Modern Biotechnology

PROPOSAL

# Different types of Akt family knockout mice generated by CRISPR/Cas9 system and their phenotype analysis to reveal the importance of Akt1

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**Abstract**

Akt is a critical regulator of multiple cellular processes, which is very important in early development and glucose metabolism. There are three isoforms of Akt in mammals: Akt1/PKB $\alpha$ , Akt2/PKB $\beta$ and Akt3/PKB $\gamma$ By generating $Akt1^{-/-}$, $Akt2^{-/-}$, $Akt3^{-/-}$, $Akt1^{-/-}Akt2^{-/-}$, $Akt1^{-/-}Akt3^{-/-}$, $Akt2^{-/-}Akt3^{-/-}$ and $Akt1^{+/-}Akt2^{-/-}$ $Akt3^{-/-}$ mice and test the normality of them respectively, I want to discover the physiological functions they take and to address the issues of isoform redundancy of the Akt family in vivo.

**Purpose/Rationale**

Akt, also known as Protein Kinase B (PKB), is a serine/threonine-specific protein kinase that functions as a critical regulator of multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration, which belongs to the cAMP-dependent protein kinase A/ protein kinase G/ protein kinase C (AGC) super family of protein kinases. These important roles Akt taken place make deregulations of Akt tightly associated with human diseases including cancer and diabetes.And there are three isoforms of Akt in mammals, termed Akt1/PKB $\alpha$ , Akt2/PKB $\beta$ and Akt3/PKB $\gamma$ . In fact, the coordination between signal specificity and kinome redundancy is a fundamental issue in cell biology but haven't been fully understood. The duplications of gene loci in vertebrates have led to the expansion of individual kinases into several homologous isoforms and made the signaling pathways more complex. Such an important kinase Akt has three isoforms

in mammals, making me wander what's the difference between these three isoforms and do they actually work separately or they are able to compensate for each other.

Nowadays, CRISPR-Cas9 system is a very hot researching field, which can be used to genome editing in mammals, including mice. In this proposal, I want to use CRISPR-Cas9 technique to generate different types of Akt knockout mice to discover the functions of three different types of Akt and their compensating relationships.

Hypothesis:

The three isoforms of Akt have differential physiological functions and can compensate for each other but may be not completely.

Specific aims:

AIM I: Using CRISPR-Cas9 technique to produce $Akt1^{-/-}$, $Akt2^{-/-}$, $Akt3^{-/-}$ mice respectively.

AIM II: Observe the phenotype and do molecular experiments of different Akt knockout mice and compare results with each other and with wild-type mice.

AIM III: Using CRISPR-Cas9 technique to produce combined-knockout mice $Akt1^{-/-}Akt2^{-/-}$, $Akt1^{-/-}Akt3^{-/-}$ and $Akt2^{-/-}Akt3^{-/-}$ respectively.

AIM IV: Observe the phenotype and do molecular experiments of these three kinds of mice in aim3 separately and compare results with each other.

AIM V: According to results above to determine the mice type needed to be produced, for example, $Akt1^{-/-}Akt2^{-/-}Akt3^{-/-}$ or $Akt1^{+/-}Akt2^{-/-}Akt3^{-/-}$.

AIM VI: Combine all results to get conclusion.

By doing this experiment, we can get better understanding of physiological functions of three isoforms of Akt and their compensating relationship, which will benefit to understanding the Akt pathway and related diseases such as diabetes and cancer.

**Background**

All three Akt/PKB isoforms share a conserved domain structure: an amino terminal pleckstrin homology (PH) domain, a central kinase domain and a carboxyl-terminal regulatory domain that contains the hydrophobic motif (Fig1)[1]

