Towards an Understanding of Protein Kinase B (PKB/Akt) Function in Mouse Development

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Summary

Protein kinase B (PKB/Akt) belongs to a subfamily of serine/threonine protein kniases called AGC protein kinases. Homologues of PKB can be found in worms, flies and mammals. Three isoforms of PKB, termed PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 that are encoded by three distinct genes, have been identified in mammals like mice and humans.

PKB can be activated by numerous growth factors, hormones, cytokines and other stimuli through a phosphatidylinositol 3-kinase (PI3K)-dependent manner. The signaling pathway of PI3K/PKB/Akt has been established and the significance of this pathway for numerous cellular and physiological processes has been recognized and widely accepted.

The understanding of developmental principles in mouse is a big challenge. How PKB contributes to mouse development and why three isoforms exist in mice have been wondering researchers in this field since the identification of these proteins in this animal.

Early mouse work using northern blotting and *in situ* hybridization showed expression of PKB/Akt in mouse embryos with isoform- and tissue-specific properties. Thus, PKB/Akt may play important roles in mouse development. In addition, the distinct tissue distribution patterns of the three isoforms suggest that these proteins have different functions.

To address these questions, we generated mouse mutant for each isoform by homologous recombination. Characterization and analyses of these mice have provided new insights into the functions of PKB/Akt in mouse development. We found that PKBα/Akt1 was the predominant isoform in placenta. PKBα/Akt1 mutant mice were born small with increased neonatal mortality. The mutant placenta displayed reduced

size and impaired development and glycogen-containing spongiotrophoblast cells are rare. More significant is a decrease in vascularization of the mutant placenta. As the size and structure of the placenta determines the growth of the fetus, we conclude that PKBα/Akt1 modulates placental development and, thus, fetal growth.

In contrast to PKBα/Akt1 mutant mice, PKBγ/Akt3 mutant mice did not show increased postnatal mortality and and grew normally. However, these mice displayed a reduced brain size by 25% after birth. This indicates that PKBγ/Akt3 is an important modulator of postnatal brain growth.

We crossed PKBα/Akt1 mutant mice with PKBγ/Akt3 mutant mice to produce compound knockout mice and found that the two proteins have different roles in the maintenance of animal survival. While $Pkb\alpha^{+/-}Pkb\gamma^{-/-}$ ($Akt1^{+/-}Akt3^{-/-}$) mice survived normally, almost all $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$($Akt1^{-/-}$ $Akt3^{+/-}$) mice died at an early age with multiple pathologies. PKBα/γ (Akt1/3) double knockout mice were embryonic lethal at around E12. The development of these mice was severely impaired, including the branchial arch arteries, the brain and the placenta. We conclude that PKBα/Akt1 is more important than PKBγ/Akt3 for animal survival but both are required for mouse development.

Introduction

1. Overview

The main vertebrate model systems for current developmental research are the frog *Xenopus*, the chick, the zebrafish and the mouse. Compared with the other three vertebrates, the mouse has more similarities to humans in biochemistry, physiology, genetics and development. The almost complete mouse genome blueprint and the huge gene homology between the mice and humans (~90%) have greatly boosted our interest in this animal (Waterston et al., 2002). The knowledge obtained and our understanding of mice will have significant implications and an invaluable impact on human life.

The study of mouse development has been facilitated extraordinarily by gene knockout techniques. The possible involvement of a gene in certain developmental events can be tested by its inactivation in mice. Meanwhile, unexpected phenotypes of various mutant mice have yielded a tremendous amount of knowledge about developmental processes and gene functions. In-depth understanding of early embryon development and organogenesis of mouse is accumulating faster than ever.=-

In this section, the whole developmental process of the mouse embryo will be summeriz. I have a great personal interest in understanding the early events of mouse development. A comprehensive description of mouse embryogenesis and organogenesis will, I hope, lead us to the principles of mouse development. This becomes increasingly necessary for the study of knockout mice.

I will divide the development of the mouse in the uterus into two sections, embryonic development and extra-embryonic, i.e. placental development. Subsequently, PKB/Akt transgenic mice will be introduced.

2. Mouse development

Mouse mating usually takes place at night. When the plug is found the next morning, it is recorded as 0.5 d.p.c (day post-coitum, or E0.5, embryonic day 0.5) meaning that fertilization is occurred half a day previously. A litter of pups is born in the morning of 19.5 d.p.c. and traditionally, the gestation period in mice is considered to be 19.5 days (Figure 1).

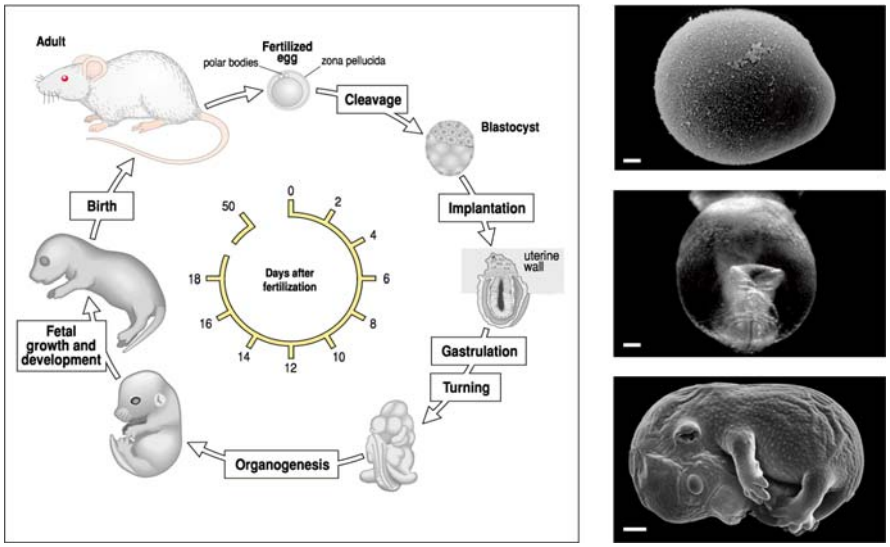


Figure 1. The life cycle of the mouse (left)**.** On the right from top to bottom, a fertilized mouse egg, a mouse embryo of 8 d.p.c. (E8.0) and a mouse embryo of 14 d.p.c. (E14) (Modified from Wolpert et al., 1998)

As shown in figure 1, embryogenesis and fetal growth from fertilization to birth can be divided into six stages, each one featuring one or more special events (Figure 1). The six stages include cleavage, implantation, gastrulation, turning, organogenesis, and fetal growth and development (Rossant and Tam, 2002; Wolpert et al., 1998).

The egg is fertilized in the oviduct, where cleavage also occurs. The blastocyst is formed by 5 days after fertilization and the interaction between blastocyst and uterus starts implantation. Placentation, a developmental process of extra-embryonic tissues,

follows implantation (Rossant and Cross, 2001). In the embryo, the events of gastrulation, turning and organogenesis occur in parallel with placentation over a period of around 7 days. The remaining period of gestation is a time of overall growth of both placenta and embryo (Wolpert et al., 1998).

Placenta is mainly an embryonic organ and the development and growth of the embryo are strongly dependent on placental function during gestation (Cross, 2000; Rossant and Cross, 2001). The development of the embryo and placenta will be described in two sections.

2.1 Embryonic development

2.1.1. **Cleavage.** It takes around 5 days from fertilization to the formation of the late blastocyst, during which the fertilized egg undergoes 5 cleavages to reach a solid ball of 32 cells called the morula (Loebel et al., 2003; O'Farrell et al., 2004). The morula cells make a two-lineage commitment to form the early blastocyst, which continues development to the late blastocyst stage prior to implantation (Figure 2).

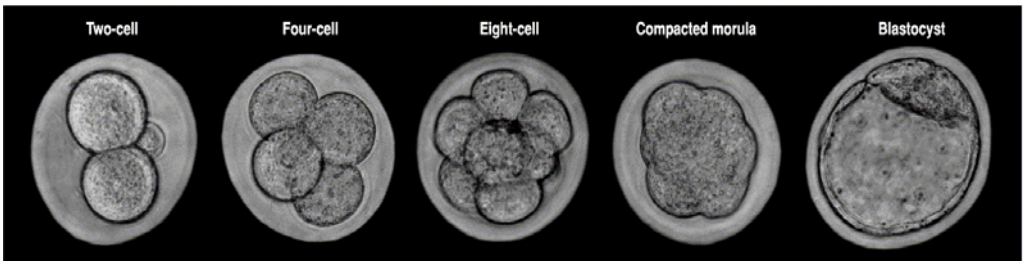


Figure 2. Cleavage and development of the early blastocyst. The fertilized egg undergoes 5 cell cycles to reach the 32-cell morula. In the early blastocyst, there are two cell lineages, trophoblast and inner cell mass. (Modified from Wolpert et al, 1998)

In contrast to *Xenopus* and *Drosophila*, the early cell cycles following fertilization are not extraordinarily fast in mice (O'Farrell et al., 2004). In fact, the first cleavage takes as long as 1.5 days to reach the 2-cell stage. The next 4 cell cycles occur with an average of 12 hours each (2 days in total) to form the morula. The universal 32 cells

then differentiate into two groups, the trophoectoderm and the inner cell mass, giving a structure termed early blastocyst (Lu et al., 2001a; O'Farrell et al., 2004). During the following 24 hours, the inner cell mass becomes divided into two regions, the primitive endoderm and the epiblast; the trophoectoderm meanwhile gives rise to the polar and mural trophoectoderm (Bard, 1994; Wolpert et al., 1998). Finally, the late blastocyst is formed, consisting of around 120 cells (Figures 2, 3).

2.1.2. Implantation. Interactions between the late blastocyst and the uterine wall trigger the mural trophectoderm to differentiate into trophoblast giant cells and the polar trophectoderm to form the ectoplacental cone (Figure 3). These are the initial events of placentation, which will be described in detail later.

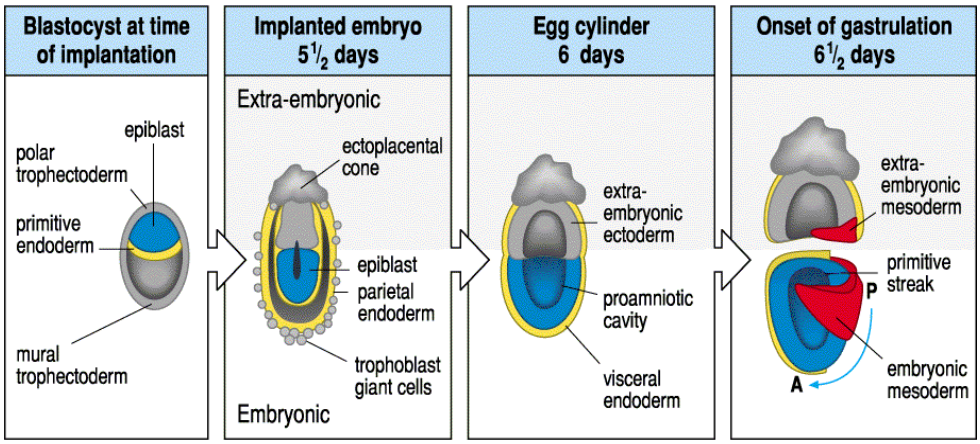


Figure 3. Early mouse embryogenesis. P→A, posterior to anterior.

(Modified from Wolpert et al, 1998)

The embryo proper develops only from the epiblast formed in the late blastocyst (Bard, 1994; Wolpert et al., 1998). The rest of the blastocyst gives rise to the future placenta. Part of the epiblast, the later extra-embryonic mesoderm, also contributes to placental development (Figure 5) (Bard, 1994; Wolpert et al., 1998).

Implantation is completed by E5.5 and the embryo joins the mother in the uterine wall like a bean bud spouting in the soil. The epiblast elongates and an internal cavity is

developed giving it a cup-shaped form (Figures 3, 4). The development of the embryo progresses to the egg cylinder (Figure 3) (Loebel et al., 2003; Lu et al., 2001a; Rossant and Tam, 2002; Wolpert et al., 1998).

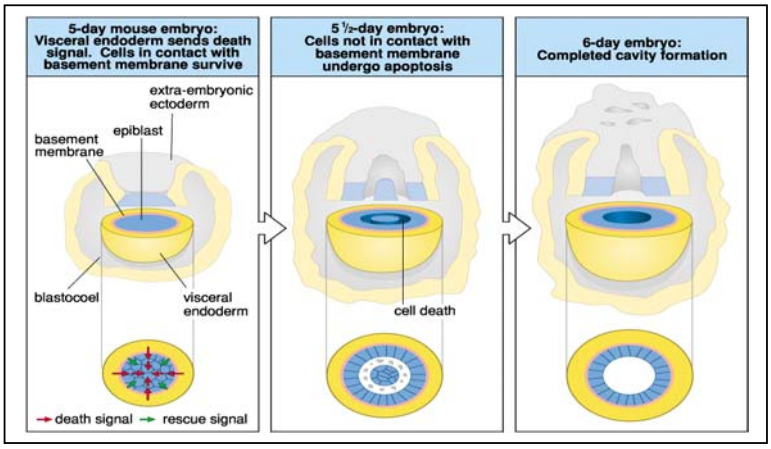


Figure 4. The formation of an internal cavity in the epiblast.

(Modified from Wolpert et al, 1998)

The cavity formation is possibly the first apoptotic event in mouse development. Originally, the epiblast is a solid structure of cells. During early embryogenesis, signals trigger the cells in the center to die creating a hollow structure (Figure 4). The cavity is filled with fluid (Wolpert et al., 1998).

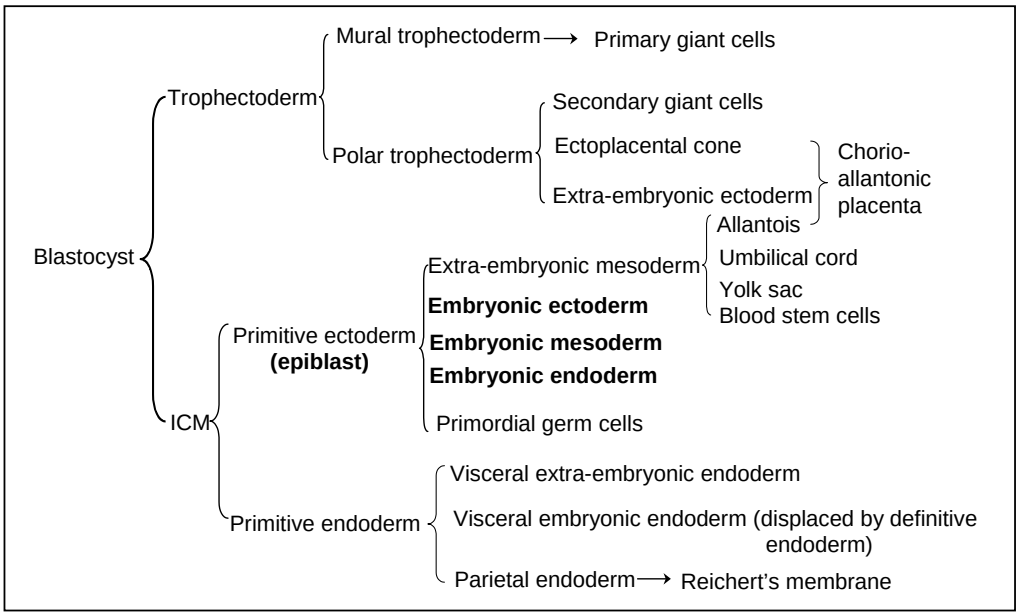


Figure 5. Cell lineage relationship, embryonic and extra-embryonic tissue origin in early mouse development. (Modified from Bard, 1994)

2.1.3. Gastrulation. Gastrulation starts at E6.5 with the formation of the primitive streak and the three germ layers, embryonic ectoderm, mesoderm and endoderm (Figures 3, 6-8). (Rossant and Tam, 2002; Wolpert et al., 1998) In the egg cylinder, there are two cell layers, the visceral endoderm and the epiblast. The epiblast is one curved (U-shaped) layer of epithelium. At a point of the posterior epiblast, cells proliferate and extend anteriorly to the bottom (tip) of the cylinder giving rise to multiple cell layers (Figures 3, 6-8). The proliferating epiblast cells migrate through the primitive streak laterally and anteriorly to form the layer or mesoderm between the ectoderm and visceral endoderm. In the end, three germ layers have formed (Figures 3, 6-8) (Merrill et al., 2004; Rossant and Tam, 2002; Sun et al., 1999; Wolpert et al., 1998).

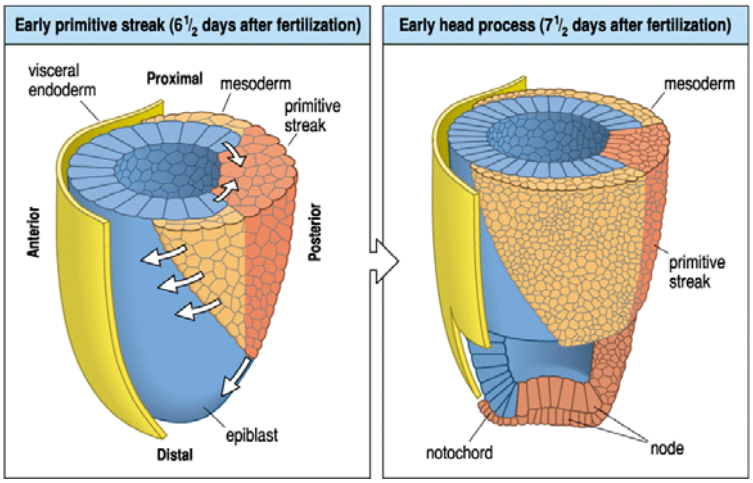


Figure 6. Formation of the three germ layers. (Modified from Wolpert et al., 1998)

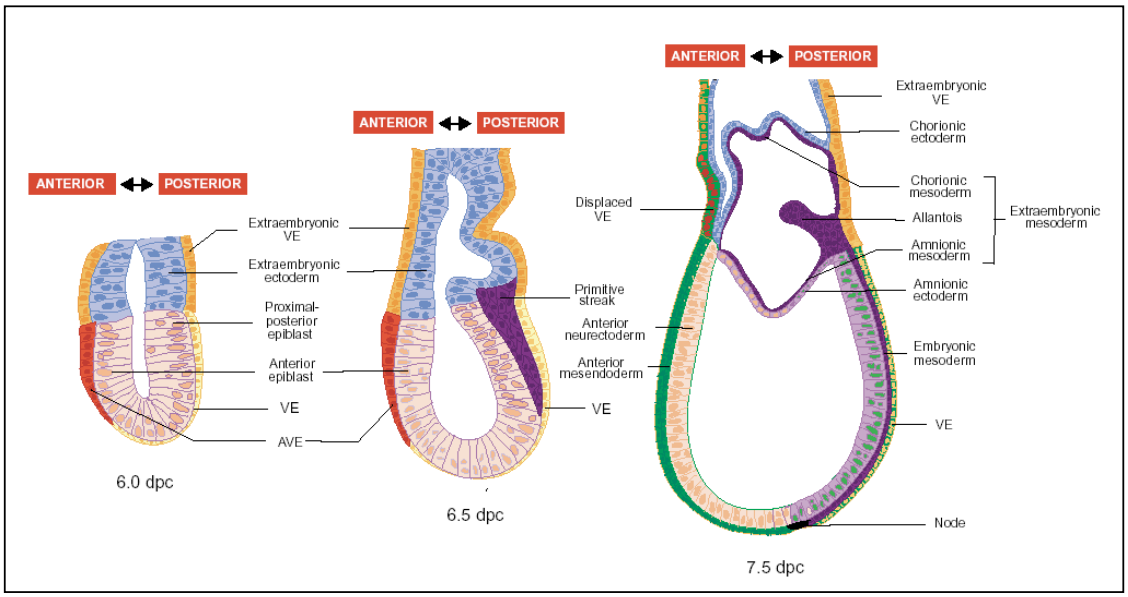
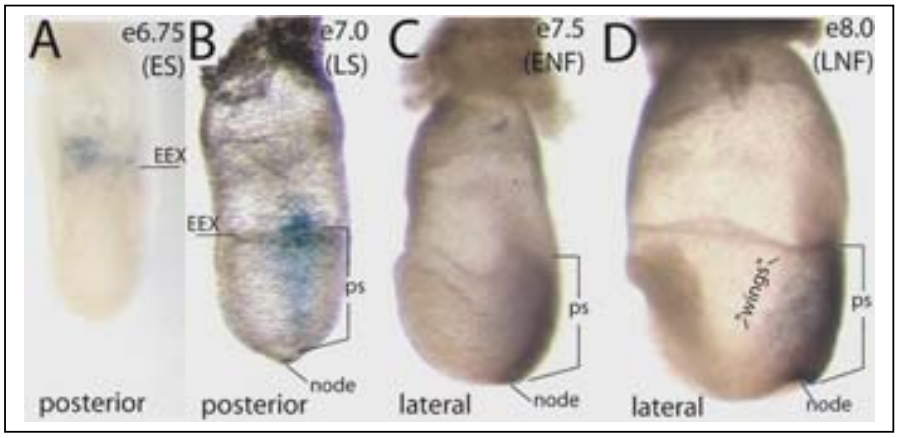
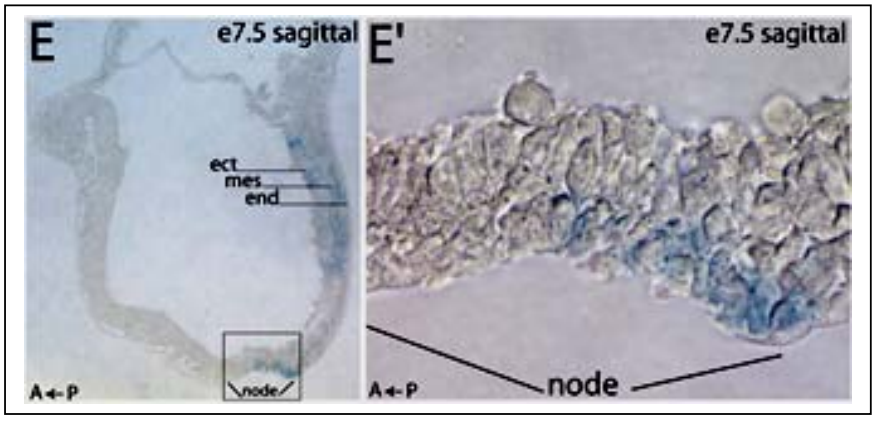


Figure 7. From egg cylinder to gastrulation. The primitive ectoderm (epiblast, in purple) develops into neural ectoderm. (Modified from Lu et al., 2001a)





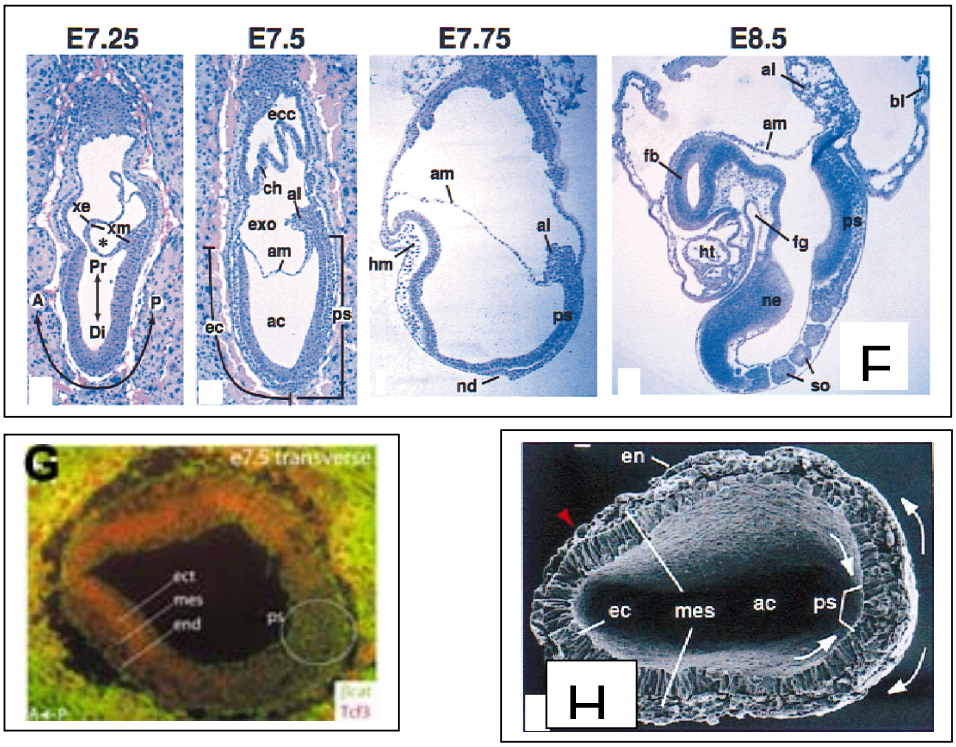


Figure 8. The primitive streak, the three germ layers in early embryos.

Abbreviations: ES, early streak stage; LS, later streak stage; EEX, embryonic/extra-embryonic border; ENF/LNF, early/late neural-fold stage; (A) Anterior; (ac) amniotic cavity; (al) allantois; (am) amnion; (bl) blood island; (ch) chorion; (Di) distal; (ec) anterior ectoderm (prospective neuroectoderm); (ecc) ectoplacental cone; (em) embryonic region; (en) endoderm; (ex) extraembryonic region; (exo) exocoelom; (fb) forebrain; (fg) foregut; (hm) head mesoderm; (ht) heart; (mes) mesoderm; (nd) node; (ne) neuroectoderm; (P) posterior; (Pr) proximal; (ps) primitive streak; (so) somite; (xe) extraembryonic ectoderm; (xm) extraembryonic mesoderm. (Modified from Merrill et al., 2004 and Sun et al., 1999)

The primitive streak migrates towards the future anterior end of the embryo. Some cells condense at the embryo's anterior end to form Hensen's node (Figures 6- 8). Cells migrating anteriorly through the node will form the notochord. Somites develope bilaterally along the notochord. Both notochord and somite are derived from mesoderm (Merrill et al., 2004; Rossant and Tam, 2002; Sun et al., 1999; Wolpert et

al., 1998). The notochord is a transient structure and its cells are eventually incorporated into the embryo column. For example, the cells overlaying the notochord develop into the brain and spinal cord. The somites give rise to the vertebrae and ribs, to the muscles of the trunk and limbs, and also develop into the dermis of the skin (Table 1).

At the late stage of gastrulation, some epiblast cells migrate through the mesoderm to enter the visceral endoderm and gradually replace it. This becomes the definite endoderm and develops into gut (Figure 9) (Bard, 1994; Sun et al., 1999; Wolpert et al., 1998).

The three germ layers will contribute to different tissues late in development. The endoderm gives rise to the gut and its derivatives of the liver and the lungs; the mesoderm develops into the skeleton-muscular system, connective tissues, kidney, heart and blood; the ectoderm develops into epidermis and nervous system (Loebel et al., 2003; Wolpert et al., 1998). It should be emphasized that the mesenchyme is developed from the mesoderm (Kalluri and Neilson, 2003; Thiery, 2002). The mesenchyme in early embryo is the origin of a variety of cells in the connective tissues, such as astrocytes, adipocytes, chondrocytes, osteoblast, muscle cells and fibroblast (Kalluri and Neilson, 2003; Thiery, 2002). The transition from mesenchyme to connective tissue cells will be described in the section on epithelial-mesenchymal transition (EMT).

Table 1. Tissue contribution of the three germ layers

Germ layers	**Organs**
Endoderm	gut, liver, lungs
Mesoderm	skeleton, muscle, kidney, heart, blood
Ectoderm	skin, nervous system

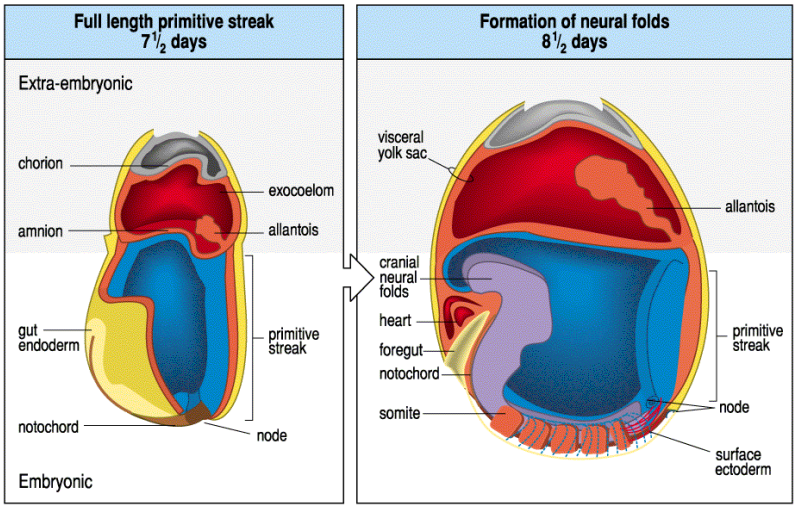


Figure 9. Development of somites and gut. (Modified from Wolpert et al, 1998)

2.1.4 Turning. During late gastrulation, the neural folds starts to form and primitive heart and liver also appear (Bard, 1994; Rossant and Tam, 2002; Wolpert et al., 1998). The embryo undergoes complicated folding and turning. The definite endoderm becomes internalized to form the gut, the heart and liver move to their final positions relative to the gut, and the head becomes distinct (Figure 10). The embryo turns to become entirely enclosed in the amnion and amniotic fluid. It is connected to the placenta by the umbilical cord (Figures 10 and 11) (Zoltewicz et al., 2004).

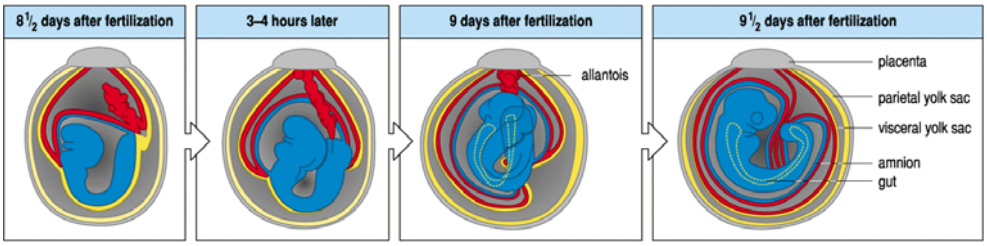


Figure 10. Final embryo turning stage of gastrulation. The embryo has a distinct head and the branchial arch and forelimb buds start to develop. (Modified from Wolpert et al, 1998)

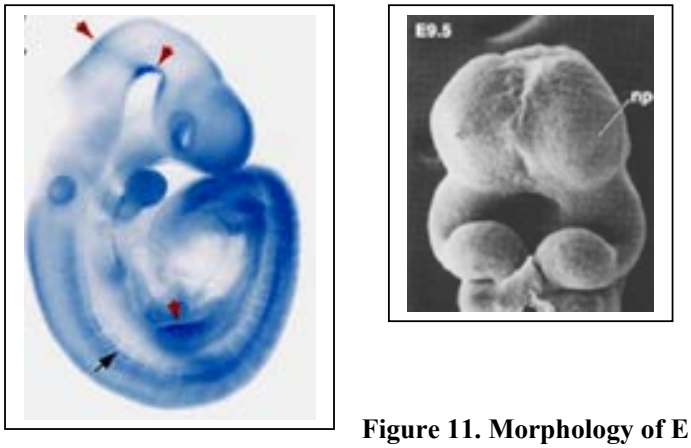


Figure 11. Morphology of E 9.5 embryos.

(Modified from Zoltewica et al., 2004)

2.1.5 Organogenesis. By the end of gastrulation, the embryo shows a distinct head and forelimb buds. The branchial arch, heart, liver and gut start to develop (Figure 11). The other organs form later (Bard, 1994; Rossant and Tam, 2002; Wolpert et al., 1998). At this stage, the majority of cell components in the embryo are various types of epithelium and mesenchymal cells. The epithelial-mesenchymal transition (EMT) is a major developmental event in the organogenesis of the heart and kidney (Kalluri and Neilson, 2003; Savagner, 2001; Thiery, 2002). This process will be described briefly here. The developmental events of nerulation, neural crest migration, and branchial arch formation are also explained because they are of great interest and are relevant to the phenotype revealed by the PKBα/γ (Akt1/3) double mutant mice.

Epithelial-mesenchymal transition (EMT) and its implications. After gastrulation, some mesoderm cells develop into a loose connective structure called mesenchyme, consisting of mesenchymal cells and extracellular matrix (Thiery, 2002). Mesenchymal cells can differentiate into a variety of cell types, including the astrocytes, adipocytes, chondrocytes, osteoblasts, muscles and fibroblasts in the connective tissue. During embryo development, the mesenchymal-epithelial transition

(MET) and epithelial-mesenchymal transition (EMT) mediate cell differentiation and organogenesis (Figure 12).

