Decreased Spermatogenesis, Fertility, and Altered *Slc2A* Expression in *Akt1*^{−/−} and *Akt2*^{−/−} Testes and Sperm

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Abstract

Akt is serine/threonine protein kinase associated with various cellular processes and 3 different isoforms exist. This work describes the reproductive phenotype of *Akt1*^{−/−} and *Akt2*^{−/−} in male mice. The seminiferous tubule diameter in *Akt1*^{−/−} testes was less than wild-type or *Akt2*^{−/−} testes. The expression of phospho-phosphatase and tensin homologue deleted on chromosome 10 (p-PTEN) and phospho-glycogen synthase kinase 3β (GSK-3β) was elevated in *Akt1*^{−/−} testes. Alterations in expression and localization to the plasma membrane of several facilitative glucose transporters (*Slc2a8*, *9a* and *9b*) were detected in these knockout compared to wild-type mice. Apoptotic sperm were more prevalent in both null mice compared to wild-type mice, whereas sperm concentration and motility were significantly lower in the null sperm. Finally, *Akt2*^{−/−} sperm had a markedly decreased fertilization rate by in vitro fertilization (IVF) and resulting embryos displayed increased fragmentation and poor growth. These results suggest that altered *SLC2A* expression and increased PTEN and GSK3β activity may be responsible for the decreased spermatogenesis, sperm maturation, and fertilization in the *Akt1*^{−/−} and *Akt2*^{−/−} male mice.

Keywords

Akt, facilitative glucose transporter, testis, sperm, fertility

Introduction

Akt is a serine/threonine protein kinase activated by insulin and various growth factors critical to many cellular processes such as glucose metabolism, transcription, cell proliferation, apoptosis, migration, cell growth, and tumorigenesis.^{1–3} Three isoforms of *Akt* have been identified in mammalian cells and are widely expressed with isotype-specific features from 3 separate genes.^{4,5} *Akt1* is the most ubiquitously expressed, and *Akt1*^{−/−} mice show increased perinatal mortality and reduced body weight. In addition, these mice show spontaneous apoptosis in testes and decreased spermatogenesis and are subfertile with a 2- to 3-fold decreased concentration of mature sperm.^{6,7} *Akt2* is predominantly expressed in insulin-responsive tissues,^{4,8} and *Akt2*^{−/−} mice demonstrate insulin resistance and diabetes but normal growth.^{7,9} They also experience decreased litter size. *Akt3* is predominantly expressed in the testes and brain,^{10,11} and *Akt3*^{−/−} mice exhibit a decreased brain weight but normal glucose homeostasis, body weight, and fertility.¹²

A recent study shows that mice with the double knockout of *Akt1* and *Akt2* exhibit multiple defects such as a severe growth retardation, skeletal muscle atrophy, impaired skin development, a delay in ossification, and small litter size and the pups that are born die just after birth.¹³ Double knockout mice of *Akt1* and *Akt3* exhibit embryonic lethality with severe impairments in growth, cardiovascular development, and organization of the nervous system.¹⁴ Double knockout mice of *Akt2*

and *Akt3* demonstrate glucose/insulin intolerance and mildly reduced body weight but normal fertility, although spontaneous apoptosis is slightly increased in the testes and testis weight is reduced by ~24%.¹⁵ In addition, *Akt2*^{−/−} adipocytes derived from immortalized mouse embryo fibroblasts display significantly reduced insulin-stimulated hexose uptake and glucose transporter GLUT4 translocation into the plasma membrane. Furthermore, hexose uptake and GLUT4 translocation are completely restored after reexpression of *Akt2* in *Akt2*^{−/−} adipocytes.¹⁶ Recently, we and others have reported that glucose utilization via GLUTs in the mouse testis and sperm is important for spermatogenesis, sperm quality, fertilization capacity, and embryo development.^{17–19} An isotype-specific function for male reproduction, however, has not yet been fully understood. Here we address reproductive characterization of *Akt1*^{−/−} and *Akt2*^{−/−} male mice. The purpose of this study is to examine spermatogenesis, sperm concentration, motility, and fertility

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in these mice and to evaluate whether deletion of *Akt1* or *Akt2* affects GLUT expression in the testes and sperm.