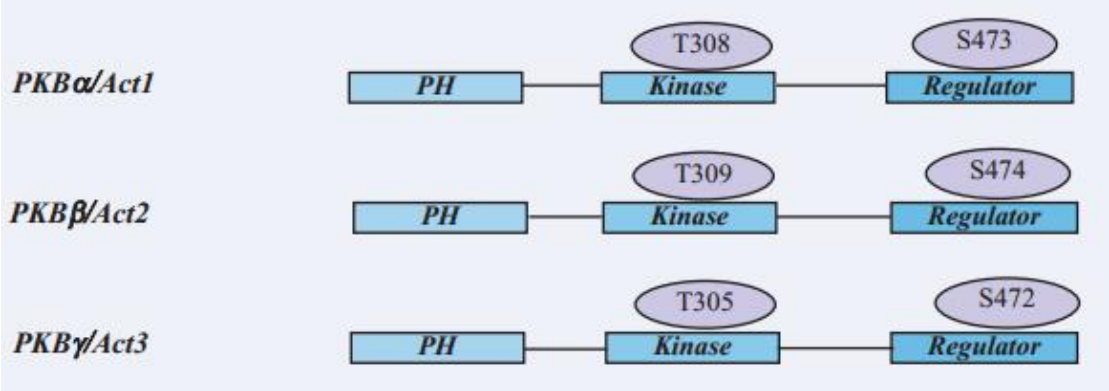


Fig1: Domain structure of human Akt/PKB isoforms.

The PH domain of these three isoforms can interact with membrane lipid products such as phosphatidylinositol(3,4,5)-trisphosphate (PIP3) produced by activated phosphatidylinositol-3-kinase (PI3K) to fully activate Akt. Akt pathway can be activated by binding of an extracellular ligand to a receptor tyrosine kinase (RTK) in the plasma membrane (Fig2). And after the activation, Akt mediates downstream responses by phosphorylating a range of intracellular proteins.

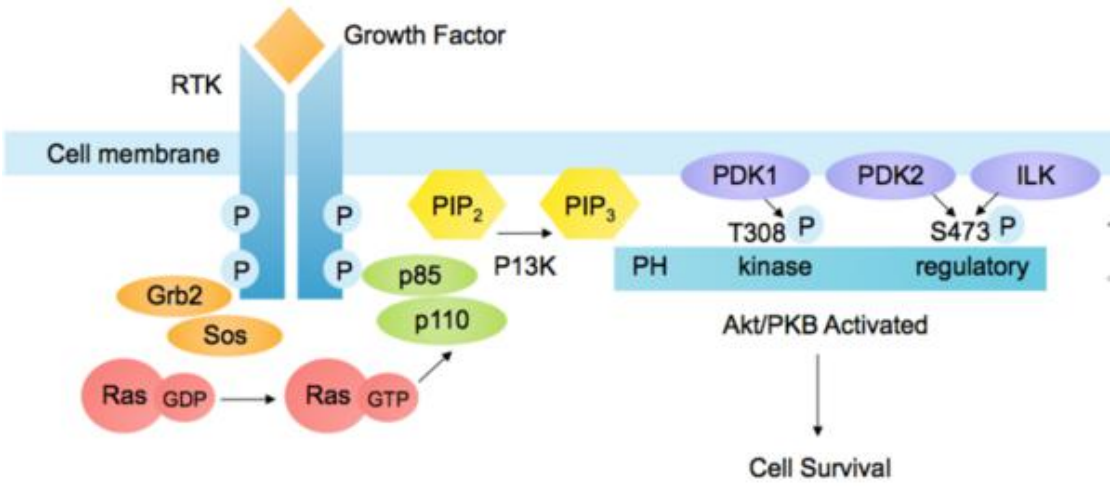


Fig2: Activation of the PI3K-Akt pathway by a Receptor Tyrosine Kinase.

Akt1 shares 81% and 83% amino acid identity with Akt2 and Akt3, respectively. Although the three isoforms show broad tissue distribution[2], Akt1 is the most ubiquitously expressed. Akt2 is expressed at a lower level than Akt1 except in insulin-responsive tissues where it predominates [3,4]. Akt3 is expressed at the lowest level except in testes and brain[5,6]. Preliminary analysis of the Akt1, Akt2, and Akt3 gene products suggest that the three isoforms have similar biochemical characteristics[7] and that phosphorylation of two sites is necessary for full activation of each isoform.

CRISPR/Cas9 system of RNA-guided genome editing, based on a nature immune system used for bacteria to protect themselves from infection by virus, is revolutionizing genetics research in a wide spectrum of organisms.