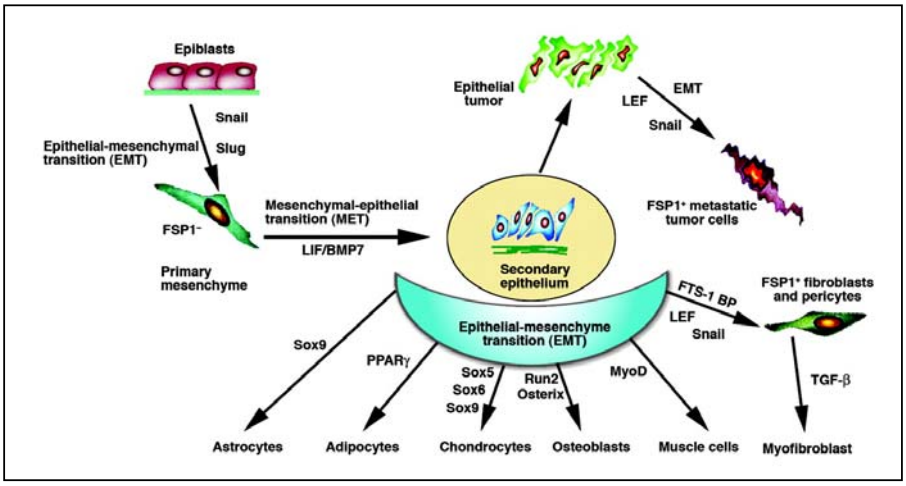


Figure 12. Mesenchymal cells can differentiate into many cell types through the MET and EMT. (Modified from Kalluri and Neilson, 2003)

Usually, the primary mesenchymal cells originate from the mesoderm. Primary mesenchymal cells are reintroduced by MET into secondary epithelium that can differentiate into different types of cells via the EMT. The secondary epithelial cells in some organs can also be transformed into primary tumors that further undergo EMT to metastasize (Kalluri and Neilson, 2003; Thiery, 2002). Therefore, EMT is fundamental to both normal development and malignant transformation of epithelial cells.

In the process of organogenesis of heart, kidney and pancreas, the EMT is essential for the generation of cardiac valves and septum, islet cells, nephric epithelium, and connective tissue (Reese et al., 2002).

Development of heart and its coronary vessels. The EMT in heart development occurs at E9.5. One day earlier at E8.5, the heart (heart tube) is composed of an outer layer of myocardium lined by a monolayer of specialized endothelial cells (Olson and

Schneider, 2003; Reese et al., 2002; Timmerman et al., 2004). The two layers are separated by a thick extracellular matrix of cardiac jelly that is secreted mainly by the myocardial cells. At E9.5, a subset of endocardial cells at the region of atrio-ventricular canal (AVC) and outflow tract undergo an EMT to form the endocardial cushion, which will further develop into cardiac valves and septum (Figures 13-15).

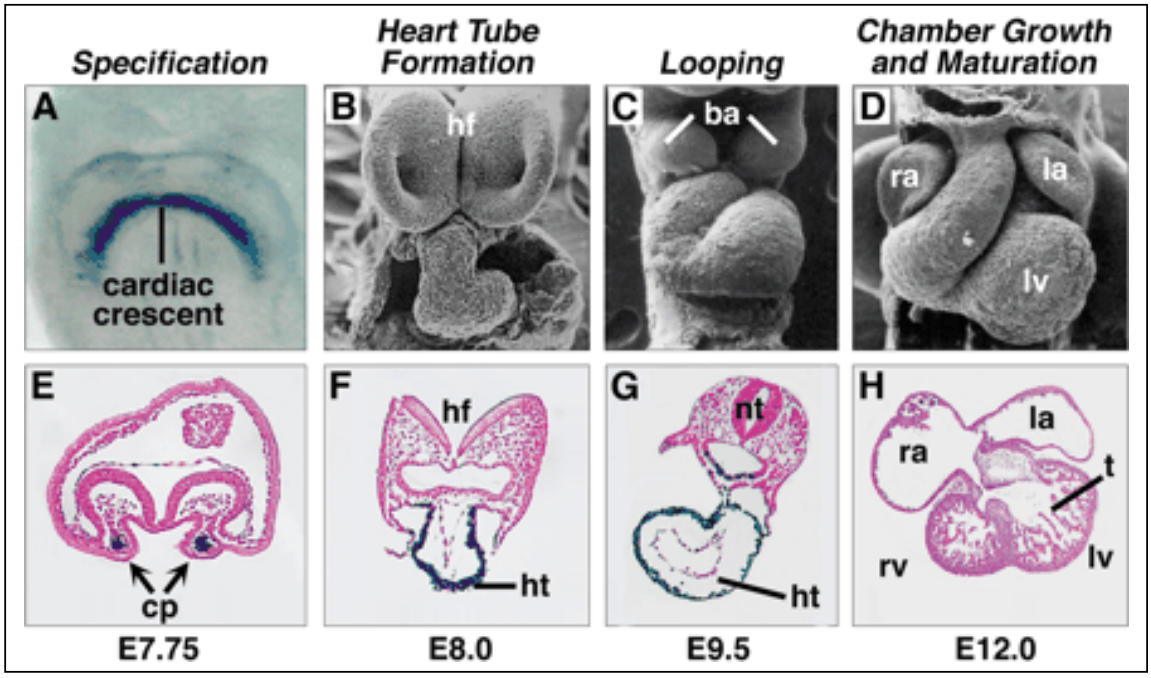


Figure 13. Heart development in mice. (cp) Cardiac progenitors; (hf) head folds; (ht) heart tube;(nt) neural tube; (ba) branchial arch; (ra) right atrium; (la) left atrium; (rv) right ventricle; (lv) left ventricle; (t) trabeculae. (Modified from Olson and Schneider, 2003)

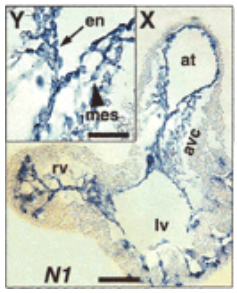


Figure 14. E9.5 heart. (en) Endocardium; (at) atrium; (rv) right ventricle; (lv) left ventricle; (avc) atrio-ventricular canal; (mes) mesenchyme. (Modified from Timmerman et al., 2004)

EMT is also critical for coronary artery development (Reese et al., 2002). At E12.5, the newly formed epicardium, a simple squamous epithelium, completely envelops the heart. The cells then migrate and undergo EMT to form capillary plexi and smooth muscle, which are remodeled into definitive arteries. Eventually, the most proximal points of the major coronal arteries link up with the aorta (Figures 15,16) (Reese et al., 2002).

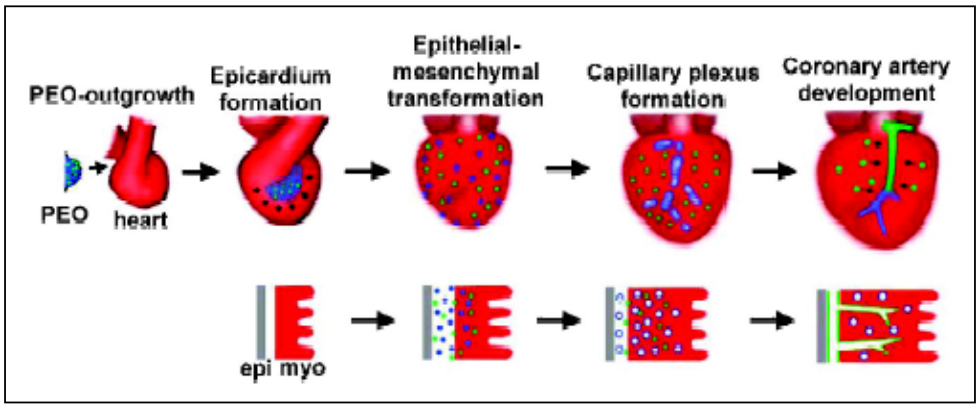


Figure 15. EMT and coronary artery development . PEO, proepicardial organ; (epi) epicardium; (myo) myocardium. (Modified from Reese et al., 2002)

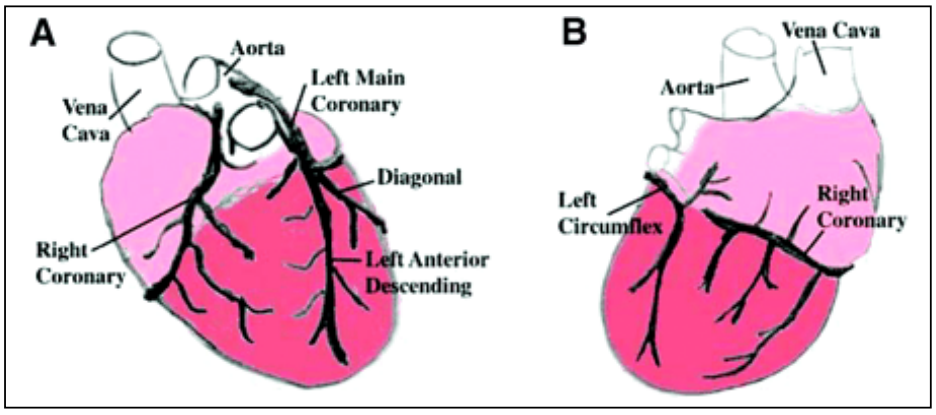
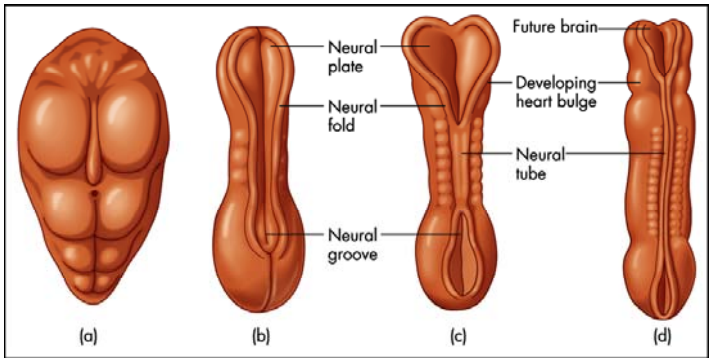


Figure 16. The coronary arteries. A. Two major arteries, the left and the right arteries can be seen on the anterior surface of heart. **B.** The posterior surface of the heart. (Modified from Reese et al., 2002)

Neurulation and neural crest. As described above, the ectoderm is the origin of the central nervous system, including the brain and the spinal cord (Bard, 1994; Wolpert et al., 1998). At as early as E7.5, the ectoderm differentiates into the neuro-epithelium of the headfold region to initiate the process of neurulation (Figures 7, 17, 18) (Copp et al., 2003; Knecht and Bronner-Fraser, 2002; Sun et al., 1999). Neurulation is the embryonic process in which the neural plate, an epithelial structure developed from a specialized region of ectoderm on the dorsal surface of the embryo, undergoes shaping and folding to form the neural tube (Copp et al., 2003; Knecht and Bronner-Fraser, 2002; Wolpert et al., 1998). Neurulation accomplishes three major events in higher vertebrates: (1) It creates the neural tube, which gives rise to the central nervous system. (2) It creates the neural crest, which migrates away from the dorsal surface of the neural tube and gives rise to a diverse set of cell types. (3) It creates the bona fide epidermis, which covers the neural tube once it is created (Copp et al., 2003; Knecht and Bronner-Fraser, 2002; Wolpert et al., 1998).

Neural crest cells originate from the edges of the neural folds and first become recognizable during neurulation (Figure 18). Later on, these cells undergo EMT and migrate away from the neural tube, giving rise to a wide variety of different cell types, including cartilage in the cranial-facial region of the head, pigment cells in the dermis, the medullary cells of the adrenal gland, glial Schwann cells, and the sensory neurons of both the peripheral and the autonomic nervous systems (Gammill and Bronner-Fraser, 2003; Ghislain et al., 2003; Knecht and Bronner-Fraser, 2002; Trainor and Nieto, 2003). The cells remaining in the neural tube develop into the brain and spinal cord,i.e. the central nervous system.



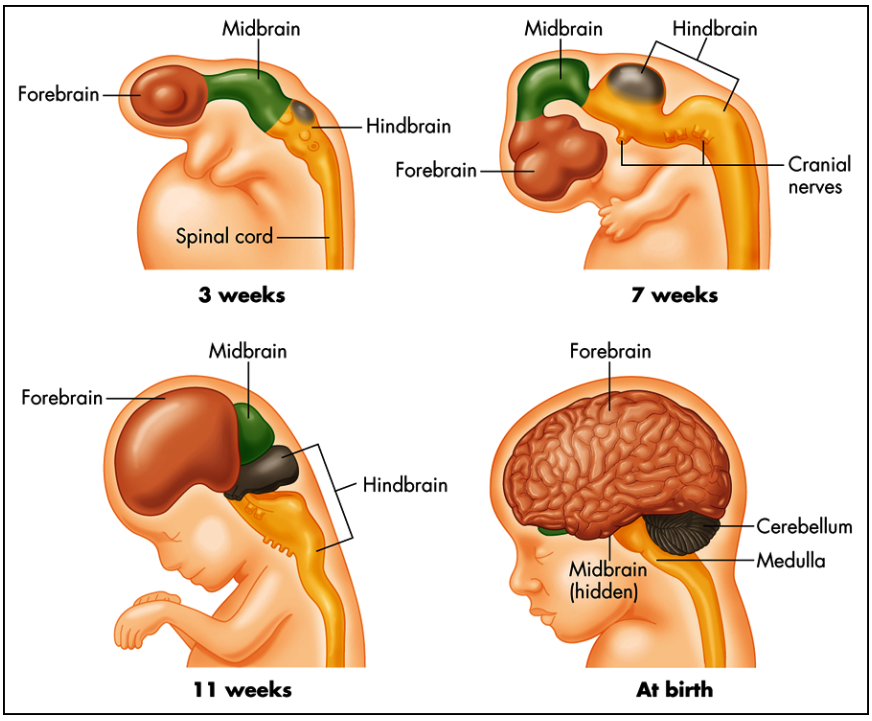


Figure 17. Neurulation and brain development. a-b, neurulation in more detail. At the bottom, different stages of brain development in humans is shown. The three parts of early brain are forebrain (proencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon).

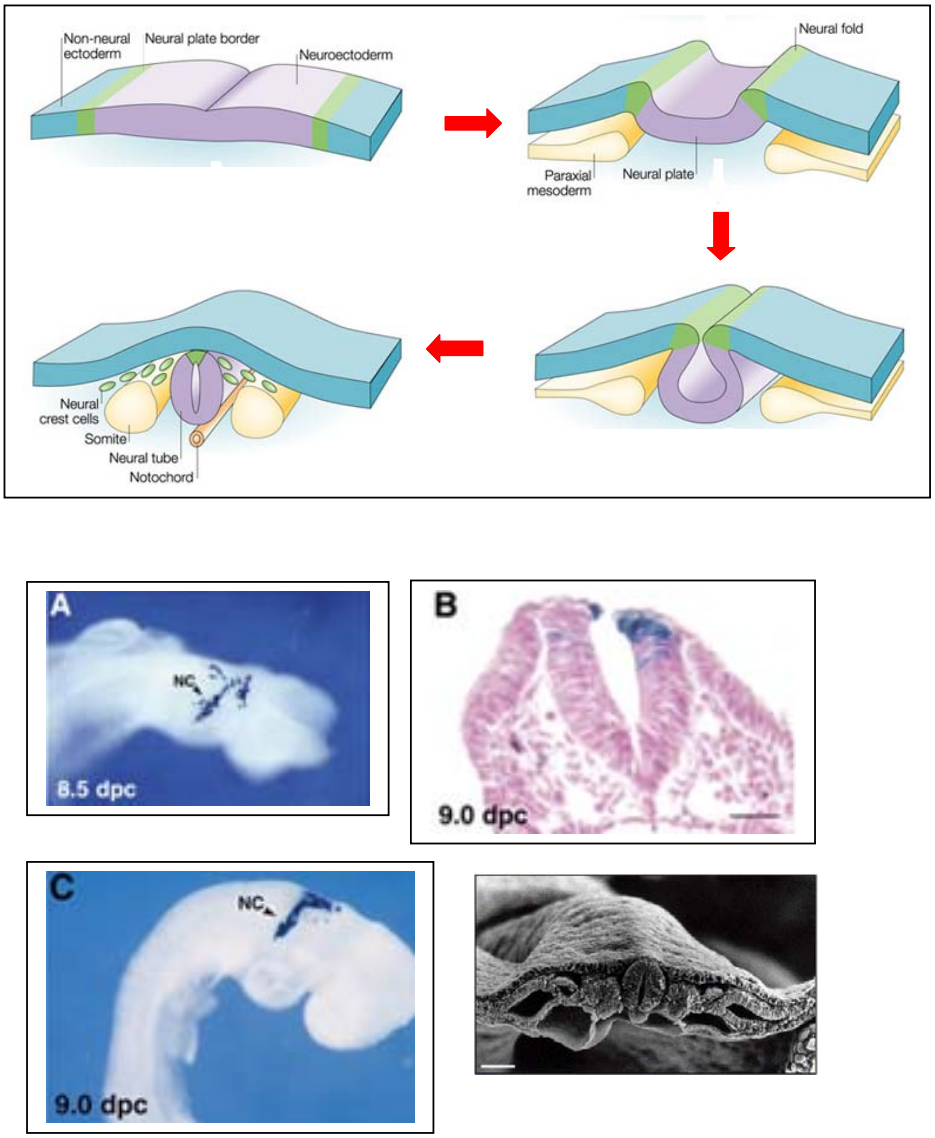


Figure 18. Neural tube formation (neurulation) and neural crest cells. NC, neural crest cells. Right: A scanning electron micrograph of chick neural tube and early somites. (Modified from Gammill and Bronner-Fraser, 2003, Ghislain et al., 2003 and Wolpert et al., 1998)

Branchial arch and neural crest cells

There are six bilaterally symmetric branchial arches (pharyngeal arches) in the mouse embryo, each of which gives rise to unique structures in the head and neck (Graham and Smith, 2001; Kaufman and Bard, 1999; Thomas et al., 1998). As mentioned above, the first branchial arch is distinct at around E9.0; the second arch is visible by E9.5 and the third, fourth and sixth branchial arches become apparent by E10 (Figures 19,20) (Graham and Smith, 2001; Kaufman and Bard, 1999; Thomas et al., 1998). The branchial arches are epithelia-covered bars of mesenchyme developed from the neural crest cells. The mesenchyme later differentiates into specific organs and structures of the head and neck (Knecht and Bronner-Fraser, 2002; Thomas et al., 1998; Trainor and Nieto, 2003). A population of the mesenchymal cells, the cardiac neural crest cells, occupies the aortic arch arteries and are essential for remodeling the arch arteries (Knecht and Bronner-Fraser, 2002; Thomas et al., 1998; Trainor and Nieto, 2003) . The cardiac neural crest cells give rise to the entire musculoconnective tissue wall of the large arteries emerging from the heart, the membranous portion of the ventricular septum, and the septum between the aorta and pulmonary artery. In addition, these cells contribute to melanocytes, neurons, cartilage, and connective tissue of the third, fourth, and sixth branchial arches. The parathyroid, thyroid, and thymus glands develop from the branchial apparatus and also have a neural crest contribution (Figures 19, 20) (Knecht and Bronner-Fraser, 2002; Thomas et al., 1998; Trainor and Nieto, 2003).

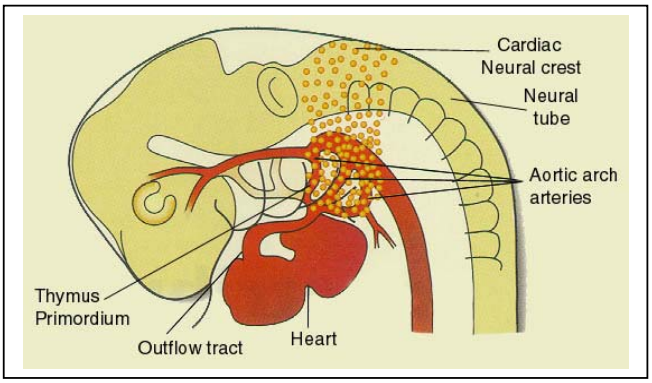


Figure 19. Migration of cardiac neural crest cells. (Modified from Bernadette C. Holdener, Dept. of Biochemistry and Cell Biology, State University of New York at Stony Brook)

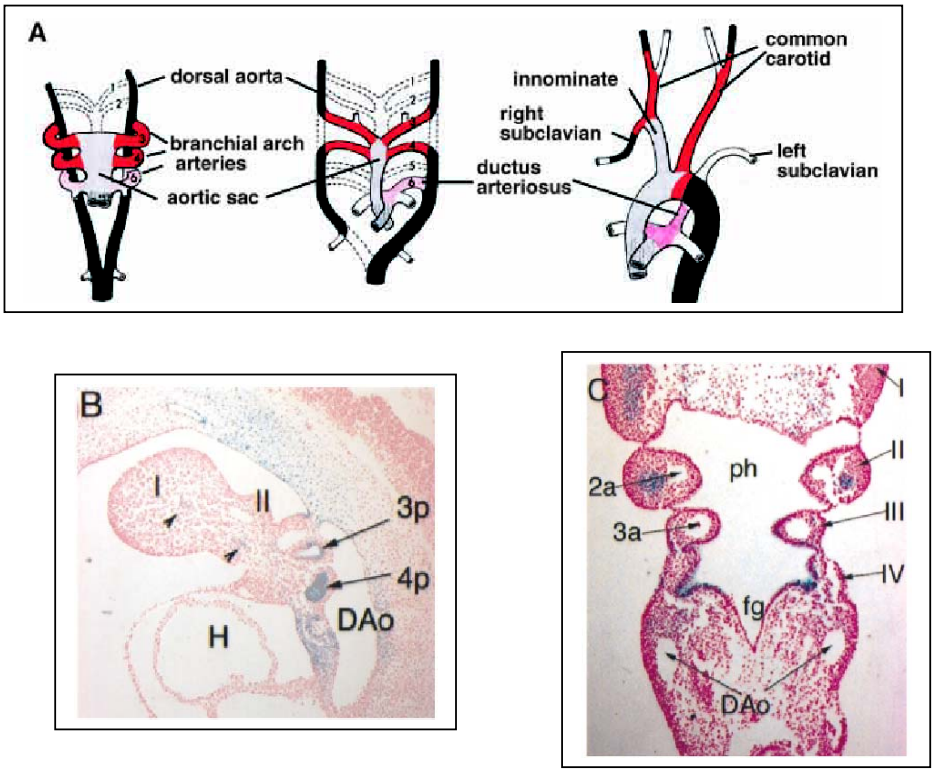


Figure 20. Branchial arches and their arteries. A. The fate and development of branchial arch arteries (from early embryo to term).B. Sagittal section of E10.5 mouse embryo. C. Coronal section of mouse embryo. I, II, III, IV, branchial arches; 2a,3a, artery; 3p,4p pouch; DAo, dorsal aorta; (fg) foregut. (Modified from Liu et al., 2002 and Vitelli et al., 2002)

2.2 Extra-embryonic development----establishment of a functional placenta

The placenta is a unique organ exclusive to mammals. It develops from the fertilized egg and is, therefore, an organ of the embryo (Alessi et al., 1996; Cross, 2000; Cross et al., 2002; Rossant and Cross, 2001; Rossant and Tam, 2002). However, it roots into the uterine wall of the mother similar to a tree into the soil, linking the fetus with the mother. During gestation, the development and growth of the fetus depends on the placenta for exchange of gases, nutrients and waste products between mother and baby. The placenta also produces pregnancy-associated hormones and growth factors and participates in the immune protection of the fetus. Any genetic or environmental alterations and insults affecting placental development or function may result in placental insufficiency and consequent impairment of fetal development and growth leading to growth retardation and malformation, even to death (Cross, 2000; Cross et al., 2002; Rossant and Cross, 2001; Rossant and Tam, 2002).

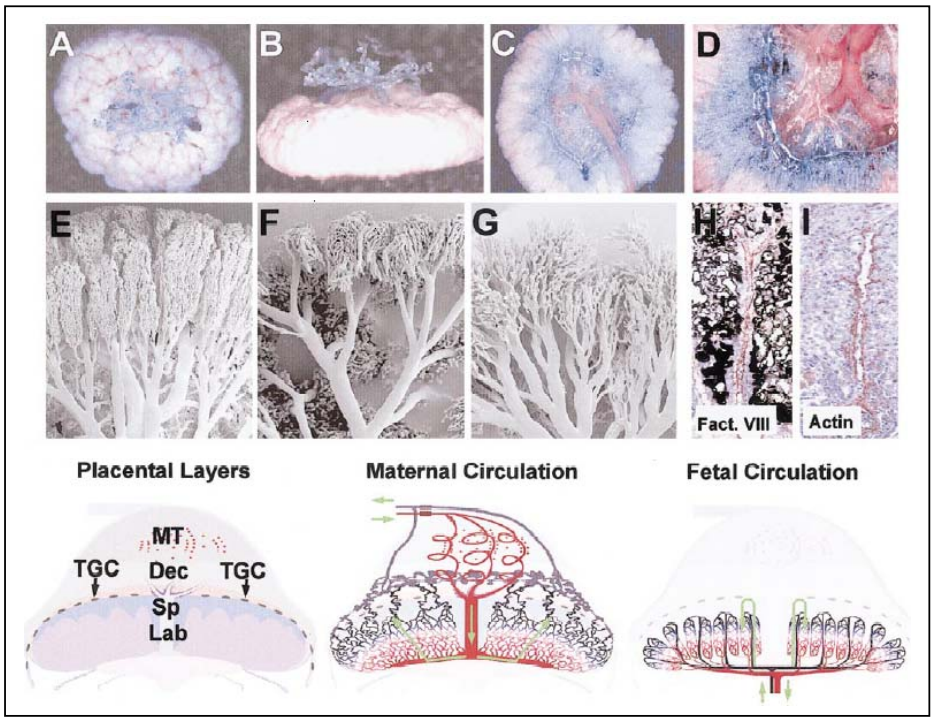


Figure 21. Vasculature and blood circulation in the placenta. (Modified from Adamson et al., 2002)

Placental development, placentation, is initiated from E3.5 and proceeds until E12.5. During this period, fetal development is rather slow. Subsequently, functional establishment of the placenta enormously improves the developmental conditions of the fetus and the increasing requirements for fetal growth are met. Fetal mass accumulates at an increasing rate. From E12.5 to 16.5, the placenta undergoes remodeling and growth together with the fetus.

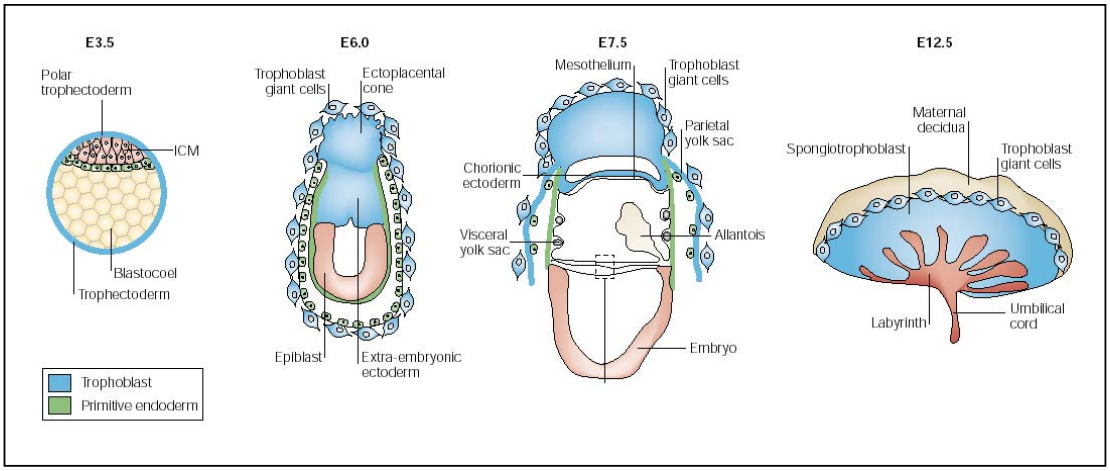


Figure 22. Establishment of a functional placenta in mouse. (Modified from Rossant and Cross, 2001)

2.2.1. Mouse placental development. As mentioned in the early embryo development, there are only two populations of cells in the early blastocyst, the inner cell mass (ICM) and the trophoblast, a mono-layer of epithelium (Figure 2). The inner cell mass will contribute mainly to the embryo proper, while the trophoblast develops into the greater part of the placenta (Rossant and Cross, 2001; Wolpert et al., 1998). In late blastocyst, a third population of cells appears, the primitive endoderm, and the trophoblast differentiates into the polar trophectoderm and mural trophectoderm (Figures 3 , 22) (Rossant and Cross, 2001; Wolpert et al., 1998). In development, the mural trophectoderm forms the primary trophoblast giant cells for implantation and the

polar trophectoderm the ectoplacental cone, a primitive placenta (Figures 3, 22). Later in gastrulation, some mesoderm cells give rise to the allantois and part of the chorion. Meanwhile, in the ectoplacental cone, the secondary trophoblast giant cells develop and the chorionononic ectoderm is also formed (Rossant and Cross, 2001; Wolpert et al., 1998). Thus, the chorion has two different origins, the mesoderm of the epiblast and the ectoderm of the ectoplacental cone (Figures 5, 7, 22). By E8.5, the allantois makes contact with the chorion, an event termed chorioallantoic fusion. After several hours of allantoic attachment, folds appear in the chorion where the fetal vascular network will be constructed (Rossant and Cross, 2001; Rossant and Tam, 2002). The trophoblast, together with its associated fetal blood vessels, subsequently undergoes extensive villous branching and remodeling to generate a densely packed structure, the labyrinth (Rossant and Cross, 2001; Rossant and Tam, 2002). At the same time, chorionic trophoblast cells begin to differentiate into the various layers of the trophoblast in the labyrinth (Figures 22, 24).

2.2.2. Placental architecture. The mature placenta consists of three main layers, maternal decidua basalis (db), junction zone (jz) and labyrinth zone (lz) (Georgiades et al., 2001). Below is a schematic representative of a sagittal section of an E15.5 placenta with the maternal side at the top and the fetal side at the bottom. The placenta is linked to the maternal uterine wall by the deciduas basalis. The labyrinth is a vascular network in which the fetal vessel capillaries are immersed in the maternal blood space (mbs) for nutrient, gas and waste product exchange. Some of the spongiotrophoblast (st) between the decidua basalis and the labyrinth synthesize glycogen (gc). The trophoblast giant cells line up between the spongiotrophoblast and the decidua basalis (tg).

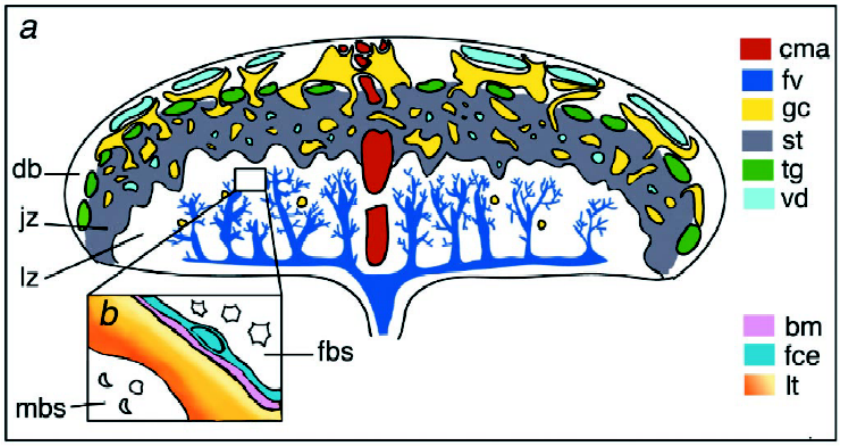


Figure 23. The architecture of the placenta. For abbreviations, see text. (Modified from Georgiades et al., 2001)

2.2.3. The trophoblasts and the labyrinth. In early embryo development, the mural trophoectoderm gives rise to primary trophoblast giant cells for implantation (Rossant and Cross, 2001). In the mature placenta, the trophoblasts fall into four categories, the secondary trophoblast giant cells (tg in Figure 23), glycogen-containing spongiotrophoblast (gc in Figure 23), non-glycogen spongiotrophoblast (st in Figure 23) and labyrinthine trophoblast (syncytiotrophoblast) (Georgiades et al., 2001). The secondary trophoblast giant cells and the spongiotrophoblast arise from the ectoplacental cone (Cross, 2000; Cross et al., 2002; Rossant and Cross, 2001), while all the other cell lineages in the placenta are derived from the epiblast (Lu et al., 2001a; Rossant and Cross, 2001). The functions of these trophoblasts are not completely clear. However, there is some evidence that the migration of trophoblast giant cells and spongiotrophoblasts are important for labyrinthine vascular expansion (Adamson et al., 2002; Cross et al., 2002; Rossant and Cross, 2001).

The inset in Figure 23 shows a higher magnification of feto-maternal interface in the labyrinth. Between the fetal blood space (fbs) and the maternal blood space (mbs) is a trilayer structure of basement membrane (bm), fetal capillary endothelium (fce) and labyrinthine trophoblast (lt, also called syncytiotrophoblast). This structure is unique in that the syncytiotrophoblast lines the maternal blood space instead of endothelial cells in common vessels. These syncytiotrophoblasts synthesize alkaline phosphatase (AP) and can be visualized by AP staining.