Materials and Methods

Animals

The homozygous *Akt1*^{-/-} and *Akt2*^{-/-} mice were a generous gift from Dr Morris Birnbaum at the University of Pennsylvania. These mice were both on C57BL/6 backgrounds and thus age-matched but nonrelated controls from this strain were used. Mice were housed according to Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Immunohistochemistry and Western Immunoblot Analysis

Immunohistochemistry of GLUTs in the testes was performed in Bouin fixed paraffin-embedded or frozen sections as previously described.^{17,20} In brief, testes were removed, fixed in Bouin solution overnight, and embedded in paraffin. Testicular sections were incubated with anti-Akt, anti-phospho-Akt(p-Akt), anti-phosphatase and tensin homologue deleted on chromosome (PTEN), anti-phospho-PTEN (p-PTEN), phosphoglycogen synthase kinase 3α (GSK3α), phospho-GSK3β, anti-Slc2a8, anti-Slc2A9a, or anti-Slc2A9b antibodies. All antibodies were generated to the mouse proteins. The anti-Akt and p-Akt antibodies detected all isoforms of Akt. The mouse GLUT antibodies were polyclonal antibodies generated in our laboratory and reported previously.^{21–23} All other antibodies were polyclonal and obtained from Cell Signaling (Danvers, Massachusetts). The sections were then incubated with a secondary antibody, Alexa Fluor 546 goat anti-rabbit immunoglobulin G. To-Pro-3 iodide (Molecular Probes, Eugene, Oregon) was used to stain the nuclei. The signals were visualized using enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Biosciences Inc., Piscataway, New Jersey) or SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, Illinois) as described previously.¹⁷ Negative controls were treated similarly but the primary antibody was not added. For experiments represented in Figures 1 to 3, blots were incubated with goat anti-mouse IRDye 680 or goat anti-rabbit IRDye 800 (1:10 000; LI-COR Biosciences, Lincoln, Nebraska) for 1 hour at room temperature. Membranes were scanned and quantitated using the Odyssey fluorescent imager (LI-COR Biosciences). Western blots were quantified, and the values were normalized to β-actin for loading variation.

Indirect Immunofluorescence of Sperm

Indirect immunofluorescence of sperm was performed as described previously.¹⁷ In brief, paraformaldehyde-fixed sperm were incubated with anti-Slc2a8, anti-Slc2A9a, or anti-Slc2A9b antibodies. Sperm were then incubated with a secondary antibody, Alexa Fluor 546 goat anti-rabbit immunoglobulin G. To-Pro-3 iodide was used to stain the nuclei.

Western blot analysis was performed on sperm as described previously.^{17,24}

In Vitro Fertilization and Computer-Assisted Sperm Analysis

Superovulation of female mice was induced by PMSG and hCG (Sigma, St. Louis, Missouri). Preparation of cauda epididymal sperm from the wild-type or *Akt2*^{-/-} male mice, insemination, and embryo culture were performed as described previously.¹⁸ Equivalent concentrations of wild-type and *Akt2*^{-/-} sperm were used to conduct the IVF. Some portions of prepared sperm were used for CASA as described.¹⁸

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Assay

After the sperm were fixed and permeabilized in 0.1% Tween-20, apoptosis was assessed using the In Situ Cell Death Detection kit (Roche, Nutley, New Jersey), according to the manufacturer's protocol. The nuclei were stained with To-Pro-3 iodide dye. After washing 3 times in phosphate-buffered saline (PBS), sperm fluorescence was observed under a confocal microscope with Nikon EZ 7.1 software (Nikon Eclipse E800; Nikon Instruments Corp, Melville, New York). The total number of nuclei and apoptotic nuclei were quantified manually. The sum of the apoptotic nuclei and the total nuclei within each group was then utilized to calculate the percentage of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive nuclei.

