Dosage-Dependent Effects of Akt1/Protein Kinase B α (PKB α) and Akt3/PKB γ on Thymus, Skin, and Cardiovascular and Nervous System Development in Mice

Zhong-Zhou Yang,¹ Oliver Tschopp,¹ Nicolas Di-Poi,² Elisabeth Bruder,³ Anne Baudry,¹ Bettina Dümmler,¹ Walter Wahli,² and Brian A. Hemmings^{1*}

Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, Basel CH-4058,¹ Center for Integrative Genomics, Biology Building, University of Lausanne, Lausanne CH-1015,² and Institute of Pathology, University of Basel, Schönbeinstrasse 40, Basel CH-4031,³ Switzerland

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Akt/protein kinase B (PKB) plays a critical role in the regulation of metabolism, transcription, cell migration, cell cycle progression, and cell survival. The existence of viable knockout mice for each of the three isoforms suggests functional redundancy. We generated mice with combined mutant alleles of *Akt1* and *Akt3* to study their effects on mouse development. Here we show that *Akt1*^{−/−} *Akt3*^{+/-} mice display multiple defects in the thymus, heart, and skin and die within several days after birth, while *Akt1*^{+/-} *Akt3*^{−/−} mice survive normally. Double knockout (*Akt1*^{−/−} *Akt3*^{−/−}) causes embryonic lethality at around embryonic days 11 and 12, with more severe developmental defects in the cardiovascular and nervous systems. Increased apoptosis was found in the developing brain of double mutant embryos. These data indicate that the *Akt1* gene is more essential than *Akt3* for embryonic development and survival but that both are required for embryo development. Our results indicate isoform-specific and dosage-dependent effects of *Akt* on animal survival and development.

In mammals, Akt1, Akt2, and Akt3 (also called protein kinase B α [PKB α], PKB β , and PKB γ) proteins have similar domain structures and can be activated by numerous growth factors in a phosphatidylinositol 3-kinase-dependent manner (1, 3, 4, 15, 23). Once activated, Akt phosphorylates and controls the activities of a diverse group of substrates involved in many cellular and physiological processes, such as cell survival, cell cycle progression, cell growth, metabolism, and angiogenesis (3, 10, 17, 23).

Although many proteins have been identified as Akt substrates, the challenge that remains is to show that they actually have an important impact on physiological processes in organisms. Recently, targeted deletion of specific isoforms of Akt in mice has proved to be a powerful tool for elucidating the physiological roles of Akt proteins (5, 7, 8, 13, 16, 25, 27, 28). Characterization of such knockout mice has yielded intriguing and surprising results. We and others found that ~40% of *Akt1* knockout mice die at a neonatal stage with growth retardation, but the other ~60% of *Akt1* knockout mice survive apparently normally. This suggests that the other two remaining *Akt* isoforms can, in part, compensate for the loss of one *Akt*. Knockout of each single isoform gives rise to a distinct phenotype. In general, *Akt1* null mice are growth retarded, which may result from placental insufficiency, while *Akt2*-deficient mice suffer from a type 2 diabetes-like syndrome and *Akt3* null mice show impaired brain development (5, 7, 8, 13, 16, 25, 27, 28). These observations indicate that the three Akt isoforms have different nonredundant physiological functions. The relatively normal de-

velopment and distinct physiological functions exhibited by single knockouts may be explained by differences in the tissue distribution and expression levels of these isoforms. We found that Akt1 is the major isoform in placenta and that placenta lacking this protein cannot form a proper vascular labyrinth; this may restrict nutrient supply to the fetus and impair growth (28). Similarly, in the major insulin-responsive tissues of fat, skeletal muscle, and liver, Akt2 is the predominant isoform (2, 7, 28). In the case of Akt3, which shows a more restricted level of expression with the brain containing the highest levels and has lower levels of expression in other tissues, ablation of this isoform affects postnatal brain development (13, 25).

We predicted that simultaneous inactivation of two *Akt* isoforms in mice would severely affect development and survival. To test this hypothesis, we crossed *Akt1* with *Akt3* knockout mice to generate compound (combined mutation of four alleles of *Akt1* and *Akt3*) and double knockout (DKO) mice. Our results show that the *Akt1* gene is more essential than the *Akt3* gene for survival and that both proteins are required for normal embryo development. Our studies demonstrate isoform-specific and dosage-dependent effects of *Akt* genes on animal survival and development.

MATERIALS AND METHODS

Preparation of murine embryonic fibroblasts (MEFs) and Western analysis. MEFs were prepared from embryonic day 10.5 (E10.5) to E13.5 embryos as previously described (14). Western analysis and the antibody that recognizes both mouse Akt1 and Akt3 have been described previously (25, 28). The isoform-specific antibodies for mouse Akt1, Akt2, and Akt3 have been described already (25). Antibodies for pSer473 were purchased from Cell Signaling Technology. Actin antibody was purchased from Neomarkers.