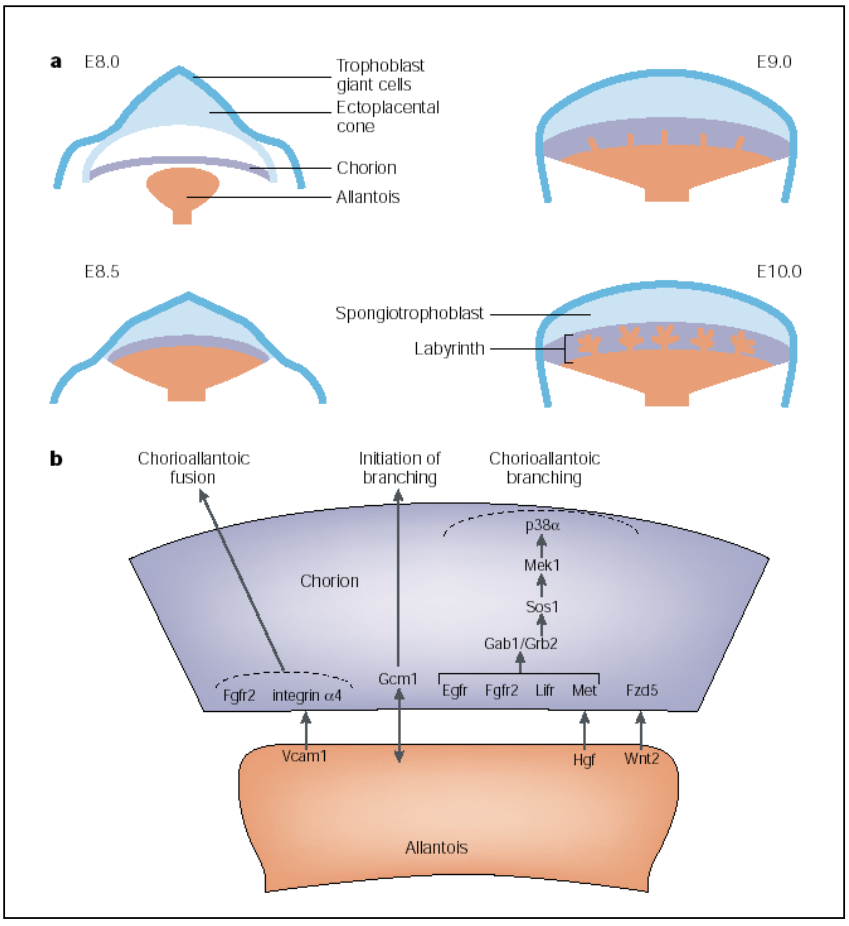


Figure 24. Morphogenesis and signaling for establishment of the labyrinth.(Modified from Rossant and Cross, 2001)

Lessons learnt from knockout mice indicate that numerous transcription factors are indispensable for differentiation of trophoblast cells (Figure 25).

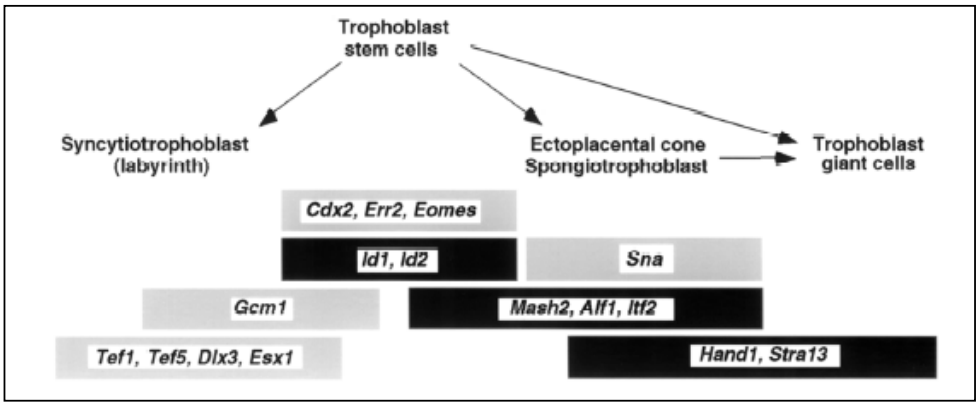


Figure 25. Critical transcription factors for trophoblast differentiation with basic helix-loop-helix (bHLH) transcription factors in black. (Modified from Cross 2000)

2.2.4. Blood circulation in the placenta. The placenta is a highly vascular structure (Figure 22). The exchange of gases, nutrients and waste products is carried out by two systems of blood circulation, the maternal and the fetal (Adamson et al., 2002). Through the central maternal artery (cma), highly oxygenated maternal blood enters the labyrinth (maternal blood space, mbs) into which less oxygenated fetal blood flows (fetal blood space, fbs) through the artery of the umbilical cord. The two blood streams do not mix but separated by the feto-maternal interface and flow in opposite directions, as displayed in Figure 22. After exchange, blood returns to the mother and fetus by the maternal placental vein and the umbilical cord vein (Adamson et al., 2002).

3. Protein kinase B (PKB/Akt) and transgenic mice

[Part of PHYSIOLOGICAL FUNCTIONS OF PKB/Akt (2004) Yang, et al. Biochemical society transactions, 32:350-354.]

Introduction

Three PKB/Akt isoforms have been identified in mice and humans (Brazil and Hemmings, 2001; Datta et al., 1999; Lawlor and Alessi, 2001; Scheid and Woodgett, 2003) . These three PKB/Akt proteins, though encoded by distinct genes localized on different chromosomes, have ~80% amino acid identity and similar domain structures. Moreover, the differences between corresponding isoforms of humans and mice are subtle (2 to 10 in ~480 amino acids), which makes it feasible to determine the functions of PKB/Akt kinases in human physiology by studying them in the mouse.

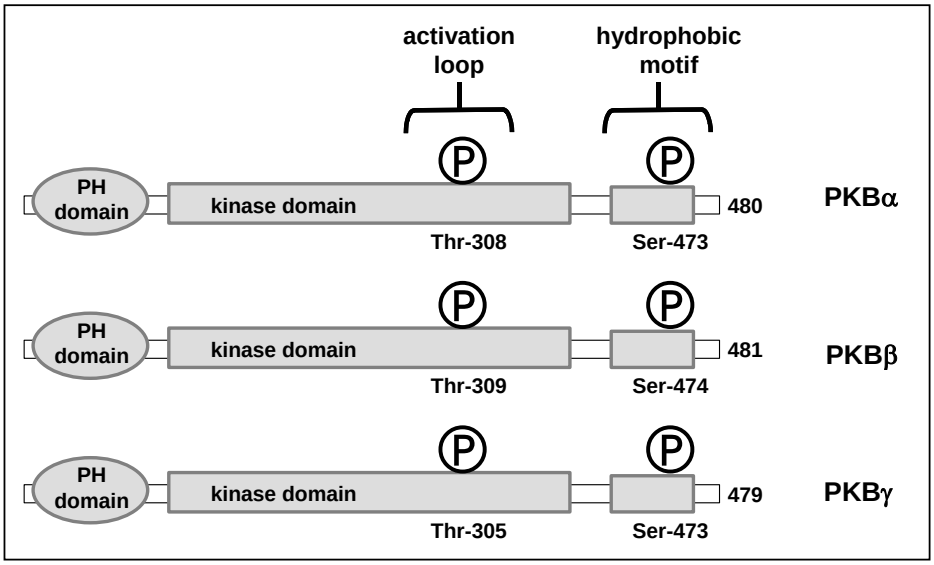


Figure 26. The three PKB isoforms in mouse.

Stimulation by numerous growth factors, cytokines, hormones and neurotransmitters can activate PKB/Akt in a phosphatidylinositol 3-kinase (PI 3K)-dependent manner (Datta et al., 1999) and (Scheid and Woodgett, 2003). Through receptor tyrosine kinases, these stimuli cause PI3K activation, and generation of the membrane

phospholipid, phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3]. PtdIns(3,4,5)P_3 then recruits PKB/Akt to the membrane, where it becomes phosphorylated at threonine 308 and serine 473 (for

Table 2 A variety of PKB/Akt transgenic mouse models

Targeting tissue	Promoter	PKB/Akt structure	Reference
Heart	a-d) α-myosin heavy chain	a) Myr-PKB-HA b) PKB (T308D/S473D) c) PKB (E40K) d) Myr-PKB	(Condorelli et al., 2002; Cook et al., 2002; Matsui et al., 2002; Shioi et al., 2002)
Thymus	a,d) CD2 b,c) Lck	a) gag-PKB b) HA-PKB; Myr-PKB-HA; HA-PKBE40K c) Myr-PKB-HA d) Myr-HA-PKB	(Jones et al., 2002; Jones et al., 2000; Malstrom et al., 2001; Na et al., 2003; Rathmell et al., 2003)
Mammary gland	a-d) MMTV-LTR	a) HA-PKB (T308D/S473D) b) HA-Myr-PKB c,d) PKB	(Ackler et al., 2002; Hutchinson et al., 2001; Schwertfeger et al., 2003; Schwertfeger et al., 2001)
Pancreas	a,b) RIP	a) Myr-PKB b) Myr-PKBΔ4-129	(Bernal-Mizrachi et al., 2001; Tuttle et al., 2001a)
Prostate	Probasin	Myr-HA-PKB	(Majumder et al., 2003)

Abbreviations: MMTV-LTR, mouse mammary tumour virus-long terminal repeat; RIP, rat insulin promotor. Myr-PKB has Lck/Src myristylation signal sequence at the amino terminus for constitutive membrane attachment and activation(similar to gag-PKB) and T308D/S473D is constitutively active.

PKBα/Akt1) by two upstream kinases, PDK1 and an as yet to be identified Ser 473 kinase. These processes of membrane targeting and activation of PKB/Akt can be facilitated and mimicked by adding the myristylation signal sequence of Lck/Src to the amino terminus of the PKB/Akt (Myr-PKB/Akt) or mutation of the two regulatory sites of PKB/Akt to acidic residues [PKB T308D/S473D (DD) in PKBα/Akt1](Alessi

et al., 1996; Scheid and Woodgett, 2003). Myristylation of PKB/Akt promotes constitutive membrane attachment and activation, and the DD double mutant of PKB is constitutively active (Alessi et al., 1996; Scheid and Woodgett, 2003). Based on these facts, Myr-PKB/Akt and PKB T308D/S473D have been commonly used for vector construction to generate transgenic mice (Alessi et al., 1996)

PKB/Akt transgenic mice

A) Overexpression of PKB/Akt in tissues

The first PKB/Akt transgenic mouse model was reported in 2000 (Jones et al., 2000). Since then, more than 10 PKB/Akt transgenic mouse lines have been produced. Constructs of PKB/Akt and the tissues targeted are summarized in Table 2. At least two mouse lines were generated for the thymus, heart, pancreas and mammary glands. A single line has been generated for prostate. Tissue-specific promoters were used to drive overexpression of PKB/Akt in these different tissues. PKB/Akt was either myristylated for membrane targeting (activation), or mutated to double D (T308D/S473D) for constitutive activation.

1) Hypertrophy and increased contractility with PKB/Akt overexpression in the heart

The α-myosin heavy chain (α-MHC) promoter has been utilized extensively to drive transgenic expression exclusively in cardiac myocytes (Shioi et al., 2000). Three of the four PKB/Akt transgenic mouse lines directly use this promoter to drive PKB/Akt overexpression in the heart. In the fourth line, PKB/Akt transcription was under the control of a tetracycline-responsive promoter that reacts to α-MHC-directed expression of tetracycline controlled transactivator (t-TA) (Condorelli et al., 2002; Cook et al., 2002; Kovacic et al., 2003; Matsui et al., 2002; Shioi et al., 2002). The most apparent phenotype of these mice was sudden death of some founders with

massive cardiac dilatation (Matsui et al., 2002). Viable derived transgenic mouse lines showed cardiac hypertrophy, with around two-fold heart weight increase. The weight increase was associated with larger cardiac myocytes (Condorelli et al., 2002; Matsui et al., 2002; Shioi et al., 2002). These mice also showed a remarkable increase in cardiac contractility and reduction in infarct size after ischemia reperfusion compared with wild-type controls (Condorelli et al., 2002; Matsui et al., 2002). Overexpression of PKB/Akt in the heart also caused higher p70S6K phosphorylation, and reduced AMP-activated protein kinase (AMPK) activity (Kovacic et al., 2003; Matsui et al., 2002; Shioi et al., 2002). The transcriptional effects of this chronic activation of PKB/Akt in the heart were analysed using DNA microarray to determine altered gene expression profiles. Of the differentially expressed genes, up-regulation of insulin-like growth factor-binding protein-5 (IGFBP-5) and down-regulation of peroxisome proliferator-activated receptor (PPAR)γ co-activator-1(PGC-1) and PPARα suggest that these may have anti-apoptotic and survival effects in the heart (Matsui et al., 2002). Overall, these results demonstrate that PKB/Akt is an important modulator of heart growth. On the other hand, this also implies that sustained or increased PKB/Akt activity in the heart is one of the causes of cardiac hypertrophy under patho-physiological conditions such as hypertension.

2) Early onset of thymic lymphomas induced by overexpression of PKB/Akt in T cells

The first reported PKB/Akt transgenic mouse line expressed a constitutively active form of PKB/Akt in T lymphocytes (Jones et al., 2000). Thereafter, two groups generated almost exactly the same mice to ask questions from different angles (Malstrom et al., 2001; Rathmell et al., 2003). Both groups reported the development of lymphomas at an early age and that the majority of the mice died between days 100

to 200. Tsichlis' group was attempting to determine the mechanism of tumour induction by constitutively active PKB/Akt while Thompson's group focused on the metabolic contributions by activation of PKB/Akt in T cells (Malstrom et al., 2001; Rathmell et al., 2003).

In their Myr-PKB/Akt transgenic mice, Tsichlis' group first observed, prior to tumour formation, that thymocytes were larger than in non-transgenic controls, although thymus size was the same because of fewer cells. Based on this, they hypothesized a thymic-intrinsic mechanism maintaining the size of the thymus by restriction of the cell proliferation driven by oncogenic Myr-PKB/Akt. Failure of this size regulation promotes cell proliferation and gives rise to an enlarged neoplastic thymus (lymphoma). To test this hypothesis, they isolated fresh thymocytes from transgenic and control thymus glands and found that the cell cycle profiles were not altered in transgenic thymocytes, which expressed low levels of cyclin D3. Nevertheless, once in culture, these transgenic thymocytes showed increases in both cell cycle progression and cyclin D3 levels, probably due to the release of thymic-intrinsic restriction mechanisms. Moreover, freshly isolated thymocytes from the lymphomas had higher levels of cyclin D3. These results supported their hypothesis for tumour induction in Myr-PKB/Akt thymus: constitutively active PKB/Akt can bypass the intrinsic size control mechanisms and cause tumorigenesis. This model of tumour development with increased PKB/Akt activity is intriguing and should help our understanding of the oncogenic functions of PKB/Akt.

Other groups found that overexpression of PKB/Akt in T cells could influence positive and negative selection of thymocytes, survival, metabolism and activation/proliferation of T cells (Jones et al., 2002; Jones et al., 2000; Na et al., 2003; Rathmell et al., 2003). Furthermore, cross-talk was demonstrated between PKB/Akt and important TCR

downstream molecules modulating the threshold of thymocyte selection and T cell activation (Na et al., 2003). These observations are similar to the phenotypes displayed by *Pten* heterozygous mice, which have high PKB/Akt activity and develop auto-immunity (production of nuclear antibodies and deposition of immune complexes in the glomerulus) and lymphomas, reinforcing the proposed role of PKB/Akt in tumorigenesis associated with the *Pten* muation (Suzuki et al., 1998).

3) Fatty milk synthesis in the mammary gland with PKB/Akt overexpression

PKB/Akt expression levels increase in the mammary gland during mouse pregnancy and more dramatically (12-fold) at the onset of lactation (Schwertfeger et al., 2003). This suggests important roles of PKB/Akt in mammary gland development and during lactation. After initiation of involution, the levels of PKB/Akt start to decrease and remain low during involution (Ackler et al., 2002; Hutchinson et al., 2001; Schwertfeger et al., 2003; Schwertfeger et al., 2001). Using the mouse mammary tumour virus (MMTV) promoter, three groups generated transgenic mice with PKB/Akt overexpression in the mammary gland. Initially, they all reported delayed involution of mammary glands in these mice (Ackler et al., 2002; Hutchinson et al., 2001; Schwertfeger et al., 2003; Schwertfeger et al., 2001). Involution results from apoptosis of the epithelium in the mammary gland, overexpression of PKB/Akt could oppose this process by promoting epithelial cell survival (Ackler et al., 2002; Hutchinson et al., 2001; Schwertfeger et al., 2003; Schwertfeger et al., 2001). Intriguingly, a subsequent study of these mice found that females produced fatty milk with excess lipids (Schwertfeger et al., 2003). The fat content of milk from these lactating transgenic mice was as high as 65-70% by volume compared with the 25-30% of wild-type mice. Overexpression of PKB/Akt in the mammary gland promoted synthesis of lipids in epithelial cells from early pregnancy until lactation. As a result of

lipid accumulation in the mammary gland, the milk was too viscous for sucking by the pups, and growth of the mice was retarded over the first 9 days of lactation. These findings suggest that PKB/Akt regulates lipid metabolism in the mammary gland (Schwertfeger et al., 2003).

Tumour formation was not detected during a long period (over 1 year) of observation of the mammary glands of these mice (Hutchinson et al., 2001). Previous studies found a correlation between increased PKB/Akt activity and transformation in the mammary gland (Perez-Tenorio and Stal, 2002). Recent reports have confirmed this relationship by showing that PKB/Akt phosphorylated p27 on threonine 157, and that phosphorylated p27 relocated from the nucleus to the cytoplasm devoid of its growth inhibitory properties, thereby allowing breast cancer cell proliferation (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002). This discrepancy suggests that other mechanisms in combination with high PKB/Akt activity cause tumorigenesis in the mammary gland.

4) Hypertrophy, hyperplasia and hyperinsulinemia of pancreas with PKB/Akt overexpression

Overexpression of PKB/Akt in islet β cells of the pancreas driven by rat insulin II promoter (RIP) has been reported by two groups and both made similar observations (Bernal-Mizrachi et al., 2001; Tuttle et al., 2001b). “Three hypers of hypertrophy, hyperplasia and hyperinsulinemia” was the most suitable and concise description of these mice, as expressed in the title of one publication (Bernal-Mizrachi et al., 2001). Transgenic expression of PKB/Akt gave rise to larger islets in the pancreas due to both increased cell size and cell number. Therefore, it was concluded that high levels of PKB/Akt caused hypertrophy and hyperplasia of islets (Bernal-Mizrachi et al., 2001; Tuttle et al., 2001b). As a result of islet mass increase, insulin secretion was elevated

and these mice were resistant to streptozotocin-induced diabetes. However, the high levels of insulin in blood had little effect on the levels of glucose. These observations indicated that PKB/Akt activation could affect islet β cell mass by altering cell size and cell number and impact insulin production (Bernal-Mizrachi et al., 2001; Tuttle et al., 2001b). This implicates PKB/Akt in insulin action and diabetes, as discussed below (Cho et al., 2001a). Moreover, PKB/Akt could possibly be used to expand islet β cells for therapeutic purposes.

5) Neoplasia induction by overexpression of PKB/Akt in the prostate

A recent report added a new mouse line to the already long list of PKB/Akt transgenic mouse models (Majumder et al., 2003). This mouse strain showed PKB/Akt overexpression in the prostate initiated by a promoter from probasin, a specific protein expressed in the prostate. This latest mouse line provides new evidence that activation of PKB/Akt in epithelial cells can be oncogenic, and supports previous studies with *Pten* mutant mice. *Pten* mutant mice have elevated PKB/Akt activity, which is possibly responsible for prostate cancer development in these mice (Majumder et al., 2003). Given the capability of PKB/Akt prostate transgenic mice to recapitulate human prostate cancer, it may be a useful model to study the role of PKB/Akt in prostate epithelial cell transformation, and to develop therapeutic strategies for this disease.

B) PKB/Akt combination tumour models and others

As mentioned above, chronic activation of PKB/Akt in mice failed to induce tumour formation in tissues such as pancreas and mammary gland, suggesting that activation of the PKB/Akt signaling pathway alone is not sufficient to initiate transformation in these tissues. It has become evident that malignant alteration involves a complex of signal transduction processes including multiple onco-proteins and tumour suppressors

such as Ras, Myc, PKB/Akt, Her-2/Neu, p53 and PTEN (Orsulic et al., 2002). Tumorigenesis results from synergistic interactions of these proteins.

The first study of transformation by use of constitutively active PKB/Akt in combination with another oncoprotein was carried out by Holland and colleagues (Holland et al., 2000). They transferred active Ras and PKB/Akt to neural progenitors in mice and found that neither Ras nor PKB/Akt was able to cause glioblastoma multiforme (GBM) in the brain. Nevertheless, together they induced high-grade gliomas with features similar to human GBMs (Holland et al., 2000). Recent research results from Varmus' group confirmed their observations (Orsulic et al., 2002). In a mouse model for ovarian carcinoma, even the combination of the three oncogenes *c-myc*, *K-ras* and *Pkb/Akt* was insufficient to induce a tumorigenic state in wild-type p53 mice. However, once introduced into p53-deficient mice, the addition of any two of the three oncogenes transformed cells (Orsulic et al., 2002).

Other experiments performed in mice involved retroviral delivery of PKB/Akt to lungs, mesenchymal stem cells and femoral arteries (Kureishi et al., 2000; Lu et al., 2001b; Mangi et al., 2003). Administration of PKB/Akt into vascular endothelial cells promoted angiogenesis (Kureishi et al., 2000), protected against oxidant-induced injury when introduced into the lung (Lu et al., 2001b), and prevented remodeling and restored the performance of infarcted hearts when delivered to stem cells (Mangi et al., 2003). These studies have unveiled novel physiological functions of PKB/Akt, and illustrate the potential of PKB/Akt for gene therapy.

To summarize these observations on transgenic mice with PKB/Akt overexpression in a variety of tissues and organs, it is obvious that PKB/Akt promotes both cell proliferation and cell growth apart from its anti-apoptotic properties. Depending on the structure and characteristics of the tissues, sustained high PKB/Akt activity alone

could be sufficient to cause transformation. Alternatively, it may contribute to tumour induction after integration with other effectors.

4. The goal of this thesis

Nearly ten years' PKB/Akt researcha has accumulated a large amount of knowledge on this kinase and unveiled some of its functions in cell biology. The findings that IGF1 and insulin can activate PKB/Akt have great implication of this kinase in growth control and insulin action. PKB/Akt was found amplified or over-activated in some human cancers, which together with its hallmark property of anti-apoptosis, suggests its important roles in animal survival and tumorigenesis. PKBβ/Akt2 mRNA is predominantly high in fat, liver and skeletal muscle implying its special involment and regulation in glucose metabolism and distinct function from the other two isoforms. The availability of gene knockout technology with mouse has made it possible to study PKB/Akt in mammals and the obtained knowledge will help understand the function of this kinase in humans. The long-term projects in the lab are generation of a variety of PKB/Akt mutant mice including single isoform knockout mice, double or triple knockout mice, to elucidate their contribution to development and physiology, and to use these mice as tools for future pharmaceutical and therapeutic studies.

As a part of the long-term projects, the goal of this thesis is, 1) generation and characterization of *Pkbα*/Akt1 knockout mice; 2) elucidation of isoform-specific functions by comparison of the three single-isoform knockout mice; 3) in-depth understanding of PKB/Akt functions in double or triple knockout mice.

Chapter I

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PKBα Regulates Placental Development and Fetal Growth

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SUMMARY

PKBα is implicated in the regulation of metabolism, transcription, cell survival, angiogenesis, cell migration, growth and tumorigenesis. Previously, it was reported that PKBα -deficient mice are small with increased neonatal mortality (Cho, H., Thorvaldsen, J.L., Chu, Q., Feng, F. and Birnbaum M.J. (2001). *J. Biol. Chem.* 276, 38349–38352. Chen, W.S., Xu, P.Z., Gottlob, K., Chen, M.L., Sokol, K., Shiyanova, T., Roninson, I., Wenig, W., Suzuki, R., Tobe, K., Kadowaki, T. and Hay, N. (2001). *Genes Dev.* 15, 2203–2208). Here we show that PKBα is widely expressed in placenta including all types of trophoblast and vascular endothelial cells. *Pkbα*$^{-/-}$ placentae display significant hypotrophy, with marked reduction of the decidual basalis and nearly complete loss of glycogen-containing cells in the spongiotrophoblast, and exhibit decreased vascularization. *Pkbα*$^{-/-}$ placentae also show significant reduction of phosphorylation of PKB and endothelial nitric oxide synthase (eNOS). These defects may cause placental insufficiency, fetal growth impairment and neonatal mortality. These data represent the first evidence for the role of PKBα and eNOS in regulating placental development and provide an animal model for intrauterine growth retardation.

INTRODUCTION

Animal size is determined by combination of cellular processes that control cell number (proliferation), cell size (mass) and cell death, including apoptosis (Conlon and Raff, 1999). Multiple genetic modifiers are involved in this process during fetal development and post-natal growth (Han and Carter, 2001; Okada and Kopchick, 2001). Hormones and growth factors play an important role in growth control. Among them insulin, insulin-like growth factor 1 (IGF-1)[1], and insulin-like growth factor 2 (IGF-2) activate the phosphoinositide 3-kinase (PI3K) signaling pathway (Blume-Jensen and Hunter, 2001; Han and Carter, 2001; Okada and Kopchick, 2001; Prada and Tsang, 1998). Recent evidence indicates that many downstream events of PI3K signaling are mediated by the serine/threonine protein kinase B (PKB, also known as Akt) (Brazil and Hemmings, 2001; Datta et al., 1999).

In mammals, there are three known PKB isoforms: PKBα/Akt-1, PKBβ/Akt-2 and PKBγ/Akt-3 encoded by separate genes. These proteins have a similar domain structure with about 80% amino acid identity (Brazil and Hemmings, 2001). PKB promotes or inhibits many cellular and physiological processes through phosphorylation of numerous substrates. Around 30 – 40 proteins are phosphorylated by PKB. These proteins are involved in glucose metabolism, transcription, cell cycle regulation, survival, inflammation, and angiogenesis. Overexpression of PKBα/Akt-1 in mouse pancreatic β cells substantially increased both cell size and total islet mass (Bernal-Mizrachi et al., 2001; Tuttle et al., 2001b). A second transgenic mouse model specifically expressing constitutively active PKBα in the heart, displayed a 2-fold increase in heart size with an increase in cardio-myocyte cell size (Matsui et al., 2002; Shioi et al., 2002). In a mouse model for ovarian carcinoma, expression of any two of the three oncogenes *c-myc*, *K-ras*, and *Pkbα* is sufficient to induce ovarian tumor

formation in a p53-deficient background (Orsulic et al., 2002). These animal models indicate that increased PKBα activity promotes both cell growth and proliferation.

To study the physiological roles of PKB, we generated PKBα–deficient mice by targeted gene disruption. We found that inactivation of PKBα caused hypotrophy and structural abnormalities of the placenta that probably contribute to placental insufficiency and subsequent impairment of fetal growth. Our results suggest a novel role for PKBα in the regulation of placental development and fetal growth.

FOOTNOTES

1 The abbreviations used are: IGF-1 and 2, insulin-like growth factor 1 and 2; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; Q-PCR, quantitative real time PCR; ICM, inner cell mass; ES, embryonic stem cells; VEGF, vascular endothelial growth factor; TSC 1 and 2, Tuberous sclerosis complex 1 and 2; S6K, ribosomal protein S6 kinase; GSK3, glycogen synthase kinase 3; eNOS, endothelial nitric oxide synthase; L-NAME, N(G)-nitro-L-arginine methyl ester; IUGR, intrauterine growth retardation.

2 Z-z. Yang, O. Tschopp and B.A. Hemmings, unpublished data.

3 O. Tschopp. D. Brodbeck. Z-z.Yang and B.A.Hemmings, unpublished data

EXPERIMENTAL PROCEDURES

Total RNA Isolation and Quantitative Real Time PCR (Q-PCR)

Total RNA was extracted and purified using Trizol Reagent (Invitrogen) and RNeasy 96 kit (Qiagen). Primer pairs and FAM/TAMRA-labeled TaqMan probes for real time PCR were designed using the Primer Express v 2.0 program (ABI PRISM, PE Biosystems). For the Q-PCR reaction, 50ng total RNA was mixed with 5' and 3' primers (T-forward, the sequences are, for α, 5'-GGCAGGAAGAAGAGACGATGG-3; for β, 5'-GAGGACGCCATGGATTACAAG-3'; for γ, 5'-CAGAGGCAAGAGGAGGAGAG-G-3' and T-reverse, the sequences are: α, 5'-CCATCTCTTCAGCCCCTGAG-3'; β, 5'-GACAGCTACCTCCATCATCTCAGA-3'; γ, 5'-TGTAGACGCATCCATCTCTTCTT

C-3'; 10 μM each), Taqman probe (the sequences are; α 5'-CTTCCGATCA-GGCTCACCCAGTGACA-3'; β, 5'-TGGCTCCCCCAGTGACTCTTCCAC-3'; γ, 5'-TGAATTGTAGCCCAACCTCACAG-ATTGATAATATAGG-3'; 5 μM each), MuLV reverse transcriptase (6.25 units, PE Biosystems), RNase inhibitor (10 units, Invitrogen) and the components of the TaqMan PCR reagent kit (Eurogentec) in a total volume of 25 μl following the TaqMan PCR reagent kit protocol (Eurogentec). Reverse transcription and real time PCR was performed in a GeneAmp Sequence Detector 5700 (PE Biosystems) as follows: 2 min reverse transcription at 50°C, 10 min denaturation at 95°C followed by 50 cycles of denaturation for 15 sec at 95°C and annealing and elongation for 1 min at 60°C. The relative quantitation of gene expression was calculated as described in the ABI PRISM 7700 user bulletin #2 (PE Biosystems).

Mouse Pkbα Gene Disruption

We isolated one positive clone from 129/SvJ BAC genomic library (Genome Systems Inc.) using a mouse *Pkbα* cDNA probe and subcloned three *BamHI* fragments of 6.5 kb, 2.8 kb and 10 kb, and a 5kb *XbaI* fragment containing all 14 exons. These subcloned fragments were completely sequenced and assembled. To construct the *Pkbα* targeting vector, we generated a 1.9 kb left-arm using PCR and fused it in frame to the 5.5 kb of *lacZ/Neo*. A 6.7 kb right-arm was ligated to the 3' end of the Neo cassette and the whole fragment was subcloned to pBluescript KS^- (Stratagene). The linearized vector (*SalI*) was introduced into 129/Ola ES cells and G418 resistant clones (96) were analyzed for homologous recombination using PCR. The primers were: (a) P684265-2, 5'-CCCACGACAGAAAGTTGTGCG-3' (b) LacZ-2, 5'-CGTCTGGCCTTCCTGTAGCC-AG-3'. Positives clones (5) were further characterized by Southern blot. Two male chimeras gave germ-line transmission. The progeny from $Pkb\alpha^{+/-}$ intercrossing have 129/Ola and C57BL/6 mixed background. Mice with a 129/Ola/Sv background were also generated. Genotyping of progeny was done by multiplex PCR with the following three primers: (a) *Pkbα* 5',5'-AGACTCTGAGCATCATCCCTGGG-3'; (b) LacZ-2 (sequence as above); (c) *Pkbα* 3', 5'-TGAAGCAGGCCTAGAGCCCCATG-3'.

Western Blot Analysis

Tissues lysates were prepared by homogenization in lysis buffer (50 mM Tris·HCl, pH 8.0; 120 mM NaCl; 1% NP-40; 40 mM β-glycero-phosphate; 100 μM Na_3VO_4; 1 μM Microcystin LR). Tissue debris was removed by centrifugation at 13000 rpm for 10 min at 4 ^oC. Protein concentrations were determined using the Bradford assay. Protein (50 μg) was fractionated by 10% SDS-PAGE and transferred

to Immobilon-P PVDF membrane. A polyclonal antibody against PKBα was obtained by immunizing rabbits with a peptide corresponding to the last 14 amino acids of human PKBα (Jones et al., 1991). The peptide sequence for generation of mouse PKBα specific antibody was VADGLKRQEEETMDFRSGSPSDNSGA. Antibodies for phospho-PKB (Ser473), p70S6K and phospho-p70S6K, GSK3 and phospho-GSK3, phospho-TSC2 and phospho-eNOS were purchased from Cell Signaling Technologies. eNOS antibody was purchased from BD Transduction Laboratories.

Histological Studies

Placentae and organs from adult mice were dissected and fixed in formalin solution (10% v/v) overnight at 4°C, then processed as follows, 30 minutes in PBS at 4°C; 30 minutes in 0.85% NaCl (in H_2O) at room temperature; 2 x 20 minutes in 50% ethanol (in 0.85% NaCl) at room temperature; 2 x 20 minutes in 70% ethanol (in PBS) at room temperature. The samples were then embedded in paraffin. Sections (5 and 10 μm) were stained with haematoxylin and eosin. Genotype of embryos was determined by PCR as above using DNA from yolk sac or from embryonic tissues. For immunohistochemistry, placentae from overnight formalin fixation were embedded in carbowax (OCT compound, Tissue Tek), cryosectioned at 20 μm, and treated with methanol for 30 minutes to inactivate endogenous peroxidase at room temperature. Sections were blocked for 30 minutes in 5% normal goat serum in PBS, and then incubated overnight at 4^{o}C with PKB antibody. The sections were then processed as described in the protocol from Vectastain ABC kit (Vector Laboratories). For alkaline phosphatase (AP) staining, 20μm cryosections were bathed in PBS for 10 minutes at room temperature, and then incubated with AP color reagents A and B (Bio-RAD). Periodic acid Schiff's staining was performed with paraffin sections.

Measurement of Fetal Vessels in Placenta

Digital pictures of wild-type and mutant placentae were taken under similar conditions with a Nikon digital camera (Nikon D1X). Vessel area and total vessel length in each placenta were measured with Image-Pro Plus software. The data were then processed for statistical analysis. The results are presented as arbitrary units.