Results

Expression of Akt/PTEN-Signaling Molecules in *Akt1*^{-/-} and *Akt2*^{-/-} Testes

Total Akt and p-Akt expressions were significantly decreased in the *Akt1*^{-/-} and *Akt2*^{-/-} testes. P-Akt expression was decreased more than total Akt expression in both *Akt1*^{-/-} and *Akt2*^{-/-} mice (Figure 1A). P-Akt in the wild-type testes appears to be predominantly localized in the spermatogenic cells, as well as Leydig cells. On the other hand, in testes from *Akt1*^{-/-} and *Akt2*^{-/-} mice, p-Akt was barely detectable. In addition, the average diameter of the seminiferous tubule of *Akt1*^{-/-} testis was significantly lower than those of wild-type or *Akt2*^{-/-} testes (*Akt1*^{-/-} 122 μm ± 14 standard deviation [SD]; wild-type 207 μm ± 10 SD; *Akt2*^{-/-} 167 μm ± 12 SD; Figure 1B). The number of sperm cells also appeared to be less in the *Akt1*^{-/-} seminiferous tubules.

Phosphatase and tensin homologue deleted on chromosome is a major regulator of the PI3K/Akt-signaling pathway, and p-Akt inhibits GSK3β activation. As shown in Figure 2A, PTEN and p-PTEN expressions were slightly increased in the *Akt1*^{-/-} testes. The expression of p-GSK3α

Figure 1. Akt and p-Akt expression in *Akt1*^{-/-} and *Akt2*^{-/-} testes. **A**, Western blot analysis of Akt and p-Akt. β-actin was used as the internal control. Note the decreased expression of Akt and p-Akt protein in the *Akt1*^{-/-} and *Akt2*^{-/-} testes. Asterisks indicate significant differences from wild-type group ($P < .001$). Values are expressed as standard error of the mean SEM. **B**, Immunohistochemistry of p-Akt. Bouin fixed paraffin-embedded testicular sections were incubated with a global anti-p-Akt antibody. The sections were then incubated with biotinylated secondary antibody and with enzyme conjugate. Coloring reaction was done using 3,3'-diaminobenzidine ([DAB] brown color), and sections were counterstained with hematoxylin (blue color). Note the decreased diameter of seminiferous tubule and the number of sperm cells in *Akt1*^{-/-} testis. All experiments represent multiple determinations (at least 3 times).

(Tyr279), which is an activated form, was slightly increased in the *Akt2*^{-/-} testes. The expression of p-GSK3β (Tyr216), which is an activated form, was greatly increased in the *Akt1*^{-/-} and *Akt2*^{-/-} testes. Phospho-PTEN in the wild-type testes was

localized predominantly in the most mature spermatogenic cell types as was also seen in the *Akt2*^{-/-} mouse. In the *Akt1*^{-/-} testes, p-PTEN expression appeared increased in the spermatogenic cells (Figure 2B).

Figure 2. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN), phospho-PTEN (p-PTEN), phospho-glycogen synthase kinase 3 α (p-GSK3 α), and p-GSK3 β expression in Akt1 $^{-/-}$ and Akt2 $^{-/-}$ testes. A, Western blot analysis of PTEN, p-PTEN (Ser380), p-GSK3 α (Tyr279), and p-GSK3 β (Tyr216). β -actin was used as the internal control. Asterisks indicate significant differences from the wild-type group ($P < .001$). Values are expressed as standard error of the mean (SEM). B, Immunohistochemistry of phospho-phosphatase and tensin homologue deleted on chromosome 10 (p-PTEN). Bouin fixed paraffin-embedded testicular sections were incubated with anti-p-PTEN antibody. The sections were then incubated with biotinylated secondary antibody and with enzyme conjugate. Coloring reaction was done using 3,3'-diaminobenzidine ([DAB] brown color), and sections were counterstained with hematoxylin (blue color). All experiments represent multiple determinations (at least 3 times).