CRISPR consists of two components: a “guide” RNA (gRNA) and a non-specific CRISPR-associated endonuclease (Cas9). The gRNA is a short synthetic RNA

composed of a “scaffold” sequence necessary for Cas9-binding and a user-defined ～20 nucleotide “spacer” or “targeting” sequence which defines the genomic target to be modified. Thus, one can change the genomic target of Cas9 by simply changing the targeting sequence present in the gRNA. The detail working principle of this technique is that the CRISPR-derived RNA and trans-activating RNA together can form a tracrRNA/crRNA complex by base pairing. This complex can guide Cas9 protein to cut the double-stranded DNA at target sequence paired with crRNA. And by designing these two kinds of RNA artificially, we can form singleguide RNA (sgRNA) with guiding role to guide the Cas9 protein to cut DNA at specific locations (Fig3)[8].

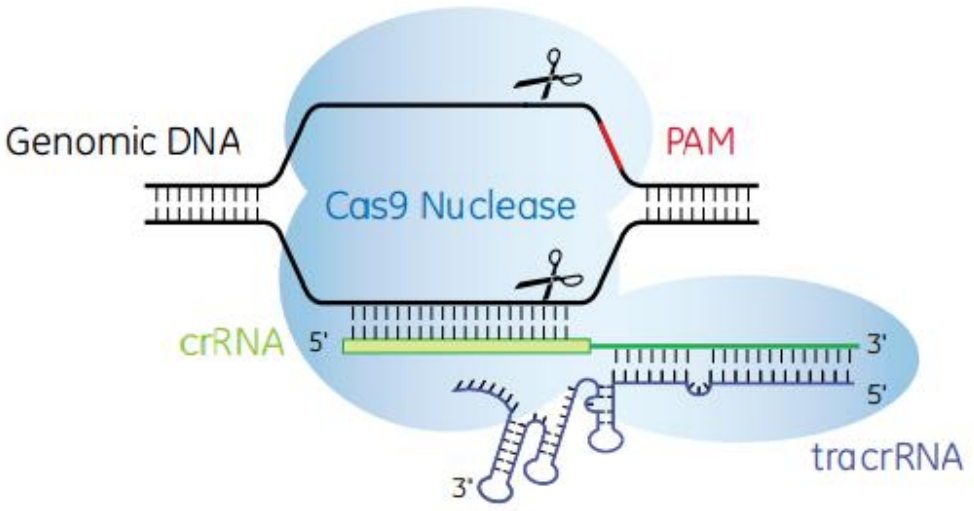


Fig3: Illustration of Cas9 nuclease (light blue), programmed by the crRNA (green); tracrRNA (purple) complex, cutting both strands of genomic DNA 5’ of the PAM (red).

**Research plan**

**Material and Methods:**

AIM I: Using CRISPR-Cas9 technique to produce Akt1$^{-/-}$, Akt2$^{-/-}$, Akt3$^{-/-}$ mice

respectively.

Use CRISPR Design Tool at http://crispr.mit.edu/. to predict gDNA seed sequences and potential off-target sites.

Prepare the targeted sgRNA by cloning annealed complementary oligos into pX330-U6-chimeric_BB-CBh-hSpCas9 at the BbsI site, generating PCR products containing a T7 promoter sequence, then performing in vitro transcription using MEGAshortscript T7 Transcription kit (Life Technologies). Products were purified using the MEGAclear kit (Life Technologies, cat. No. AM1908).[9]

Produce capped polyadenylated Cas9 mRNA by in vitro transcription too.

(Actually we can also purchase Cas9 mRNA and sgRNA in the market.)

Before the formal coinjection, determine the optimal concentration of Cas9 mRNA for targeting in vivo is necessary. We can microinject varying amounts of Cas9-encoding mRNA with Akt1/Akt2/Akt3 targeting sgRNA separately at constant concentration, such as 20ng/ml into pronuclear (PN) stage one-cell mounse embryos and assess the frequency of altered alleles at the blastocyst stage using the RFLP assay. Then we can get an amount that have most efficiency but less toxicity to be our final amount to inject[10].

Then coinject Cas9 mRNA with sgRNAs into zygote.

Then blastocysts derived from the injected embryos can be transplanted into foster mothers.

Extract DNA from tails of mice and using PCR to detect whether the knockout is successful or not.

AIM II: Observe the phenotype and do molecular experiments of different Akt knockout mice and compare results with each other and with wild-type mice.

For all three kinds of knockout mice and wild-type:

Use western blot to analyze protein lysates derived from mouse embryo fibroblasts (MEFs) isolated from wild-type embryos and homozygous knockout embryos of three isoforms respectively to confirm the amount of Akt protein expression.