Mice. Mice were housed in accordance with the Swiss Animal Protection Ordinance in groups with 12-h dark-light cycles and with free access to food and

* Corresponding author. Mailing address: Friedrich Miescher Institute for Biomedical Research, P.O. Box 2543, Maulbeerstrasse 66, Basel CH-4002, Switzerland. Phone: 41-61-697-4872. Fax: 41-61-697-3976. E-mail: brian.hemmings@fmi.ch.

FIG. 1. Expression Akt isoforms in neonates, Akt1/Akt3 localization in E11.5 to E12.5 embryos, generation of *Akt1/Akt3* compound, and double knockout mice. (A) Expression patterns of Akt isoforms in neonates. Four wild-type mice from two litters were sacrificed, and their tissues were pooled for lysate preparation. Forty micrograms of total protein was used for Western analysis with the indicated isoform-specific antibodies. (B) Akt1/Akt3 antibody specificity. Mouse Akt1, Akt2, and Akt3 cDNAs cloned in pCMV5 were expressed in HEK293 cells, and cell lysates were prepared for Western analysis. Immunoblot analysis was carried out with an affinity-purified Akt1/Akt3 antibody that recognizes Akt1 and Akt3. Actin levels were determined to control for equal loading. (C) Absence of Akt1 and Akt3 proteins in DKO mouse embryonic fibroblasts (MEFs). MEFs were prepared from wild-type, *Akt1/Akt3* DKO, or *Akt2/Akt3* DKO embryos, and cell lysates derived from these cells were used for Western analysis using the antibody as described in the legend to panel B with actin as control. (D) PCR genotyping for *Akt1* and *Akt3* loci. WT, wild type; DKO, double knockout. (E) Absence of Akt1 and Akt3 proteins in DKO mouse tissue (placenta). Total phospho-Akt is substantially decreased. (F to I) HE and immunohistochemical staining of E11.5 and E12.5 embryos with the Akt1/Akt3 antibody. Panels F and G are for the E11.5 embryo and H and I are for the E12.5 embryo; F and H show hematoxylin and eosin staining, and G and I show immunohistochemical staining. Akt1 or Akt3 (or both) is expressed in both the central and peripheral nervous systems of brain, neural tube, and heart. Arrowheads indicate various ganglia. Abbreviations: HB, hindbrain; MB, midbrain; FB, forebrain; BA, branchial arch; H, heart; NT, neural tube; CG, cranial ganglia; DRG, dorsal root ganglia; Li, liver; T, tongue.

TABLE 1. Progeny of *Akt1*^{+/-} *Akt3*^{+/-} matings^a

Genotype	<i>l</i> ^{+/+} <i>3</i> ^{+/+}	<i>l</i> ^{+/+} <i>3</i> ^{+/-}	<i>l</i> ^{+/+} <i>3</i> ^{-/-}	<i>l</i> ^{+/-} <i>3</i> ^{+/+}	<i>l</i> ^{+/-} <i>3</i> ^{+/-}	<i>l</i> ^{+/-} <i>3</i> ^{-/-}	<i>l</i> ^{-/-} <i>3</i> ^{+/+}	<i>l</i> ^{-/-} <i>3</i> ^{+/-}	<i>l</i> ^{-/-} <i>3</i> ^{-/-}
No. of animals	27	34	20	31	74	37	11	3	0
%	11.3	14.3	8.4	13	31	15.5	4.6	1.3	0
Theoretical %	6.3	12.5	6.3	12.5	25	12.5	6.3	12.5	6.3

^a Total number analyzed, 237.

FIG. 2. Defects in the thymus from *Akt1*^{-/-} *Akt3*^{+/-} mice. (A) Thymi from P3 *Akt1*^{+/+} *Akt3*^{+/-} and *Akt1*^{-/-} *Akt3*^{+/-} littermates. (B to E) HE staining of the thymus from panel A. Panels B and C are lower-magnification images of panels D and E. Arrowheads in panel E indicate the Hassall's corpuscle-like structures in the *Akt1*^{-/-} *Akt3*^{+/-} mice which are not found in wild-type mouse thymus. Cap, capillary blood vessel. Magnification for panels B and C, ×40; for panels D and E, ×400.

water. All procedures were conducted with relevant authority approval. *Akt1*^{-/-} and *Akt3*^{-/-} mice have been described previously (25, 28). All mice had a 129 Ola and C57BL/6 mixed background.

BrdU incorporation. E10.5 to E11.5 pregnant mice were injected intraperitoneally with bromodeoxyuridine (BrdU) (1 mg per 20 g of body weight). The mice were sacrificed 2 h later, and embryos together with placenta were dissected (25, 28).