RESULTS

Expression of PKB Isoforms in Mouse Tissues

The expression profile of the mRNA encoding the three PKB isoforms was determined by quantitative PCR using total RNA obtained from 3 male and 3 female wild-type mice. For this comparative analysis, the level of PKBα in the brain was set at 100% and all other tissues were compared to this value for each isoform (Fig.1.1A). PKBα was expressed in all organs and tissues examined but the levels in pancreas and skeletal muscle was very low. However, in most tissues and organs, PKBβ levels were the highest and PKBγ levels were the lowest. Notably, PKBβ mRNA was highly abundant in the insulin-responsive tissues such as fat, skeletal muscle and liver as previously reported (Altomare et al., 1998b; Cho et al., 2001a). PKBγ levels were high in the brain and testis, lung, mammary gland and fat, but were extremely low in other tissues. The amount of PKBα protein was also investigated by Western blot with an isoform-specific antibody (Fig.1.1B). PKBα protein was most abundant in brain, thymus and testis, with slightly lower levels observed in heart, lung, pancreas, spleen, and fat. Significantly, the lowest levels of PKBα protein were found in skeletal muscle, liver, and kidney. Similar results were obtained with a second α-specific antibody directed against residues 106-131 of mouse PKBα[2]. There were marked differences between the mRNA and protein levels of PKBα in some tissues, which may be due to mRNA translation differences and/or protein turnover.

Pkbα Gene Structure and Disruption

We sequenced approximately 24 kb of the mouse *Pkbα* locus that contains 13 coding exons (Bellacosa et al., 1993) and found a non-coding exon (termed exon 0) and the

promoter region of the gene (Fig. 1.2A). In order to ablate the *Pkbα* gene, a targeting vector (Fig. 1.2B) was

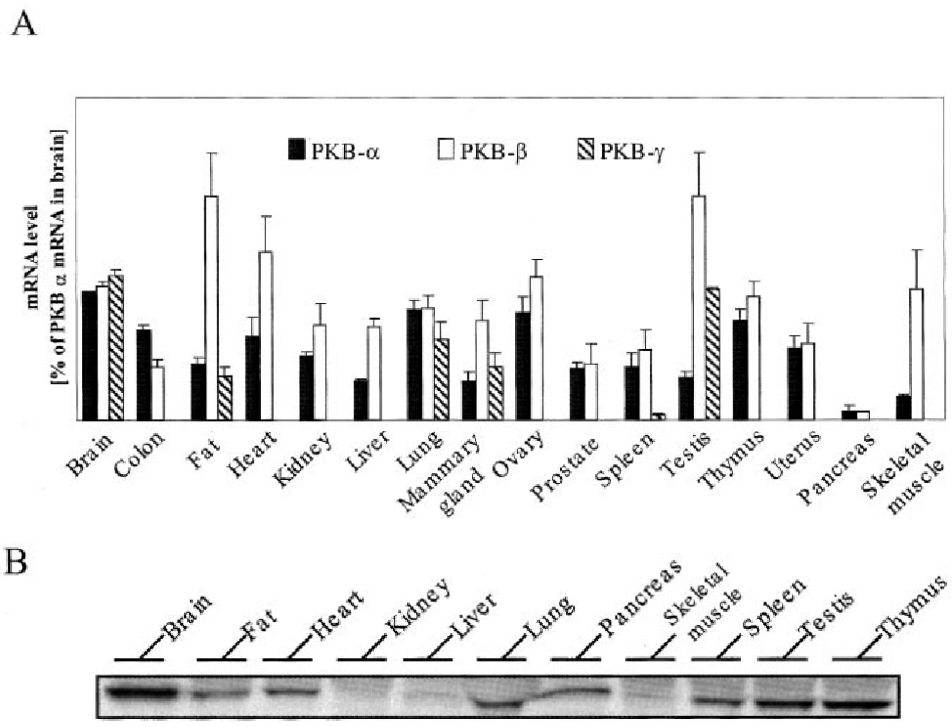


Fig. 1.1. mRNA expression profiles of PKB isoforms and Western blot analysis of the α isoform.

A, Quantitative PCR analysis of mRNA encoding the three PKB isoforms from wild-type mice. We dissected 16 organs and tissues from three wild-type males and three wild-type females for total RNA isolation. Results are expressed as the mean ± SD. The results were normalized using pre-developed TaqMan assay reagents for detection of human 18S rRNA as an internal control. ***B,*** Western blot analysis of PKBα from wild-type mouse tissues. The experiment was repeated with three wild-type mice and gave similar results.

constructed by insertion of a *lacZ/Neo* cassette into exon 1 to replace its coding segment, which encodes the first 15 N-terminal amino acids, thereby disrupting its transcriptional organization (Leitges et al., 2001). Correctly targeted embryonic stem

cell clones were identified using a PCR-based strategy (Fig. 1.2C). One of the clones possessing the correctly targeted allele was used for blastocyst aggregation, and germ-line transmission was achieved. Heterozygous (*Pkbα* $^{+/-}$) mice were mated to produce

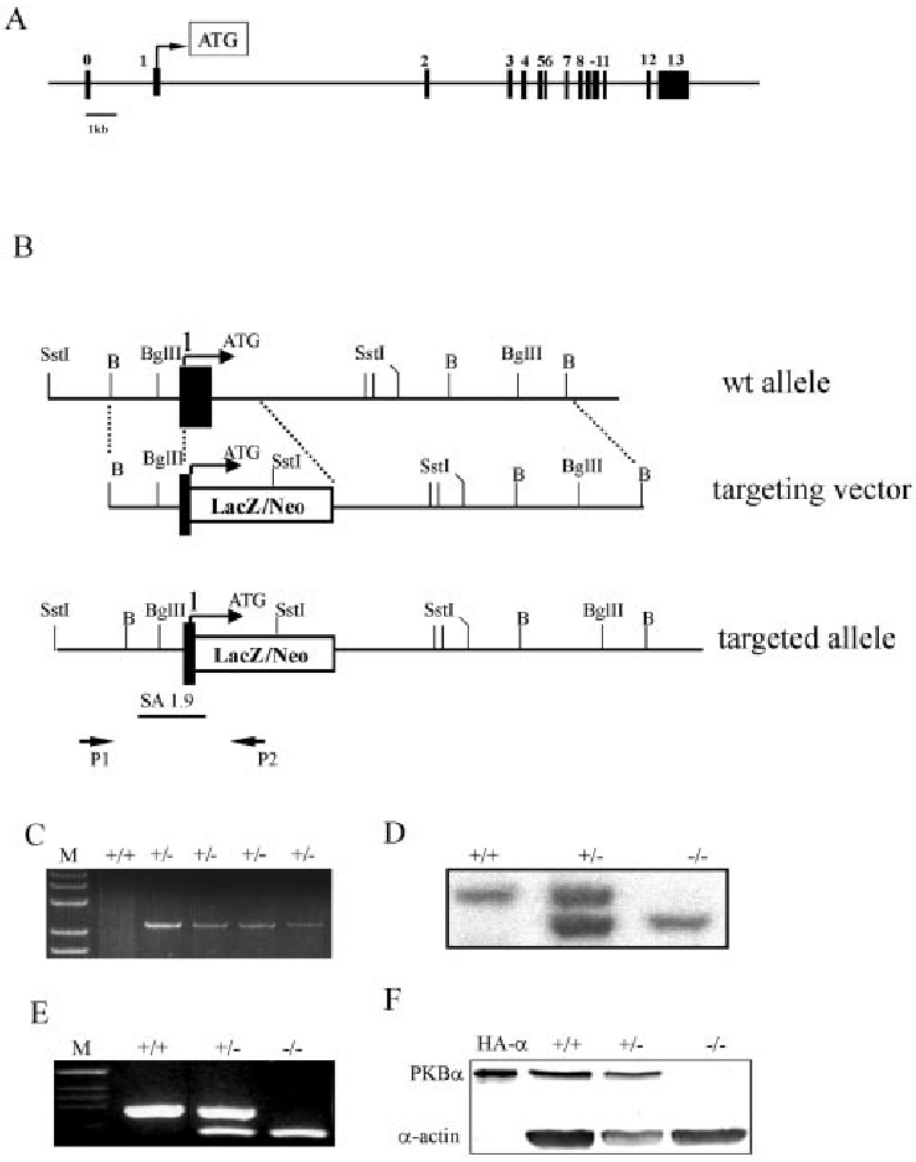


Fig. 1.2. *Pkbα* gene disruption.

A, Structure of the *Pkbα* gene. The translation start codon ATG is indicated by an arrow, coding exons are numbered from 1 to 13 and exon 0 is a non-coding exon. ***B***, Schematic representation of the mouse *Pkbα* allele (top), targeting vector (middle) and targeted allele

(bottom). A *LacZ/Neo* cassette was introduced at the start ATG in exon 1 to disrupt translation. B indicates *BamHI*. P1 (P684265-2) and P2 (lacZ-2) are two primers used for ES clone screening by PCR. SA 1.9 was used as a probe for Southern analysis. ***C,*** PCR screening for positive clones. The amplified product is ~2.2 kb. ***D,*** Southern blot analysis of the progeny of a *Pkbα* $^{+/-}$ intercross. The wild-type band is 5.3 kb and the targeted band is 4.5 kb (*Sst*I digestion). ***E,*** PCR genotyping of progeny. The wild-type and targeted bands are 300 and 220 bp, respectively. ***F,*** Western blot analysis of the PKBα protein in *Pkbα* $^{+/+}$, *Pkbα* $^{+/-}$ and *Pkbα* $^{-/-}$ mice. Protein extracts were prepared from heart and lysates from HEK293 cells transfected with HA-tagged mouse PKBα was used as a positive control.

homozygous (*Pkbα* $^{-/-}$) offspring that were genotyped by PCR and characterized by Southern analysis (Fig. 1.2D and E). Western blot analysis using an isoform specific antibody, confirmed the absence of PKBα protein (Fig. 1.2F). Using several different PKB antibodies we did not find any evidence for truncated protein products in heart, thymus and spleen confirming that our targeted disruption strategy led to a null phenotype (data not shown)

These mice have 129/Ola and C57BL/6 mixed genetic background. The phenotype described in this article is similar between mice with the 129/Ola/Sv or 129/Ola/C57BL/6 mixed background.

Increased Neonatal Morbidity and Mortality with Pkbα $^{-/-}$ ***Mice***

We genotyped by PCR approximately 600 pups from *Pkbα* $^{+/-}$ intercrosses at 2–4 weeks after birth. The percentage of *Pkbα* $^{-/-}$ offspring obtained was ~ 17% (Table 1.1), indicating that about 40% of *Pkbα* $^{-/-}$ mice were already lost. Therefore, embryos were isolated at different stages of gestation and genotyped using PCR. The number of *Pkbα* $^{-/-}$ embryos from embryonic ages of E14 to E18 was consistent with the expected

Mendelian ratio, excluding early embryonic lethality as a cause of decreased *Pkbα*$^{-/-}$ progeny (Table 1.1). *Pkbα*$^{-/-}$ intercrosses (homozygous intercrosses) revealed that *Pkbα*$^{-/-}$ mice were fertile, but produced markedly smaller litters compared to *Pkbα*$^{+/-}$ intercrosses (6.7 ± 2.5, n=40, vs. 9.7 ± 3.3, n=40, respectively; $p<0.01$). Furthermore,

Table 1.1

Frequency of progeny from Pkbα$^{+/-}$ *intercrosses*

Male and female of *Pkbα*$^{+/-}$ were mated for a short time (4 hours). Plugging was checked at the end of mating and was determined as E0.

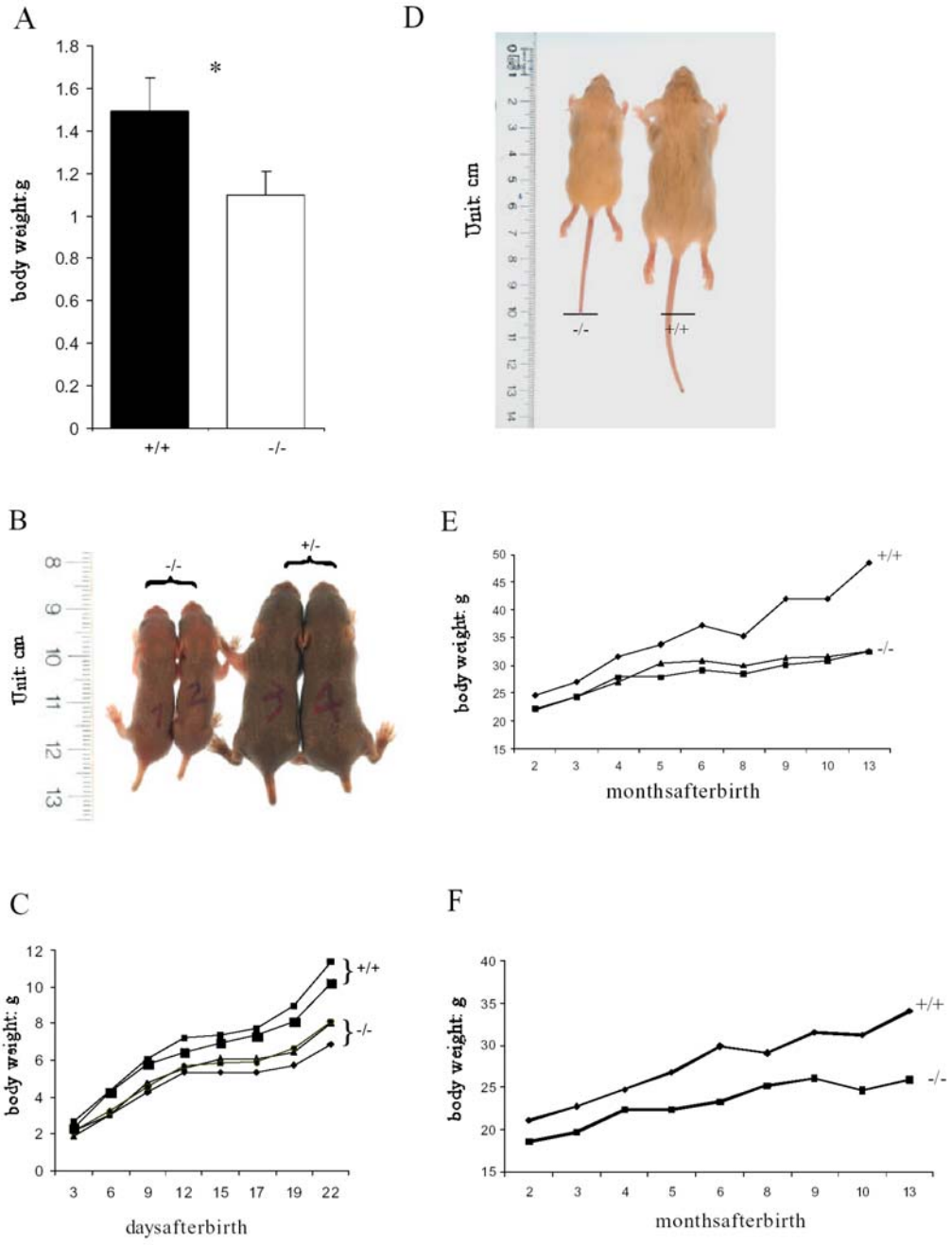
Stage	Genotype			Total Number
	Pkbα$^{+/+}$	***Pkbα***$^{+/-}$	***Pkbα***$^{-/-}$	
E14	23 (21%)	58 (53%)	29 (26%)	110
E15	26 (21%)	69 (54%)	32 (25%)	127
E16	39 (32%)	62 (51%)	21 (17%)	122
E17	20 (21%)	53 (56%)	22 (23%)	95
E18	23 (28%)	36 (45%)	22 (27%)	81
2-4 weeks	166 (28%)	323 (55%)	100 (17%)	589

increased mortality, mainly within the first 3 days after birth, was observed in litters generated from *Pkbα*$^{-/-}$ intercrosses (62 of 268 mice, 23.1%) compared to litters from *Pkbα*$^{+/-}$ intercrosses (30 from 389 mice, 7.7%). *Pkbα*$^{-/-}$ newborns appeared small, weak and pale and frequently the majority of pups from *Pkbα*$^{-/-}$ intercross litters died within 10 days. The precise cause of death was not determined. Intercrosses of *Pkbα*$^{+/-}$ males with *Pkbα*$^{-/-}$ females revealed an intermediate phenotype in terms of litter size at birth (7.5 ± 2, n=22) and postnatal mortality of the pups (18 from 166 mice, 10.8%).

Pkbα*$^{-/-}$ *Mice Exhibit Reduced Body Weight and Delayed Growth

At birth, *Pkbα*$^{-/-}$ mice (from heterozygous intercrosses) were distinguishable from their wild-type littermates by their smaller size (see Supplementary figure). The body weight of mutant newborns was approximately 70% of wild-type littermates (body weight: wild-type, 1.49 ± 0.16 g, n=15, vs. mutant, 1.1 ± 0.11 g, n=12, $p<0.01$).

Surviving *Pkbα* $^{-/-}$ mice grew slowly after birth, and some of these mice were extremely stunted and lean. One litter of mice (11 pups) from a *Pkbα* $^{+/-}$ intercross was observed from birth until 13 months of age. The growth curve indicated that *Pkbα* $^{-/-}$



Supplementary figure. Post-natal growth retardation of *Pkbα* $^{-/-}$ mice.

A, Mean body weight of wild-type and *Pkbα* $^{-/-}$ newborns. 15 wild-type and 12 *Pkbα* $^{-/-}$ newborns from 5 litters were weighed (* $p<0.01$). ***B***, *Pkbα* $^{+/-}$ and *Pkbα* $^{-/-}$ pups from one litter at 5 days after birth. ***C***, Representative growth curve of wild-type and *Pkbα* $^{-/-}$ littermates from birth to adulthood. ***D***, Representative picture of wild-type and *Pkbα* $^{-/-}$ littermates at 20 days after birth. ***E, F***, Growth curves of wild-type and *Pkbα* $^{-/-}$ littermates from 2 to 13 months old. ***E***, male; ***F***, female.

mice maintained their small size from birth to adulthood. At 13 months, two *Pkbα* $^{-/-}$ males weighed 32.5 g and 32.7 g, respectively, while a wild-type male littermate weighed 48.5 g; one *Pkbα* $^{-/-}$ female and one wild-type female littermate weighed 26 g and 34.1 g, respectively.

The major organs and tissues, including brain, heart, liver, spleen, thymus, skin, bone marrow, pancreas, testis, ovary, lung, kidney, fat, skeletal muscle, and stomach, from 5 wild-type and 5 *Pkbα* $^{-/-}$ mice (2 males and 3 females of each genotype) were dissected and examined at 2 months of age. Organ weight reduction, with the exception of the brain and pancreas, in *Pkbα* $^{-/-}$ mice was proportional to the body weight reduction. Histological examination did not reveal any major difference between wild-type and *Pkbα* $^{-/-}$ mice apart from a modest reduction in the subcutaneous fat in the *Pkbα* $^{-/-}$ mice[2]. In addition, biochemical examination of metabolites, enzymes and electrolytes did not reveal any significant difference between wild-type and *Pkbα* $^{-/-}$ mice[2].

Placental Hypotrophy and Fetal Growth Impairment in Pkba $^{-/-}$ ***Mutant Mice***

Following dissection of E14.5 embryos, we found that the mutant embryos were 20% smaller than their wild-type littermates (embryo weight: wild-type, 291 ± 45 mg, n=15; *Pkbα* $^{-/-}$, 235 ± 49 mg, n=12, $p<0.01$; Fig. 1.3A). In addition, we found that the placental weight of mutant embryos was 24% lower than that of wild-type littermates

(placenta weight at E14.5: wild-type, 121 ± 18 mg, n=15; *Pkbα* $^{-/-}$, 92 ± 17 mg, n=12; $p<0.01$; Fig. 1.3A). Macroscopically, the placentae from mutant embryos were not only small but also thin. These differences were also observed at later embryonic stages: at E16.5 the weight of wild-type embryos was 707 ± 86 mg (n=16) and that of mutants 586 ± 67

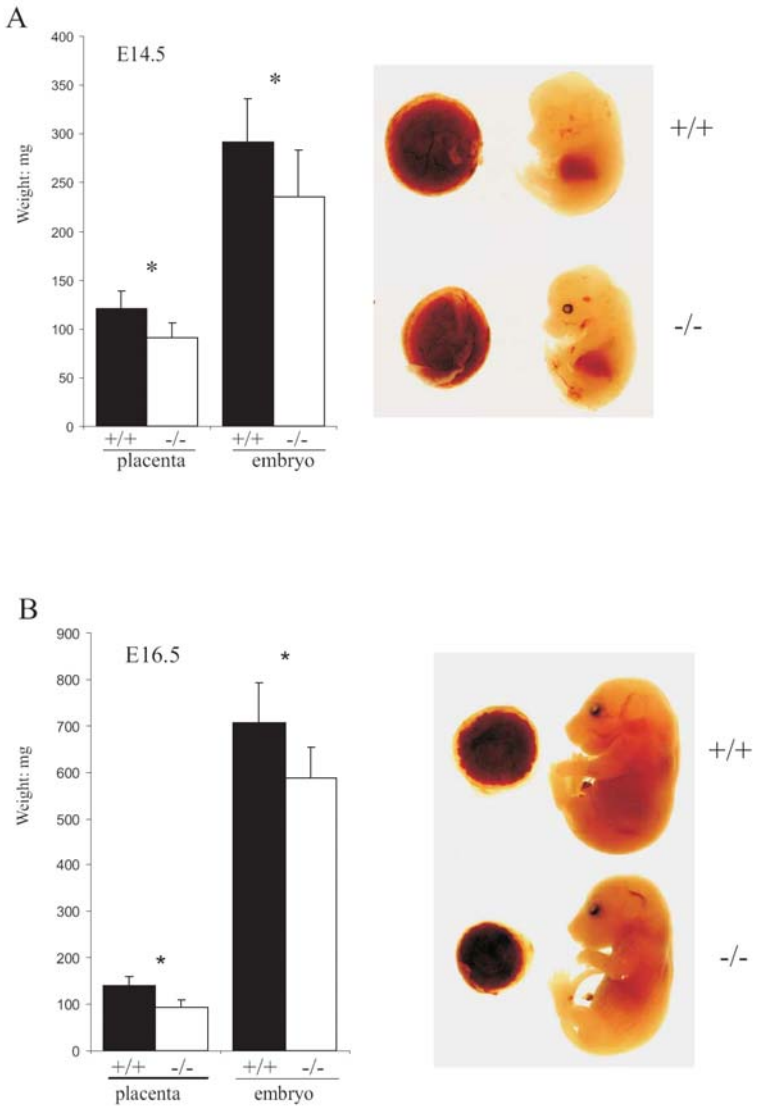


Fig. 1.3. Placental hypotrophy and fetal growth impairment of *Pkbα* $^{-/-}$ mice.

A, Reduced size of E14.5 *Pkbα* $^{-/-}$ embryos and their placentae. Embryos and their placentae were dissected from *Pkbα* $^{+/-}$ intercrosses at E14.5. After weighing the embryos and placentae,

tissues from the embryos or yolk sac were dissected and used for DNA isolation and genotyping by PCR. Data from wild-type and *Pkbα* $^{-/-}$ embryos and placentae were collected for statistical analysis as shown in the left (* p<0.01). The right picture shows the smaller size of *Pkbα* $^{-/-}$ embryo and its placenta compared to those of wild-type at this gestational age. ***B,*** Similar results were obtained at E16.5 (left, weight of wild-type and *Pkbα* $^{-/-}$ embryos and placentae,* p<0.01; right, picture of wild-type and *Pkbα* $^{-/-}$ embryos and placentae at this age.)

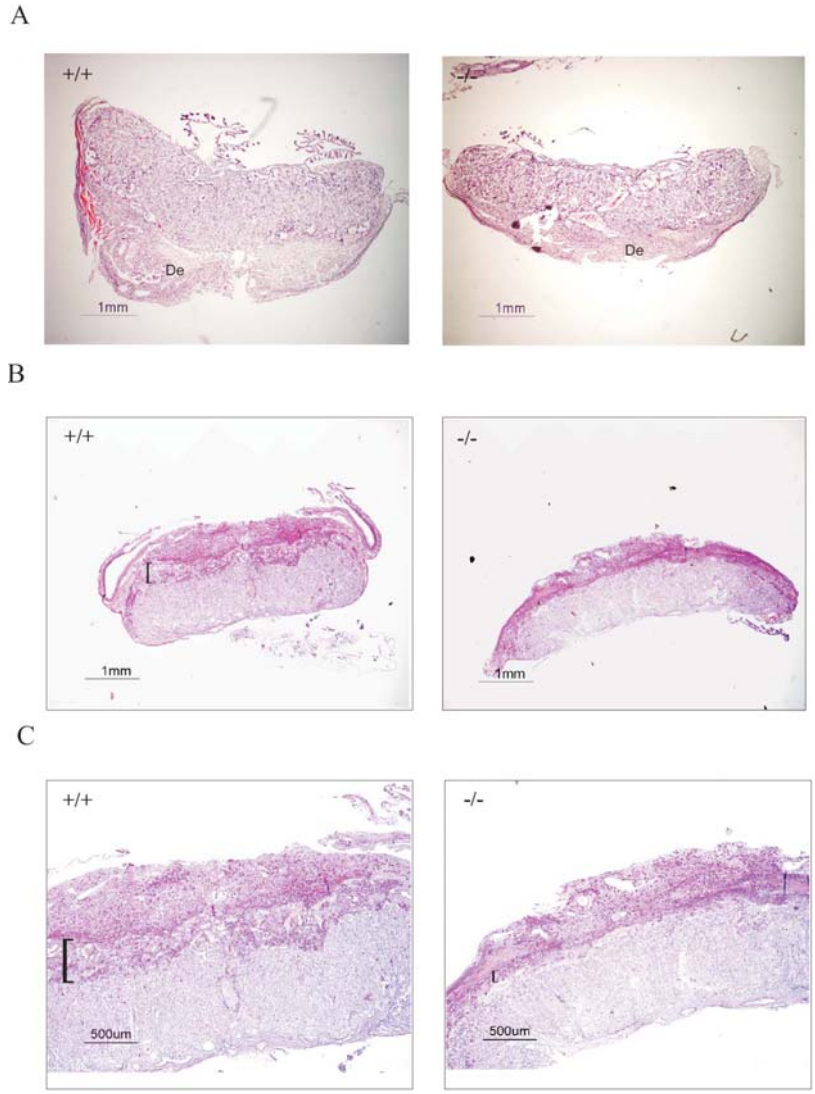
mg (n=12) (*Pkbα* $^{-/-}$ /wild-type=83%; p<0.01; Fig. 1.4B); the weight of wild-type and mutant placentae was 142 ± 18 mg (n=16) and 94 ± 17 mg (n=12), respectively (*Pkbα* $^{-/-}$ /wild-type=66%. p<0.01, Fig. 3B). From E14.5 to E16.5, the wild-type placentae increased in weight (mean weight from 121 mg to 142 mg) while the weight of mutant placentae did not change (mean weight from 92 mg to 94 mg). It thus appears that the growth and development of both mutant placenta and embryo were impaired.

Altered Development of Pkbα Mutant Placentae

Next, we performed histological analysis to study the structure of wild-type and mutant placentae. Haematoxylin and eosin staining showed that all three major layers of placenta, decidua, spongiotrophoblast and labyrinth, were smaller in mutant placentae compared to the wild-type (Fig. 1.4A). In addition, the decidua was disproportionately decreased or apparently missing in some mutant placentae (Fig.1.4A). Periodic acid Schiff's staining showed a marked reduction of glycogen-containing cells in the spongiotrophoblast in mutant placentae (Fig.1.4B and C). We also performed alkaline phosphatase staining to display these cells in order to study fetal vascular structure. We found that the outline of labyrinth was easily discerned after staining and observed that fetal vasculature was disordered with fewer vessels (Fig.1.4D to F).

Expression and Subcellular Distribution of PKBα in Placentae

Disruption of the *Pkbα* gene apparently causes severe hypotrophy and developmental changes of placenta, suggesting that PKBα is required for normal placental development. To test this, we first examined the protein levels of PKBα in placenta compared to brain where its levels are high. Western blot with a PKBα specific antibody showed that, in three wild-type placentae from one litter of E14.5 embryos, the amount of PKBα was similar and significantly higher than that in brain (Fig.1.5A). We performed immunhistochemical staining with a PKBα specific antibody to study its localization in E14.5 wild-type placenta. The results show that PKBα was present in all types of trophoblast cells and vessel endothelial cells except in the decidua where



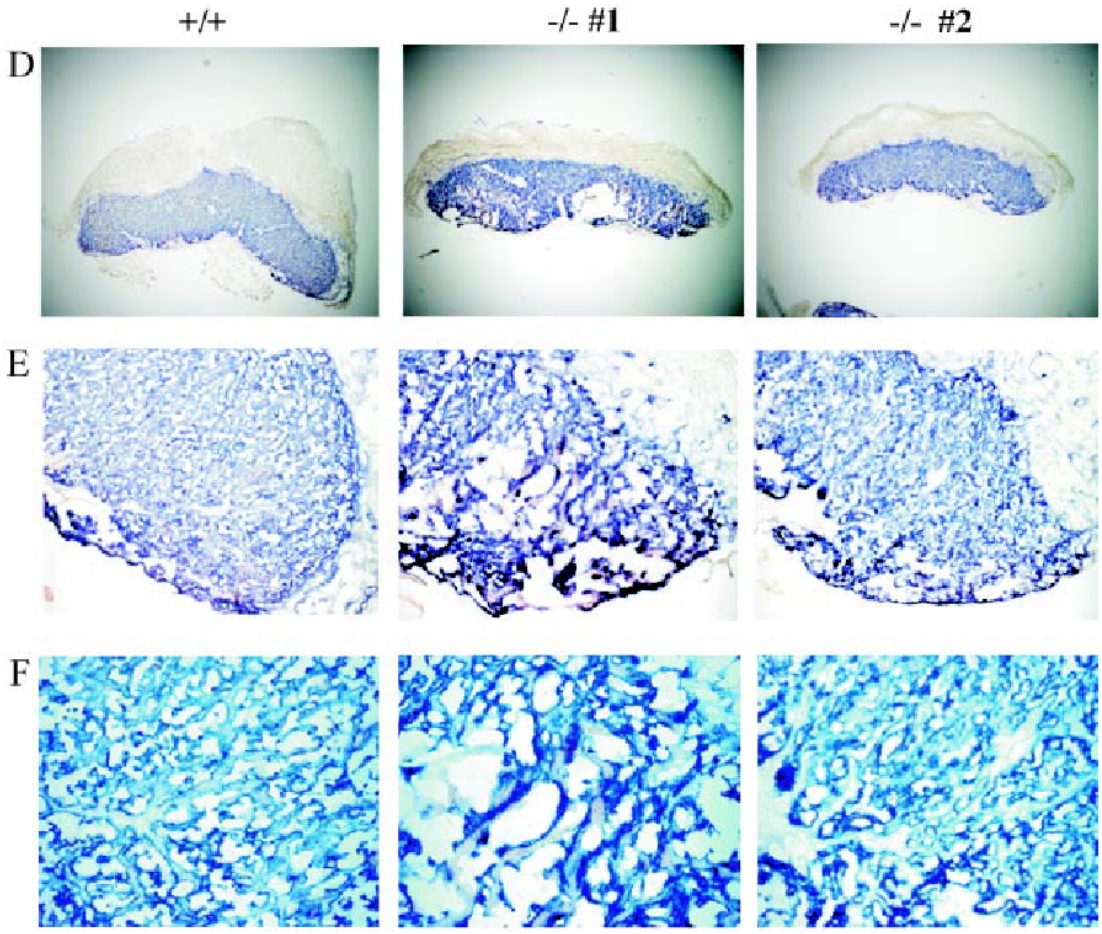


Fig. 1. 4. Structural abnormalities of E14 mutant placentae.

A, Haematoxylin and eosin staining shows that in mutant placenta, the major layers of decidua, spongiotrophoblast and labyrinth were reduced but the decrease of decidua was more significant than other layers. Magnification: 20X. De, decidua. ***B,*** Periodic acid Schiff's staining shows a marked reduction of glycogen-containing cells in the spongiotrophoblast in mutant placentae. Magnification: 20X. ***C,*** Higher magnification of ***B***, 40X. Bracket indicates the glycogen-containing cells in the spongiotrophoblast. ***D*** to ***F,*** Structure of the labyrinth revealed by alkaline phosphatase staining. ***D,*** The outline of the labyrinth is clearly shown (blue color). Note that the vasculature is disordered in mutant 1. Magnification: 20X. ***E,***

Higher magnification of ***D*****,** 40X**.** Fetal erythrocytes are stained brown in the blood vessels. Note that in mutant 1, fetal blood vessels were dilated and the total blood vessel area was greatly reduced. Mutant 2 shows reduced and disordered vasculature. ***F,*** Higher magnification (100X) of labyrinth.

the amount was relatively low. In contrast, with mutant placenta we could not detect any PKBα (Fig. 1.5B to D). This was consistent with the immunoblot analysis that revealed an absence of PKBα from mutant placenta from heterozygous intercrosses (Fig. 1.5E). In this type of mating, the decidua in the mutant mice was derived from the $Pkb\alpha^{+/-}$ maternal uterus (whereas the other parts of the placenta are derived solely from the embyo). Indeed we also set up matings between homozygous males and females and collected data for placentae and embryos. We found that placentae and embryos from homozygous matings were smaller than those from heterozygous matings (data not shown). This suggests that the genotype of the mother contributes to the phenotype.