Compare the size of the wild-type mice and knockout mice at the same age.

Do the oral glucose-tolerance test and glucose measurements for each group. Male and female wild-type and Akt1/2/3-null mice at 7 weeks of age can be fasted overnight respectively. Then collect the blood sample from the retro-orbital sinus with a micropipette (0.025ml) immediately before administration of a glucose load (1g gluose per kilogram of body weight) by oral gavage using a syringe equipped with a murine oral-feeding needle (20 gauge; Popper & Sons Inc., New Hyde Park, New York, USA). Blood samples were taken at 30, 60, and 120 minutes following glucose administration. Blood was immediately diluted into 100ul of 0.025% heparin in normal saline on ice. Red cells were pelleted by centrifugation at top speed in a Beckman Microfuge 12 for 2 minutes. Glucose was determined in the supernatants using the Roche/Hitachi 912 clinical chemistry analyzer (Roche Diagnostics Corp, Indianapolis, Indiana, USA).

Besides these experiments:

For Akt2, micro-CT scanning can be used to assess the size of multiple adipose

depots.

For Akt3, as we already know that it's expressed large level in brain, brain weight measurement will be done and compared with wild-type mice.

AIM III: Using CRISPR-Cas9 technique to produce combined-knockout mice $Akt1^{-/-}Akt2^{-/-}$, $Akt1^{-/-}Akt3^{-/-}$ and $Akt2^{-/-}Akt3^{-/-}$ respectively.

Only difference compared to AIM I is that here we coinject Akt1 and Akt2/ Akt1 and Akt3/ Akt2 and Akt3 sgRNAs together with Cas9 mRNA into zygotes.

AIM IV: Observe the phenotype and do molecular experiments of these three kinds of mice in aim3 separately and compare results with each other.

For $Akt1^{-/-}Akt2^{-/-}$, $Akt1^{-/-}Akt3^{-/-}$ mice, because of their early death we will not discuss them for further experiment.

For $Akt2^{-/-}Akt3^{-/-}$ mice, all experiments have already commonly done in all three single knockout mice will be done.

After getting the result that these mice are relatively normal except the smaller size, I want to know whether upregulation of the Akt1 in these mice is the reason of the normality. So immunoblotting of protein extracts from various tissues of 4-day-old $Akt2^{-/-}Akt3^{-/-}$ mice with an isoform-specific antibody against Akt1 will be done.

AIM V: According to results above to determine the mice type needed to be produced, for example, $Akt1^{-/-}Akt2^{-/-}Akt3^{-/-}$ or $Akt1^{+/-}Akt2^{-/-}Akt3^{-/-}$.

Because $Akt1^{-/-}Akt2^{-/-}$ and $Akt1^{-/-}Akt3^{-/-}$ mice both exhibit severe development deficiency and die early, $Akt1^{-/-}Akt2^{-/-}Akt3^{-/-}$ seems do not need to be produced.

After ruling out the possibility that Akt1 upregulation is the reason why $Akt2^{-/-}Akt3^{-/-}$ mice can be relatively normal, I think produce $Akt1^{+/-}Akt2^{-/-}Akt3^{-/-}$ mice to see whether Akt1 is dose-dependent affects mouse development or not is necessary. Mating $Akt1^{-/-}$ mice and $Akt2^{-/-}Akt3^{-/-}$ mice can get $Akt1^{+/-}Akt2^{+/-}Akt3^{+/-}$ mice. Then cross $Akt1^{+/-}Akt2^{+/-}Akt3^{+/-}$ mice with $Akt2^{-/-}Akt3^{-/-}$ mice can get our targeted $Akt1^{+/-}Akt2^{-/-}Akt3^{-/-}$ mice.

**Data analysis:**

AIM I: Using CRISPR-Cas9 technique to produce $Akt1^{-/-}$, $Akt2^{-/-}$, $Akt3^{-/-}$ mice respectively.

All three knockout mice are expected to be viable.

AIM II: Observe the phenotype and do molecular experiments of different Akt knockout mice and compare results with each other and with wild-type mice.

The expected result of all three isoforms of Akt is shown below in Fig4 - the knockout mice should show no detectable level of Akt1/Akt2/Akt3 protein respectively[11]. Thus, the targeted disruption resulted in a functionally null allele.