Expression of Slc2a8, Slc2a9a, and Slc2a9b in Akt1 $^{-/-}$ and Akt2 $^{-/-}$ Testes and Sperm

To address whether Akt1 or Akt2 deficiency affects glucose transporter (SLC2A) expression, we analyzed Slc2a8, Slc2a9a,

and Slc2a9b expression in the testes and sperm of the Akt1 $^{-/-}$ and Akt2 $^{-/-}$ male mice by Western blot analysis. As shown in Figure 3, Slc2a8 expression in whole testes was equal between wild-type and null mice from either strain. However, Slc2a9a was not detected in the Akt1 $^{-/-}$ and Akt2 $^{-/-}$ testes.

Figure 3. Glucose transporter (*Slc2a*) expression in *Akt1*^{-/-} and *Akt2*^{-/-} testes by Western blot analysis. β -actin was used as the internal control. Note the absence of *Slc2a9a* and increased *Slc2a9b* in *Akt1*^{-/-} and *Akt2*^{-/-} testes. Asterisks indicate significant differences from the normal group ($P < .001$). All experiments represent multiple determinations (at least 3 times). Values are expressed as standard error of the mean (SEM).

Interestingly, *Slc2a9b* expression was increased in the *Akt1*^{-/-} and *Akt2*^{-/-} testes. In the absence of staging, images are representative of the largest tubules present in each testis section. Confocal microscopy showed that *Slc2a8* localization was decreased in the plasma membrane of the *Akt2*^{-/-} spermatocytes. In the *Akt1*^{-/-} and *Akt2*^{-/-} testes, *Slc2a9a* was not detected, and *Slc2a9b* was increased in the seminiferous tubules (Figure 4). As shown in Figure 5, *Slc2a8* was similarly localized in the acrosome, mid-piece, and principal piece of the normal and *Akt1*^{-/-} sperm. In the *Akt2*^{-/-} sperm, *Slc2a8* expression was decreased in the acrosome and was not detected in the principal piece. *Slc2a9a* was not detected in the *Akt1*^{-/-} and *Akt2*^{-/-} sperm. *Slc2a9b* was similarly localized in the mid-piece and principal piece of the wild-type, *Akt1*^{-/-}, and *Akt2*^{-/-} sperm.

Increased Apoptosis in *Akt1*^{-/-} and *Akt2*^{-/-} Sperm

It has previously been reported that male *Akt1*^{-/-} mice display increased germ cell apoptosis,^{25,26} however no studies have characterized the sperm that are produced by these mice

and no reports have described the *Akt2*^{-/-} fertility phenotype. To examine whether the deletion of *Akt1* or *Akt2* affects sperm apoptosis, TUNEL assay was performed. The percentage of apoptotic sperm were higher in the *Akt1*^{-/-} (37.9%) and *Akt2*^{-/-} (8.6%) male mice than in the wild-type male mice (4.8%); (Figure 6).

Sperm Concentration, Motility, and Fertility in *Akt1*^{-/-} and *Akt2*^{-/-} Mice

To examine the quality and fertility of the *Akt1*^{-/-} and *Akt2*^{-/-} sperm, computer-assisted sperm analysis (CASA) and in vitro fertilization (IVF) were performed. As shown in Figure 7A, cauda epididymal sperm concentration was significantly lower in the *Akt1*^{-/-} and *Akt2*^{-/-} sperm than in the wild-type sperm. *Akt1*^{-/-} mice had severe oligozoospermia and the sperm concentration of *Akt2*^{-/-} mice was decreased by nearly 50% of the wild-type sperm concentration. In the *Akt1*^{-/-} and *Akt2*^{-/-} male mice, the percentage of motile sperm and of motile sperm with progressive motility was significantly decreased. In addition, the percentage of rapidly moving sperm was lower, and an increase in the percentage of static sperm was higher in the *Akt1*^{-/-} and *Akt2*^{-/-} male mice than in the wild-type male mice.

Next, we performed IVF with a wild-type cumulus-oocyte complex combined with equivalent concentrations of wild-type versus *Akt2*^{-/-} sperm. *Akt1*^{-/-} mice were not included due to their severely low sperm concentration and motility. The wild-type sperm group exhibited an 88.5% fertilization rate, with 64.5% of fertilized embryos developing to the blastocyst stage. On the other hand, sperm isolated from *Akt2*^{-/-} mice showed a markedly decreased fertilization rate (52.5%), with 13.5% of the fertilized embryos developing to the blastocyst stage (Figure 7B). In addition, oocytes fertilized with *Akt2*^{-/-} sperm showed increased fragmentation during cleavage stages and overall poor embryo quality.