Hematoxylin-eosin (HE) and immunohistochemistry (IHC). The HE and IHC protocols were as described previously (25, 28). For IHC, sections were incubated at 4°C overnight with antibodies for Akt1 and Akt3, BrdU (AbCam Ltd.), platelet endothelial cell adhesion molecule (PECAM) (Pharmingen), skin markers of keratin 10 (K10) and K14, and involucrin (BABCO). The sections were then processed as described in the protocol of the Vectastain ABC kit (Vector Laboratories).

Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL staining). Paraffin-embedded sections were treated with 20 μ g/ml proteinase K for 10 min at 37°C (omitted for cryosections). Endogenous peroxidase was inactivated by treatment with 3% H₂O₂ in 100% methanol for 30 min at room temperature. The sections were then equilibrated with 1 $\times$ terminal deoxynucleotidyl transferase (TdT) buffer for 15 min at room temperature and incubated with TdT and biotinylated dUTP (TdT reaction) for 1 h at 37°C. The reaction was stopped by washing with 1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate). A Vectastain ABC kit (Vector Laboratories) was used as described by the manufacturer for color development.

Quantification and statistics. Quantification of skin thickness and hair follicles was performed with three double-heterozygous (DH) (*Akt1*^{+/-} *Akt3*^{+/-}) and three *Akt1*^{-/-} *Akt3*^{+/-} mice as described previously (11). For the TUNEL assay of heart, five fields of each section were counted for apoptotic cells. The *t* test was used for statistical analysis. Similar scoring was performed for placental vasculature.

RESULTS

Tissue distribution of Akt isoforms in neonates and mid-gestation embryos. We first investigated the expression patterns of the three isoforms in neonates and surprisingly show that the expression of the Akt3 isoform is more widely expressed than it is in adults (Fig. 1A). Similar to adult mice, Akt3 is highest in the brain and Akt2 is highest in fat as reported previously (7, 13, 25, 28). However, Akt1 and Akt3 are abundant in kidney and skeletal muscle, whereas they are nearly absent in adult mice (Fig. 1A). The ubiquitous expression pattern of the three Akt proteins in neonates indicates that all three isoforms are apparently required for early development.

These isoform-specific antibodies could not be used for immunohistochemistry (IHC) because of nonspecific background staining. Therefore, we used an antibody that specifically recognized both Akt1 and Akt3 (labeled Akt1/Akt3 antibody in Fig. 1B and C) that was originally developed against the carboxyl-terminal peptide sequence of porcine Akt1 (18). This affinity-purified antibody recognized mouse Akt1 and Akt3 but not Akt2 (Fig. 1B) and could be used for IHC.

At E11.5, the level of Akt1 or Akt3 protein (or both) is high in the brain, neural tube, and heart (Fig. 1F and G). At E12.5, the signals are predominantly localized in the developing brain and various ganglia, and the levels (indicated by the staining

signal) increased compared to that at E11.5 (Fig. 1H and I). The tissue distribution patterns indicate that the two proteins are important for brain and cardiovascular development at E11.5 to E12.5.

Generation of Akt1 and Akt3 double mutant mice. *Akt1*, *Akt2*, and *Akt3* genes have been successfully targeted in our laboratory by homologous recombination (25, 28; B. A. Dümmler, unpublished data). In the present work, we first mated *Akt1*^{-/-} males with *Akt3*^{-/-} females to generate *Akt1*^{+/-} *Akt3*^{+/-} (DH) mice. Intercrossing of these DH mice gave rise to offspring with nine genotypes (Table 1). Genotyping of these mice was carried out by independent PCR for the *Akt1* and *Akt3* loci as previously described (25, 28) (Fig. 1D). Western blot analysis confirmed the absence of the two proteins in the MEFs and tissues from *Akt1*/*Akt3* DKO mice (Fig. C and 1E).

***Akt1*^{-/-} *Akt3*^{+/-} mice die at an early age and *Akt1*/*Akt3* double knockout mice are embryonic lethal.** Among the nine genotypes, only *Akt1*^{-/-} *Akt3*^{+/-} and DKO mice had survival problems, whereas all others were produced in numbers consistent with the expected Mendelian ratio (Table 1). More than 90% of *Akt1*^{-/-} *Akt3*^{+/-} mice were lost by the time of genotyping, and we did not observe any DKO mice at the time of genotyping (Table 1). We next determined at what stage *Akt1*^{-/-} *Akt3*^{+/-} mice and DKO mice die. For *Akt1*^{-/-} *Akt3*^{+/-} mice, we set up matings between *Akt1*^{+/-} *Akt3*^{-/-} males and *Akt1*^{-/-} females. Theoretically, offspring of this mating are half *Akt1*^{-/-} *Akt3*^{+/-} and half DH (1:1). Of the 20 newborns collected from four litters, 8 were DH and 12 were *Akt1*^{-/-} *Akt3*^{+/-}, which is consistent with the expected Mendelian ratio. Therefore, *Akt1*^{-/-} *Akt3*^{+/-} mice appear to develop to term. In a follow-up study, we found that the majority of *Akt1*^{-/-} *Akt3*^{+/-} mice died several days after birth.