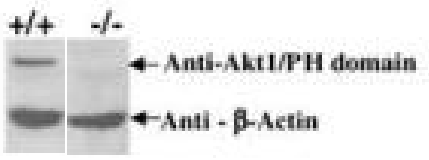


Fig4: Western blot analysis of proteins extracted from mouse embryo fibroblasts (MEFs) isolated

from wild-type (+/+) embryos and homozygous knockout (-/-) embryos.

For Akt1, the Akt1$^{-/-}$ mice are expected to be viable but increased perinatal mortality may occur. And the body weight of knockout mice is expected to be about 20% smaller than wild-type, like shown in Fig5 and Fig6. And they are not expected to display a diabetic phenotype according to the oral glucose-tolerance test and glucose measurements.


Fig5: Side view of 5-week-old mice.

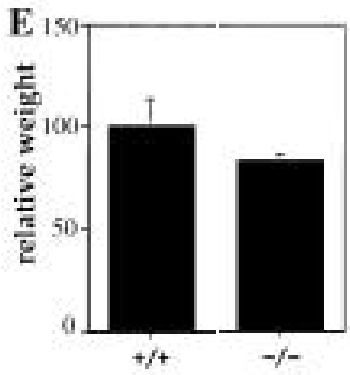


Fig6: Relative body weight of wild-type and Akt1 knockout mice 30days after birth.

For Akt2, the Akt2$^{-/-}$ mice are expected to be viable and born in the expected Mendelian ratio. Both male and female Akt2-null mice exhibited a mild growth deficiency. And adipose tissue mass is expected to decrease in Akt2 knockout mice, like shown in Fig7.[12]

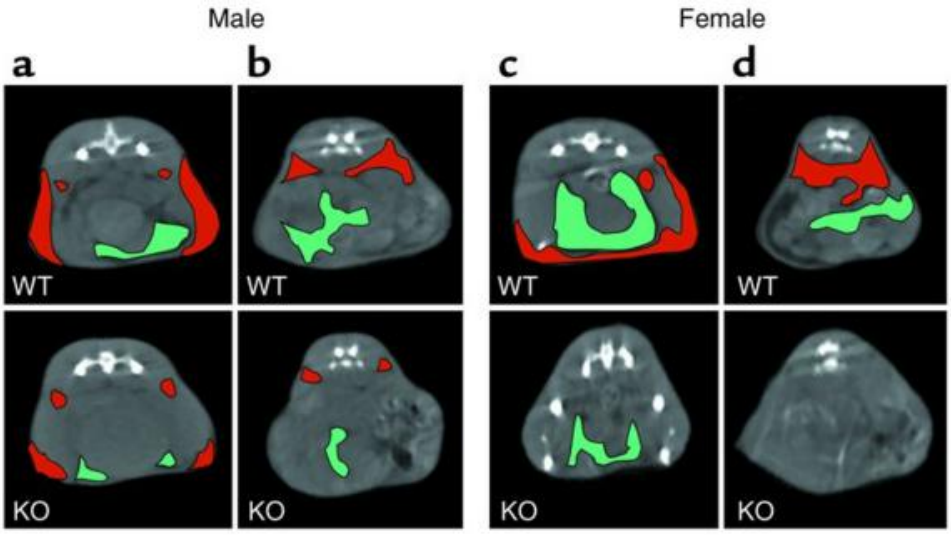


Fig7: Adipose tissue mass is decreased in Akt2-null mice. Representative cross-sectional images of wild-type (WT) and Akt2-null (KO) male (a and b) and female (c and d) mice subjected to micro-CT analysis of in situ adipose tissue mass. (a and c) The inguinal subcutaneous (red) and epididymal/gonadal (green) depots and (b and d) the retroperitoneal (red) and mesenteric (green) depots are demarcated for illustration of the gross effect of the Akt2 deficiency on adipose tissue mass.

Akt2 knockout mice are expected to exhibit fasting hyperglycemia and glucose intolerance in the oral glucose-tolerance test, shown in Fig8.