Significant Reduction of Phosphorylation of PKB and eNOS in Mutant Placentae with Decreased Vascularization

The status of the other two PKB isoforms, β and γ from wild-type placenta was analyzed by Western blot using isoform-specific antibodies. We found that both were expressed in mature wild-type placenta, with no apparent up-regulation in mutant placenta at the protein level[2]. Furthermore, we analyzed the phosphorylation levels of PKB using an antibody specific for the hydrophobic motif phosphorylation site (Ser473 in PKBα) that recognizes all three phosphorylated isoforms. Three wild-type and three mutant placentae from one litter were examined for this purpose. We found

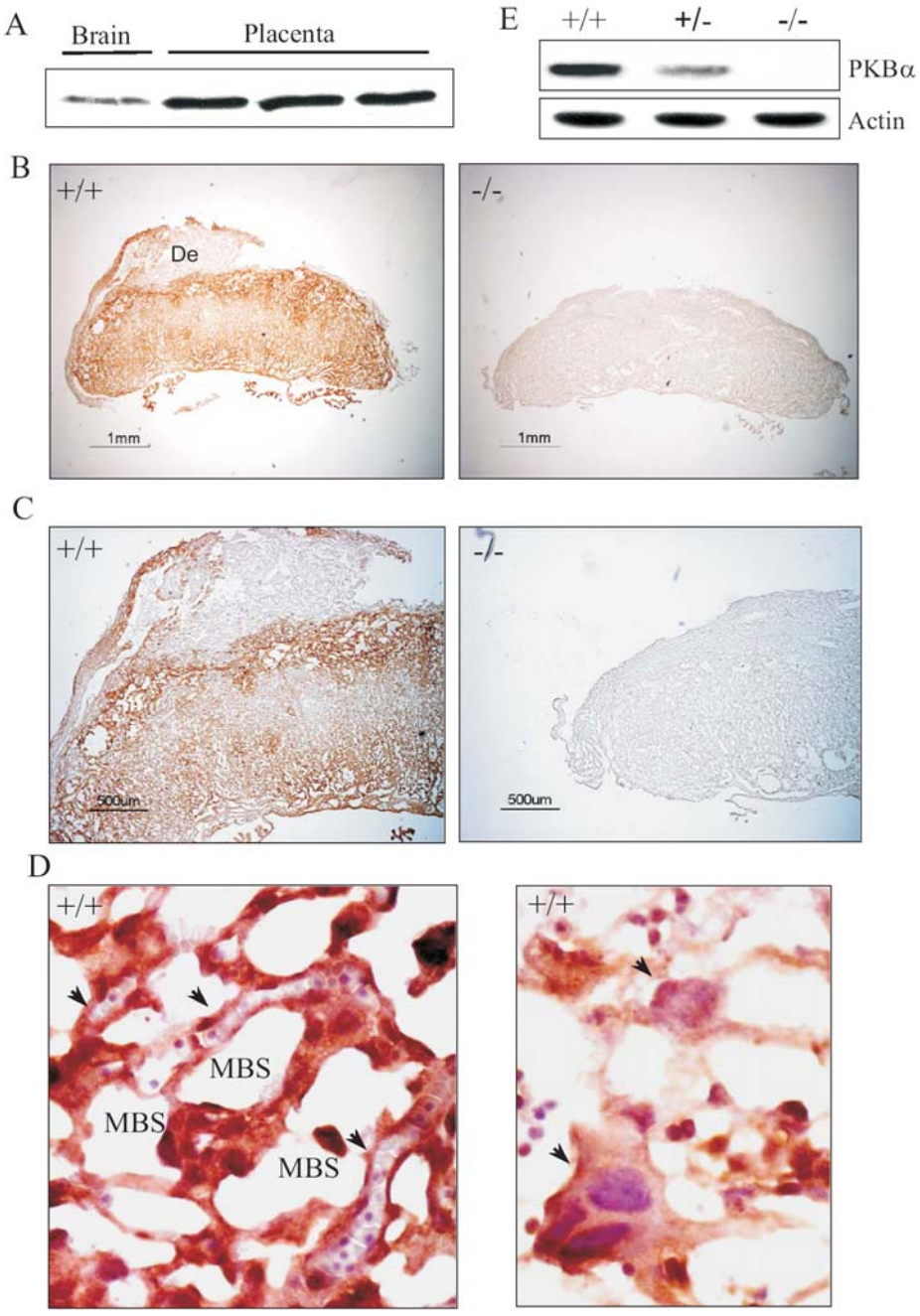


Fig 1.5. Immunohistochemical and Western analysis of PKBα in placenta.

A, Western blot analysis of wild-type E14.5 placenta and whole adult brain lysates for PKBα. ***B*** to ***D,*** Immunohistochemical analysis of placental PKBα. ***B,*** PKBα is detected in all placental cells types except decidua but is absent in mutant placenta. Magnification: 20 X. De, decidua. ***C,*** Higher magnification of ***B***: 40 X. ***D,*** Arrowheads indicate the endothelium of fetal

capillaries on the left and trophoblast giant cells on the right**.** MBS, maternal blood space. ***E,*** Western blot analysis of genotyped placenta obtained from a *Pkbα* $^{+/-}$ intercross.

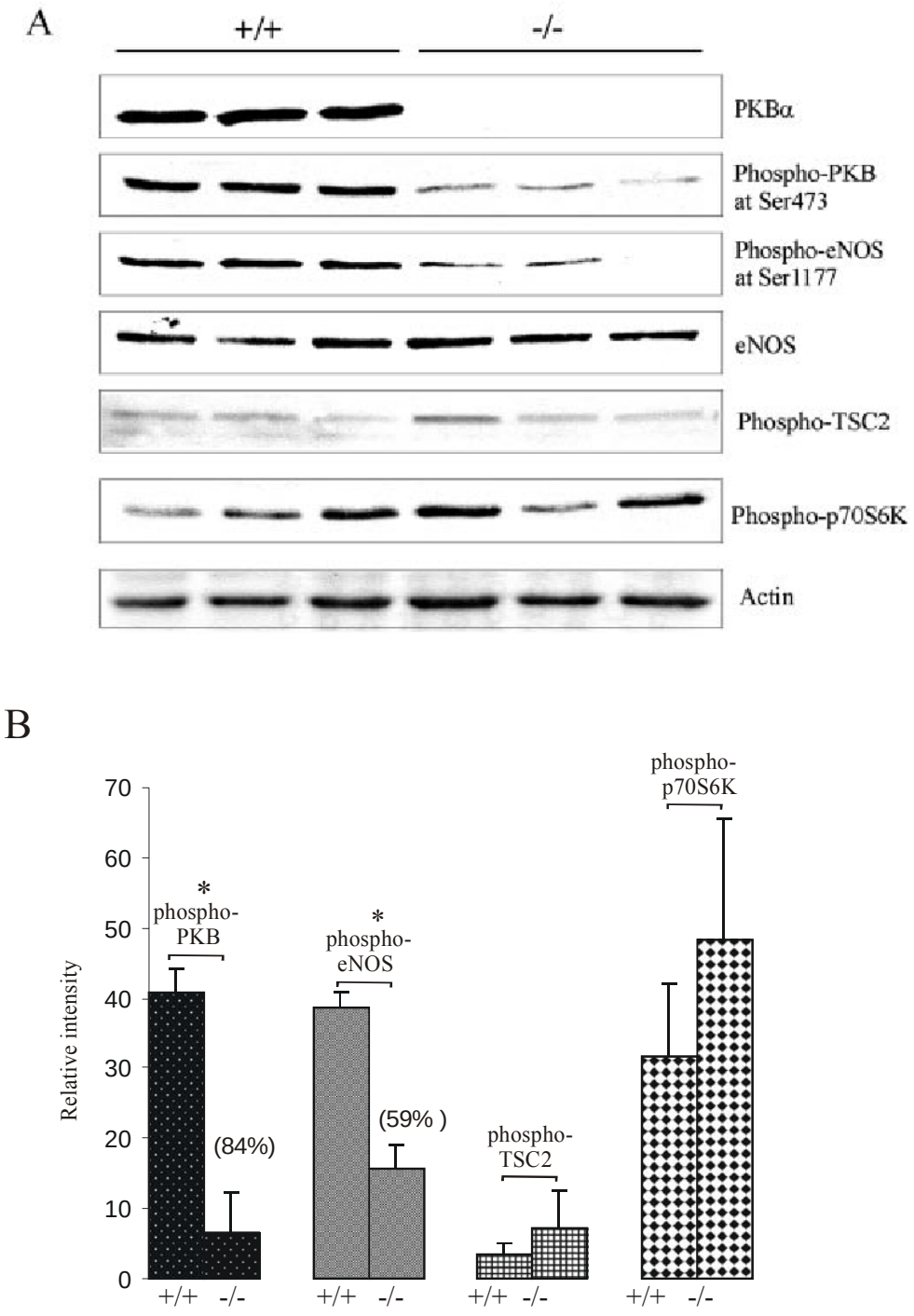


Fig. 1. 6. Phospho Western blot analysis of PKB, eNOS, TSC2 and p70S6K of placentae.

A, Placentae from 3 wild-type and 3 mutant littermates were analyzed for phosphorylation status of proteins involved in PKB signaling with the indicated antibodies. An actin blot is included as a loading control. ***B,*** Quantitation of the Western blot signals. The sum of the relative absorbance intensities for the three samples was compared for the wild-type and *Pkbα* $^{-/-}$ placentae.

that the phosphorylation levels of PKB at Ser473 in all three mutant placentae were reduced markedly (84% decrease) compared with wild-type indicating that PKBα represents the major phosphorylated (active) isoform in placenta (Fig. 1.6A and B). Phosphorylation levels of tuberous sclerosis complex 2 (TSC2) and p70S6K were slightly increased in mutant placentae compared with wild-type (Fig. 1.6A). However, the phosphorylation status of eNOS phosphorylation on Ser1177, were substantially reduced (59% decrease) in mutant placentae (Fig. 1.6A and B). Because eNOS phosphorylation by PKB is important for angiogenesis (Kurieishi et al., 2000), we analyzed the vasculature on the fetal side of placenta from E14.5 wild-type and mutant embryos. Microscopically, we found that in mutant placentae there were significantly less blood vessels and the vessels were smaller and shorter compared to wild-type. The vascular branching was scarce and the micro-vessel covering area was also markedly decreased in mutant placentae (Fig. 1.7A). To compare the difference quantitatively, digital pictures of placentae were taken and used to measure the vessels with image-processing software. Two parameters were measured to analyze the vascularization of wild-type and mutant placentae: total visible vessel length and the vessel-covering area. In total, we examined 5 mutant and 11 wild-type placentae from 5 litters of embryos of heterozygous crosses. The results revealed significant differences between wild-type and mutant placenta (total vessel length per placenta: mutant, 894 ± 654; wild-type,

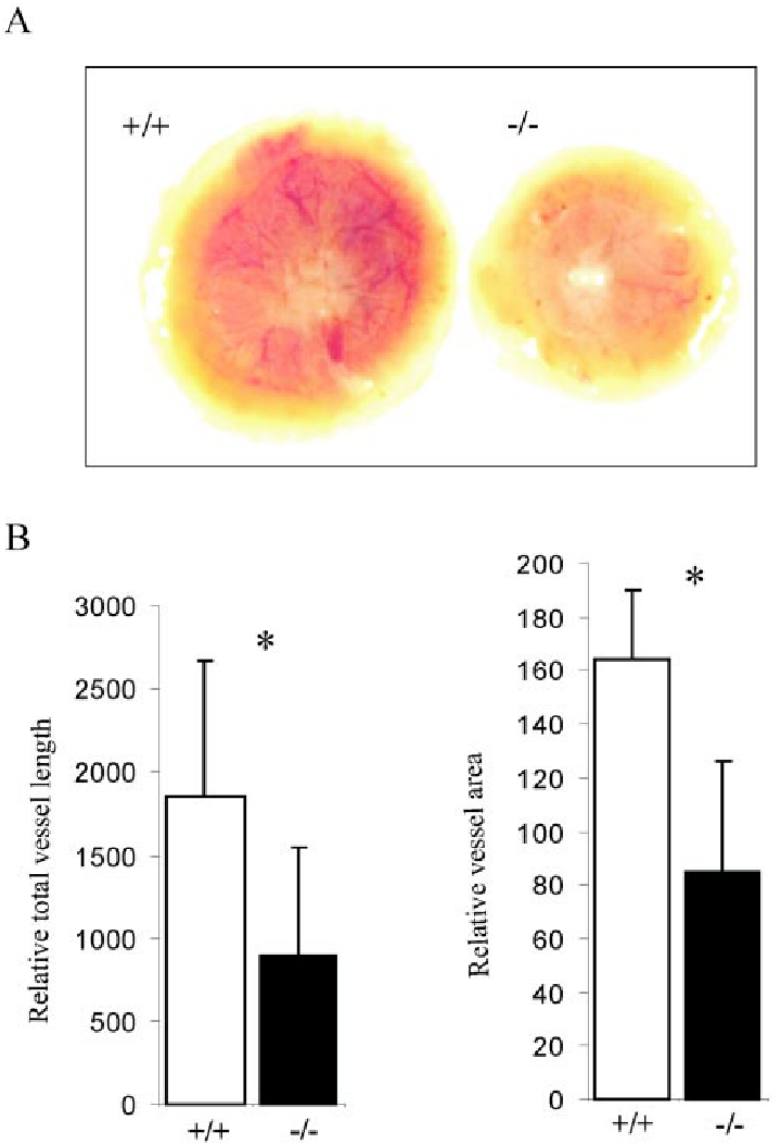


Fig. 1.7. Reduced vascularization in *Pkbα*$^{-/-}$ placentae

A, A representative picture showing vascular decrease of a mutant placenta. ***B,*** Quatitation of vessel length and vessel area in mutant placenta (* $p< 0.05$ for vessel length and * $p< 0.01$ for vessel area).

1848 ± 817, [mutant/wild-type=48%] $p<0.05$; vessel-covering area per placenta: mutant, 84912 ± 41131; wild-type, 163846 ± 26264 [mutant/wild-type=52%] $p<0.01$

Fig. 1.7B). The percentage decrease of total vessel length (52%) and vessel-covering area (48%) was greater than that of weight reduction of the placenta (29%). Thus, the vascular decrease is more severe than the weight reduction.

DISCUSSION

Our results suggest that PKBα is a major component of the signal transduction apparatus responsible for placental development and fetal growth in mice. Loss of PKBα results in a marked reduction of total phosphorylation levels of PKB leading to a defect in the growth and development of placenta and suggest that fetal growth retardation of PKBα null mice is apparently due to defects in placental function and subsequent insufficiency. The results also suggest that the β and γ isoforms of PKB can only partially compensate for the loss of the α.

In early mouse embryo development, the differentiation of trophoblast into trophectoderm parallels the formation of the inner cell mass (ICM) in the blastocyst by E3.5. The ICM gives rise to the embryo itself plus extra-embryonic membranes, such as the allantois and the amnion, while the trophectoderm develops into the ectoplacental cone, which eventually becomes the placenta (Ihle, 2000; Rossant and Cross, 2001). PKBα can be detected in murine ES cells derived from the ICM (Rathjen and Rathjen, 2001; Williams et al., 2000)[2]. This indicates that PKBα may also be expressed in trophoblasts during early embryogenesis. Our findings that PKBα is highly expressed in the mature placenta with widespread distribution in all type of trophoblast cells reinforce this possibility. Coincidentally, both IGF-1 and IGF-2 are expressed during early embryogenesis (Lopez et al., 1996; van Kleffens et al., 1998) and suggests that the insulin/IGF/PI3K/PKB-signaling pathway may function at this stage of embryonic development promoting mitosis, differentiation, and cell growth. Recently, Kamei *et al.* reported that the PI3K/PKB signaling pathway regulates the

development of the differentiated trophoblast giant cell phenotype using trophoblast stem cell lines (Kamei et al., 2002).

PKBβ knockout mice are insulin-resistant and display diabetes mellitus-like symptoms indicating that PKBβ is essential for glucose metabolism and insulin signal transduction, but apparently does not affect fetal growth (Cho et al., 2001a). *Pkbγ* gene has been also successfully targeted in our lab and the size of these mice is normal[3]. Overall, these results reinforce the important and distinct role of PKBα in placental development and fetal growth control. Although the levels of PKBβ and γ are low compared with that of α, all three are necessary for placental function since decrease of γ levels together with absence of α ($Pkb\alpha^{-/-}\gamma^{+/-}$) intensify the small phenotype of placenta and embryo, and *Pkbα/γ* double knockout mice ($Pkb\alpha^{-/-}\gamma^{-/-}$) are embryonic lethal[2]. This raises the possibility that the growth retardation of PKBα null mice is a combination of placental insufficiency and autonomous fetal growth impairment. The observation that the mutant mice sustain their small size during life suggests that growth retardation may be imprinted. To test this hypothesis tetraploid rescue experiments need to be performed. Generation of mice with placenta-specific (extra-embryonic tissue-specific) knockout of PKBα will also help address this question**.**

PKB has been well documented as a key mediator of cell and organ growth in *Drosophila* and mouse (Leevers et al., 1996a; Stocker et al., 2002; Tuttle et al., 2001b; Verdu et al., 1999a). Recently, the protein product of the tumor suppressor gene TSC2 was identified as substrate of PKB implicated in regulating p70S6K activity (Gao et al., 2002; Inoki et al., 2002; Potter et al., 2002). PKB phosphorylates TSC2 and promotes disassembly of the TSC1-TSC2 complex relieving the inhibition of p70S6K. Therefore, we checked whether the PKB-TSC2-p70S6K pathway was altered in mutant placenta where total PKB phosphorylation was very low. Adamson and Cross

found that placental growth continues until E17.5 (Adamson et al., 2002) and our results are consistent with these observations (wild-type placental weight increased from 121 ± 18 mg at E14.5 to 142 ± 18 mg at E16.5). Based on this, we speculate that the growth control pathway of PKB-TSC2-p70S6K may be required in placenta during this period. However, when we analyzed the phosphorylation levels of TSC2 and p70S6K in E14.5 placentae, we could not find any significant difference between wild-type and mutants.

During placental development, extensive angiogenesis and vascularization take place to form new blood vessels, which then dilate and become modified (Cross et al., 2002; Rossant and Cross, 2001). In mature placenta, maternal and fetal vascular structures are interconnected to ensure efficient nutrient and gas exchange between mother and fetus (Adamson et al., 2002; Cross, 2000; Rossant and Cross, 2001). These facts prompted us to examine the VEGF-PKB-eNOS signaling pathway. Our results demonstrate that there was no change in the level of transcripts encoding VEGF (data not shown) and in the protein levels of eNOS in mutant placenta. However, when we evaluated the phosphorylation of eNOS on Ser1177, we found significant reduction of eNOS phosphorylation in mutant placenta. As phosphorylation of eNOS at this site is important for its activation (Dimmeler et al., 1999; Fulton et al., 1999), reduced phosphorylation may result in decreased activity of this enzyme in mutant placenta. The cellular and physiological effects of eNOS activation by PKB have been demonstrated recently using mouse and rabbit endothelial cells (Hafezi-Moghadam et al., 2002; Luo et al., 2000; Rossant and Cross, 2001; Scotland et al., 2002). First, eNOS phosphorylation on Ser-1177 by PKB is required for endothelial cell migration induced by VEGF, and is also important for basal and stimulated NO release in both endothelial cells and in intact blood vessels. Second, delivery of constitutively active

PKB to rabbit femoral arteries significantly promote new vessel formation, increased the resting diameter and blood flow while L-NAME, an eNOS inhibitor, blocked this vasodilatation. In contrast, endothelium-dependent vasodilatation in response to acetylcholine is reduced in vessels transfected with dominant-negative PKB (Luo et al., 2000; Scotland et al., 2002). Third, corticosteroids improve vasorelaxation and blood flow and thus generate acute and rapid cardiovascular and stroke protective effects, through PI3K/PKB signaling-dependent activation of eNOS (Kurieishi et al., 2000; Limbourg et al., 2002). Thus, these results demonstrate that the PKB-dependent activation of eNOS is important and necessary for endothelial survival, new vessel formation, vasorelaxation and dilatation, and blood flow. Therefore, it is reasonable to attribute the phenotype of *Pkbα* mutant placenta to the reduced eNOS activity. The decreased angiogenesis, vasodilatation and blood flow restrict the growth and development of *Pkbα* mutant placenta. Indeed, mice with eNOS disruption display not only hypertension but also abnormal prenatal and postnatal development including placental hypotrophy, fetal growth restriction and reduced survival (Hefler et al., 2001).

Another significant result obtained in this study was the nearly complete loss of glycogen-containing spongiotrophoblast cells in mutant placenta. This result is consistent with the role of PKB in regulating GSK3 and indirectly glycogen synthase. Decrease of PKBα would lead to constitutive activation of GSK3 and therefore inhibit glycogen synthesis. Coincidentally, mice lacking eNOS also show insulin resistance and decreased insulin-stimulated glucose uptake which indicates that eNOS is important for the control not only of arterial pressure but also of glucose and lipid homeostasis (Duplain et al., 2001).

Intrauterine growth retardation (IUGR) is a major problem affecting infant mortality and morbidity, accounting for about 8% of human pregnancies (Anthony et al., 2001; Regnault et al., 2002). The etiologies for IUGR are complicated and are frequently associated with abnormalities in placental growth, structure and function (Pardi et al., 2002; Regnault et al., 2002). These abnormalities adversely affect placental function, and restrict the nutrient supply to the fetus, eventually giving rise to slow growth. The phenotypes of *Pkbα*$^{-/-}$ mice are strongly reminiscent of human IUGR. The mechanisms responsible for IUGR at the molecular level are still not clear. The results from the PKBα deficient mouse implicate PKBα-eNOS signaling inefficiency as one of the possible causes of this condition.

In conclusion, our studies have confirmed and extended two previous reports on the phenotype of PKBα knockout mice (Chen et al., 2001; Cho et al., 2001b), and provides novel insights into the function of PKBα in placental development and fetal growth. The results demonstrate a major role for PKBα in regulating mouse embryo development, probably mediated by insulin, IGF-1 and IGF-2. Significantly, our findings reinforce the significance of PKB-eNOS signaling in placentation. Loss of PKBα also profoundly affects postnatal growth. This study also provides novel insights into the understanding of human IUGR, and may have applications for the development of therapeutic approaches for this condition.

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The GenBank accession number for the mouse *Pkb*α genomic sequence is AF534134.

Chapter II

Reduced brain size in protein kinase Bγ (PKBγ/Akt3) null mutant mice

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Running title: Reduced brain size in PKBγ knock out mice

Abstract

Protein kinase B (PKB /Akt) is implicated in many critical cellular processes such as metabolism, cell proliferation, survival and apoptosis. Three closely related isoforms have been identified and termed PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3. To elucidate the physiological role of PKBγ mice with targeted disruption were generated. In contrast to PKBα-deficient mice, *Pkbγ*$^{-/-}$ mice are viable and do not present a growth retardation syndrome. In adult *Pkbγ* mutant mice, the brain size and weight were dramatically reduced by about 25%. MRI analysis of *Pkbγ*$^{-/-}$ brains revealed a uniformly reduced size, affecting all major brain regions. Histological analysis of *Pkbγ*$^{-/-}$ brains displayed smaller structures, but a structurally unaltered organisation. The reduced brain weight of *Pkbγ*$^{-/-}$ mice is caused, at least partially, by a significant reduction of the cell size and cell number. Our results provide novel insights into the function of PKBγ and suggest a critical role in the postnatal brain development.

Introduction

Protein kinase B (PKB, also named Akt) is a second messenger-regulated kinase, which has been implicated in many critical cellular processes such as glucose metabolism, cell proliferation, apoptosis and angiogenesis (Brazil and Hemmings, 2001). In mammalian cells, three closely related isoforms of the PKB family have been identified and termed PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (Brodbeck et al., 1999; Cheng et al., 1992; Jones et al., 1991; Masure et al., 1999; Nakatani et al., 1999). These proteins are products of three separate genes located on distinct chromosomes and are broadly expressed, but with some isoform-specific features, e.g. PKBβ is expressed at high levels in insulin-sensitive tissues (Altomare et al., 1998a; Yang et al., 2003) . All three members of the PKB family contain a highly conserved amino-terminal pleckstrin homology (PH) domain, a central catalytic domain and a short carboxy-terminal regulatory domain (Brazil and Hemmings, 2001). In addition, a minor C-terminal splice variant lacking the hydrophobic motif of PKBγ was identified (Brodbeck et al., 2001).

PKB is activated by a large set of stimuli, including growth factors, hormones and cytokines (Brazil and Hemmings, 2001; Datta et al., 1999). Activation of PKB occurs in response to signaling via phosphoinositide 3-kinase (PI-3K) and requires the membrane-bound second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) (Burgering and Coffer, 1995; Cross et al., 1995). The current model for PKB regulation proposes that PIP3, generated following PI-3K activation, interacts with the PH domain of PKB, recruiting the kinase to the plasma membrane and promoting a conformational change that allows phosphorylation. Phosphorylation occurs on two regulatory sites by upstream kinases on the plasma membrane. One of these critical phosphorylation sites resides in the activation loop of the kinase domain (Thr-308 in

PKBα) and the other is located in the C-terminal regulatory domain (Ser-473 in PKBα) (Alessi et al., 1996; Brodbeck et al., 1999; Meier et al., 1997). The recent results describing the crystal structure of the inactive and active forms of PKBβ have revealed a novel mechanism for the regulation of PKB by hydrophobic motif phosphorylation (Yang et al., 2002a; Yang et al., 2002b).

The upstream kinase which phosphorylates Thr-308 in the activation loop of the kinase domain of PKBα in a PIP3-dependent-manner has been identified and termed 3-phosphoinositide-dependent kinase-1 (PDK-1) (Vanhaesebroeck and Alessi, 2000). Thr-308 phosphorylation is necessary and sufficient for PKB activation; however, maximal activation requires additional phosphorylation at Ser-473 by a kinase that has been characterized biochemically but the molecular identity of which remains undetermined (Alessi et al., 1997; Hill et al., 2002). Recently, a negative regulator of PKB was identified and termed carboxy-terminal modulator protein (CTMP). CTMP inhibits the phosphorylation of PKB and thus blocks downstream signaling (Maira et al., 2001).

PKB was shown to phosphorylate numerous substrates involved in the regulation of cell metabolism, survival and growth such as glycogen synthase kinase-3 (GSK3), Forkhead transcription factor and caspases (Brazil and Hemmings, 2001; Datta et al., 1999).

All three PKB isoforms are implicated in tumor formation (Nicholson and Anderson, 2002). The *Pkbα* gene is amplified in human gastric cancer and displays an increased activity in breast and prostate cancer (Staal, 1987). PKBβ has been over-expressed in ovarian, pancreatic and colon tumors (Cheng et al., 1992). Upregulation of PKBγ has been found in estrogen-receptor negative breast tumor and in androgen-insensitive prostate cancer cell lines (Nakatani et al., 1999).

Recently, mice with targeted disruption of *Pkbα*, *Pkbβ* or both genes were obtained. *Pkbα* mutant mice display an approximately 30% reduction of body size and a partial neonatal lethality (Chen et al., 2001; Cho et al., 2001b; Yang et al., 2003). Moreover, loss of *Pkbα* led to a placental hypotrophy with impaired vascularization (Yang et al., 2003). Glucose metabolism was not altered in $Pkb\alpha^{-/-}$ mice. In contrast, *Pkbβ* deficient mice exhibit a diabetes-like syndrome with elevated fasting plasma glucose, hepatic glucose output and peripheral insulin resistance, but only mild growth retardation syndrome (Cho et al., 2001a; Garofalo et al., 2003). Furthermore, the adipose tissue mass was significantly reduced (Garofalo et al., 2003). Mice lacking both isoforms die after birth, probably due to respiratory failure (Peng et al., 2003). Additionally, P*kb* α/β double mutant newborns display a severe body weight reduction (≈50%) and a prominent atrophy of the skin and skeletal muscle, as well as an impaired adipogenesis and a delayed ossification.

In this article, we report the generation and characterization of mice with targeted disruption of the *Pkbγ* gene. In contrast to $Pkb\alpha^{-/-}$ mice, *Pkbγ* mutant mice are viable without an increased perinatal mortality and do not display a growth retardation syndrome. Loss of PKBγ profoundly affects the postnatal brain growth. Brains from adult *Pkbγ* mutant mice show a dramatic reduction of size and weight, affecting all major parts of the brain. Taken together, our results suggest a novel and important role of PKBγ in postnatal brain development.

Methods

Targeted disruption of the PKBγ gene by homologous recombination

A ~11 kb *Hind III* fragment was subcloned which contain *Pkbγ* exon 4 and 5. An *Nco I* site was generated into exon 4 for insertion of a ~5 kb IRES-LacZ-Neo cassette. The targeting vector was linearized with *Sal I* and electroporated into 129/Ola ES cells. An external probe was used for ES cell screening following Xba I digestion. An internal probe and a LacZ-Neo probe were used for characterization of ES clones positive for homologous recombination (data not shown). Correctly targeted ES cells were used to generate chimeras. Male chimeras were mated with wild type C57BL/6 females to obtain $Pkb\gamma^{+/-}$ mice, which were intercrossed to produce *Pkbγ* homozygous mutants. The progeny from $Pkb\gamma^{+/-}$ intercrosses have a mixed genetic background of 129/Ola and C57BL/6. Genotyping of progeny was done 3-4 weeks after birth by multiple PCR with the following three primers: (a) *Pkbγ* 5', GGTTCTGTGGGAGGTAGTTCTC; (b) New-neo-2, GCAATCCATCTTGTTCAATGGCCG; and (c) *Pkbγ* 3', CCATCGGTCGGCTACGGCTTGG.

Animals

Mice were housed according the Swiss Animal Protection Ordinance in groups with 12 h dark-light cycles and with free access to food and water. All procedures were conducted under relevant authority approval.

Quantitative real time PCR

The levels of PKB isoforms in wildtype and mutant mice were determined by Q-RT-PCR. The experiment was performed as previously described (Yang et al., 2003). Briefly, total RNA was purified using Trizol Reagent (Invitrogen). For the Q-PCR reaction, 50 ng total RNA was mixed with 5' and 3' primers, Taqman probe, MuLV reverse transcriptase, RNase inhibitor and the components of the TaqMan PCR reagent

kit (Eurogentec) in a total volume of 25 µl following the TaqMan PCR reagent kit protocol.

Western blot analysis

For Western blot analysis, protein lysates were prepared by homogenization of various organs in lysis buffer (50 mM Tris-HCl, pH 8.0; 120 mM NaCl; 1% NP-40; 40 mM β-glycero-phosphate; 100 µM Na_3VO_4; 1 µM Microcystin LR). Homo-genates were centrifuged twice (13000 rpm for 10 min at 4 ^oC) to remove cell debris. Protein concentrations were determined using the Bradford assay. Protein (50 µg per sample) was separated by 10% SDS-PAGE and transferred to Immobilon-P PVDF membrane (Millipore). PKB isoform specific antibodies were obtained by immunizing rabbits with isoform specific peptides as previously described (Yang et al., 2003). To purify antibodies, sera were preabsorbed on membranes loaded with protein lysates from *Pkbγ*$^{-/-}$ mice. Antibodies against phospho-PKB (Ser473), phospho-GSK-3α/β(Ser21/9)phospho-TSC2 (Thr1462) and phospho-p70S6K were purchased from Cell Signaling Technologies. Antibodies against p27 and ERK were purchased from Santa Cruz Biotechnology. Antibody against phospho-ERK (Thr202/Tyr204) was purchased from Promega. Pan actin antibody was purchased from NeoMarkers.

Histological examination

For histological analysis, animals were perfused with phosphate-buffered saline and 4% paraformaldehyde in phosphate-buffered saline. Organs were dissected and kept in the same fixation solution overnight at 4 ^oC. Samples were embedded in paraffin following dehydration in ethanol. Tissues were cut in 6 µm thick sections and stored for staining. For hematoxylin-eosin (company) and cresyl-violet (Sigma) stainings, sections were deparaffinazed and stained as previously described (Yang et al., 2003). For immunohistochemistry, brains were fixed as described above, kept in sucrose

solution overnight, embedded in carbowax (O.C.T. compound, Tissue Tek) and 20 μm sections were prepared. Sections were blocked for 30 minutes in 5% normal goat serum in phosphate-buffered saline and then incubated overnight at 4 ^{o}C with the primary antibodies. The sections were subsequently processed according to the manufacturers` protocol from Vectastain ABC kit (Vector Laboratories).

Cell number determination

To determine the number of cells in a whole brain, the DNA content was determined as described by Labarca and Paigen (Labarca and Paigen, 1980). Briefly, the method is based on the enhancement of fluorescence seen after binding of Bisbenzimid (Riedel-de Haen) to DNA. A linear standard curve (1 μg/ml to 10 μg/ml) was determined to calculate the DNA concentration (data not shown).

Magnetic resonance imaging (MRI)

Adult PKBγ wild type and mutant mice (n=5 per group, female 4 months old) underwent MRI examinations at 2.35 T using a MRBR 4.7/400 mm magnet (Magnex Scientific, Abingdon, England) equipped with BGA20 gradients (100 mT m 1) driven by a DBX system (Bruker Biospin, Ettlingen, Germany). For in vivo studies the animals were treated as previously described (Natt et al., 2002). Subsequently, mice were perfused as described and brains were analysed histologically.