Discussion

In this study, we hypothesized that glucose utilization regulated by *Akt* signaling may affect normal spermatogenesis, sperm motility, and fertilization via *Slc2a8*, *Slc2a9a*, and *Slc2a9b*. Our findings support this hypothesis in that sperm from these mice demonstrate decreased glucose transporter expression, increased apoptosis, and impaired function as defined by abnormal fertilization and embryo competence.

Slc2a8 localization was decreased in the plasma membrane and acrosome of the *Akt2*^{-/-} spermatocytes and was not detected in the principal piece of the *Akt2*^{-/-} sperm (Figures 4 and 5). We have shown previously that *Slc2a8* localization in the plasma membrane of the testes is slightly decreased in the streptozotocin (STZ)-induced diabetic mice and markedly decreased in the Akita mice, which have significant hyperglycemia, resulting from a single amino acid substitution in the insulin 2 gene.¹⁸ We have also shown that surface *Slc2a8* expression is regulated by the insulin/insulin-

Figure 4. Localization of *Slc2a8*, *Slc2a9a*, and *Slc2a9b* in *Akt1*^{-/-} and *Akt2*^{-/-} testes by immunofluorescence. Paraformaldehyde-fixed testicular sections were incubated with primary antibodies. The sections were then incubated with a secondary antibody, Alexa Fluor 546 goat anti-rabbit immunoglobulin G (red fluorescence). To-Pro-3 iodide was used to stain the nuclei (blue fluorescence). *Slc2a8* expression was decreased in the plasma membrane of the *Akt2*^{-/-} spermatocytes. *Slc2a9a* was not detected, and *Slc2a9b* was increased in the *Akt1*^{-/-} and *Akt2*^{-/-} testes.

like growth factor 1 (IGF-1) signaling pathways through the IGF-1 receptor in the mouse blastocyst.^{21,27} In different diabetic models, hepatic *Slc2a8* has shown altered expression patterns.²⁸ Taken together, these findings suggest that *Akt2* signaling induced by insulin and/or growth factors may play an important role in *Slc2a8* translocation to the plasma membrane in the mouse testes.

Recently, we have also shown that *Slc2a9a* and *Slc2a9b* are expressed in the mouse testis and differentially localize in the sperm.¹⁷ Here we showed that *Slc2a9a* is not detected in the *Akt1*^{-/-} and *Akt2*^{-/-} testes. Similarly, we reported the same lack of *Slc2a9a* in the insulin-deficient Akita mice.¹⁸ These findings suggest that the absence of posttranscriptional events regulated by insulin-Akt signaling may lead to decreased *Slc2a9a* protein expression or increased degradation as reported in other cell types.^{29,30} Conversely, in the *Akt1*^{-/-} and *Akt2*^{-/-} testes, *Slc2a9b* was increased in the seminiferous

tubules. Consistent with this finding, we have previously discovered that *Slc2a9b* protein expression is significantly increased in the kidney and liver from STZ-induced diabetic mice compared with nondiabetic mice.²⁰ Finally, we discovered that the number of apoptotic sperm was higher in the testis of *Akt1*^{-/-} (37.9%) and *Akt2*^{-/-} (8.6%) mice than in the wild-type mice (4.8%). Prior studies have demonstrated increased germ cell apoptosis in testes, but sperm characteristics have not been studied.^{25,26} Our findings suggest that a lack of Akt expression may result in decreased glucose transporter expression and altered glucose utilization and/or insulin signaling, leading to decreased sperm competence.