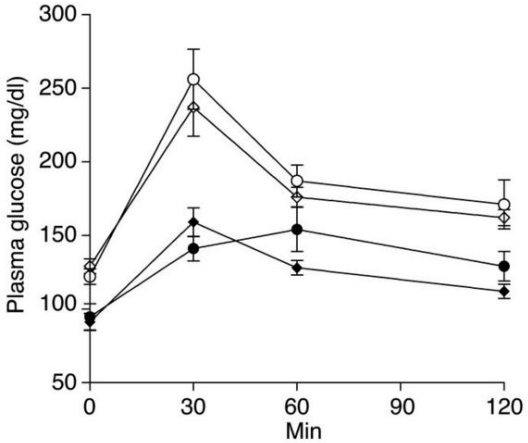


Fig8: Seven-week-old Akt2-null mice exhibit fasting hyperglycemia and glucose intolerance in an oral glucose-tolerance test. Blood samples were taken from overnight-fasted Akt2-null (open symbols) and wild-type (filled symbols) mice at time zero. Mice were immediately given an oral dose of glucose (1 g/kg), and blood was sampled at the indicated times. Plasma glucose levels were significantly elevated in both male and female Akt2-null mice (open circles and diamonds, respectively) relative to wild-type male and female mice (filled circles and diamonds, respectively) at time zero and 30 minutes following the glucose load.

For Akt3, knockout mice are expected to be born at a Mendelian frequency and not exhibit any overt abnormalities in growth. But the reduction in brain weight is expected to exhibit (Fig9), without abnormality in glucose homeostasis and body weights (Fig10)[13].

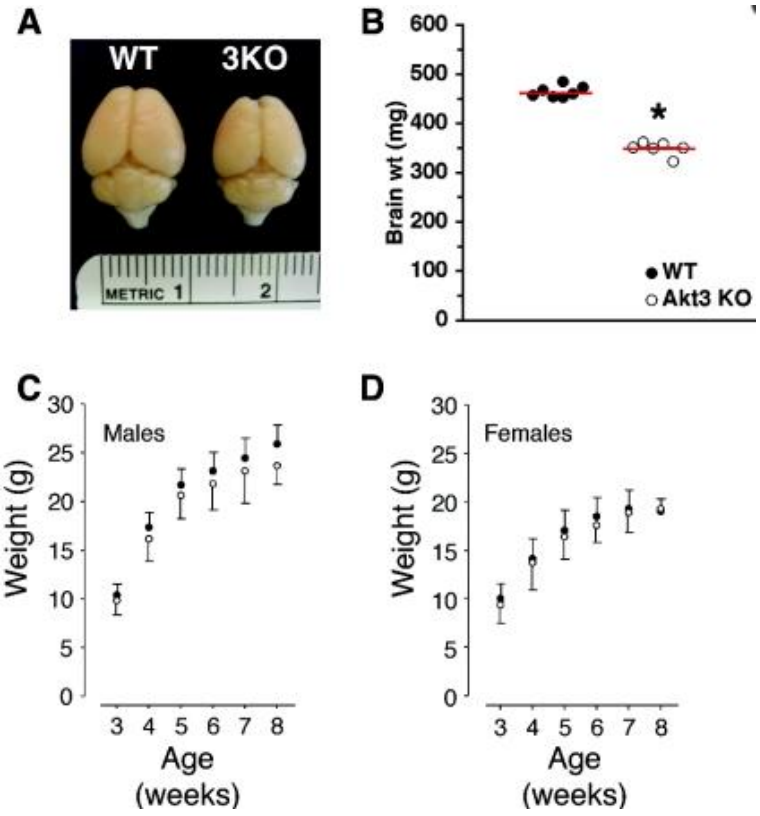


Fig9: Selective reduction of brain size in Akt3-deficient mice. (A) Brains were dissected from 30-week-old wild-type (WT) and Akt3-deficient (3KO) mice. (B) Plot of adult brain weights from Akt3 knockout mice and littermate controls ($N$ = 6 or 7). The mean value is shown by a horizontal red bar. The asterisk indicates that $P$< 0.001 for wild-type versus Akt3-deficient brains. Male (C) and female (D) mice were weighed periodically during the first 8 weeks of life. Filled circles represent wild-type mice; open circles depict Akt3-deficient mice. Values are means ± standard errors of the means (SEM) ($N$ = 15 to 20).