Statistical analysis

To compare body weight, brain weight and volume, brain/body weight ratio, DNA content and cell number between *Pkbγ*$^{+/+}$ and *Pkbγ*$^{-/-}$, an unpaired Students t-test was performed. P-values below 0.05 were considered as significant and p-values below 0.01 as highly significant.

Results

Targeting strategy and confirmation of genotype

Pkbγ$^{-/-}$ mice were generated by targeted disruption of exon 4 thereby abolishing protein translation (Fig.2.1 A). The ablation of PKBγ was confirmed by PCR, Q-RT-PCR and Western blot analysis using brain tissue samples from *Pkbγ* wild type, heterozygous and mutant mice (Fig.2.1 B and C). To confirm the absence of PKBγ, Western blot analysis was performed with an isoform specific anti-PKBγ antibody. In contrast to the *Pkbγ*$^{+/+}$ brain tissue samples, we did not find a detectable level of PKBγ in the samples derived from mutant mice (Fig.2.1 C). Furthermore, quantitative RT-PCR displayed a complete ablation of the PKBγ mRNA in brain samples of *Pkbγ* mutant mice (data not shown). Additionally, Western blot analysis with antibodies specific for PKBα and PKBβ, respectively did not reveal a compensatory upregulation of PKBα and/or PKBβ in the brain of *Pkbγ*$^{-/-}$ mice (Fig.2.1 D).

Distribution of PKBγ in various wild type tissues

It has been reported that the PKBα and β isoforms are broadly expressed among all organs, but with some isoform specific features (Yang et al., 2003 Altomare et al., 1998). Less is known about the tissue distribution of the PKBγ isoform. Previous reports suggest, that the PKBγ isoform has a more restricted pattern, with high levels in the adult brain and fetal heart and low levels in liver or skeletal muscle (Brodbeck et al., 1999; Yang et al., 2003; Masure et al., 1999). Therefore, the tissue distribution of PKBγ was assessed by quantitative RT-PCR and Western blot analysis, respectively, in 15 major tissues. The mRNA levels of PKBγ were normalized to the level of PKBγ in

the brain. PKBγ mRNA was found at highest levels in brain and testis and at lower levels in lung, mammary gland, fat and spleen (Fig.2.2 A).

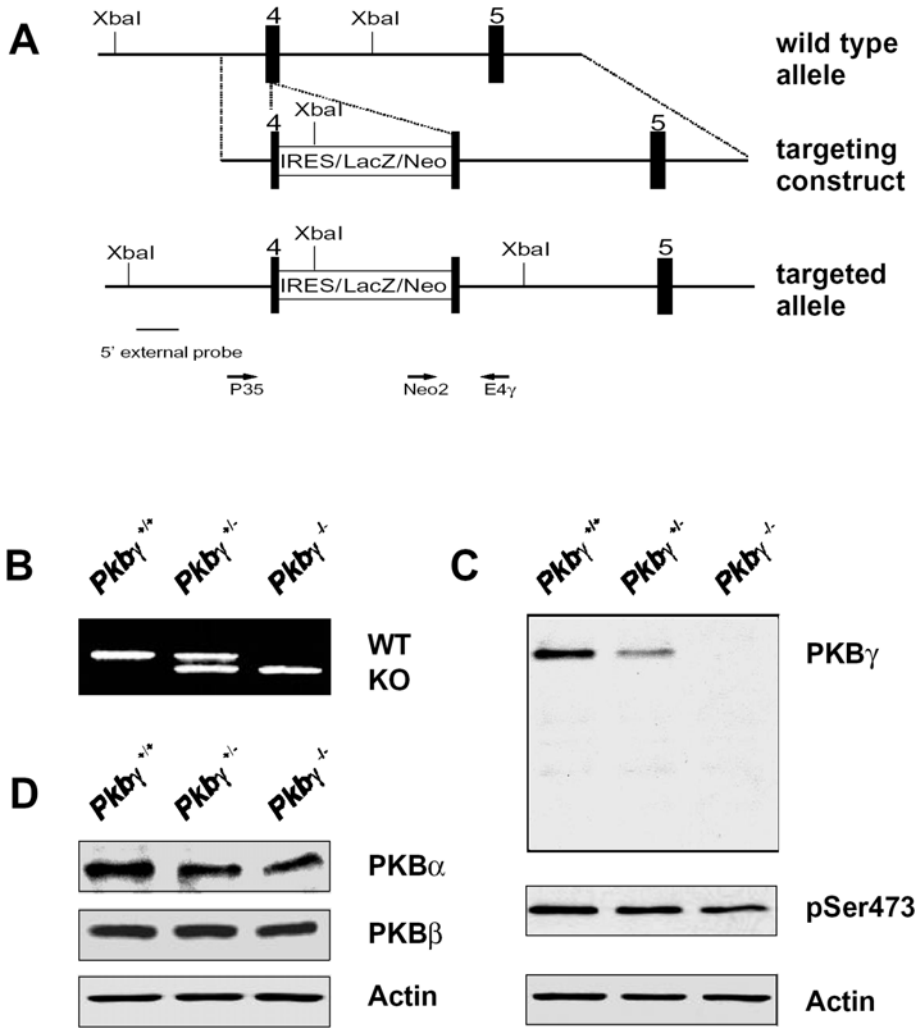


Figure 2.1 Targeting strategy and confirmation of genotype

(A) The genomic organization of the *Pkb*γ wild type allele (top) was disrupted using a targeting vector with an IRES-LacZ-Neo-cassette (middle). Targeting of the wild type allele leads to the disruption of exon 4 of the *Pkb*γ gene (bottom). Arrowheads indicate the localization of the primers for the PCR reaction.

(B) The genotype of mice was confirmed using a PCR-based strategy. A representative result from $Pkb\gamma^{+/+}$ (≈500bp), $Pkb\gamma^{+/-}$ and $Pkb\gamma^{-/-}$ (≈350 bp) mice are shown.

(C) The level of PKBγ in the brain of *Pkbγ* $^{+/+}$, *Pkbγ*$^{+/-}$ and *Pkbγ*$^{-/-}$ mice, respectively, was determined by Western blot analysis using a PKBγ specific antibody (50 µg protein per lane).

(D) The levels of PKBα and PKBβ, respectively, were determined in brain lysates from *Pkbγ* $^{+/+}$, *Pkbγ*$^{+/-}$ and *Pkbγ*$^{-/-}$ mice, respectively, using PKBα or PKBβ specific antibodies (50 µg protein per lane).

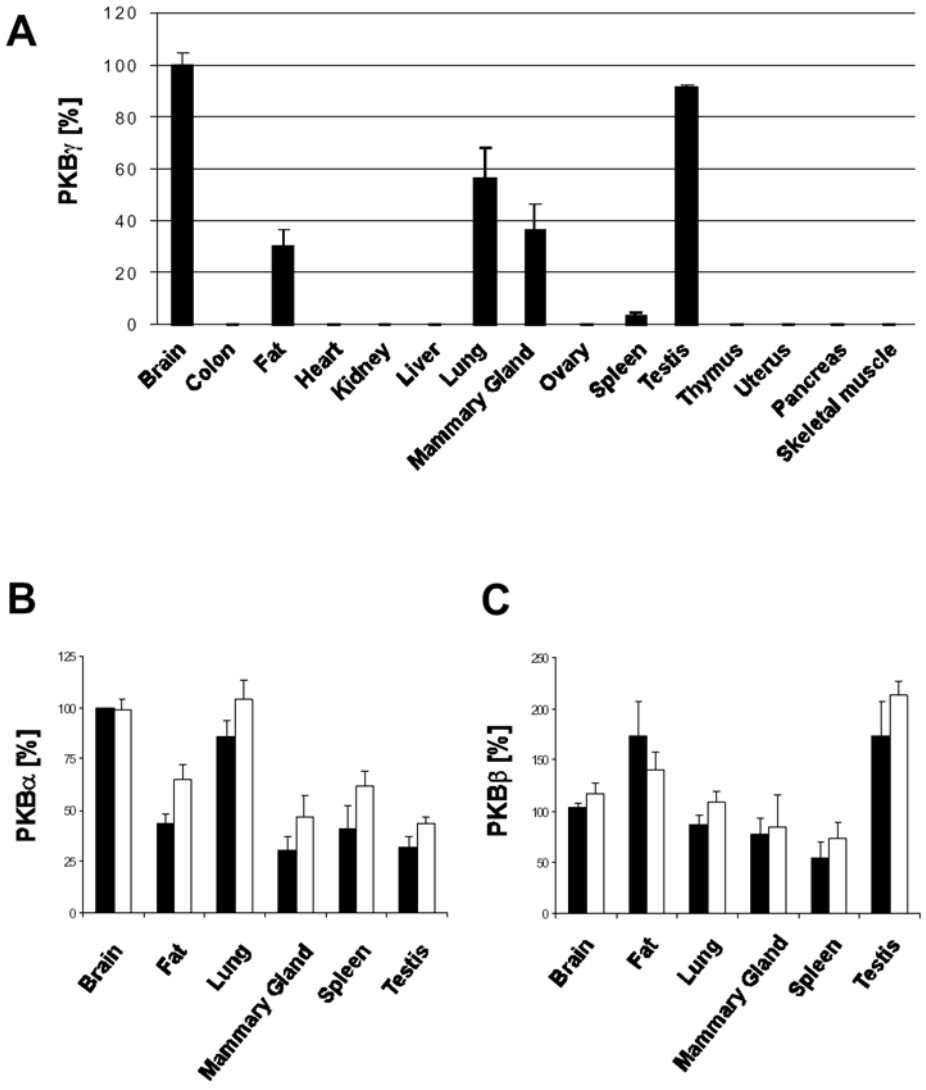


Figure 2. 2 Tissue distribution of PKBγ and levels of PKBα and β in PKBγ mutant mice

(A) The mRNA level of PKBγ was determined in 15 different organs from PKBγ $^{+/+}$ mice. Total RNA was isolated from 3 adult mice and the levels were normalized to the level of PKBγ in the brain (100%).

(B) mRNA levels of PKBα and **(C)** PKBβ, respectively, were determined using total RNA from 6 different organs of adult PKBγ wild type (n=3; black bars) and mutant mice

To investigate whether the ablation of PKBγ leads to compensatory increase of PKBα and/or PKBβ, total RNA was isolated from brain, testis, lung mammary gland, fat and spleen of 3 PKBγ wild type and 3 mutant mice and a quantitative RT-PCR was performed as described (Yang et al., 2003). The levels of PKBα and β were normalized to the level of PKBα in the wild type brain and set as 100%. Overall, no marked up-regulation of PKBα and/or PKBβ was observed, including the brain (Fig.2.2 B and C). These results are consistent with the findings of the Western blot analysis with protein extracts of brains from PKBγ wild type and mutant mice (Fig.2.1 D).

Dispensable role of PKBγ for postnatal survival, fertility and body weight

Recently, it has been shown by independent groups that mice with targeted disruption of the PKBα gene display an increased neonatal mortality (Chen et al., 2001; Cho et al., 2001b; Yang et al., 2003). Therefore, we investigated whether $Pkb\gamma^{-/-}$ mice were born at/in the expected Mendelian ratio. Analysis of more than 400 pups (age: 3-4 weeks) from PKBγ heterozygous matings revealed no evidence for an increased mortality of PKBγ $^{-/-}$ mice after birth ($Pkb\gamma^{+/+}$**:** $Pkb\gamma^{+/-}$**:** $Pkb\gamma^{-/-}$ = 104 (25.6%)**:** 206 (50.7%)**:** 96 (23.6%)). Since PKBγ is highly expressed in the testis, fertility of mutant mice was tested using male $Pkb\gamma^{-/-}$ x female $Pkb\gamma^{+/+}$ and female $Pkb\gamma^{-/-}$ x male $Pkb\gamma^{+/+}$ matings, respectively. Both male and female knock out mice matings gave normal pregnancies and births, indicating that fertility is not impaired in either male or female PKBγ mutant mice. PKB has been implicated in cell and organ growth (Yang et al., 2004). Previously it has been reported that, besides the increased mortality, mice with *Pkbα*, *Pkbβ* and *Pkbαβ* null mutations also present a mild to severe growth retardation

syndrome (Chen et al., 2001; Cho et al., 2001b; Garofalo et al., 2003; Peng et al., 2003; Yang et al., 2003). Therefore, body weight was measured in male *Pkbγ* knock out mice and wild type littermate controls (n=5-8 per group) at different time points and the obtained results are shown in Fig.2.3 A. The body weight did not differ significantly between *Pkbγ* mutant mice and wild type controls at any time point. A similar result was observed using female *Pkbγ* $^{-/-}$ mice and *Pkbγ* $^{+/+}$ controls (n=5-8, data not shown), indicating a dispensable role of PKBγ for body weight control.

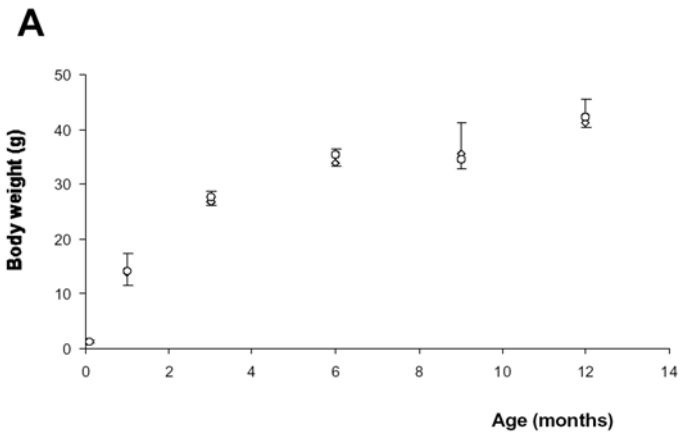
Essential role of PKBγ for postnatal brain development

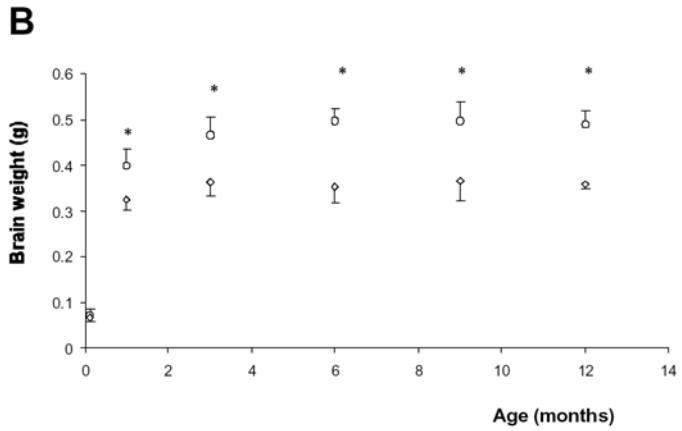
For further analysis, *Pkbγ* $^{+/+}$ and *Pkbγ* $^{-/-}$ mice were dissected and all major organs were investigated macroscopically. Compared to *Pkbγ* $^{+/+}$ controls, the overall size of the brain from adult *Pkbγ* $^{-/-}$ mice was strikingly reduced. A representative example is shown in Fig.2.3 C-E. Subsequently, the weights of freshly dissected brains of *Pkbγ* wild type and *Pkbγ* mutant mice were measured at different ages and the results are shown in Fig.2.3 B. Compared to age and gender matched wild type littermate controls, brains from adult *Pkbγ* $^{-/-}$ mice exhibit a highly significant reduction ($p<0,01$) of brain weight. In adult *Pkbγ* $^{-/-}$ mice the brain weight was reduced about 25% (range: 22%-29%) affecting both male and female mice. But in contrast to *Pkbα*, *Pkbβ* or *IGF-I* null mutant mice, the brain/body weight ratio of *Pkbγ* mutant mice was also significantly reduced to a similar extent (Beck et al., 1995; Cheng et al., 1998; Powell-Braxton et al., 1993).The reduction of brain size and weight was first observed at the age of 1 month, but less pronounced (18%). Interestingly, at birth the brain weight did not differ significantly between *Pkbγ* $^{+/+}$ and *Pkbγ* $^{-/-}$ (Fig.2.3B).

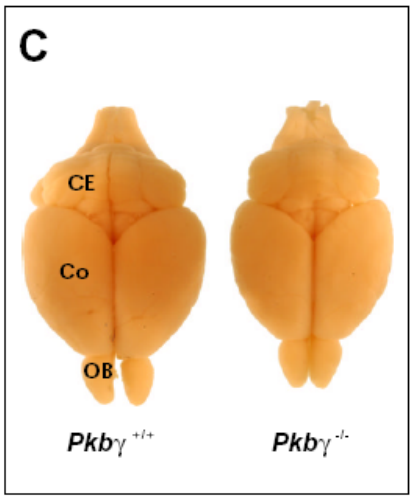
In vivo Magnetic resonance Imaging (MRI) of *Pkbγ* mutant mice

MRI has been shown to be an excellent tool to study brain anatomy in transgenic and mutant mice (Kooy et al., 1999; Lin et al., 2003; Natt et al., 2002). For further

characterization of the *Pkbγ* mutant brain anatomy, 5 adult female knock out and 5 adult female wild type littermate controls were examined using conventional MRI.







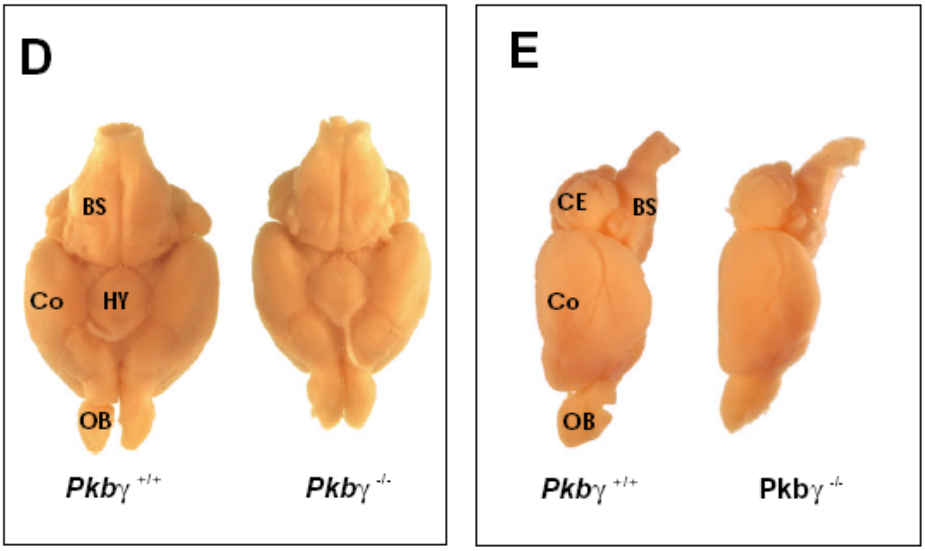


Figure 2.3 Reduced brain size in adult PKBγ mutant mice

(A) Body weight of male *Pkbγ*$^{+/+}$ and *Pkbγ*$^{-/-}$ mice was measured at different time points (n=5-8 per genotype).

(B) Weight of freshly dissected brains of male *Pkbγ*$^{+/+}$ and *Pkbγ*$^{-/-}$ mice were measured at different time points (n=5-8 per genotype). P-values below 0.05 were indicated with an asterisk.

(C) Cranial, **(D)** caudal and **(E)** lateral view of brains from adult PKBγ wild type (left side) and knock out (right side) mice. Various brain structures are labeled as followed: CO= cortex, CE= cerebellum, OB= olfactory bulb, BS= brainstem, HY= Hypothalamus

Mice were anesthetized and intubated, and T_2-weighted MRI were obtained as previously described (Natt et al., 2002). Representative images of sagital, horizontal and coronal section from *Pkbγ*$^{+/+}$ and Pkbγ $^{-/-}$, respectively, are shown in Fig.2.4 A-F. The in vivo MRI confirmed the dramatic reduction of brain size in all 5 PKBγ $^{-/-}$ mice. Compared to wild type littermates, the volume of the whole brain from PKBγ $^{-/-}$ mice was significantly reduced about 25% (513±14 vs. 391±16 mm^3, p<0.01). MRI

confirmed that various brain regions were affected, including cortex, cerebellum, hippocampus, olfactory bulb and ventricular spaces. But no alteration in the structural organization of the brain was observed, confirming the macro-pathological findings. Furthermore, an external hydrocephalus was excluded as a cause of the reduced brain size. Interestingly, in all 5 PKBγ $^{-/-}$ mice, but in none of the 5 wild type controls, an impressive reduction of the anterior part of the corpus callosum was observed (Fig. 2.4 C-F). Other commissures such as anterior, posterior or hippocampal commissure were not or less severely affected.

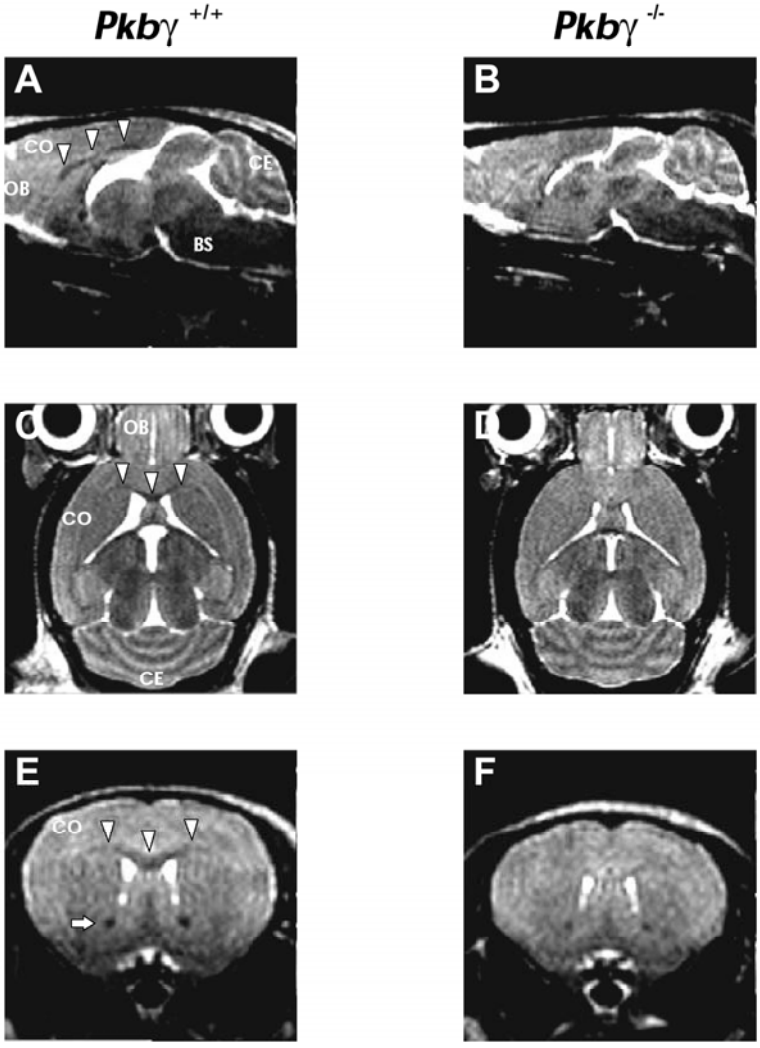


Figure 2.4 Magnetic resonance images of brains from adult PKBγ mutant mice

PKBγ wild type and mutant littermate controls (n=5 per group, all female, 4 months old) underwent MRI and T_2-weighted data were obtained. Representative pictures from *Pkbγ* $^{+/+}$ **(A, C** and **E**) and PKBγ$^{-/-}$ mice (**B, D** and **F**) are shown in a sagital (**A** and **B**), horizontal (**C** and **D**) and coronal (**E** and **F**) plane. Various parts of the brain were labeled as followed: CO= cortex, CE= cerebellum and OB= olfactory. The corpus callosum is marked with arrowheads and the anterior commissure with an arrow.

Histology of PKBγ wild type and mutant mice

To investigate changes on the microscopical level, a histological examination of *Pkbγ*$^{-/-}$ brain was performed. Various brain regions, including cerebellum, hippocampus, cortex and corpus callosum, were examined on Hematoxilin-Eosin stained sections and representative pictures are shown in Fig.2.5 A-H. Histological examinations did not reveal any abnormalities in the overall structure of the different brain regions, but with the exception of the reduced size of all regions. As seen in the MRI, the thickness of the corpus callosum in PKBγ mutant mice was impressively reduced (data not shown). Additionally, a myelin staining with Luxol fast blue performed (Fig.2.5 G-L). Myelin staining revealed not only a reduced thickness of the corpus callosum but also a less intensively stained structure.

Cell number determination in the brain of in PKBγ mutant mice

Since PKB is a key player involved in cell growth, proliferation and apoptosis, we assessed the cell number in the central nervous system by measuring the DNA content of whole brains from *Pkbγ* $^{+/+}$ and *Pkbγ* $^{-/-}$ mice. The DNA content is considered a parameter for cell number and the DNA content per gram of tissue is considered a parameter of cell density (and inverse correlated to cell size) (Labarca and Paigen, 1980; Zamenhof and van Marthens, 1976). The DNA content was determined in brains

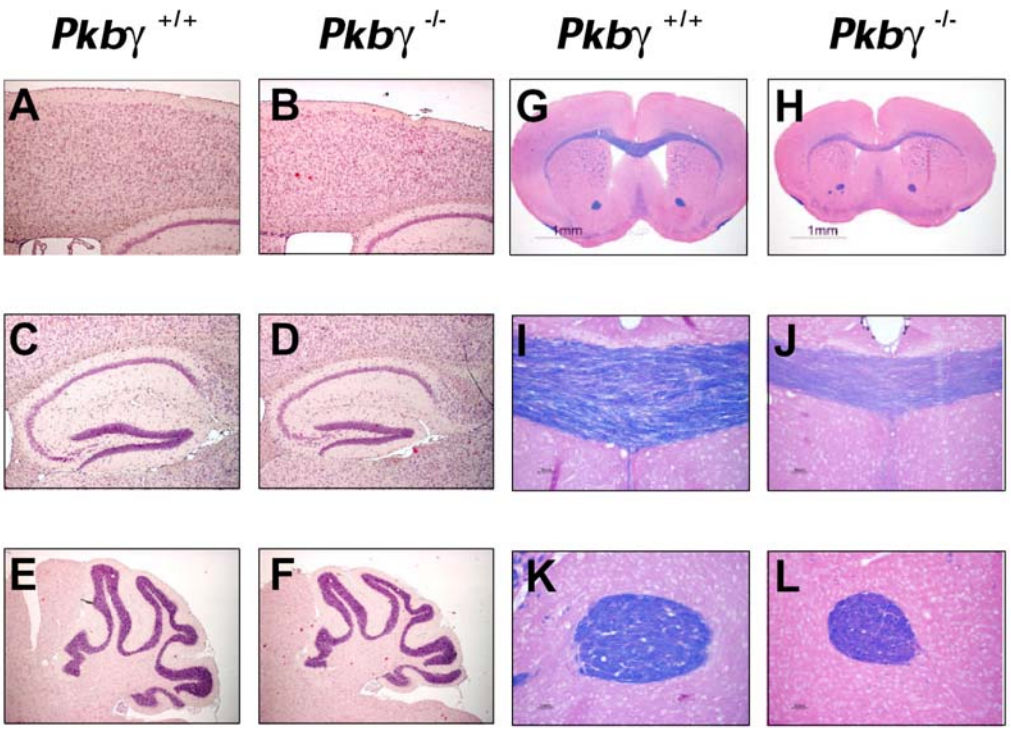


Figure 2.5 Histology of brains from PKBγ mutant mice

Hematoxylin/Eosyin (HE) stained parasagital sections of the cortex **(A and B)**, hippocampus **(C and D)** and cerebellum **(E and F)** from *Pkbγ* $^{+/+}$ (**A, C** and **E**) and *Pkbγ* $^{-/-}$ mice (**B, D** and **F**) are shown. Brains of adult mice were dissected and stained for myelin (blue) with Luxol-Fast blue and Eosin Y. Coronal section of whole brain (**G** and **H**), corpus callosum (**I** and **J**) and anterior commissure (**K** and **L**) from *Pkbγ* $^{+/+}$ (**G, I** and **K**) and *Pkbγ* $^{-/-}$ mice (**H, J** and **L**) are shown.

derived from newborn and 1-month-old mice. At 1 month the cell number in the brains from PKBγ mutant mice was slightly, but significantly, reduced when compared to PKBγ $^{+/+}$ controls. In contrast, the DNA content in the brains of newborns did not differ significantly between PKBγ wild type and mutant mice. Additionally, at the stage of 1 month, the DNA content per gram of tissue was significantly increased in samples derived from *Pkbγ* $^{-/-}$ when compared to wild type littermate controls. Taken

together, the results suggest that both cell size and cell number contribute to the reduction of brain size observed in 1-month old mutant mice (table 2.1).

Table 2.1 Cell number in the brain of *Pkbγ* wild-type and mutant mice

	Newborns		1 month old	
	Pkbγ$^{+/+}$	*Pkbγ*$^{-/-}$	*Pkbγ*$^{+/+}$	*Pkbγ*$^{-/-}$
Body weight (g)	1.21±0.23	1.31±0.14 (107%)	14.1±2.5	13.0±2.3 (92%)
Brain weight (g)	0.071±0.001	0.069±0.006 (98%)	0.41±0.03	0.32±0.03 (78%)
Brain/body weight ratio	0.059±0.011	0.054±0.008 (90%)	0.030±0.004	0.025±0.003 (85%)
DNA/brain (mg)	0.59±0.04	0.56±0.12 (96%)	1.38±0.03	1.29±0.07 (93%)*
DNA/g of tissue (mg)	8.53±1.64	8.11±1.29 (95%)	3.38±0.20	4.04±0.47 (119%)*

P-values below 0.05 were indicated with an asterisk.

Myelination in PKBγ mutant mice

Several recent studies emphasize the importance of insulin-like growth factor I (IGF-I) signaling in the survival and proliferation of oligodendrocytes and myelination in the central nervous system (D´Ercole et al., 2002). It has been reported that mice lacking IGF-1 have also a reduced brain weight with notable hypomyelination in the cortex, corpus callosum and anterior commissure (Beck et al., 1995; Ye et al., 2002). Therefore, a Western blot analysis with cell lineage specific markers was performed using antibodies against myelin basic protein, glial fibrillary acidic protein and M-Neurofilament. Interestingly, no obvious difference was observed (n=6 per genotype, data not shown)

Signaling in PKBγ mutant mice

To further elucidate the mechanisms which could lead to a decreased brain size in PKBγ mice, the phosphorylation state of PKB itself and its substrates was determined in brains from *Pkbγ*$^{+/+}$ and *Pkbγ*$^{-/-}$ mice using phospho-specific antibodies. First, we analyzed the phosphorylation/activation state of PKB in protein extracts from brain of *Pkbγ*$^{+/+}$ and *Pkbγ*$^{-/-}$ using an antibody specific for the phosphorylation site (anti phospho-Ser 473) in the hydrophobic motif of the regulatory domain of all 3 isoforms (Fig.2.6). As expected, the total level of activated PKB in the *Pkbγ*$^{-/-}$ sample was

markedly reduced, but not completely abolished, when compared to the samples of *Pkbγ*$^{+/+}$ littermate controls. The phosphorylation levels of glycogen synthase kinase (GSK), tuberous sclerosis complex 2 (TSC2), ERK, p27 and p70S6K were only slightly, if at all, changed in the *Pkbγ*$^{-/-}$ samples.

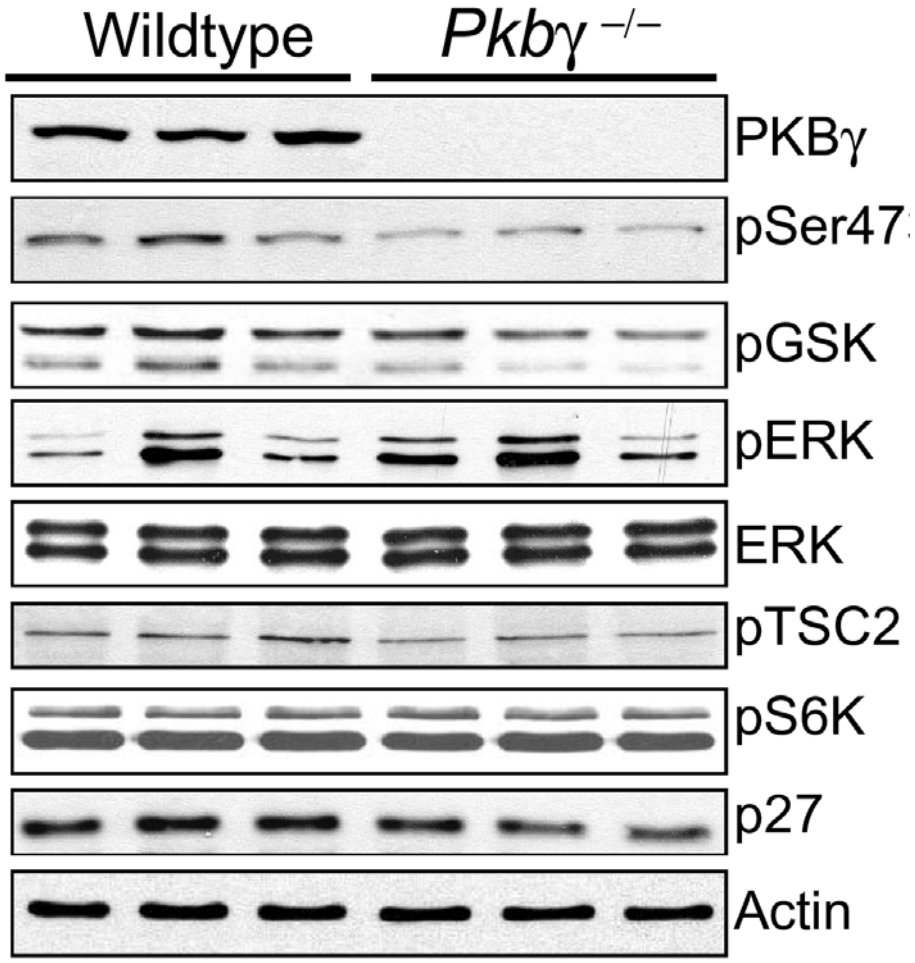


Figure 2.6 Phospho-Western blot analysis of brains from PKBγ mutant mice

To check the phosphorylation status of proteins involved in PKB signaling, lysates from 3 wild type and 3 mutant brains were analyzed using various antibodies as indicated. Actin served as loading control (50 μg protein/lane).