We next determined the stage of DKO embryonic lethality. No double knockout embryos were found at E14.5. At E12.5, some double knockout embryos had already decayed, leaving only the placenta and body remnants (progeny of *Akt1*^{+/-} *Akt3*^{-/-} intercross mice were the following: for genotype *Akt1*^{+/-} *Akt3*^{-/-}, 15 mice; for genotype *Akt1*^{+/-} *Akt3*^{-/-}, 12 mice; for genotype *Akt1*^{-/-} *Akt3*^{-/-}, 13 mice [7 of the *Akt1*^{-/-} *Akt3*^{-/-} mice were dead and partially decayed). DKO embryos dissected at E11.5 displayed severely retarded development (data not shown). When E10.5 embryos were dissected and genotyped, double knockout embryos were found with morphology comparable to that of *Akt1*^{+/-} *Akt3*^{-/-} littermates. These data indicate that double knockout mutants die around E11 to E12.

Thus, Akt3 appears to be redundant for the survival of Akt1 mutant mice. In contrast, Akt1 mutant mice (*Akt1*^{-/-}) showed a 40% loss after birth, with the mortality of *Akt1*^{-/-} *Akt3*^{+/-} mice increased to more than 90%.

FIG. 3. Structural defects in the heart from *Akt1*^{-/-} *Akt3*^{+/-} mice. (A) The hearts of *Akt1*^{-/-} *Akt3*^{+/-} mice are smaller than those of the controls, but the atria are as big as those of the controls. (B to D) Sagittal sections of the hearts. (B to D) HE staining of hearts from panel A. (C and D) Enlargement of the right side of *Akt1*^{-/-} *Akt3*^{+/-} hearts and apparent atria septum defects in *Akt1*^{-/-} *Akt3*^{+/-} hearts (P3). The wall of the right atrium of the *Akt1*^{-/-} *Akt3*^{+/-} heart is thin due to enlargement (C and D). The arrowhead in panel D indicates atria septum defects. (E and F) TUNEL assay. Arrowheads indicate apoptotic cells. (G) Quantification of apoptosis. Error bars indicate standard deviation. **, *P* < 0.01. RA, right atrium; RV, right ventricle; LA, left atrium. Magnification for panels B to D, $\times 40$; for panels E and F, $\times 400$.

Multiple defects in the thymus, heart, and skin of *Akt1*^{-/-} *Akt3*^{+/-} mice. Nearly all *Akt1*^{-/-} *Akt3*^{+/-} mice died within a few days after birth. To determine possible causes of death, we first performed systemic anatomic organ analysis of postnatal day 3 (P3) viable *Akt1*^{-/-} *Akt3*^{+/-} mice and their DH littermates (the latter mice survive normally and were, therefore, used as the control). Generally, most organs from *Akt1*^{-/-} *Akt3*^{+/-} mice analyzed were proportionally smaller than those of DH controls.

Strikingly, *Akt1*^{-/-} *Akt3*^{+/-} mice also displayed nonproportional hypotrophy of the thymus, heart, and skin. We have studied the six thymi from two litters of P3 mice (three *Akt1*^{-/-} *Akt3*^{+/-} and three DH). The thymus of *Akt1*^{-/-} *Akt3*^{+/-} mice is hypotrophic and much smaller than the control (Fig. 2A to C). There are many Hassall's corpuscle-like structures (arrowheads in Fig. 2E) formed in the thymus of *Akt1*^{-/-} *Akt3*^{+/-} mice, but they were not seen in the DH control (Fig. 2D).

To analyze heart development, we used eight *Akt1*^{-/-} *Akt3*^{+/-} and three *Akt1*^{-/-} mice (similar numbers for control) from newborn to P8. The hearts of *Akt1*^{-/-} *Akt3*^{+/-} mice were smaller than those of controls, the difference being mainly in the ventricles (Fig. 3A). Further histological study of these P3 hearts and other P3 hearts from wild-type mice revealed that *Akt1*^{-/-} *Akt3*^{+/-} hearts had dilated (enlarged) atria and ventricles, especially on the right side (Fig. 3C and D). In addition, we found a range of defects in *Akt1*^{-/-} *Akt3*^{+/-} hearts, including atria septum defect, muscular ventricle septum defect, ventricle septum vascular anomaly, and thickened valves (data not shown). Small ventricle size and insufficient contractility of cardiomyocytes of *Akt1*^{-/-} *Akt3*^{+/-} hearts may lead to the retention of blood in the right side of the heart and cause the atria septum to open. One *Akt1*^{-/-} *Akt3*^{+/-} mouse that died after 8 days exhibited a significantly enlarged right atrium and ventricle (data not shown). Trichrome staining revealed a large area of necrosis (data not shown). TUNEL staining of the heart identified a large number of apoptotic cells outside of the necrotic area (Fig. 3F and G). Histological structure of the lung of *Akt1*^{-/-} *Akt3*^{+/-} mice was similar to that of controls (data not shown). The data presented are representative of the phenotype observed for these mice.