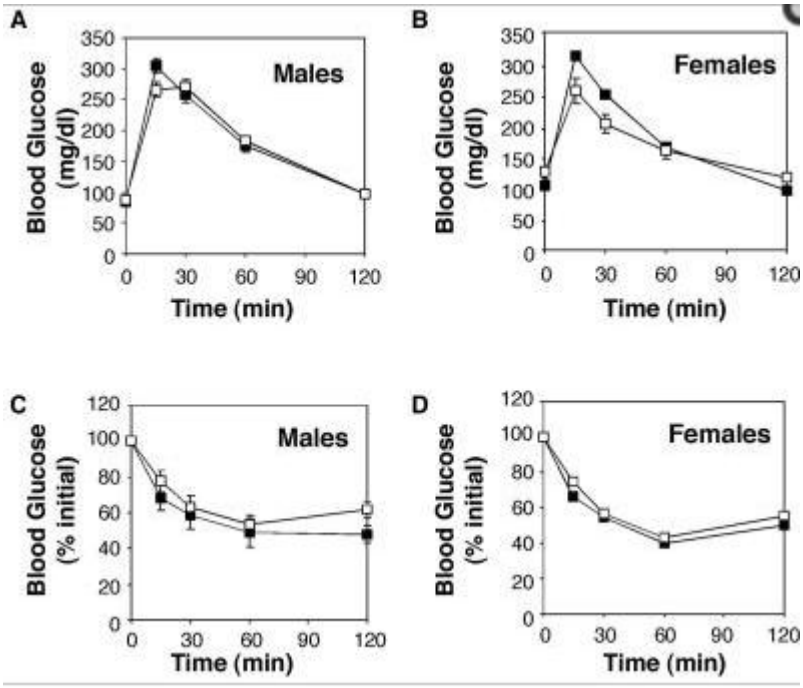


Fig10: Metabolism in Akt3-deficient mice. (A and B) Glucose tolerance tests were performed with male (A) and female (B) mice as described in Materials and Methods. Values represent means ± SEM (*N* = 8 to 10). Filled squares represent wild-type mice; open squares depict Akt3-deficient mice. (C and D) Insulin tolerance tests were performed with male (C) and female (D) mice as described in Materials and Methods. Values represent means ± SEM (*N* = 8 to 10). Filled squares represent wild-type mice; open squares depict Akt3-deficient mice.

AIM III: Using CRISPR-Cas9 technique to produce combined-knockout mice $Akt1^{-/-}Akt2^{-/-}$, $Akt1^{-/-}Akt3^{-/-}$ and $Akt2^{-/-}Akt3^{-/-}$ respectively.

$Akt1^{-/-}Akt2^{-/-}$ mice are expected to die shortly after birth and display multiple defects[14].

$Akt1^{-/-}Akt3^{-/-}$ mice are expected to die around embryonic day 12[15].

$Akt2^{-/-}Akt3^{-/-}$ mice are expected to be viable.

AIM IV: Observe the phenotype and do molecular experiments of these three kinds of mice in aim3 separately and compare results with each other.

$Akt2^{-/-}Akt3^{-/-}$ mice are expected to survive with no other obvious abnormality but

relatively smaller size compared to wild-type mice(Fig11)[16]. And Akt1 level in $Akt2^{-/-}Akt3^{-/-}$ mice in different tissue types show no signs of upregulation (Fig12), which rules out the possibility that the compensatory upregulation of Akt1 make $Akt2^{-/-}Akt3^{-/-}$ mice show this normal phenotype.


Fig11: Top view of wild-type (WT), *Akt2*−/− *Akt3*−/− (DKO) (12-week-old male mice).