Furthermore, our findings indicate that the average seminiferous tubule diameter and the density of sperm cells of *Akt1*^{-/-} testis were much lower than those of wild-type or *Akt2*^{-/-} testes. In the *Akt1*^{-/-} testes, p-PTEN expression was increased in the spermatogenic cells. In addition, the expression of active p-

Figure 5. Immunofluorescence of *Slc2a8*, *Slc2a9a*, and *Slc2a9b* in the *Akt1*^{-/-} and *Akt2*^{-/-} sperm. Paraformaldehyde-fixed sperm were incubated with primary antibodies. Sperm were then incubated with a secondary antibody, Alexa Fluor 546 goat anti-rabbit immunoglobulin G (red fluorescence). To-Pro-3 iodide was used to stain the nuclei (blue fluorescence). *Slc2a8* expression in the *Akt2*^{-/-} sperm was decreased in the head and was not detected in the principal piece. *Slc2a9a* was not detected in the *Akt1*^{-/-} and *Akt2*^{-/-} sperm. h, head; m, mid-piece; p, principal piece.

GSK3 β (Tyr216) was greatly increased in both the *Akt1*^{-/-} and *Akt2*^{-/-} testes. Phosphatase and tensin homologue deleted on chromosome 10 phosphatase, is a tumor suppressor implicated in a wide variety of human cancers.³¹ Phosphatase and tensin homologue deleted on chromosome 10 encodes a 403 amino acid polypeptide originally described as a dual-specificity protein phosphatase.³² Phosphatase and tensin homologue deleted on chromosome 10 is a major negative regulator of the PI3K/Akt-signaling pathway^{33,34} and possesses a carboxy terminal, noncatalytic regulatory domain with 3 phosphorylation sites (Ser380, Thr382, and Thr383) that regulate PTEN stability and may positively affect its biological activity.^{35,36} Glycogen synthase kinase 3 was initially identified as an enzyme that regulated glycogen synthesis in response to insulin.³⁷ Glycogen synthase kinase 3

is a ubiquitously expressed serine/threonine protein kinase that phosphorylates and inactivates glycogen synthase. Glycogen synthase kinase 3 is a critical downstream element of the PI3 kinase/Akt cell survival pathway, and its activity can be inhibited by Akt-mediated phosphorylation at Ser21 of GSK-3 α and Ser9 of GSK-3 β .^{38,39}

Our finding of an increased amount of p-PTEN in the testes of *Akt1*^{-/-} mice is not unexpected since recent studies have shown that inhibition of Akt phosphorylation/activity leads to PTEN phosphorylation (Ser380) and GSK3 β (Ser9) phosphorylation, with subsequent activation of both.⁴⁰⁻⁴² Specifically, zebrafish embryogenesis relies on a counterbalance between prosurvival, proliferation PI3K-Akt signaling and the proapoptotic, antiproliferation activity of PTEN isoforms.

Figure 6. Apoptosis analysis in the *Akt1*^{-/-} and *Akt2*^{-/-} sperm by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. **A**, Representative pictures for TUNEL assay of sperm. Apoptotic nuclei were depicted in red. To-Pro-3 iodide was used to stain the nuclei (blue fluorescence). **B**, Apoptotic sperm were higher in the *Akt1*^{-/-} (37.9%) and *Akt2*^{-/-} (8.6%) male mice than in the wild-type male mice (4.8%). Asterisks indicate significant differences from wild-type group ($P < .001$). All experiments represent multiple determinations (at least 3 times). Values are expressed as standard error of the mean (SEM).

Disrupting steady-state PTEN levels via deficiency of Akt activity leads to altered gastrulation movements and consequent developmental defects in zebrafish. The conclusion of these studies was that coordinate regulation of PTEN activity by PI3K-Akt signaling is an adaptation allowing cells to modulate growth factor activation of the PI3 K pathway under

normal conditions and, if necessary, to cope with environmental stress during embryogenesis. The same balance of Akt/PTEN/GSK3 could be operating in the process of spermatogenesis, as in our study. How this link between deficiency of Akt expression and increased PTEN/GSK3 β activation leads to decreased sperm competence, characterized by decreased

