas fructose derived from glucose is not transported into maternal blood; (*iii*) the placenta is the site of conversion of glucose to fructose; (*iv*) fructose is produced continuously by the placenta independent of glucose concentration in maternal or fetal blood; and (*v*) the flux of glucose from the maternal to the fetal circulation can be as much as 70 mg/min in ewes made hyperglycemic (6, 12–14). These results were confirmed in studies using radiolabeled glucose to demonstrate its conversion to radiolabeled fructose by the placenta of pigs (15).

The role of fructose remains unclear. Therefore, it has been ignored in studies of metabolic pathways associated with metabolism of hexose sugars, because glucose, but not fructose, is metabolized via the glycolytic pathway (16–20). Nevertheless, fructose can be used for synthesis of nucleic acids and generation of reducing equivalents in the form of NADPH H^+ in the fetal pig (21) and in HeLa cells (22). However, it has been reported that neither fructose nor glucose is metabolized via the pentose phosphate pathway in the ovine placenta (23). Fructose and glucose are equivalent as substrates for entering metabolic pathways leading to synthesis of neutral lipids and phospholipids in heart, liver, kidney, brain, and adipose tissue of fetal lambs, refuting general statements that fructose is not metabolized in fetal tissues of domestic animals (24). In adults rats the activities of glucose-6-phosphate dehydrogenase, malic enzyme, and acetyl-CoA carboxylase in liver are stimulated by glucose; this stimulation increases lipogenesis (25), and fructose enters adipocytes by both insulin-independent and insulin-insensitive mechanisms (26).

Fructose is the primary sugar in blood, allantoic fluid, and amniotic fluid of the fetal pig, but it decreases in allantoic fluid as glucose increases between days 82 and 112 of the 114-d period of gestation (27). The rapid clearance of fructose from blood of piglets by 24 h postpartum indicates that the neonatal piglet is unable to use fructose as an energy source (28, 29).

Because of the lack of understanding of the role of fructose, the most abundant hexose sugar in the pregnant uterus, we conducted experiments to elucidate fructose metabolism using

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our established porcine trophectoderm (pTr) cell line. Our results demonstrated that fructose is involved actively in stimulating cell proliferation and mRNA translation via the activation of mechanistic target of rapamycin (MTOR) cell signaling and synthesis of glycosaminoglycans, specifically hyaluronic acid, via the hexosamine metabolic pathway. Specifically, this study focused on MTOR complex 1 (MTORC1), which includes the regulatory-associated protein of MTOR (Raptor), mammalian LST8/G protein β-subunit–like protein (mLST8/GβL), and their AKT1 substrate 1, proline-rich (AKT1S1) and disheveled, Egl-10, and pleckstrin domain-containing protein 6 (DEPDC6) that is involved with nutrient and energy sensing and protein synthesis and is responsive to insulin, growth factors, serum, phosphatidic acid, amino acids, and oxidative stress (30).

Results

Glucose and Fructose Stimulate pTr Cell Proliferation. We first investigated biological effects of glucose and fructose on proliferation of pTr cells. Treatment of pTr cells with glucose in customized medium containing 5% serum for 48 h and 96 h increased pTr cell numbers by 1.8- fold ($P < 0.01$) and 3.2-fold ($P < 0.01$), respectively (Fig. 1*A*). Similarly, at 48 h and 96 h of fructose treatment, pTr cell numbers increased by 2.7-fold ($P < 0.01$) and 5.3-fold ($P < 0.01$), respectively (Fig. 1*B*). However, there was no evidence that treatment of pTr cells with both glucose and fructose had either additive or synergistic effects ($P > 0.10$) (Fig. 1*C*). These results indicate that fructose and glucose stimulate pTr cell proliferation, but the fold-increase was greater for fructose.

Glucose and Fructose Activate MTOR Signaling in pTr Cells. The pTr cells deprived of glucose and fructose for 6 h after serum starvation were treated with 4 mM glucose or 4 mM fructose. Western blot analyses of whole pTr cell extracts indicated that glucose increased phosphorylated ribosomal protein S6K (p-RPS6K), p-eukaryotic translation initiation factor 4E-binding protein 1 (p-EIF4EBP1), and p-ribosomal protein S6 (p-RPS6) by 2.7-fold ($P < 0.01$), 2.4-fold ($P < 0.01$), and 6.1-fold ($P < 0.01$), respectively, over basal levels within 30 min and that this level of activation was maintained to 120 min after treatment (Fig. 2 *A–C*). Likewise, fructose increased the abundance of p-RPS6K, p-EIF4EBP1, and p-RPS6 by 2.9-fold ($P < 0.01$), 2.3-fold ($P < 0.01$), and 4.2-fold ($P < 0.01$), respectively, over basal levels within 30 min, and this level of activation was maintained to 120 min after treatment (Fig. 2 *D–F*). These results indicate that both fructose and glucose activate the MTOR-RPS6K–RPS6 or MTOR–EIF4EBP1 pathways, stimulating proliferation and growth of pTr cells.

We next performed immunofluorescence analyses to determine cellular locations of p-RPS6K, p-EIF4EBP1, and p-RPS6 in response to treatment of pTr cells with 4 mM glucose or 4 mM fructose. The abundance of p-RPS6K protein increased in the nucleus after treatment with glucose and fructose but not in untreated pTr cells (Fig. 3*A*). The p-EIF4EBP1 protein was localized predominantly in the nucleus but also, to a much lesser extent, was found in the cytoplasm of pTr cells in response to glucose and fructose (Fig. 3*B*). The p-RPS6 protein was abundant in the cytoplasm of pTr cells treated with glucose and fructose (Fig. 3*C*). These results indicate that each effector protein involved in mRNA translation downstream of MTOR