Discussion

In this article we report the phenotypic consequences of the ablation of the PKBγ gene. We demonstrated that the targeted disruption of the PKBγ gene by homologous recombination results in a complete ablation of the gene product. Furthermore, the inactivation of the PKBγ gene leads to a considerable reduction of the total phosphorylated/activated PKB in the mutant brain. Interestingly, the levels of the α and β isoform of PKB assessed on the mRNA and protein level in PKBγ mutant brains are not elevated and they fail to fully compensate for the loss of PKBγ. This result is consistent with the findings in PKBα and PKBβ mutant mice, where no compensatory up regulation of the other isoforms was found (Yang et al., unpublished observation, Cho et al., 2001, Garofalo et al., 2003). Our results show that, in contrast to PKBα and PKBβ mutant mice, PKBγ mice are viable and do not suffer from a growth retardation syndrome, suggesting a dispensable role in embryonic and postnatal body growth (at least not this particular genetic background).

It has been recognized that the IGF-I/PI3-K/PKB pathway plays a crucial role in the mammalian brain development. Mice with targeted disruption of the IGF-I and insulin-receptor substrate gene display a brain phenotype with a marked reduction of the brain mass (Beck et al., 1995; Schubert et al., 2003; Ye et al., 2002). In contrast, an increased brain mass was observed in mice overexpressing IGF-1 (Mathews et al., 1988; Ye et al., 1995). Moreover, mice with brain specific deletion of PTEN, a negative regulator of the PI3-K/PKB pathway, exhibit an enlarged brain with seizures and ataxia resembling Lhermitte-Duclos disease (Backman et al., 2001; Kwon et al., 2001). Unfortunately, less is known about the consequences of the inactivation of PKBα and PKBβ for the mouse brain development. PKBα $^{-/-}$ and PKBβ mice show

only a slight diminution of brain weight, but both present an increased brain/body weight ratio (Yang et al.,, unpublished observation, Garofalo et al., 2003). However, inactivation of the PKBγ gene results in impressive weight and size reduction of the brain in adult mice. In contrast to the PKBα, PKBβ and IGF-I mutant mice, the brain/body weight ratio was also significantly reduced. Interestingly, PKBγ deficiency did not affect the general anatomical organization of the brain and a missing brain region could be excluded by MRI analysis as the main cause of the weight reduction. MRI revealed various affected brain regions, including cerebellum, cortex, hippocampus or ventricular spaces.

PKBαγ double mutant mice provide supplementary information of the crucial role of PKB in brain development. Besides the complete embryonic mortality and the severe growth retardation, they exhibit a defective development of brian (Yang et al., unpublished observation).

To determine the specific role of the different PKB isoforms in brain development, mice with a brain specific deletions (Cre/Lox system) of a single isoform will be generated and help to answer these questions. Although we have not observed obvious differences in the behavior between PKBγ wild type and mutant mice, sophisticated behavioral studies are needed to test behavior under more challenging conditions (e.g. rotarod for motor function) and for less obvious brain functions (e.g. memory).

As mentioned above, mice with targeted disruption of a single PKB isoform were generated and all demonstrate a distinct phenotype. Interestingly, the PKBβ knock out mice exhibit a diabetes-like syndrome with hyperglycemia and impaired insulin action in fat, liver and skeletal muscle and, depending of the genetic background, mild growth retardation (Cho et al., 2001, Garofalo et al., 2003). However, mice deficient in PKBα revealed a normal glucose metabolism, but display an approximately 30% reduction in

body size and partial neonatal lethality, which might be caused by placental insufficiency (Cheng et al., 2001, Cho et al., 2001, Yang et al., 2003). Recently, Peng and colleagues reported the successful generation of PKBαβ double mutant mice. They exhibit a severe intrauterine growth retardation syndrome and they die briefly after birth. Moreover, they present multiple defects in the skin, bone and fat (Peng et al., 2003). It has been speculated that the distinct phenotypes of PKBα and PKBβ mutant mice are due to specific and separate functions of the disrupted PKB isoform. Previously, Peng and colleagues proposed an alternative explanation for the individual phenotypes (Peng et al., 2003). They hypothesized that the individual phenotype could be due to the loss of the dominant isoform in a special tissue, which leads to a significant reduction of the total activated PKB below a critical level. For example, the observed diabetes mellitus-like syndrome of PKBβ $^{-/-}$ mice could be due the ablation of the dominant isoform (PKBβ) in the classic insulin responsive tissue like fat, liver and muscle. Additionally, the loss of a second isoform would have an additive effect in reducing the total PKB and therefore would intensify the phenotype. This proposition is further supported by our initial observation from the PKBαγ double knock out mice. The additional ablation of a single copy of PKBγ in PKBα deficient mice leads to an even more increased perinatal mortality, and the ablation of both PKBγ alleles leads to intrauterine death of PKBαγ double knock out (Yang et al., unpublished data). However, it cannot be ruled out yet that the observed phenotypes are due to a combination of both the reduced total PKB level and the loss of an isoform-specific function.

It has been shown that the PI3-K/PKB signaling pathway plays a crucial role in the determination of cell size (Scanga et al., 2000; Shioi et al., 2002; Tuttle et al., 2001a; Verdu et al., 1999b). Results from Transgenic mice over-expressing PKB showed an

increased size of cardiac myocytes, larger thymocytes or hypertrophy and hyperplasia in the pancreas (Kovacic et al., 2003; Mangi et al., 2003). In mice with brain specific deletion of PTEN an increased neuronal soma size was observed (Backman et al., 2001; Kwon et al., 2001). In contrast, in PKBαβ double mutant mice the size of skeletal muscle cells was dramatically reduced (Peng et al., 2003). Our results show that both cell number and cell size are affected, but the relative contributions of the cell size reduction seems to be more prominent than the reduction of cell number. Additionally, it has been shown that the mTor signaling pathway is also involved in the determination of cell size (Montagne et al., 1999; Oldham et al., 2000; Zhang et al., 2000). PKB modulates mTor activity by phosphorylating TSC2 with a subsequent disassembling of the TSC1-TSC2 complex (Inoki et al., 2002, Manning et al., 2002, Potter et al., 2002). However, we observed only a slight reduction of the phosphorylation level of TSC2 and p70S6Kinase in the brains of PKBγ mutant mice. Taken together, we demonstrated that PKBγ deficient mice display a distinct phenotype when compared to PKBα and PKBβ mutant mice. We emphasized the importance of PKB in determination of cell size and number. Our study provides novel insights into the function of PKBγ and suggests a critical role in the postnatal brain development in mammals.

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Chapter III

PKBα/Akt1 is more important than PKBγ/Akt3 for mouse survival but both are required for mouse development

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SUMMARY

The three protein kinase B (PKB/Akt) isoforms —PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 display multiple similarities in domain structure and cellular function. Given that PKB is essential for cell survival, the existence of viable knockout mice for each gene suggests redundancy of the three proteins. Nevertheless, each deletion causes distinct phenotypes, indicating different physiological roles. Here we show that *Pkbα*$^{-/-}$*Pkbγ*$^{+/-}$ compound mutant mice display multiple organ/tissue pathology and the majority die early, whilst *Pkbα*$^{+/-}$ *Pkbγ*$^{-/-}$ mice survive normally. Double knockout (*Pkbα*$^{-/-}$*Pkbγ*$^{-/-}$) causes embryo lethality at around E12, with developmental defects in the placenta, brain and branchial arch arteries. These data indicate that PKBα is more essential than PKBγ for animal survival, but that both are required for placenta and embryo development. There are also suggestions of isoform-specific and dosage-dependent effects of PKB on animal survival and development.

INTRODUCTION

Protein kinase B (PKB, also known as Akt) belongs to a subfamily of serine/threonine protein kinases called AGC protein kinases that includes protein kinase C (PKC), ribosomal S6 kinase (S6K), the cyclic-AMP-dependent protein kinase (PKA), the serum and glucocorticoid-induced protein kinase (SGK) and others (Brazil and Hemmings, 2001; Lawlor and Alessi, 2001). In mammals such as mouse, rat and human, the PKB family consists of three isoforms, PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3, encoded by three independent genes on distinct chromosomes (Brazil and Hemmings, 2001; Datta et al., 1999; Lawlor and Alessi, 2001; Scheid and Woodgett, 2001). In mouse and humans, PKBα, β and γ proteins are made up of 480, 479 and 481 amino acids respectively, and have similar domain structures: N-terminal pleckstrin homology (PH) domain, intermediate catalytic domain and C-terminal regulatory domain. The amino acid constitution of the catalytic domain in these proteins is almost identical. All three proteins can be activated by numerous growth factors in a phosphatidylinositol 3-kinase (PI 3K)-dependent manner at the cell membrane (Brazil and Hemmings, 2001; Datta et al., 1999; Lawlor and Alessi, 2001; Scheid and Woodgett, 2001).

Approximately30-40 proteins have been reported to be substrates of PKB. The majority of these substrates have a consensus motif of RXXRXS/T, where X can be any amino acid and S/T is the serine/threonine phosphorylation site of PKB (Brazil and Hemmings, 2001; Lawlor and Alessi, 2001). Determination of this motif in a protein and its conservation in several vertebrate homologue can be used to predict PKB substrates. Based on this, Yaffe and Cantley developed a computer programme to score all known proteins in a database search for PKB targets, which can facilitate the discovery of novel proteins in the PKB signalling pathway and help to understand

PKB functions (Yaffe et al., 2001). The functions of numerous identified or predicted substrates indicated that PKB is involved in many cellular and physiological processes such as cell survival, cell cycle progression, cell growth, metabolism and angiogenesis. Although many proteins have been identified as PKB substrates through *in vitro* biochemical studies and *in vivo* cell transfection assays, the challenge that remains is to show that they actually have an important impact on physiological processes in organisms. Studies in Drosophila of PKB (dAkt) overexpression and mutation have recognized its importance in organimal growth regulation and have confirmed the predicted functions of PKB (Leevers et al., 1996b; Stocker et al., 2002; Verdu et al., 1999a). Recently, targeted deletion of specific isoform of PKB in mouse has proved to be a powerful tool for elucidating the physiological roles of PKB proteins (Chen et al., 2001; Cho et al., 2001a; Cho et al., 2001b; Garofalo et al., 2003; Yang et al., 2003). Characterization of such knockout mice has yieled intriguing and surprising results. First, we and others have found that inactivation of a single PKB isoform does not affect embryo development of animal, despite the fact that ~ 40% of *Pkbα* knockout mice die at a neonatal stage. This suggests that the other two PKB isoforms can compensate for the absence of PKBα (Chen et al., 2001; Cho et al., 2001b; Yang et al., 2003). However, no upregulation of the other two isoforms was observed at the protein level. Second, knockout of each single isoform gives rise to a distinct phenotype. In general, PKBα null mice are small in size, which may result from placental insufficiency, while PKBβ-deficient mice develop a type 2 diabetes-like syndrome (Chen et al., 2001; Cho et al., 2001a; Cho et al., 2001b; Garofalo et al., 2003; Yang et al., 2003). These observations, on the other hand, indicate that the three PKB isoforms have different non-redundant physiological functions. As mentioned above, PKB proteins have a very high homology with similar domain structures, and show identical

functions in cell transfection assays. The relatively normal development and distinct physiological functions demonstrated by single knockouts may be explained by differences at the tissue distribution and expression levels of these isoforms. For example, in the major insulin responsive tissues of fat, skeletal muscle and liver, PKBβ is the predominant isoform (Altomare et al., 1998a; Cho et al., 2001a; Yang et al., 2003). Inactivation of this isoform in mouse may greatly affect glucose metabolism and causes diabetes. Similarly, we found that PKBα is the major isoform in placenta and that placenta lacking this protein can not form a proper vascular structure; this may restrict nutrient supply to the foetus and impair growth (Yang et al., 2003).

Thus, one could expect that simultaneous inactivation of two PKB isoforms in mice would severely affect development and survival. Recently, it was shown that double knockout mice of PKBα/β die shortly after birth (Peng et al., 2003). These mice displayed a more obvious phenotype than single knockout mice, including dwarfism, impaired skin development, skeletal muscle atrophy and abnormal bone development. However, the results are surprising in that one isoform (PKBγ) is still sufficient for animals to develop to term.

We crossed Pkbα/Pkbγ knockout mice to generate compound and double knockout mice. Our observation shows that PKBα is more important than PKBγ for survival and that both proteins are required for normal embryo development. Our studies demonstrate isoform-specific and dosage-dependent effects of PKB proteins in physiology, animal survival and development.

METHODS

Mice

The animal work was performed in accordance with the Swiss law on animal welfare and protection. *Pkbα* $^{-/-}$ mice have been described previously (Yang et al. 2003). The generation of *Pkbγ* null mice was described elsewhere (Tschopp et al. submitted). *Pkbα* $^{-/-}$ males were mated with *Pkbγ* $^{-/-}$ females to produce double heterozygous mice (*Pkbα* $^{+/-}$*Pkbγ* $^{+/-}$). Intercross of double heterozygous mice gave rise to compound mice with 9 genotypes that were analysed by PCR. Primers used for genotyping were as described previously (Yang et al., 2003; Tschopp et al., submitted). Matings between *Pkbα* $^{+/-}$*Pkbγ* $^{-/-}$ males and *Pkbα* $^{-/-}$ females were set up in order to examine the postnatal survival of *Pkbα* $^{-/-}$ *Pkbγ* $^{+/-}$ mice. The progeny were all *Pkbγ* $^{+/-}$ and half of them were *Pkbα* $^{-/-}$ (half *Pkbα* $^{-/-}$ *Pkbγ* $^{+/-}$). To increase the number of double knockout mice, we set up matings between *Pkbα* $^{+/-}$*Pkbγ* $^{-/-}$ males and females. The percentage of double knockout mice was 25%. All these mice had 129 Ola and C57BL/6 mixed background.

BrdU incorporation

E10.5 to E11.5 pregnant mice were injected intraperitoneally (IP) with BrdU (1mg/20g body weight). The mice were sacrificed 2 h later and embryos together with placentas were dissected. After washing with PBS, the samples were fixed in formalin at 4^{o}C overnight. Cryosections were prepared for staining.

Histology

After dissection, embryos and tissues were treated by, fixation in 10% formalin at 4°C overnight; washing in PBS at 4°C for 30 min, washing in 0.85% NaCl (in H_2O) at room temperature for 30 min, washing twice in 50% EtOH (in 0.85% NaCl) at room temperature for 20 min, washing twice in 70% EtOH (in PBS) at room temperature for

20 min. Treated samples were processed for paraffin embedding and 10 μm sections cut for haematoxylin and eosin staining.

The immunohistochemistry protocol was as described previously (Yang et al., 2003). Briefly, tissues or embryos were fixed in formalin at 4°C overnight, transferred to 0.5 M sucrose/PBS solution at 4°C overnight, and washed in PBS. Then the samples were embedded in OCT compound (Tissue Tek) and 20 μm sections cut. The sections were treated with 0.3% H_2O_2 in methanol for 30 min to inactivate endogenous peroxidase, blocked in 5% normal goat serum in PBS for 30 min, and then incubated at 4°C overnight with antibody for PKBα and γ (Yang et al., 2003) as well as BrdU, and PECAM (Pharmingen). The sections were then processed as described in the protocol of the Vectastain ABC kit (Vector Laboratories).

RESULTS

Dominant role of PKBα over PKBγ in postnatal mouse survival

Pkbα and *Pkbγ* genes have been successfully targeted by homologous recombination in our lab (Yang et al., 2003;Tschopp, submitted). Previously, we observed that the *Pkbα*$^{-/-}$ mice develop to term and that around 60% of these mice survive normally (Yang et al., 2003). Similarly, *Pkbβ*$^{-/-}$ and *Pkbγ*$^{-/-}$ mice also develop normally during gestation. Nevertheless, *Pkbβ*$^{-/-}$ and *Pkbγ*$^{-/-}$ mice did not show increased postnatal mortality (Cho et al., 2001a; Garofalo et al., 2003). It is well known that PKB proteins play pivotal roles in multiple cellular and physiological processes, such as cell survival and angiogenesis, that are essential for normal embryo development. However, the existence of viable single knockout mice for each isoform suggestss functional redundancy of the three proteins. The presence of any two PKB proteins can meet the developmental requirements of mice. Given that around 40% of *Pkbα*$^{-/-}$ mice die

neonatally, it is highly possible that inactivation of PKBβ or PKBγ together with PKBα has a severe impact on animal development, and intensifies morbidity and increases mortality of the mice. To test this, we crossed *Pkbα* and *Pkbγ* mutant mice to produce various compound mice. We first mated $Pkb\alpha^{-/-}$ males with $Pkb\gamma^{-/-}$ females to generate $Pkb\alpha^{+/-}Pkb\gamma^{+/-}$ (double heterozygous) mice. Except for mild growth retardation (~10% smaller than wild-type at birth), these mice had no apparent defects and grew normally. Intercross of these double heterozygous mice gave rise to offspring with 9 genotypes (Table 3.1) genotyped at around 1 month old. The number of $Pkb\alpha^{+/-}$ $Pkb\gamma^{-/-}$ mice was in accordance with the expected Mendelian ratio and these mice appeared normal (Table 3.1).

Table 3.1. Progeny of $Pkb\alpha^{+/-}Pkb\gamma^{+/-}$ Matings

Genotype	$\alpha^{+/+}\gamma^{+/+}$	$\alpha^{+/+}\gamma^{+/-}$	$\alpha^{+/+}\gamma^{-/-}$	$\alpha^{+/-}\gamma^{+/+}$	$\alpha^{+/-}\gamma^{+/-}$	$\alpha^{+/-}\gamma^{-/-}$	$\alpha^{-/-}\gamma^{+/+}$	$\alpha^{-/-}\gamma^{+/-}$	$\alpha^{-/-}\gamma^{-/-}$
Number of animals	27	34	20	31	74	37	11	3	0
Percentage	11.3	14.3	8.4	13	31	15.5	4.6	1.3	0
Theoretical percentage	6.3	12.5	6.3	12.5	25	12.5	6.3	12.5	6.3

Total number:237

However, very few $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ mice (3 vs 37 of $Pkb\alpha^{+/-}Pkb\gamma^{-/-}$) survived at the time of genotyping. These results supported our hypothesis that haplo-insufficiency of PKBγ in $Pkb\alpha^{-/-}$ mice intensifies the phenotype and reduces the survival of the mice. Actually, the mortality increased from ~ 40% in single $Pkb\alpha^{-/-}$ mice to ~90% in $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ mice, while $Pkb\gamma^{-/-}$ and $Pkb\alpha^{+/-}Pkb\gamma^{-/-}$ mice did showed no increase in mortality. These results suggested that PKBα is more important that PKBγ for animal survival.

$Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ mice display severe growth retardation and multiple organ/tissue pathology

As approximately 90% of the $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ mice were lost before genotyping at about 1 month old, we determined at what stage these mice die. Matings were set up between $Pkb\alpha^{+/-}Pkb\gamma^{-/-}$ males and $Pkb\alpha^{-/-}$ females. Theoretically, offspring of this mating are half $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ and half $Pkb\alpha^{+/-}Pkb\gamma^{+/-}$ (1:1). Of the 20 newborns collected from 4 litters, 8 were $Pkb\alpha^{+/-}Pkb\gamma^{+/-}$ and 12 $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$, which is consistent with the Mendelian ratio. Therefore, $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ can develop to term. Bodyweight of $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ newborns was comparable to that of $Pkb\alpha^{-/-}$ ($Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ 1.064 ± 0.11g; $Pkb\alpha^{-/-}$ 1.099 ± 0.11g, P=0.46; Table 3.2). This indicates that haplo-insufficiency of PKBγ in a PKBα-null background has little effect on the growth of embryos. Follow-up observations of the $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ mice revealed severe growth deficiency and these mice died within 10 days (Fig 3.1A, Table 3.2). Newborns and 3-day-old $Pkb\alpha^{+/-}Pkb\gamma^{+/-}$ and $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ littermates were sacrificed and major organs/tissues were dissected for macroscopic and histological studies. The stomachs of $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ mice contained milk, indicating ability to suck (Fig 3.1E). The thymuses of

Table 3.2 Body Weight of *WT*, $Pkb\alpha^{-/-}$, $Pkb\alpha^{+/-}\gamma^{+/-}$ and $Pkb\alpha^{-/-}\gamma^{+/-}$ Mice

Body weight: g	*WT*	$Pkb\alpha^{-/-}$	$Pkb\alpha^{+/-}\gamma^{+/-}$	$Pkb\alpha^{-/-}\gamma^{+/-}$
Newborn (P1) (mean ± SD)	1.49 ± 0.16 (n=15)	1.099 ± 0.11 (n=11)	1.33 ± 0.1 (n=8)	1.064 ± 0.11 (n=11) a
P3	N/A	N/A	2.29 ± 0.31 (n=4)	1.465 ± 0.33 (n=6) b

a) The difference in body weight of $Pkb\alpha^{-/-}$ and $Pkb\alpha^{-/-}\gamma^{+/-}$ newborns was not significant (P=0.46).
b) $Pkb\alpha^{-/-}\gamma^{+/-}$ mice grew slower than $Pkb\alpha^{+/-}\gamma^{+/-}$ littermates.

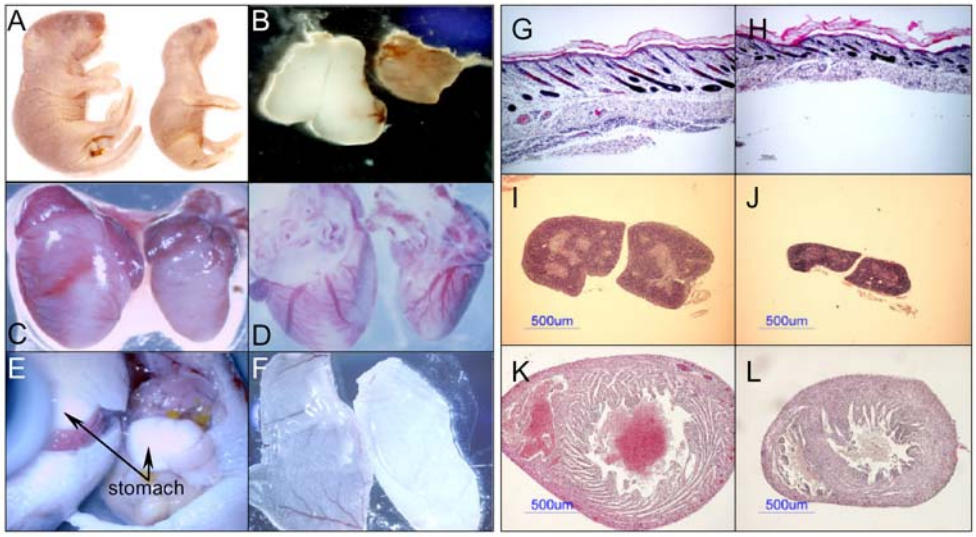

Figure 3.1. Severe Growth Deficiency and Multiple Pathology of *Pkbα*$^{-/-}$*γ*$^{+/-}$ Mice.

(A-F) Three-day-old *Pkbα*$^{+/-}$*γ*$^{+/-}$ and *Pkbα*$^{-/-}$*γ*$^{+/-}$ littermates together with their organs. In each panel, left is *Pkbα*$^{+/-}$*γ*$^{+/-}$ and right *Pkbα*$^{-/-}$*γ*$^{+/-}$.

(A) Mice. (B) Thymus. (C) Heart, anterior side. (D) Heart, posterior side. (E) Stomach. Both are full of milk. (F) Skin, interior side (from the back, the heart level).

(G-L) Histological studies of the skin, thymus and heart from *Pkbα*$^{+/-}$*γ*$^{+/-}$ and *Pkbα*$^{-/-}$*γ*$^{+/-}$ mice. (G) (I) (K) *Pkbα*$^{+/-}$*γ*$^{+/-}$ and (H) (J) (L) *Pkb α*$^{-/-}$*γ*$^{+/-}$. (G,H) Skin. x100. (IJ) Thymus. x40. (KL) Heart, transverse sections. x40.

Pkbα$^{-/-}$*Pkbγ*$^{+/-}$ mice were atrophied (Fig. 3.1B). Haplo-insufficiency of PKBγ together with PKBα loss mainly affected the size of heart ventricles; atria size was similar to that of *Pkbα*$^{+/-}$*Pkbγ*$^{+/-}$ littermates (Fig 3.1C, D). The skin of *Pkbα*$^{-/-}$*Pkbγ*$^{+/-}$ mice was loose and thin with scarcely visible subcutaneous vasculature (Fig. 3.1D). The density of adnexal structure was similar in *Pkbα*$^{-/-}$*Pkbγ*$^{+/-}$ mice and wild-type, but dermal and subcutaneous mesenchymal tissue was diminished in the mutant, particularly subcutaneous fat and adnexal structures positioned close to each other (Fig. 3.1G and

1H). Hassal's corpuscles formed in the thymus of *Pkbα*$^{-/-}$*Pkbγ*$^{+/-}$ mice and starry sky cortical apoptosis was apparent; this is attributable to increased stress of asphyxia (Fig. 3.1I,J). Intramyocardial vessel density was reduced in the hearts of *Pkbα*$^{-/-}$*Pkbγ*$^{+/-}$ mice. Myocardial muscle fibres were shorter and not as well aligned as in *Pkbα*$^{+/-}$ *Pkbγ*$^{+/-}$ mice. In addition, trabecular muscle was less compact in *Pkbα*$^{-/-}$*Pkbγ*$^{+/-}$ hearts (a minor degree of 'non-compaction').

PKBα and PKBγ are required for mouse development

Genotyping of progeny from *Pkbα*$^{+/-}$*Pkbγ*$^{+/-}$ double heterozygous intercrosses at the time of weaning showed that no double knockout mice survived (Table 3.1). Because the majority of the *Pkbα*$^{-/-}$*Pkbγ*$^{+/-}$ mice die young, it is highly possible that double knockout mice were lost during gestation. To test this, we set up matings between *Pkbα*$^{+/-}$*Pkbγ*$^{-/-}$ males and females to increase the number of double knockout progenies (1 in 4 is a double knockout). Dissection at E14 detected no double knockout embryos. E11 embryos were dissected and genotyped and double knockout embryos were found with morphology comparable to *Pkbα*$^{+/+}$*Pkbγ*$^{-/-}$ littermates. The isolation of embryos one day later at E12 showed that some double knockout embryos had either already disappeared, leaving only the placenta, or they had abnormal morphology (Table 3.2, and Fig. 3.2A-C). These data indicate that double knockout mutants die between E11 and E12.

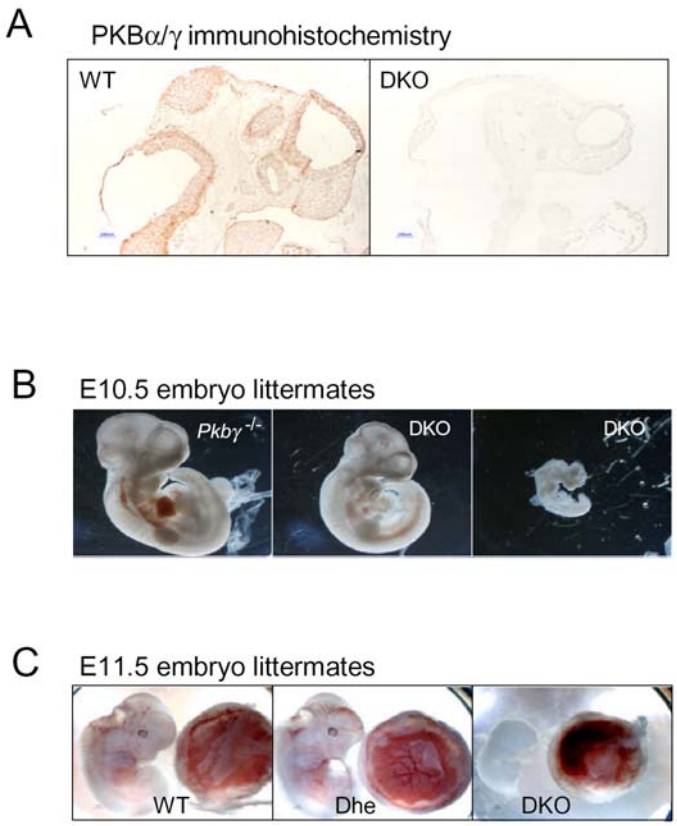

Figure 3.2. E10.5 and E11.5 Embryos.

(A) Immunohistochemistry with PKBα/γ antibody showing the absence of the two proteins in E10.5 double knockout embryos.

(B) E10.5 littermates. Note the abnormal development and morphology of one double knockout embryo.

(C) E11.5 littermates. Only some remnants of the double knockout embryos are left.

WT, wild-type; DKO, double knockout; Dhe, double heterozygous.

Tissue distribution of PKBα/γ in E11.5 and 12.5 embryos

Because *Pkbα*$^{-/-}$*Pkbγ*$^{-/-}$ mice die between E 11 and 12, we studied the localization of the two isoforms in wild-type embryos at these stages to determine where they function. Immunohistochemical staining was performed on cryosections with an antibody recognizing both mouse PKBα and γ but not β. At E11.5, these two proteins were mainly in the central nervous system and heart (Fig. 3.3A-F). At E12.5, they

were expressed at high levels in the central and peripheral nervous system, including the brain and various ganglia (Fig. 3.3G-J). These tissue distribution patterns may indicate that the two proteins are important for brain and cardiovascular development at E11.5 to 12.5.

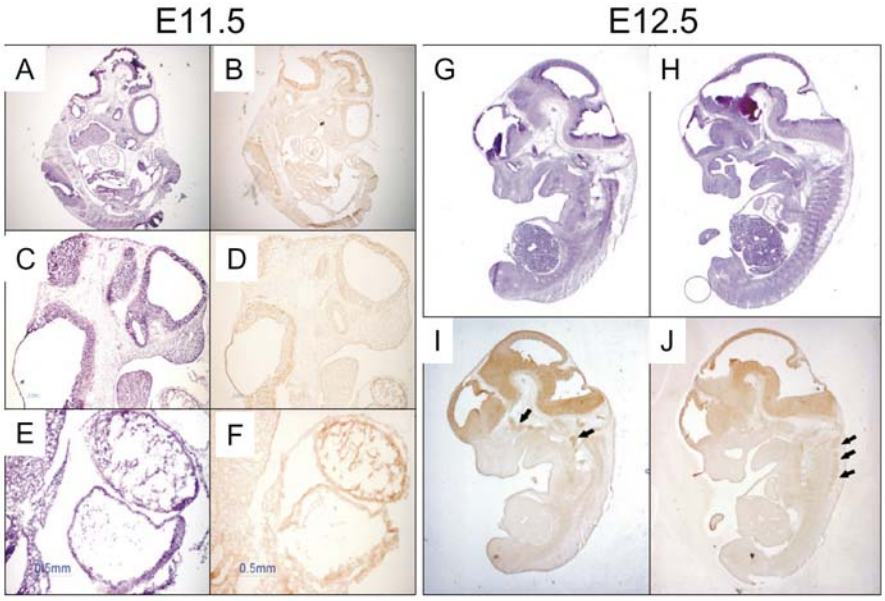

Figure 3.3. PKBα/γ Distribution in E11.5 and E12.5 Mice.

(A-F) E11.5. (A) (C) (E) H&E staining and (B) (D) (F) immunohistochemical staining with PKBα/γ antibody. (E) (F) showing the heart.

(G-J) E12.5. (G) (H) H&E staining and (I) (J) Immunohistochemical staining with PKBα/γ antibody. The arrows indicate various ganglia.

Abnormal development in double knockout embryos

At E11, wild-type mice showed branchial arch development corresponding to 11 days, with prominent bulging of branchial arch contours (Fig. 3.4A). One or two larger and many smaller vascular channels were visible within the mesenchyme of each of the first two to three branchial arches, with delicate branching into multiple small vascular channels in the subcoelomic area. The delicate small vessels were not well defined in conventional histology but were highlighted by PECAM staining (Fig. 3.4A). The dorsal aorta ran along the mesenchyme, separating the dorsal aspect of the neural tube

towards the caudal somites from the coelomic cavity, and showed a medium lumen diameter (not shown). The forebrain, midbrain and hindbrain displayed a regular circular structure with multiple layers of cells (Fig. 3.4C).