Previously, we observed impaired hair follicle development in *Akt1*^{-/-} mice by HE staining (11). We performed immunohistochemical and HE analyses to evaluate the structure and development of the skin of *Akt1*^{-/-} *Akt3*^{+/-} mice. We have analyzed 11 *Akt1*^{-/-} *Akt3*^{+/-} mice (and a similar number for control) from E16.5 to P8 for skin development. The thin-skin phenotype observed was found in nearly all the mice examined.

Immunohistochemical staining showed Akt1 and Akt3 to be localized mainly in the keratinocytes of epidermis and follicles

at P2 (Fig. 4A), which coincided with phospho-Ser473 staining (Fig. 4B). HE staining revealed a reduction in the thickness of *Akt1*^{-/-} *Akt3*^{+/-} skin at P2 and P8 (Fig. 4C). Thickness of the hypodermis and total skin, total number of hair follicles (or hair follicle density), and hair morphogenesis of skin samples from three *Akt1*^{-/-} *Akt3*^{+/-} mice and three controls (*Akt1*^{+/-} *Akt3*^{+/-}) at P3 were all significantly different (Fig. 4G). Cell differentiation is normal in *Akt1*^{-/-} *Akt3*^{+/-} skin based on keratin 14 (K14), keratin 10 (K10), and involucrin staining (data not shown). Although all skin layers were present in *Akt1*^{-/-} *Akt3*^{+/-} mice, individual skin layers were much thinner than in the wild type. Thus, the skin as a whole was much thinner. Hair follicles were distinctly reduced in size in *Akt1*^{-/-} *Akt3*^{+/-} animals, and there were fewer (black) hair fibers (data not shown). The impaired skin development of *Akt1*^{-/-} *Akt3*^{+/-} mice may be attributable to decreased vasculature revealed by PECAM (CD31) staining (24) (Fig. 4E and F). This provides further evidence for Akt1- and Akt3-dependent regulation of angiogenesis and vascular formation.

Abnormal development of DKO embryos. At E11.5, some DKO embryos displayed apparent vascular defects and hemorrhages. E10.5 DKO embryos displayed morphology comparable to that of the control. Therefore, we studied embryonic development of E10.5 DKO embryos to understand the ensuing embryo lethality. We analyzed four to five mutant embryos (similar for control) at E10.5 and E12.5. The data presented are representative of the phenotype observed.

Vascular structure was studied using PECAM staining (24). We found a significant decrease in vasculature of DKO embryos compared with controls (Fig. 5A to H). Fine (capillary) vessels were abundant in the head (neuroepithelium) and body trunk of the control but were not apparent in DKO embryos (Fig. 5A to D). Furthermore, DKO embryos displayed hypoplastic branchial arches (Fig. 5G), fused or dilated arch arteries, and the absence of fine vessels (Fig. 5H).

Previously, we observed reduced vasculature in *Akt1*^{-/-} placenta (28), and a more significant vascular defect was found in DKO placenta (Fig. 5J and K). In addition, reduced cell proliferation was detected in DKO placenta (Fig. 5M).

The predominant expression of Akt1 or Akt3 (or both) in the nervous system at E11.5 to E12.5 suggests an important role of these two proteins in the maintenance of brain development. However, there was no significant difference between DKO and wild-type control mice with cell proliferation displayed by BrdU staining (data not shown).

An increase of apoptotic cells was detected using the TUNEL assay in the neuroepithelium and in the neurons of various ganglia in DKO embryos compared to the wild type (Fig. 6A to H). These results indicate that Akt1 and Akt3 contribute to the development of the nervous system by maintenance of cell survival.