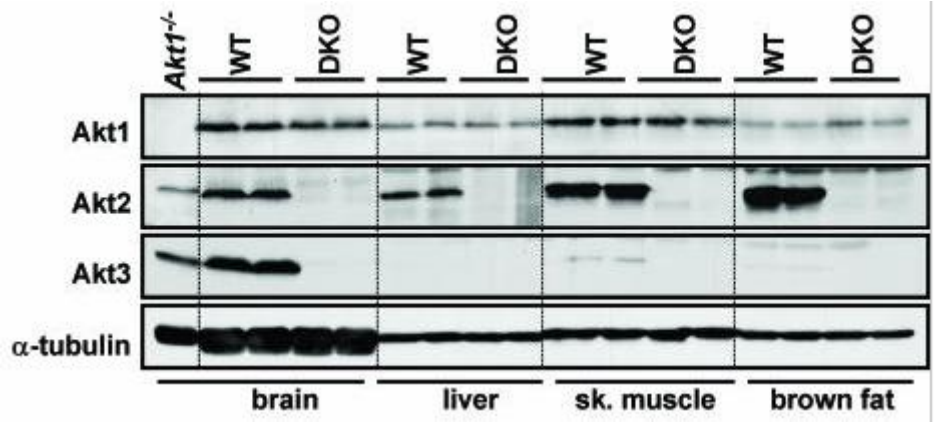


Fig12: Akt1 protein levels in 4-day-old wild-type (WT) and *Akt2*−/− *Akt3*−/− (DKO) pups. The indicated tissues from wild-type and DKO pups were lysed and blotted with antibodies specific for Akt1, Akt2, and Akt3. As a loading control, the expression of α-tubulin was detected on the same membrane. Duplicates are from independent samples.

AIM V: According to results above to determine the mice type needed to be produced, for example, $Akt1^{-/-}Akt2^{-/-}Akt3^{-/-}$ or $Akt1^{+/-}Akt2^{-/-}Akt3^{-/-}$.

Reduction of total Akt is expected in $Akt1^{+/-}Akt2^{-/-}Akt3^{-/-}$ mice(Fig13). Smaller size and relatively normality still are expected in this type of mice.

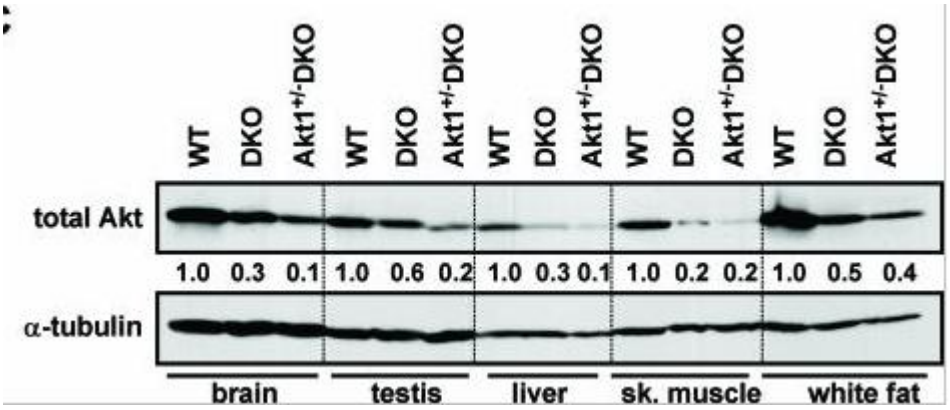


Fig13: Stepwise reduction of total Akt levels in wild-type (WT), *Akt2−/− Akt3−/−* (DKO), and *Akt+/− Akt2−/− Akt3−/−* (Akt+/−DKO) mice. Tissues from 3-month-old mice of the indicated genotypes were lysed and blotted with an Akt antibody that recognizes all three isoforms. As a loading control, the expression of α-tubulin was detected on the same membrane. Bands were quantified, and indicated values represent total Akt expression relative to wild-type expression levels, normalized to the α-tubulin control.

AIM VI: Combine all results to get conclusion.

Mice lacking Akt1 exist increased perinatal mortality and reductions in body weight.

Differently, mice lacking Akt2 are born in the expected Mendelian ratio still with slighter abnormal growth. Moreover, they exhibit a diabetes-like syndrome with an elevated fasting plasma glucose level and peripheral insulin resistance.

And Akt3-deficient mice exhibit a reduction in brain weight but maintain normal glucose homeostasis and body weights.

These results indicate that these three Akt isoforms have differential physiological functions, leading us to the experiments disrupting two of them at the

same time.

The result that $Akt1^{-/-}Akt2^{-/-}$ mice and $Akt1^{-/-}Akt3^{-/-}$ mice cannot even survive but the $Akt2^{-/-}Akt3^{-/-}$ mice and $Akt1^{+/-}Akt2^{-/-}Akt3^{-/-}$ mice can be relatively normally grown up demonstrate that the Akt1 is very important in development. Akt1 is sufficient enough, even in the case where only one allele is present, to enable successful embryonic development and postnatal survival in mice lacking Akt2 and Akt3.

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