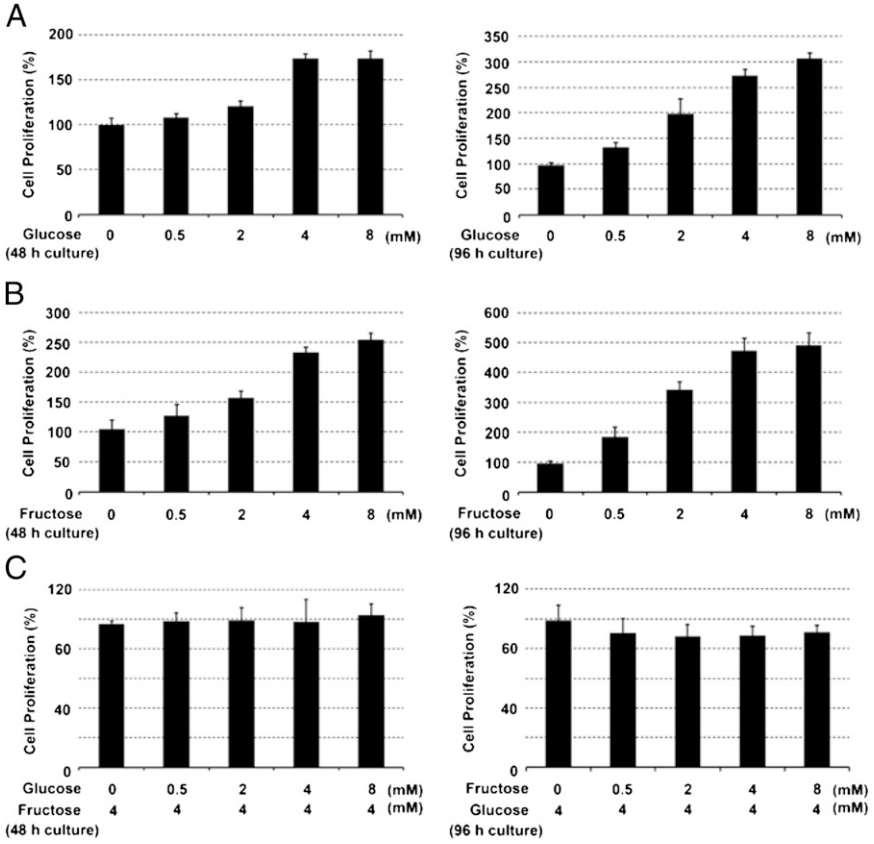

Fig. 1. Dose-dependent effects of glucose (*A*), fructose (*B*), and glucose plus fructose (*C*) on the proliferation of pTr cells. The pTr cells were seeded at 30% confluence in wells and cultured with the indicated doses of glucose and fructose. Cell numbers were determined after 48 h (*Left*) or 96 h (*Right*) of incubation, and data are expressed as a percentage relative to nontreated control (100%). All quantitative data are presented as the LSM with overall SEM. See *Materials and Methods* for a complete description.

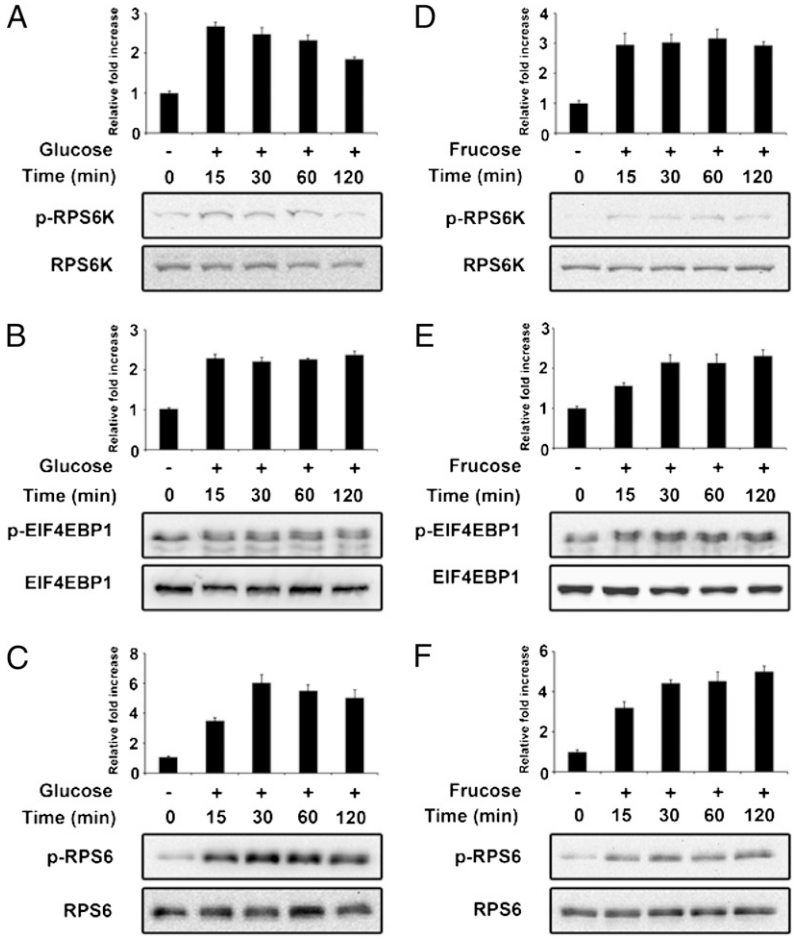

Fig. 2. Analysis of time-dependent phosphorylation of RPS6K (*A* and *D*), EIF4EBP1 (*B* and *E*), and RPS6 (*C* and *F*) in response to glucose (*A*, *B*, and *C*) and fructose (*D*, *E*, and *F*) in pTr cells at 0, 15, 30, 60, and 120 min after treatment. Monolayers of 80% confluent pTr cells were serum starved for 24 h, then deprived of either glucose or fructose for 6 h, and then treated with physiological concentrations of glucose (4 mM) or fructose (4 mM). Blots were imaged to calculate the normalized values as levels of phosphorylated protein relative to total protein. See *Materials and Methods* for a complete description.

was localized in and presumably exerted its functional role in different intracellular compartments of pTr cells to stimulate cell proliferation.

MTOR Signal Transduction Induced in pTr Cells by Glucose and Fructose Involves PI3K/AKT1 Proteins. To determine the cell-signaling pathways mediating effects glucose and fructose on PI3K/