Figure 7. Functional analysis of *Akt1*^{-/-} and *Akt2*^{-/-} sperm. **A**, Computer-assisted sperm analysis (CASA) of *Akt1*^{-/-} and *Akt2*^{-/-} sperm. Mean sperm concentration, motility, and progressive motility were significantly lower in the *Akt1*^{-/-} and *Akt2*^{-/-} sperm than in the wild-type sperm. Static sperm were significantly higher in the *Akt1*^{-/-} and *Akt2*^{-/-} sperm than in the wild-type sperm. Asterisks indicate significant differences from wild-type group ($P < .001$). **B**, In vitro fertilization (IVF) using *Akt2*^{-/-} sperm. Percentage of fertilized embryos with normal oocytes and percentage of fertilized embryos developed to blastocyst were much lower in the *Akt2*^{-/-} sperm group than in the wild-type sperm group.

motility/fertilization and abnormal expression of glucose transporters, is the question raised by this study. Elsewhere in the male reproductive tract PTEN/GSK3 β are inversely linked to androgen receptor (AR) activity and Wnt/beta-Catenin activation. In prostate, an increase in PTEN/GSK3 β results in decreased AR activity and abnormal Wnt pathway signaling.⁴³ It is conceivable that this same inactivation of AR and

downstream events could be responsible for the changes seen in sperm and seminiferous tubules in the *Akt1*^{-/-} and *Akt2*^{-/-} mice. Future studies will test this hypothesis.

In regard to the Akt isotype-specific function in male reproduction, sperm concentration and motility were all significantly lower in the *Akt1*^{-/-} and *Akt2*^{-/-} sperm. *Akt1*^{-/-} mice were so severely oligospermic that IVF could not be performed. In

Akt2^{−/−} mice, IVF was possible, however, this sperm had much lower fertilization capacity than wild-type mice and of the embryos that formed, and developmental competence was significantly impaired. We have previously demonstrated that male insulin-dependent diabetes leads to male subfertility by altering steroidogenesis, sperm motility, and *Slc2a* expression.¹⁸ Original observations of AKT1 and 2 null mice suggest that the isotypes impart distinct phenotypes, with *Akt1*^{−/−} impairing the growth of the organism while maintaining similar glucose sensitivity to wild-type mice, whereas AKT2 null mice present with impaired glucose utilization. Although isotype distribution varies among tissues and even species, it is known that each isotype triggers signaling of a different pathway despite their similar protein structure resulting in the distinct phenotypes as evidenced by the example of severe oligospermia noted in the AKT1 ^{−/−} versus AKT2 ^{−/−} mice. The results of the present study suggest that *Akt1* and *Akt2* signaling may differentially affect GLUTs expression and thus ultimately leading to decreased spermatogenesis, sperm maturation, and fertilization in the *Akt1*^{−/−} and *Akt2*^{−/−} testes and sperm. It is well known that the fertilization capability of male gametes is directly linked to glucose metabolism^{44,45} and that spermatogenesis is disturbed in diabetes perhaps contributing, in part to infertility in these males.⁴⁶ In addition, many studies have documented abnormalities in testicular function and spermatogenesis in diabetic animals.^{47–49}

Male reproductive alterations have also been widely reported in individuals with diabetes, both type 1 and 2.^{50,51} In men affected by insulin-dependent diabetes, sperm have severe structural defects,⁵² significantly lower motility, and lower ability to penetrate hamster eggs.⁵³ Although, the molecular mechanisms of reduced spermatogenesis, motility, and fertilization capacity in *Akt1*^{−/−} and *Akt2*^{−/−} male mice remain to be elucidated, *Akt1* and *Akt2* signaling may play an important role in GLUT expression and PTEN/GSK3 β activity, all directly affecting sperm number and quality.

In summary, this study is the first to determine that the phenotype of male infertility in male *Akt1*^{−/−} and *Akt2*^{−/−} diabetes appears to be related to the expression of glucose transporters, possibly due to decreased glucose utilization as well as decreased prosurvival signaling and increased apoptotic signaling during sperm development via the Akt and PTEN/GSK3 β pathways, respectively. Improving insulin sensitivity and glucose uptake in diabetic males or directly activating the Akt pathway during IVF may improve not only sperm parameters but also fertilization and embryo/pregnancy outcomes.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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