FIG. 4. Histology of *Akt1*^{-/-} *Akt3*^{+/-} skin. (A and B) Immunohistochemical staining of Akt1/Akt3 and phospho-Akt (Ser473) in control skin. (A) Akt1/Akt3 localization. The two proteins are mainly localized in the keratinocytes of the epidermis (Ep) and hair follicles (arrowheads). (B) Phospho-Akt (Ser473) in control skin. Localization of phospho-Akt is similar to that of Akt1/Akt3. (C and D) HE staining. Skin of *Akt1*^{-/-} *Akt3*^{+/-} mice is much thinner than that of control littermates and has fewer hair follicles (arrowheads). (E and F) PECAM (CD31) staining to display the vasculature. *Akt1*^{-/-} *Akt3*^{+/-} skin has fewer vessels than the control. Insets in all panels are lower magnification images showing a larger part of the skin. (G) Quantification of skin thickness and hair follicles. HE staining sections from three *Akt1*^{-/-} *Akt3*^{+/-} mice and three *Akt1*^{+/-} *Akt3*^{+/-} littermates (P3) were measured for thickness and hair follicle density. Error bars are standard deviation, and two asterisks indicate a significant difference between the two genotypes ($P < 0.05$). Magnification, $\times 200$.

FIG. 5. Analysis of the vascular system of DKO embryos. (A to H) Left panels are HE staining to show the morphology, and the right panels are PECAM staining to display vasculature. (A to D) Fine vessels (capillary) in the neuroepithelium of hindbrain. Arrowheads indicate the small vessels (capillary). Small vessels are abundant in the control and are evenly distributed, while these vessels are scarce in DKO embryos. (E to H) Branchial arches and their arteries. In the control, first, second, and third arch arteries can be seen (arrowheads), and small vessels are rich in mesenchymal tissue (F). However, the branchial arches of DKO mice are hypoplastic, with little mesenchymal tissue, and the large arch arteries cannot be distinguished (arrowhead). Small vessels are also scarce in DKO branchial arches. Abbreviations: NE, neuroepithelium; BA, branchial arch; WT, wild type. Magnification, $\times 100$. (I to M) Histological analysis of DKO placenta. (I and J) E12.5 placentas. PECAM staining displays fetal vessels in the labyrinth. In the wild type (I), fetal vessels are well formed and filled with fetal nucleated erythroid cells (arrowheads), while in DKO mice (J) these vessels are rare, although the epithelial cells can be seen. (K) Quantification of fetal vessels in wild-type and DKO placenta. The double asterisks indicates a significant difference ($P < 0.05$). (L and M) E10.5 placentas. BrdU staining. DKO placenta shows a substantial reduction of proliferation, especially in the spongiotrophoblast layer (Sp). De, decidua; Lab, labyrinth. Magnification, $\times 40$.

DISCUSSION

With neonatal mice, we found that the three Akt proteins are widely expressed in contrast to results obtained in the adult mice, where the Akt3 protein expression is quite restricted. Our findings provide important insights and help better under-

stand the developmental defects in *Akt1/Akt3* compound mutant mice and *Akt1/Akt2 Akt1/Akt3* DKO mice. In the single knockouts of Akt isoforms, *Akt2* and *Akt3* null mice have no neonatal survival disadvantages (7, 16, 25, 27). Nevertheless, we and others have observed that 40% of *Akt1* null mice fail to

FIG. 6. Apoptosis in wild-type (WT) and DKO embryos. The left panels are HE staining, and the right panels are TUNEL staining. In the wild type, very few apoptotic cells can be seen in the neuroepithelium, but there are many in DKO neuroepithelium (arrowheads indicate apoptotic cells). (A to D) E10.5; (E to H) E9.5. Magnification for panels A and B, $\times 40$; for panels C to F, $\times 200$.

survive past the neonatal period (8). This raises the intriguing possibility that the *Akt1* gene is the most critical isoform of the three for early mouse development. Nearly all *Akt1*^{-/-} *Akt3*^{+/-} mice died at an early age, while *Akt1*^{+/-} *Akt3*^{-/-} mice survived normally. This suggests that the *Akt1* gene is more essential than *Akt3* for mouse survival. In a recent report, *Akt1/Akt2* double knockout mice developed to term but died shortly after birth with severe developmental defects, including dwarfism, skeletal deformity, and skin abnormalities (22). We found that *Akt1*^{-/-} *Akt3*^{+/-} mice have severe growth deficiencies, and nearly all died young with multiple organ and tissue pathologies. Mice lacking both *Akt1* and *Akt3* genes died at approximately E11 to E12 and displayed multiple fatal developmental defects. *Akt1/Akt2* double knockout mice with only *Akt3* developed to term (22), but *Akt1/Akt3* DKO mice (i.e., only *Akt2* was expressed) did not develop beyond the midterm (E12). This indicates a more critical role for *Akt3* than *Akt2* in early animal development and survival. Thus, the order of apparent importance of the three *Akt* genes in mouse development and survival is *Akt1* > *Akt3* > *Akt2*. The phenotypes observed with *Akt1/Akt3* DKO and *Akt1/Akt2* DKO mice are largely due to loss of *Akt1* function. Taken together, these results indicate that the three *Akt* genes have isoform-specific and dosage-dependent effects on mouse survival and development.