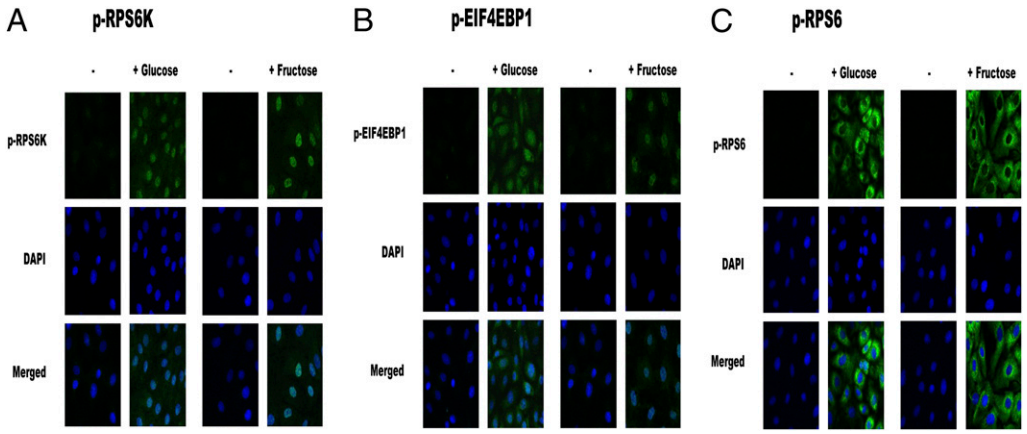

Fig. 3. Immunofluorescence localization of p-RPS6K (*A*), p-EIF4EBP1 (*B*), and p-RPS6 (*C*) proteins in pTr cells. Immunoreactive p-RPS6K protein was localized predominantly in the nucleus of pTr cells treated with glucose or fructose, p-EIF4EBP1 protein was localized in the cytoplasm and nucleus of pTr cells treated with glucose and fructose, respectively; p-RPS6 protein was localized primarily in the cytoplasm of pTr cells treated with glucose or fructose. (Magnification: 40×.). See *Materials and Methods* for a complete description.

AKT1, RPS6K, and EIF4EBP1, pTr cells were pretreated with pharmacological inhibitors of either PI3K (25 μM LY294002) or MTOR kinase (25 nM rapamycin) for 2 h. Results indicated that phosphorylation of both RPS6K and EIF4EBP1 in response to glucose was inhibited by both PI3K and MTOR inhibitors ($P < 0.01$ or $P < 0.001$, respectively; Fig. 4 *A* and *C*). Likewise, induction of phosphorylation of both RPS6K and EIF4EBP1 by fructose was inhibited by both PI3K and MTOR inhibitors ($P < 0.01$ or $P < 0.001$, respectively; Fig. 4 *B* and *D*). These results suggest that activation of the PI3K–AKT1 pathway by fructose is required for transcriptional activation in the fructose-induced cell signaling cascade from cytoplasm to nucleus to enhance proliferation and growth of pTr cells.

MTOR Cell Signaling in Response to Glucose and Fructose Is Mediated by Glutamine-Fructose-6-Phosphate Transaminase 1 Activity. To examine whether glutamine-fructose-6-phosphate transaminase 1 (GFPT1) is both an upstream effector of MTOR and a nutrient sensor in the hexosamine biosynthesis pathway, we used azaserine (an inhibitor of GFPT1) to assess whether GFPT1 activity is required for glucose and fructose to activate pTr cell proliferation. Azaserine inhibited ($P < 0.01$) the ability of both glucose (Fig. 5*A*) and fructose (Fig. 5*B*) to stimulate proliferation of pTr cells. In particular, the rate of pTr cell proliferation decreased 70–80% with the addition of 1 μM azaserine compared with effects of glucose alone (Fig. 5*A*) or fructose alone (Fig. 5*B*). Even in the presence of both fructose and glucose, azaserine inhibited ($P < 0.01$) proliferation of pTr cells (Fig. 5*C*). These results indicate that the nutrient-sensing system in pTr cells is more sensitive to fructose than to glucose.

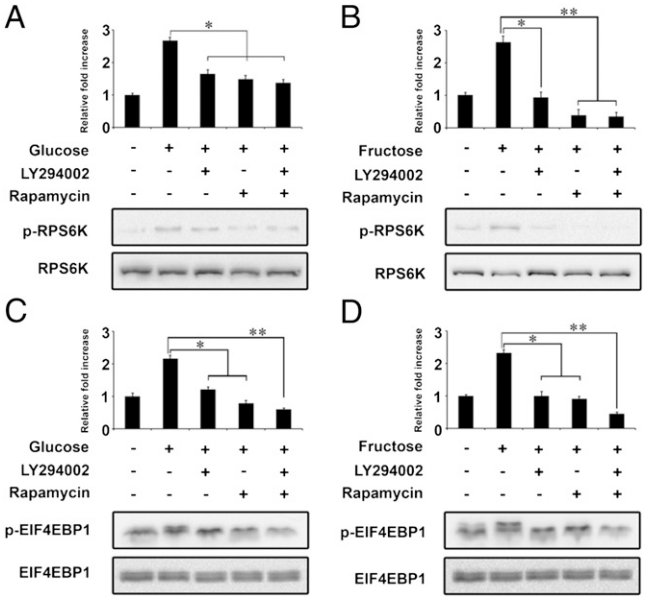

Fig. 4. Inhibition of RPS6K (*A* and *B*) and EIF4EBP1 (*C* and *D*) phosphorylation. Monolayers of 80% confluent pTr cells were serum starved for 24 h, deprived of glucose and fructose for 6 h, and then pretreated with 25 μM LY294002 or 25 nM rapamycin for 30 min. After stimulation with 4 mM glucose (*A* and *C*) or 4 mM fructose (*B* and *D*) for 60 min, total cell lysates were subjected to SDS/PAGE followed by Western blotting. Both rapamycin and LY294002 inhibited increases in p-RPS6K and p-EIF4EBP1. Blots were imaged to calculate the normalized values presented as levels of phosphorylated protein relative to total protein. Asterisks denote an effect of treatment (*$P < 0.01$; **$P < 0.001$). All quantitative data are presented as the LSM with overall SEM. See *Materials and Methods* for a complete description.

The next experiment used Western blot analyses of whole pTr cell extracts treated with azaserine to determine if inhibition of GFPT1 decreased the abundance of p-RPS6K and p-EIF4EBP1 proteins (Fig. 6 *A* and *B*). Interestingly, the phosphorylation of RPS6K and EIF4EBP1 proteins was inhibited more by treatment with the combination of azaserine and LY294002 than by treatment with azaserine alone when pTr cells were stimulated with either glucose or fructose (Fig. 6 *C* and *D*). These results suggest that fructose stimulation of the MTOR signaling cascade is affected by both the AKT1- and GFPT1-dependent cell-signaling pathways for stimulation of proliferation and growth of pTr cells.

The next step was to perform a GFPT1-knockdown experiment using GFPT1-specific siRNAs. As illustrated in Fig. 7*A*, GFPT1 protein expression was inhibited by 70, 80, and 81% 48 h after transfection with GFPT1 siRNA at 10, 25, and 50 nM, respectively. Cells transfected with the GFPT1-specific siRNA had less GFPT1 than did naive and mock-treated cells ($P < 0.001$) or cells transfected with control siRNA ($P < 0.001$). To verify these results, we performed immunofluorescence analyses and compared the expression patterns of GFPT1 protein in pTr cells cultured in the presence of control and GFPT1 siRNA (Fig. 7*B*). Immunoreactive GFPT1 protein was most abundant in the nucleus of pTr cells treated with control siRNA and naive cells but was barely detectable in cells transfected with GFPT1 siRNA. When combined with GFPT1 siRNA, glucose inhibited pTr cell proliferation by 60%, and fructose inhibited pTr cell proliferation by 72% ($P < 0.01$). Also, when both fructose and glucose plus GFPT1 siRNA were used to culture pTr cells, cell proliferation decreased by about 50% ($P < 0.01$) (Fig. 7 *C–E*). Collectively, these results demonstrate that stimulation of the MTOR–RPS6K and MTOR–EIF4EBP1 signaling pathways by glucose and fructose is mediated through GFPT1 and the hexosamine pathway.

Glucose and Fructose Stimulate Hyaluronic Acid Synthesis. Hyaluronic acid, a glycosaminoglycan synthesized via the hexosamine biosynthesis pathway, may play an essential role in angiogenesis and other aspects of cell function, particularly in the placenta during early pregnancy (31). Therefore, we determined whether fructose stimulates hyaluronic acid synthesis. As illustrated in Fig. 8*A*, glucose increased the amount of secreted hyaluronic acid by 6.2-fold at 48 h and by 11.3-fold at 96 h, and this effect was inhibited by azaserine in a dose-dependent manner. Pericellular hyaluronic acid also was increased by glucose treatment and inhibited by azaserine. Similarly, fructose increased secreted hyaluronic acid by 10.5- and 12.0-fold ($P < 0.01$) and increased pericellular hyaluronic acid by 7.0- and 7.3-fold ($P < 0.01$) at 48 h and 96 h of culture, respectively, and the effects of fructose were inhibited by azaserine in a dose-dependent manner (Fig. 8*B*). These results indicate that fructose and glucose stimulate the production of hyaluronic acid via GFPT1 and the hexosamine biosynthesis pathway to stimulate growth and development of the porcine trophoblast and perhaps the embryo/fetus during pregnancy.

Discussion

This study provides evidence for key roles of fructose in cellular functions and detailed analyses of the actions of fructose on cell-signaling pathways affecting proliferation and mRNA translation of cells (in this case, pTr cells). The results support our hypothesis that fructose is metabolized to glucosamine-6-PO4 by GFPT1 of the hexosamine biosynthesis pathway and stimulates the MTOR cell-signaling pathway to increase proliferation and mRNA translation of porcine conceptus trophectoderm cells.

Researchers focusing on intrauterine growth restriction and on the subsequent adult onset of metabolic disease in various ungulate species have ignored fructose as an important metabolic substrate, perhaps because fructose is not metabolized via the glycolytic pathway in the placenta, fetus, or neonate (16–20).

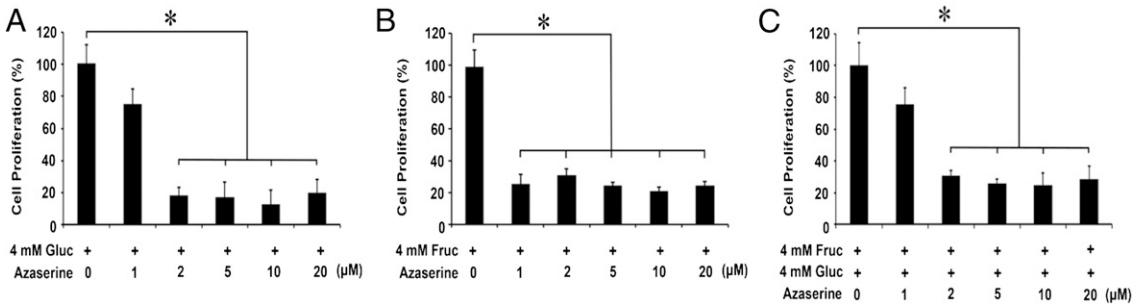

Fig. 5. Inhibition of GFPT1 activity. pTr cells pretreated for 2 h with the indicated doses of azaserine were stimulated with 4 mM glucose (*A*), 4 mM fructose (*B*), or 4 mM glucose and 5 mM fructose (*C*) or for 96 h. The cell-culture medium was changed every 48 h. Cell numbers were determined after stimulation, and data are expressed as a percentage relative to nontreated control (100%). Asterisks denote an effect of treatment (*$P < 0.01$). All quantitative data are presented as the LSM with overall SEM. See *Materials and Methods* for a complete description.

In ewes, for example, the maximum concentration of glucose in allantoic fluid is 1.1 mM between days 35 and 140 of pregnancy, whereas the concentration of fructose is between 11.1 and 33 mM during the same period (32). Therefore, in accordance with the results shown in Fig. 1, fructose exerts its maximum effects on cell proliferation at molar concentrations well below those in allantoic fluid. Glucose, on the other hand, was tested at physiological concentrations and at concentrations above those found in allantoic fluid. Therefore, fructose is the hexose sugar most likely to stimulate the MTOR nutrient-sensing pathway and to be metabolized via the hexosamine pathway to stimulate growth and development of the conceptus.

Fructose also is the primary sugar in blood, allantoic fluid, and amniotic fluid of the fetal pig; it increases in allantoic fluid until day 80 of gestation and then decreases as glucose increases between days 82 and 112 of pregnancy (27). Fructose is cleared rapidly from blood of newborn piglets, falling to almost undetectable levels by 24 h postpartum (27). Further, the neonatal piglet is unable to use fructose as an energy source (27, 28).

The present study revealed that the stimulatory effect of fructose on pTr cell proliferation is equivalent to or greater than that of glucose. These results explain the role of fructose [which has been called "the most enigmatic aspect of embryonic carbohydrate metabolism" (7)] in the development of the conceptus (embryo/fetus and its associated placenta). Furthermore, we found that fructose induces phosphorylation of RPS6K, RPS6, and EIF4EBP1 in pTr cells. In general, MTOR–RPS6K cell signaling relays signals stimulated by nutrients such as amino acids and mitogens to stimulate cell proliferation, differentiation, and gene expression (33). MTOR also acts in parallel with the