In this study, we found that *Akt1* and *Akt3* have essential roles in cardiovascular development and function. The probable cause of sudden death of *Akt1*^{-/-} *Akt3*^{+/-} mice is heart failure, possibly as a result of defective heart development and cardiomyocyte function (6). *Akt1/Akt3* DKO mice embryonic lethality could also be attributed to cardiovascular anomalies. These embryos died at around E12, with significant vascular reduction in embryo and placenta; large vessels in the branchial arches and dorsal aorta were also abnormal. As these large branchial arch and dorsal aorta arteries are converted to aortic artery and other large arteries, the developmental defects of these vessels at E12 inhibits further development of mice and enhances morbidity (9, 20, 21).

Our studies of *Akt1/Akt3* DKO mice also indicate that *Akt1* and *Akt3* are critical for nervous system development. Previously, we found that Akt3 protein levels are the highest in the brain, and deletion of this isoform substantially impaired brain development (13, 25). In *Akt1/Akt3* DKO embryos, many apoptotic cells were found in the neuroepithelium and neurons of ganglia. Therefore, *Akt1* and *Akt3* are indispensable for nervous system development in the embryo through their maintenance of cell survival (12, 29, 30).

Another interesting finding is the Hassall's corpuscle-like structures in *Akt1*^{-/-} *Akt3*^{+/-} thymus, as this structure is well developed in the thymus of humans and guinea pigs but poorly developed in the thymus of mice and rats (26). In humans, the Hassall's corpuscles have been proposed to remove apoptotic thymocytes (26). Previously, Hay's group found increased apoptotic thymocytes in *Akt1* null mice (5). The formation and function of these structures in *Akt1*^{-/-} *Akt3*^{+/-} thymus need to be studied further.

In conclusion, our results indicate that *Akt1* and *Akt3* genes apparently regulate multiple developmental processes in mice. *Akt1* and *Akt3* genes share some redundant functions required to maintain mouse development and survival. However, these

two proteins seem to play distinct roles based on the phenotypes of the individual knockouts. To rigorously understand the causes of the different phenotypes, it will be necessary to identify the specific Akt substrates involved in the development of the different organs.

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REFERENCES

- Alessi, D., M. Andjelkovic, B. Caudwell, P. Cron, N. Morrice, P. Cohen, and B. A. Hemmings. 1996. Mechanism of activation of protein kinase B by insulin and IGF1. *EMBO J.* 15:6541-6551.
- Altomare, D. A., G. E. Lyons, Y. Mitsuuchi, J. Q. Cheng, and J. R. Testa. 1998. Akt2 mRNA is highly expressed in embryonic brown fat and the AKT2 kinase is activated by insulin. *Oncogene* 16:2407-2411.
- Brazil, D. P., and B. A. Hemmings. 2001. Ten years of protein kinase B signalling: a hard Akt to follow. *Trends Biochem. Sci.* 26:657-664.
- Burgering, B. M., and P. J. Coffey. 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* 376:599-602.
- Chen, W. S., P.-Z. Xu, K. Gottlob, M.-L. Chen, K. Sokol, T. Shiyanova, I. Roninson, W. Weng, R. Suzuki, K. Tobe, T. Kadowaki, and N. Hay. 2001. Growth retardation and increased apoptosis in mice with homozygous disruption of the akt1 gene. *Genes Dev.* 15:2203-2208.
- Chien, K. R., and E. N. Olson. 2002. Converging pathways and principles in heart development and disease: CV@CSH. *Cell* 110:153-162.
- Cho, H., J. Mu, J. K. Kim, J. L. Thorvaldsen, Q. Chu, E. B. Crenshaw III, K. H. Kaestner, M. S. Bartolomei, G. I. Shulman, and M. J. Birnbaum. 2001. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB β). *Science* 292:1728-1731.
- Cho, H., J. L. Thorvaldsen, Q. Chu, F. Feng, and M. J. Birnbaum. 2001. Akt1/PKB α is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J. Biol. Chem.* 276:38349-38352.
- Conway, S. J., A. Kruzynska-Frejtag, P. L. Kneer, M. Machnicki, and S. V. Koushik. 2003. What cardiovascular defect does my prenatal mouse mutant have, and why? *Genesis* 35:1-21.
- Datta, S. R., A. Brunet, and M. E. Greenberg. 1999. Cellular survival: a play in three acts. *Genes Dev.* 13:2905-2927.
- Di-Poi, N., C. Y. Ng, N. S. Tan, Z. Yang, B. A. Hemmings, B. Desvergne, L. Michalik, and W. Wahli. 2005. Epithelium-mesenchyme interactions control the activity of peroxisome proliferator-activated receptor β/δ during hair follicle development. *Mol. Cell. Biol.* 25:1696-1712.
- Dudek, H., S. R. Datta, T. F. Franke, M. J. Birnbaum, R. Yao, G. M. Cooper, R. A. Segal, D. R. Kaplan, and M. E. Greenberg. 1997. Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* 275: 661-665.
- Easton, R. M., H. Cho, K. Roovers, D. W. Shineman, M. Mizrahi, M. S. Forman, V. M.-Y. Lee, M. Szabolcs, R. de Jong, T. Oltersdorf, T. Ludwig, A. Efstratiadis, and M. J. Birnbaum. 2005. Role for Akt3/protein kinase B γ in attainment of normal brain size. *Mol. Cell. Biol.* 25:1869-1878.
- Feng, J., R. Tamaskovic, Z. Yang, D. P. Brazil, A. Merlo, D. Hess, and B. A. Hemmings. 2004. Stabilization of Mdm2 via decreased ubiquitination is mediated by protein kinase B/Akt-dependent phosphorylation. *J. Biol. Chem.* 279: 35510-35517.
- Franke, T. F., S. I. Yang, T. O. Chan, K. Datta, A. Kazlauskas, D. K. Morrison, D. R. Kaplan, and P. N. Tsichlis. 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 81:727-736.
- Garofalo, R. S., S. J. Orena, K. Rafidi, A. J. Torchia, J. L. Stock, A. L. Hildebrandt, T. Coskran, S. C. Black, D. J. Brees, J. R. Wicks, J. D. McNeish, and K. G. Coleman. 2003. Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB β . *J. Clin. Invest.* 112:197-208.
- Hanada, M., J. Feng, and B. A. Hemmings. 2004. Structure, regulation and function of PKB/AKT—a major therapeutic target. *Biochimica et Biophysica Acta Proteins Proteom.* 1697:3-16.
- Jones, P., T. Jakubowicz, F. Pitossi, F. Maurer, and B. Hemmings. 1991. Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. *Proc. Natl. Acad. Sci. USA* 88:4171-4175.
- Lawlor, M. A., and D. R. Alessi. 2001. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J. Cell Sci.* 114:2903-2910.