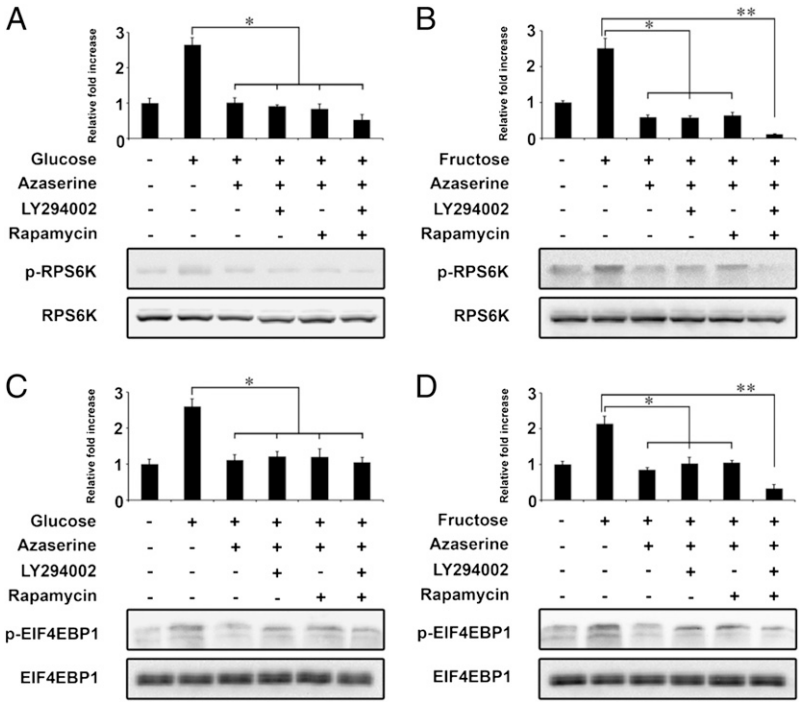

Fig. 6. Inhibition of GFPT1 decreased the abundance of RPS6K and EIF4EBP1 phosphorylation. Monolayers of 80% confluent pTr cells were serum starved for 24 h, deprived of glucose and fructose for 6 h, and then were pretreated with 25 μM LY294002 or 25 nM rapamycin for 30 min and azaserine for 2 h to inhibit PI3K, MTOR, and GFPT1, respectively. Panels *A* and *B* indicate effects of inhibitors of PI3K, MTOR, and GFPT1 in p-RPS6K. Panels *C* and *D* indicate effects of the same respective inhibitors on p-EIF4EBP1. After stimulation, total cell lysates were subjected to SDS/PAGE followed by Western blotting. Blots were imaged to calculate the normalized values presented as levels of phosphorylated protein relative to total protein. Asterisks denote an effect of treatment (*$P < 0.01$; **$P < 0.001$). All quantitative data are presented as the LSM with overall SEM. See *Materials and Methods* for a complete description.

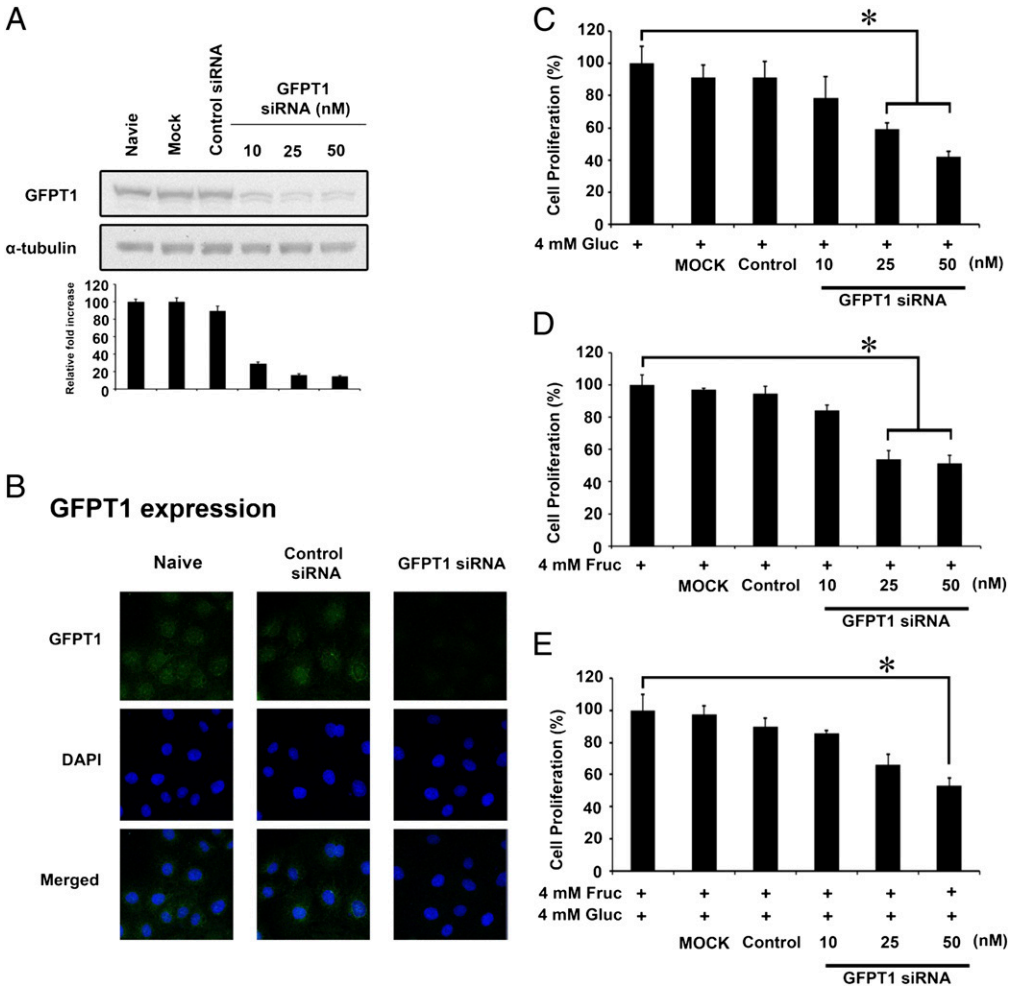

Fig. 7. GFPT1 knockdown decreased proliferation of pTr cells. (*A*) GFPT1 protein levels were measured in the control group (naive, mock, and control siRNA treatment) and in the GFPT1-silenced group (GFPT1 siRNA) in a dose-dependent manner. Blots were imaged to calculate the normalized values presented as levels of GFPT1 protein relative to TUBA protein. (*B*) Immunofluorescence microscopy detected GFPT1 protein in naive and control siRNA treatments but not in the GFPT1-silenced group. (*C*–*E*) Effects of glucose (*C*), fructose (*D*), and fructose plus glucose (*E*) combined with GFPT1 siRNA treatment on proliferation of pTr cells. Asterisks denote an effect of treatment (*$P < 0.01$). All quantitative data are presented as the LSM with overall SEM. See *Materials and Methods* for a complete description.

PI3K–AKT1 pathway to transduce growth factor signals and regulate common downstream effectors such as RPS6K and EIF4EBP1. However, little is known about the roles of the members of MTOR–RPS6K or PI3K–AKT pathways in pTr development during early pregnancy. The present study demonstrates that fructose and glucose activate RPS6K, RPS6, and EIF4EBP1 and that LY294002 and rapamycin inhibit that activation. Thus, fructose likely influences porcine fetal/placental development by activating the MTOR–RPS6K and/or PI3K–AKT pathways during the periimplantation period of conceptus development.

In the present study, stimulation of the MTOR cell-signaling pathway by glucose and fructose was mediated via GFPT1 and the hexosamine pathway. As a nutrient sensor in the hexosamine biosynthesis pathway and an MTOR upstream effector, fructose-mediated GFPT1 activity was inhibited by azaserine, which decreased pTr cell proliferation and levels of p-RPS6K and p-EIF4EBP1 proteins in pTr cells. In addition, with respect to effects mediated by either glucose or fructose, the phosphorylation of RPS6K and EIF4EBP1 proteins was inhibited more by the combination of azaserine plus LY294022 than by the combination of azaserine plus LY294002 and rapamycin. Therefore, we suggest that fructose stimulates both MTOR–RPS6K and MTOR–EIF4EBP1 signaling cascades for trophoblast cell growth. Other metabolic pathways for activation by fructose remain to be explored to elucidate its role in the pregnant uterus and in other organ systems.

The pentose phosphate pathway is responsible for the generation of reducing equivalents, in the form of NADPH, used in reductive biosynthesis reactions within cells (e.g., fatty acid synthesis), production of ribose-5-phosphate used in the synthesis of nucleotides and nucleic acids, and production of erythrose-4-phosphate used in the synthesis of tyrosine, citrulline, and proline. There is evidence that fructose is used for the synthesis of nucleic acids and reducing equivalents in the form of NADPH H^+ in the fetal pig (21), in ovine placenta (22), and in HeLa cells cultured with fructose as the only hexose sugar (23).

Fructose and glucose are equivalent as substrates in entering metabolic pathways leading to synthesis of neutral lipids and phospholipids in the heart, liver, kidney, brain, and adipose tissue of fetal lambs, refuting general statements that fructose in not metabolized in fetal tissues of domestic animals (24). In adult rats the hepatic activities of glucose-6-phosphate dehydrogenase, malic enzyme, and acetyl-CoA carboxylase are stimulated by glucose, which increases lipogenesis (25), and fructose enters adipocytes by both insulin-independent and in-

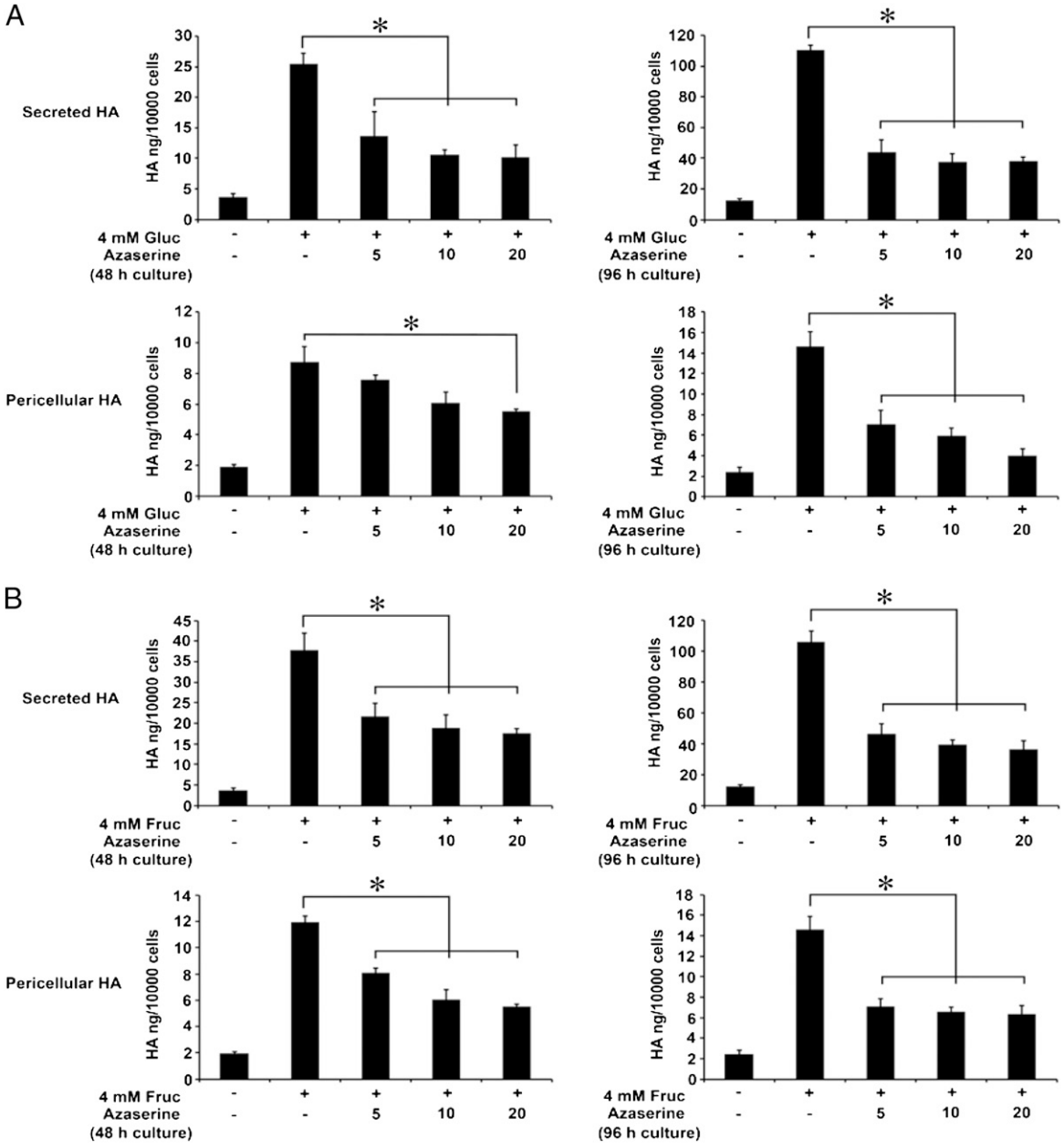

Fig. 8. Glucose and fructose stimulate hyaluronic acid (HA) synthesis and secretion in porcine trophectoderm cells. pTr cells were serum starved for 24 h and pretreated with different concentrations of azaserine for 2 h and then were treated or not treated with glucose (*A*) or fructose (*B*) for 48 h (*Left*) or 96 h (*Right*). (*A*) Quantitation of secreted (*Upper*) and pericellular (*Lower*) hyaluronic acid by glucose. (*B*) Quantitation of secreted (*Upper*) and pericellular (*Right*) hyaluronic acid by fructose. The amount of secreted and pericellular hyaluronic acid in each extract was measured by ELISA based on the specific interaction of hyaluronic acid with hyaluronic acid-binding protein. Asterisks denote an effect of treatment ($*P < 0.01$). See *Materials and Methods* for a complete description.