20. Liu, C., W. Liu, J. Palie, M. F. Lu, N. A. Brown, and J. F. Martin. 2002. Pitx2c patterns anterior myocardium and aortic arch vessels and is required for local cell movement into atrioventricular cushions. *Development* **129**: 5081–5091.
21. Papaioannou, V. E., and R. R. Behringer. 2005. Mouse phenotypes, a handbook of mutation analysis. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. Peng, X.-d., P.-Z. Xu, M.-L. Chen, A. Hahn-Windgassen, J. Skeen, J. Jacobs, D. Sundararajan, W. S. Chen, S. E. Crawford, K. G. Coleman, and N. Hay. 2003. Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. *Genes Dev.* **17**:1352–1365.
23. Scheid, M. P., and J. R. Woodgett. 2001. PKB/AKT: functional insights from genetic models. *Nat. Rev. Mol. Cell Biol.* **2**:760–768.
24. Stainier, D. Y., R. K. Lee, and M. C. Fishman. 1994. Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31): alternatively spliced, functionally distinct isoforms expressed during mammalian cardiovascular development. *Development* **120**:2539–2553.
25. Tschopp, O., Z.-Z. Yang, D. Brodbeck, B. Duemmler, M. Hemmings-Mieszczak, T. Watanabe, T. Michaelis, J. Frahm, and B. A. Hemmings. 2005. Essential role of protein kinase B γ (PKB γ /Akt3) in postnatal brain development, but not for glucose homeostasis. *Development* **132**:2943–2954.
26. Watanabe, N., Y. H. Wang, H. K. Lee, T. Ito, Y. H. Wang, W. Cao, and Y. J. Liu. 2005. Hassall's corpuscles instruct dendritic cells to induce CD4⁺ CD25⁺ regulatory T cells in human thymus. *Nature* **436**:1181–1185.
27. Yang, Z.-Z., O. Tschopp, A. Baudry, B. Duemmler, D. Hynx, and B. A. Hemmings. 2004. Physiological function of PKB/Akt. *Biochem. Soc. Trans.* **32**:350–354.
28. Yang, Z.-Z., O. Tschopp, M. Hemmings-Mieszczak, J. Feng, D. Brodbeck, E. Perentes, and B. A. Hemmings. 2003. Protein kinase B α /Akt1 regulates placental development and fetal growth. *J. Biol. Chem.* **278**:32124–32131.
29. Yuan, J., M. Lipinski, and A. Degterev. 2003. Diversity in the mechanisms of neuronal cell death. *Neuron* **40**:301–413.
30. Yuan, J., and B. A. Yankner. 2000. Apoptosis in the nervous system. *Nature* **407**:802–809.