sulin-insensitive mechanisms (26). In studies with HeLa cells in medium containing 1 mM glucose or less, 80% of the glucose is metabolized via glycolysis, but only 4–5% of the glucose enters the tricarboxylic acid (TCA) cycle. When 2 mM fructose was present in the medium, about 100 times fewer molecules of fructose than glucose were used per mass of cells, and glycolytic activity was reduced about 900-fold. Almost all fructose entered the pentose shunt to produce reducing equivalents and nucleic acids necessary for biosynthetic processes. With fructose in the medium, HeLa cells used glutamine to provide about 98% of the energy, but fructose had no effect on intracellular levels of ATP or TCA-cycle intermediates.

It is possible that the developing conceptus uses fructose in a manner similar to that used by HeLa cells. That is, fructose may be metabolized via the oxidative arm of the pentose shunt to provide the necessary substrate for high rates of cell proliferation and associated biosynthetic processes. Energy may be derived from glutamine catabolism, and it is important to note that glutamine is required for conversion of fructose-6-PO_4 to glucosamine-6-PO_4 (34). A study to determine amino acid concentrations in ovine allantoic fluid and amniotic fluid, as well as in fetal and maternal plasma in pregnant ewes between days 30 and 140 of gestation, revealed that alanine, glutamine, glycine, and serine contributed 50% of total α-amino acids in fetal plasma (35). Interestingly,

concentrations of alanine, citrulline, and glutamine in allantoic fluid increased 20-, 34-, and 18-fold, respectively, between days 30 and 60 of gestation and were 24.7, 9.7, and 23.5 mM, respectively, during this period of rapid placental growth and development (32, 35). Remarkably, in sheep, alanine, citrulline, and glutamine accounted for ~80% of total α-amino acids in allantoic fluid during early gestation, when both the concentration and total amount of fructose increase rapidly (35).

Unique features of fructose may be its production by the placenta and its sequestration in fetal blood and fetal fluids to be metabolized along with glutamine via the hexosamine pathway. This pathway leads to synthesis of glycosaminoglycans, e.g., hyaluronic acid, uridine diphosphate-*N*-acetyl glucosamine, and uridine diphosphate-*N*-acetyl galactosamine, that are involved in the synthesis of glycolipids, glycosaminoglycans, and proteoglycans critical to cell and tissue functions. Hyaluronic acid and hyaluronidase increase in the uterine lumen of pigs in response to progesterone (36). This may stimulate angiogenesis (31) and/or morphogenesis and tissue remodeling of the placenta as reported for the human placenta (37). Wharton's jelly, which is composed primarily of hyaluronic acid and also supports fibroblasts and stem cells (38), accumulates in the placentas of most mammals and localizes primarily in the umbilical cord but also, to a lesser extent, in placental blood vessels (39). It is clear that angiogenesis is critical to conceptus development in all species, and results of the present study indicate that fructose is used for synthesis of glycosaminoglycans such as hyaluronic acid that support angiogenesis, particularly in the placenta.

Uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) is involved extensively in intracellular signaling as a substrate for O-linked *N*-acetylglucosamine transferases, nuclear pore formation and nuclear signaling, and the glucose-sensing mechanism, as well as for cellular insulin sensitivity (40). Uridine diphosphate-*N*-acetylgalactosamine is another sugar-donor metabolite; its addition to serine or threonine residues represents the first step in the biosynthesis of mucins such as O-glycans that impart unique structural features to mucin glycoproteins and membrane receptors and resistance to thermal change and proteolytic attack to a number of proteins. The O-linked carbohydrate side chains function as ligands for receptors for lymphocyte and leukocyte homing and as signals for protein sorting. Overactivity of the hexosamine pathway results in increased UDP-hexosamines; this increase is an important mechanism by which hyperglycemia causes insulin resistance.

Glucose was reported to induce proliferation of human trophoblast cells through MTOR signaling in a PI3K-independent mechanism that involves activation of MTOR by metabolites of the GFPT1 pathway, particularly UDP-GlcNAc: (41). They proposed that UDP-GlcNAc is responsible for phosphorylation of TSC2, a GTPase-activating protein, and p70S6K1, a protein kinase downstream of MTOR, to stimulate trophoblast cell proliferation in response to the metabolism of glucose to glucose-6-PO_4, fructose-6-PO_4, and glucosamine-6-PO_4. Results from the present study indicate that fructose can stimulate the MTOR cell-signaling pathway to affect cell proliferation and also can be used in the hexosamine pathway to synthesize hyaluronic acid that can affect angiogenesis and other aspects of fetal/placental development during pregnancy (Fig. 9).

In summary, the placenta converts available glucose from the mother to fructose, which is sequestered in blood and fluids (allantoic and amniotic) of the conceptus. The literature indicates that fructose enters cells and is converted to fructose-1-PO_4, but it also may be converted to fructose-6-PO_4, which then can be metabolized by various tissues of the conceptus via either the pentose phosphate pathway or the hexosamine pathway. In the mammalian conceptus, fructose plays a role in activating MTOR cell signaling via the hexosamine-synthetic pathway, thereby promoting embryonic/fetal growth and development. Thus, the unusual abundance of fructose in fetal blood and fetal fluids has a physiological role during gestation that must be taken into account in studies of intrauterine development of the conceptus.

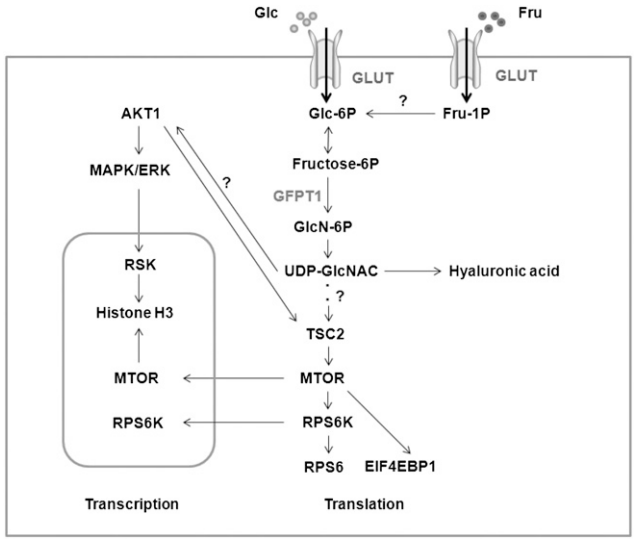

Fig. 9. Schematic diagram of the GFPT1-mediated MTOR cell-signaling pathway affected by glucose and fructose in pTr cells. Fructose stimulates GFPT1 in the hexosamine biosynthesis pathway and activates MOTR–RPS6K and MTOR–EIF4EBP1 signal transduction cascades for stimulation of pTr cell proliferation and growth. AKT1, proto-oncogenic protein kinase Akt; EIF4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; Fru, fructose; Fru-1P, fructose-6-phosphate (fructose-6P); GlcN-6P, *N*-acetylglucosamine-6-phosphate; GFPT1, glutamine-fructose-6-phosphate transaminase 1; Glc, glucose; Glc-6P, glucose-6-phosphate; GLUT, glucose/fructose transporter; MTOR, mechanistic target of rapamycin; RPS6, ribosomal protein S6; RPS6K, ribosomal protein S6K; TSC2, tuberous sclerosis 2; UDP-GlcNAC, UDP-*N*-acetylglucosamine.

Materials and Methods

Cell Culture. An established mononuclear pTr cell line from day 12 pig conceptuses was used in the present in vitro studies, as described previously (42,43). For each experiment, the design was replicated in three independent experiments.

Proliferation Assay. The pTr cells (20% confluent) were subcultured in 24-well plates in growth medium until the monolayer reached up to 30% confluence and then were switched to serum- and insulin-free customized medium. After starvation for 24 h, cells were deprived of glucose and fructose for 6 h. Then either glucose or fructose was added to each well (n = 3 wells per treatment) with 5% (vol/vol) serum at the indicated concentration. Cells grown in serum-free specialized medium and specialized medium containing 10% FBS served as negative and positive controls, respectively. Cell numbers were determined as described previously (44–48).

Western Blot Analyses. Whole-cell extracts and immunoblot assays were prepared and performed as described previously (44). Immunoreactive proteins were detected using rabbit anti-human polyclonal antibodies against p-RPS6 IgG at a 1:1,000 dilution and RPS6 IgG at a 1:2,000 dilution and 10% (wt/wt) SDS/PAGE gel; rabbit anti-human polyclonal p-RPS6K IgG at a 1:1,000 dilution and RPS6K IgG at a 1:2,000 dilution and 10% SDS/PAGE gel; and rabbit anti-human polyclonal p-EIF4EBP1 IgG and EIF4EBP1 IgG, each at a 1:1,000 dilution, and 15% (wt/wt) SDS/PAGE gel. As a loading control, mouse anti–α-tubulin (TUBA) IgG was used after the proteins were detected on the Western blots. All antibodies were from Cell Signaling Technology. Multiple exposures of each Western blot were performed to ensure linearity of chemiluminescent signals.

Immunofluorescence Analyses. The effects of glucose and fructose on phosphorylation of RPS6K, EIF4EBP1, and RPS6 were determined by immunofluorescence microscopy as previously described (44, 45). Cells probed with rabbit anti-human polyclonal p-RPS6K IgG (Thr421/Ser424) at a 1:200 di-

lution, rabbit anti-human polyclonal p-RPS6 IgG at a 1:200 dilution, or rabbit anti-rat polyclonal p-EIF4EBP1 IgG at a 1:200 dilution (antibodies were from Cell Signaling Technology) were incubated with goat anti-rabbit IgG Alexa 488 (Chemicon) at a 1:200 dilution for 1 h at room temperature. Cells then were washed and overlaid with Prolong Gold Antifade with DAPI. For each primary antibody, images were captured with identical microscope and detector settings to facilitate comparisons of spatial distribution and fluorescence intensity among treatments.

Target-Specific siRNAs for GFPT1 Knockdown. For mRNA sequences of porcine *GFPT1* (NM_001194979.1), three potential siRNA target sites were determined using the Invitrogen design program. The most effective target sequence (CAG AUC AUG AAG GGC AAC U) was selected and synthesized. Silencer Negative Control siRNA (Cell Signaling Technology), which does not cause specific degradation of any cellular mRNA, was used as a negative control. Down-regulation of GFPT1 expression was confirmed by Western blotting analyses and immunofluorescence.

Transfection. The pTr cells were treated with specific GFPT1 siRNA; controls included cells that received naive treatment (no siRNA or Lipofectamine 2000) or mock treatment (Lipofectamine 2000 only). Transfection of siRNA was performed according to the manufacturer's procedure. To analyze the effects of azaserine on glucose- and fructose-induced proliferation in pTr cells, glucose (4 mM) and/or fructose (4 mM) with azaserine was added to the culture medium 48 h after transfection, and the incubation was continued for another 48 h. Using green fluorescein conjugate control siRNA duplexes (Cell Signaling Technology), we estimated that more than 95% of the cells were transfected successfully.

Quantitation of Hyaluronic Acid. The amount of hyaluronic acid was measured as described previously (37), with minor modifications. After treatment, cell culture medium was centrifuged at 8,000 × *g* at 4 °C for 5 min, and the supernatant was assayed to determine secreted hyaluronic acid. Then cells were washed three times with PBS and treated with Trypsin-EDTA (0.25%) solution at 37 °C for 5 min; the reaction was stopped by the addition of culture medium just before cell numbers were determined by using a hemocytometer. The cells then were centrifuged, and the supernatant was used for quantitation of pericellular hyaluronic acid. The cell pellet was digested with proteinase K (120 μg/mL) and centrifuged, and the supernatant was analyzed for intracellular hyaluronic acid. The amount of hyaluronic acid in each different extract was measured by ELISA based on the specific interaction of hyaluronic acid with hyaluronic acid-binding protein as determined using the hyaluronic acid detection kit (R&D Systems). To serve as the negative control, some samples were pretreated with hyaluronidase (2 units/mL at 37 °C for 2 h) before being subjected to the ELISA.

Statistical Analyses. All quantitative data were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System (SAS Institute, Inc.). Percent changes in responses were analyzed following log transformation, because percentages are not distributed normally. Data from Western blotting were corrected for differences in sample loading using TUBA data as a covariate. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. Significance was considered to be $P \leq 0.05$. Data are presented as the least-squares mean (LSM) and SEM.

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