In contrast, the PKBα/γ double knockout mice demonstrated hypoplastic, hardly scalloped branchial arches with only little mesenchyme (Fig. 3.4A). Vessels within the branchial arches were fewer than in the wild-type mice but had diameters twice that of the wild-type vessels. Subcoelomic ramifications of these vessels in the branchial arches were scarce and only little mesenchyme was formed around the blood vessels. Similarly, the dorsal aorta was larger than in wild-type mice (not shown). The neural tube appeared multiloculated in the double knockout mice and the perineural mesenchyme was permeated by multiple irregular dilated and thin-walled vascular channels. Brain structure was disrupted in some embryos (Fig. 3.4B). This phenotype was more prominent in E12 embryos (Fig. 3.4C). The delicate edges of the brains were curled and folded, with extremely low cellularity (Fig. 3.4B, C). BrdU staining of E11 embryos showed no large differences between wild-type and double knockout mice (Fig. 3.4B). However, the decreased and altered cell layers in the brain indicate abnormal differentiation of the neural cells in double knockouts.

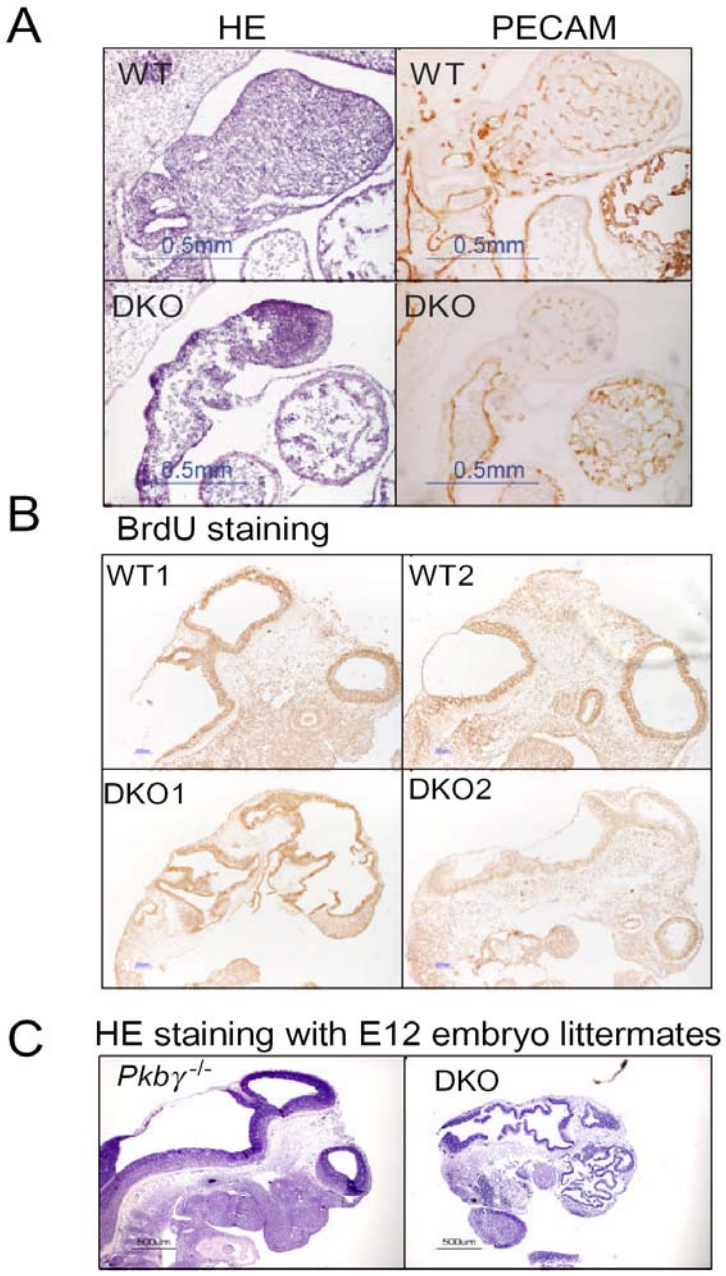

Figure 3.4. Histological Analysis of E11 and E12 Embryos.

(A) Branchial arch and its vasculature in E11 embryos. Note that the branchial arch of double knockout embryo is hypoplastic with a dilated artery, and that there are fewer micro-vessels. (B) BrdU staining of E11 embryos. In the DKO1 embryo, the brain structure is disrupted with fewer cell layers. In the DKO2 embryo, the cellularity in the brain is low.

(C) E12 embryos. The DKO embryo shows irregular and curled brain architecture, especially in the forebrain.

Altered placental development in double knockout mutant mice

Previously, we found defects in placental architecture in PKBα null mice, including hypotrophy, loss of glycogen-containing spongiotrophoblast cells and reduced vascularization (Yang et al., 2003). As placental growth and development proceed until E17.5, we performed BrdU staining with E11 placentas to study cell proliferation. In the wild-type placenta, the spongiotrophoblast cells showed prominent proliferation signals, while the signals were much weaker and the spongiotrophoblast layer could not be discerned in double knockout placenta (Fig. 3.5A-F). In E12 placentas, alteration of the labyrinth was apparent with double knockout placenta. The total area was reduced significantly (Fig. 3.5G-I) and both H&E and PECAM staining showed massive reduction in foetal vessels (Fig. 3.5J-O). In wild-type placenta, foetal vessels were broadly and evenly distributed within maternal blood spaces and filled with foetal immature nucleated erythroid cells. In the double knockout placenta, there were many endothelial cells but not in the form of vessel structures.

DISCUSSION

Here we report observations of PKBα/γ compound knockout mice. Loss of PKBα together with haplo-insufficiency of PKBγ caused severe post-natal survival consequences in these mice. The mice showed serious growth deficiency and nearly all died young with multiple organ/tissue pathology. Mice lacking both proteins were embryo lethal at around E12 and displayed fatal developmental defects. These results differ from those with PKBα/β compound knockout mice and have significant implications.

Isoform-specific and dosage-dependent effects of PKB on mouse survival

In the single knockouts of PKB isoforms, PKBβ and PKBγ null mice had no neonatal survival disadvantages. Nevertheless, we and others observed that around 40%

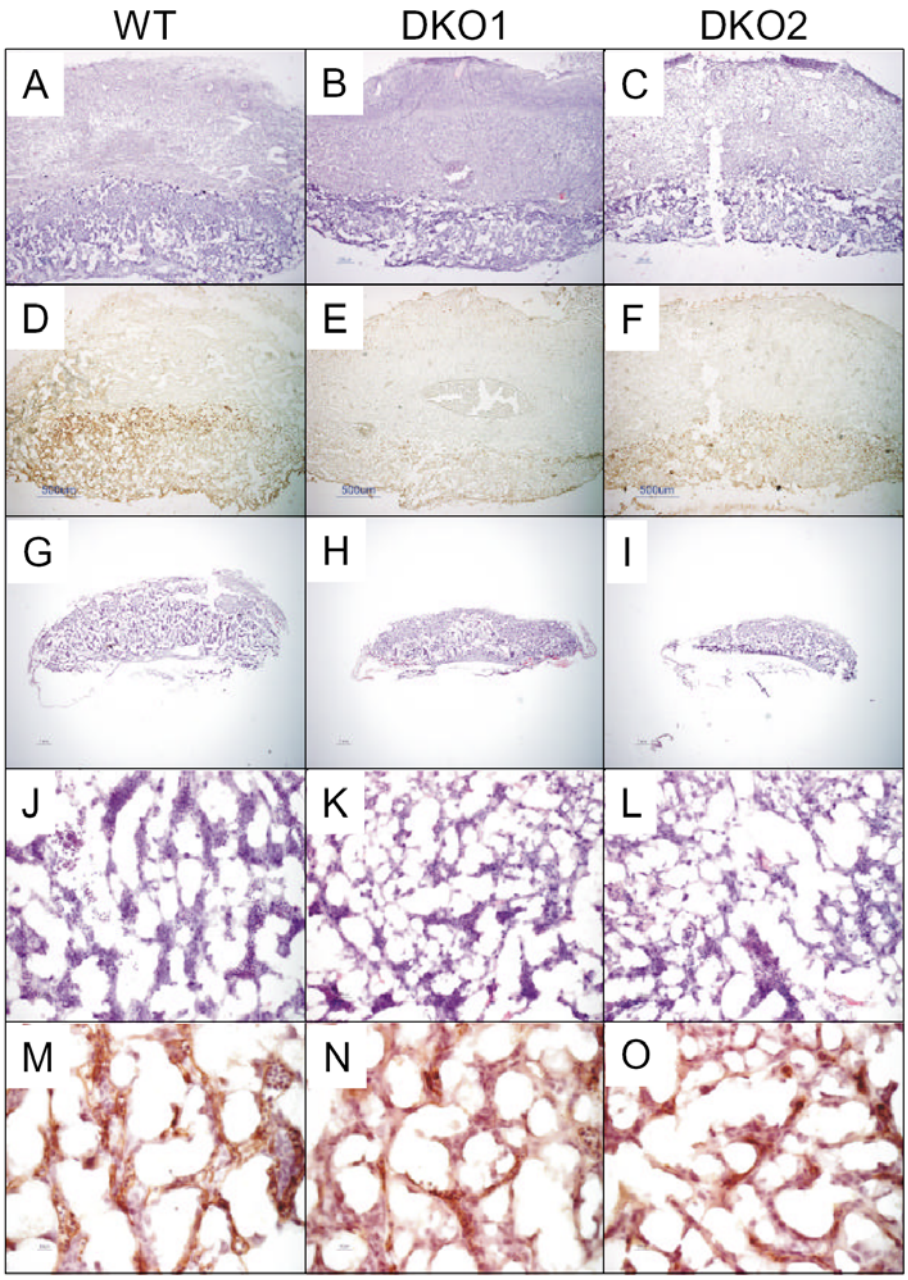

Figure 3.5. Histological Studies of E11 and E12 Placentas.

(A-F) E11 placentas. (A) (B) (C) H&E staining and (D) (E) (F) BrdU staining. In wild-type placenta, the layer of spongiotrophoblasts shows strong proliferation signals, whilst the signals are dramatically reduced in the double knockout placentas.

(G-O) E12 placentas. (G)-(L) H&E staining and (M) (N) (O) PECAM staining. (G-I) In the double knockout placentas, the total area of labyrinth is significantly reduced compared with the wild-type.

(J-L) Higher magnification of (G-I).

(M-O) In the wild-type placenta, foetal vessels are distributed among the maternal blood spaces but in the double knockout placentas, very few foetal vessels were found.

of PKBα null mice fail to survive the neonatal age (Cho et al., 2001b; Yang et al., 2003). This raises the intriguing possibility that PKBα is the most critical isoform of the three for animal survival.

No indications of post-natal survival difficulties was found with $Pkb\alpha^{-/-}Pkb\beta^{+/-}$ mice (Peng et al., 2003). However, we found that $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ mice have increased post-natal mortality from ~40% in single $Pkb\alpha^{-/-}$ mice to ~90%. Therefore, PKBγ also contributes to animal survival. This hypothesis is supported by the developmental status of PKBα/β and PKBα/γ double knockout mice. PKBα/β double knockout mice with only PKBγ develop to term. But in the presence of only PKBβ in PKBα/γ double knockout mice, the mice do not develop beyond the mid-term. This indicates a more important role for PKBγ than PKBβ in animal development and survival. Based on this, given the presence of PKBα in PKBβ/γ double knockout mice, these animals should survive the whole gestation.

Previously, we examined the expression levels of the three PKB mRNA in mouse tissues and organs by quantitative PCR. At least two isoforms were expressed in each tissue/organ (Yang et al., 2003). Earlier comprehensive and intensive studies using cell transfection assays showed that the three PKB proteins have similar properties in terms of their activation by growth factors such as insulin and insulin-like growth factors,

and subsequent phosphorylation of substrate proteins like GSK3 (Brazil and Hemmings, 2001). Therefore, the isoform-specific effects observed in single and double knockout mice may result from differences in tissue distribution and expression levels of the three isoforms, which are determined by the organisms according to physiological necessity. In another word, the non-isoform-specific effects of the three PKB proteins in cell transfection studies become isoform-specific in physiology.

The essential role of PKBα/γ in mouse development

In the past, extensive studies of PKB proteins produced the undisputed conclusion that PKB is a powerful opponent of apoptosis. This may oversimplify the roles of multi-faceted PKB in physiology. The phenotype manifested by PKBβ null mice has clarified this point. Nothing related to apoptosis was observed in these mice but, instead, the pivotal roles of PKB in regulating glucose metabolism and insulin action have been confirmed and are now well accepted.

In this study, we have demonstrated that PKBα and PKBγ are essential for embryogenesis and development. In our previous study with PKBα null mice, we showed that this protein is an important regulator of placental development and foetal growth. The development of the placenta occurs at an early stage of embryogenesis. Here , with $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ mice, we observed impaired development of the skin, thymus and ventricle myocardium. Mice lacking PKBα and PKBγ do not survive beyond the mid-term and show multiple developmental defects. Therefore, PKB is involved in a variety of processes of embryogenesis and organogenesis.

Placental insufficiency and embryo intrinsic mechanisms in PKBα/γ double knockout mice

PKBα/γ double knockout mice are embryo lethal at around E12. The causes of death fall into two categories, placental insufficiency and embryo intrinsic mechanisms.

Previously, characterization and analysis of PKBα null mice led us to the conclusion that developmental abnormalities and small size of placenta restrict the growth of the embryo. However, we do not exclude the possibility of embryo intrinsic mechanisms in the control of foetal growth in the absence of PKBα. In addition, all three PKB proteins can be detected in wild-type placenta by Western blotting, albeit that PKBα is the predominant isoform. This suggests that the development of placenta is impaired further in PKBα/γ double knockout mice and that the double knockout embryo is in more unfavourable conditions. Increasing evidence has shown placental insufficiency to be the primary cause of embryo lethality in many knockouts, e.g. in the case of *Rb* deletion and SOCS3 inactivation (Takahashi et al., 2003; Wu et al., 2003). James Ihle recently wrote a review on placental defects-dependent embryo lethality in numerous knockout mice showing placenta as a critical determining factor for death (Ihle, 2000). Frequently, it is seen that foetal vascularization is not properly established in the labyrinth of these knockout placentas; as a result, the growth and development of the foetus is suppressed. Under these adverse conditions, the foetus develops abnormally and eventually dies (Rossant and Cross, 2001).

The phenotypes manifested by $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ mice, on the other hand, strongly support the view that embryo intrinsic mechanisms are responsible for the embryo lethality. The 50% loss of PKBγ does not affect the growth of embryos and, the bodyweight of these mice at birth is comparable to that of single PKBα null mice. Nevertheless, the $Pkb\alpha^{-/-}Pkb\gamma^{+/-}$ mice die within 10 days of their birth and display developmental abnormalities of skin, thymus and heart. Because after birth the development, growth and remodelling of organs/tissues become independent of the placenta, these effects may result from the complete absence of PKBα and 50% loss of PKBγ. In the ventricle myocardium, there are three layers of muscles with distinct

fibre orientations, outer spiral, middle circular and inner longitudinal. The organization of these muscle fibres determines the contractility of the heart. The observed alteration in myocardiac architecture in the ventricle of *Pkbα*$^{-/-}$*Pkbγ*$^{+/-}$ mice suggests that these mice die from heart failure. This is consistent with the heart-specific deletion of PDK1 mice (Mora et al., 2003).

Transcriptional regulation by PKB during development

The multiple organ/tissue defects in *Pkbα*$^{-/-}$*Pkbγ*$^{+/-}$ mice and the embryo lethality of PKBα/γ double knockout mice led us to the hypothesis that certain transcription factors essential for development are PKB substrates or are modulated by PKB. Phosphorylation of these transcription factors by PKB, directly or indirectly, regulates their transcriptional activities and subsequent developmental events. The phenotypes displayed by the placenta and branchial arch arteries in PKBα/γ double knockout mice resemble those of Hand1(eHAND) and Hand2 (dHAND) knockout mice (Firulli et al., 1998; Riley et al., 1998; Yamagishi et al., 2000; Yanagisawa et al., 2003). The appearance of *Pkbα*$^{+/-}$*Pkbγ*$^{+/-}$ mice is similar to Twist null mice (Sosic et al., 2003). Hand1, Hand 2 and Twist proteins belong to the basic helix-loop-helix (bHLH) transcription factor family, which is involved in numerous essential developmental processes (Firulli et al., 1998; Riley et al., 1998; Yanagisawa et al., 2003). Recently, it was reported that Trachealess (Trh), a bHLH-PAS transcription factor required for development of the trachea and other tubular organs in *Drosophila*, is a new PKB/Akt target (Jin, et al.2001). Phosphorylation of Trh by PKB is essential for its nuclear localization and regulation of branching morphogenesis. In another report, it was suggested that PKB regulates the interaction between neural bHLH proteins and the CBP/p300 to promote neuronal differentiation (Vojtek, et al. 2003). More recent work showed that Hand proteins can be phosphorylated by PKC and PKA and that

phosphorylation of these proteins affects chicken development. Coincidentally, one phosphorylation site seems to have a PKB consensus motif (Firulli et al., 2003). Further work to investigate the connection between PKB proteins and bHLH in development is needed.

In summary, we found that PKBα is more important than PKBγ for animal physiology and survival, and that both proteins are required for normal mouse development.

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Final discussion

The aim of the work described in this thesis was to examine the physiological and developmental roles of PKB/Akt proteins in mice by loss of function analysis through gene targeting technology. Complete knockout mice for each isoform have been produced, as well as mice with floxed alleles of *Pkbα/Akt1* and *Pkbβ/Akt2* for future analyses. Compound and double knockout mice of PKB/Akt were produced by crossing single knockout mice. Characterization of these mice has unveiled novel functions of PKB/Akt in development and physiology. These results will help improve our future understanding of these kinases.

This thesis concerns in particular the single knockout of PKBα/Akt1 and of PKBγ/Akt3, and the compound knockout of PKBα/Akt1 and PKBγ/Akt3. Studies of PKBα/Akt and PKBγ/Akt3 mutant mice should help determine the specific role of these two isoforms in mice and reveal functional differences between all three isoforms. Analyses of PKBα/Akt1 and PKBγ/Akt3 compound and double knockout mice have provided insights into the role of PKB/Akt in mouse development. The generation and characterization of the PKBα/Akt1 mutant mouse line, as the first of its type in our laboratory has defined methodology and expertise that will be of use for studies of further mutant mice.

I) PKB/Akt and postnatal animal survival

The hallmark function of PKB/Akt, which has been established by experiments with primary cerebellar neuron cultures, is the promotion of cell survival (Brunet et al., 1999; Datta et al., 1997; Dudek et al., 1997). Survival factors such as IGF1 can suppress apoptosis by activation of PKB/Akt, which subsequently phosphorylates BAD and forkhead transcription factors and contribute to cell survival(Brunet et al., 1999; Datta et al., 1997; Dudek et al., 1997). Subsequent experiments using numerous

types of cells in a similar manner demonstrated that the IGF1/insulin/PI3K/PKB/Akt pathway is relevant to the survival of all cell types (Datta et al., 1999). This led to prediction of the early lethality with PKBα/Akt1 mutant mice, supported by the observation that only PKBα/Akt1 and PKBγ/Akt3 are detectable in the ES cells and that the PKBα/Akt1 level is markedly higher than PKBγ (Williams et al., 2000). ES cells are the population of inner cell mass (ICM), that together with the trophoblasts constitutes the very early embryo (blastocyst). If the PI3K/PKB/Akt pathway is indispensable for ES cell survival, deletion of PKBα should at the very least disrupt the growth of these cells and impair mouse development. To test this, we generated PKBα/Akt1 null ES cells using heterozygous ES cells. In the absence PKBα/Akt1, the growth of ES cells was comparable to that wild-type (data not shown). This result suggests that PKBγ/Akt3 alone is sufficient for cell survival or that this pathway is simply not essential. On the other hand, the strong potential of ES cells for proliferation may mean that these cells can overcome survival disadvantages.

With PKBα/Akt1 null ES cells almost intact, it is not surprising that PKBα/Akt1 mutant mice develop to term (Yang et al., 2003). Nevertheless, these mice do display increased neonatal mortality and nearly half of them die after birth (Cho et al., 2001b; Yang et al., 2003). Although the exact cause of death is not clear, $Pkb\alpha^{-/-}\gamma^{+/-}$ ($Akt1^{-/-}3^{+/-}$) mice provides some hints. The $Pkb\alpha^{-/-}\gamma^{+/-}$ ($Akt1^{-/-}3^{+/-}$) mice manifest structural abnormalities in thymus and heart. Previously, young PKBα/Akt1 mice were found to have very small thymuses (data not shown). Given that the PKBα/Akt1 level is normally higher than PKBγ/Akt3, the defects in $Pkb\alpha^{-/-}\gamma^{+/-}$ ($Akt1^{-/-}3^{+/-}$) mice may be attributable mainly to PKBα/Akt1 loss (Yang et al., 2003). It is, thus, highly possible that the PKBα/Akt1 mutant mice die from heart failure or immune deficiency. On the

other hand, retarded growth of PKBα/Akt1 mutant mice, resulting from either placental insufficiency or an embryo intrinsic mechanism, may also impair their survival. This is commonly seen in humans, where the organs of pre-mature babies are incapable of proper function.

Increased postnatal mortality is only seen with PKBα/Akt1 null mice; PKBγ/Akt3 null mice survival normally (Tschopp et al., unpublished data). There has been no indication of postnatal death of PKBβ/Akt2 (Cho et al., 2001a; Garofalo et al., 2003). Thus, of the three PKB/Akt proteins, PKBα/Akt1 displays a specific and important role in animal survival. Furthermore, PKB/Akt appears to play a pro-survival role not only in cells but also in the whole animal. Whether this is indeed related to an anti-apoptosis effect needs further investigation.

II) PKB/Akt and metabolism

The pioneering demonstration of PKB/Akt activation by IGF1 and insulin greatly improved our understanding of the role of this kinase in metabolism and has many implications (Alessi et al., 1996). Both insulin and IGF1 play important roles in glucose, fat and protein metabolism in all human cells. In current models, the two growth factors/hormones trigger two main signalling pathways, Ras-Raf-MAPK and PI3K/PKB(Akt)/S6K. The Ras-Raf-MAPK pathway promotes mitosis and the PI3K/PKB(Akt)/S6K pathway regulates metabolism.

a) Glucose metabolism. The role of PKB/Akt in glucose metabolism cannot be better demonstrated than in PKBβ/Akt2 null mice. In the absence of PKBβ/Akt2, mice develop a type 2 diabetes-like syndrome (Cho et al., 2001a; Garofalo et al., 2003). Insulin action is blocked in these mice, although the insulin concentration is high in serum (hyperinsulinemia) and glucose cannot be consumed (with consequent hyperglycemia) (Cho et al., 2001a; Garofalo et al., 2003). In addition, PKBβ/Akt2-

deficient mice exhibit glucose intolerance and impaired muscle glucose uptake (Cho et al., 2001a; Garofalo et al., 2003).

It is interesting to compare insulin action and glucose metabolism in pancreatic PKB/Akt overexpression mice and PKBβ/Akt2-deficient mice. Both display hyperinsulinemia but glucose levels are normal and all three PKB/Akt proteins are intact in the transgenic mice (Bernal-Mizrachi et al., 2001; Cho et al., 2001a; Garofalo et al., 2003; Tuttle et al., 2001a). Excess insulin in serum from the transgenic mice fails to reduce glucose levels, suggesting a mechanism to control insulin signalling. However, insulin signalling is disrupted in PKBβ/Akt2-deficient mice and high insulin levels cannot rescue the defect in this signalling pathway (Bernal-Mizrachi et al., 2001; Cho et al., 2001a; Garofalo et al., 2003; Tuttle et al., 2001a).

Glucose metabolism is normal in PKBα/Akt1 mutant mice, suggesting functional differences between PKBα/Akt1 and PKBβ/Akt2 in this regard (Chen et al., 2001; Cho et al., 2001b; Yang et al., 2003). However, we found a population of trophoblasts, the glycogen-containing spongiotrophoblasts, are almost completely absent from PKBα/Akt1 mutant placenta (Yang et al., 2003). This may be evidence that PKBα/Akt1 is also involved in glucose metabolism. As with PKBα/Akt1 mutant mice, PKBγ/Akt3 mutants show normal glucose utilization.

The mechanism of the PKB regulation of glucose metabolism is not clear, but there are some reasonable hypotheses. One is that, phosphorylation by PKB negatively regulates GSK3 activity, which is crucial for glucose metabolism. PKB was also found to promote glucose transport into cells via Glut4, thus facilitating glucose utilization (Whiteman et al., 2002).

b) Protein synthesis. Recent reports highlight PKB/Akt regulation of protein synthesis through phosphorylation of TSC2 (Tuberous sclerosis complex 2) (Gao et al., 2002;

Inoki et al., 2002; Potter et al., 2002). mTOR (mammalian target of rapamycin) is regarded as a central modulator of protein synthesis by activation of S6K1 (p70 ribosomal protein S6 kinase) and inhibition of 4E-BP1(eukaryotic initiation factor 4 binding protein1).

mTOR activity is promoted by Rheb (Ras homologue enriched in brain), a small G protein like Ras. As a GTPase-activating protein (GAP), TSC2 can inactivate Rheb and, therefore, inhibit mTOR signalling. PKB/Akt can phosphorylate and inhibit TSC2, leading to activation of the mTOR signalling pathway and protein synthesis (Li et al., 2004).

We measured the phosphorylation levels of TSC2 in PKBα/Akt1 mutant placenta but found little difference between the wild-type control and the knockout (Yang et al., 2003). However, a reduction in phospho-TSC2 was seen in double knockout murine embryonic fibroblasts (MEFs) of PKBα/β (Akt1/2) (Peng et al., 2003).

The elucidation of how the PKB/Akt-TSC2-Rheb-mTOR-S6K pathway controls protein synthesis should help answer the question of how PKB/Akt promotes cell growth (increase in cell size).

c) Fat metabolism. In humans, glucose is utilized for three main purposes: the production of ATP as energy, fat synthesis and glycogen synthesis. Th fat in adipocytes and the glycogen in hepatocytes and myocytes are source of stored energy. Therefore, it is not surprising that PKB/Akt increased lipid synthesis as seen in the transgenic mice with PKB/Akt overexpression in the mammary gland (Schwertfeger et al., 2003).

We found reduced subcutaneous fat in PKBα/Akt1 mutant mice (Yang et al., 2003) and age-dependent loss of adipose tissue is prominent in PKBα/Akt2 mutant mice (Garofalo et al., 2003).

To summarize, PKB/Akt is involved in the metabolism of the three major nutrients, glucose, protein and fat. The functions of PKB/Akt proteins are thus important for homeostasis of metabolism and critical for survival.

III) PKB/Akt and development

PKB/Akt not only plays a central role in metabolism but also has an impact on development. Alterations in mice lacking different PKB/Akt isoforms indicate that PKB/Akt is indispensable for normal animal development. For example, the most prominent phenotype displayed by PKBα/Akt1 and PKBα/β (Akt1/2) mutant mice is short stature (Chen et al., 2001; Cho et al., 2001b; Peng et al., 2003). PKBα/Akt1 mutant mice also have an abnormal placental structure and the brain of PKBγ/Akt3 mutants are smaller than wild-type (Yang et al., 2003; Tschopp et al., unpublished data). PKBβ/Akt2 mutant mice display age-dependent loss of adipose tissues and mildly reduced body size (Garofalo et al., 2003).

Mice lacking PKBα/β (Akt1/2) show impaired skin development, atrophic skeletal muscle and abnormal bone development. Adipogenesis is also affected (Peng et al., 2003). PKBα/γ (Akt1/3) double knockout mice are embryo lethal with developmental defects in placenta, branchial arch and arteries, and in the brain. $Pkb\alpha^{-/-}\gamma^{+/-}$ ($Akt1^{-/-}3^{+/-}$) mice die at an early age and have multiple pathology of skin, heart and thymus.

IV) Control of size and adipogenesis by PKB/Akt: coupling of metabolism and development

An intriguing question that has puzzled researchers for many years is how metabolism affects development. For example, when Drosophila grow under nutrient-limitated conditions, adults are smaller than well-fed controls (Kramer et al., 2003). Organism size is determined by both cell number and cell size (Conlon and Raff, 1999). Cell size or cell growth is dependent on protein synthesis, which is regulated by

PKB/Akt, as discussed above. Several transgenic mouse models have confirmed PKB/Akt function in cell size regulation. For example, overexpression of PKB/Akt in cardiac myocytes, lymphocytes and pancreatic β cells increased cell size, giving rise to heart, thymus and islets larger than inwild-type controls (Condorelli et al., 2002; Malstrom et al., 2001; Matsui et al., 2002; Shioi et al., 2002). Thus, PKB/Akt is involved in organ growth via regulation of protein metabolism, i.e. PKB/Akt integrates metabolism with development.

PKB/Akt plays a central role in glucose and fat metabolism. Overexpression of PKB/Akt in the mammary gland increases lipid synthesis (Schwertfeger et al., 2003). Deletion of PKBα, PKBβ or both impairs adipose tissue development (Garofalo et al., 2003; Peng et al., 2003; Yang et al., 2003). In PKBα/β (Akt1/2) double knockout MEFs, the transcription of PPARγ, a master gene in regulating fat synthesis cannot be induced properly (Peng et al., 2003). These observations indicate that in the absence of PKB/Akt, lipid synthesis is affected; and as a result, the adipose tissue cannot develop normally.

Therefore, through regulation of glucose, fat and protein metabolism, PKB/Akt modulates developmental events such as size control and adipogenesis. PKB/Akt couples metabolism with development.

V) PKB and the cell cycle

PKB/Akt affects not only cell growth but also cell number (cell proliferation) by promoting cell cycle progression. PKB/Akt facilitates G1/S transition and the initiation of M phase. Two important substrates of PKB/Akt, FOXO and MDM2 are involved in the G1/S transition. FOXO enhances transcription of p27 and downregulates cyclin D mRNA levels (Collado et al., 2000; Medema et al., 2000; Schmidt et al., 2002). Inhibition of CDK2 by p27 and decreased cyclin D levels prohibits cell cycle

progression iN G1. Phosphorylation of FOXO by PKB/Akt promotes its cytoplasmic translocation, resulting in decreased p27 transcription and increased cyclin D levels (Collado et al., 2000; Medema et al., 2000; Schmidt et al., 2002). Once phosphorylated by PKB/Akt, MDM2 moves to the nucleus and promotes p53 degradation. Induction of p21 transcription by p53 is reduced and the inhibition of the cell cycle in G1 by p21 is released (Ashcroft et al., 2002; Mayo and Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001). Phosphorylation of GSK3 by PKB/Akt also increases cyclin D levels for the G1/S transition (Diehl et al., 1998).

Phosphorylation of CHFR and Myt1 by PKB/Akt initiates M phase (Okumura et al., 2002; Shtivelman, 2003; Shtivelman et al., 2002).

VI) PKB/Akt and neural crest cell development.

In E12.5 embryos, levels of PKBα/γ (Akt1/3) are particularly high in the brain and a variety of ganglia. Thus, PKBα/γ (Akt1/3) appear to be markers of the central and peripheral nervous systems. If it is true that 'where it exists, there it functions', these two PKB isoforms may play important roles in the nervous system. As ganglia originate from neural crest cells, there may be some relation between these proteins and neural crest cells, perhaps in connection with their development. In the PKBα/γ (Akt1/3) double knockout embryos, we found hypoplastic branchial arches and abnormal branchial arch arteries at around E11. At this stage, the majority of the cells comprising branchial arches are mesenchymal cells that are derived from neural crest cells. We also found alterations in $Pkb\alpha^{-/-}\gamma^{+/-}$ ($Akt1^{-/-}3^{+/-}$) mice of other neural crest derivatives, such as thymus and heart septum. All these changes indicate PKB/Akt involvement in neural crest cell development. To test this possibility further, it would be interesting to follow the migration of neural crest cells in PKBα/γ (Akt1/3) mutant mice during early development using various markers, or to culture neural crest cells

for induction of differentiation. The elucidation of PKB/Akt function here would help understand the formation of specific tissues.

VII) PKB/Akt and the cardiovascular system.

Our finding of reduced vascularisation in PKBα/Akt1 and PKBα/γ (Akt1/3) double mutant placentas, together with the developmental defects observed in PKBα/γ (Akt1/3) double mutant branchial arch arteries suggest that PKB/Akt regulates vascular structure (angiogenesis and vascularisation). In addition, the malformation of ventricle myocardium in $Pkb\alpha^{-/-}\gamma^{+/-}$ ($Akt1^{-/-}3^{+/-}$) mice suggests that PKB/Akt modulates heart development; abnormal PKB/Akt signalling may contribute to heart failure. Detailed study of hearts in PKBα/γ (Akt1/3) double mutant embryos could unveil the underlying regulatory mechanisms.

VIII) PKB/Akt mice and tumour formation. PKB/Akt promotes cell cycle progression and cell growth. Therefore, it is not difficult to imagine why increased PKB/Akt activity gives rise to tumour formation. Angiogenesis (nes vessel formation) is critical for tumour development, and PKB/Akt may also contribute to tumour development through stimulation of novel vessel development. In the pharmaceutical industry, much effort is expended in a search for PKB/Akt inhibitors and, thus, for tumour growth inhibitors. However, our observations of PKBβ/Akt2 mice suggest that inhibition of tumour growth could be at the expense of changes in regulation leading to diabetes?

Thus, a more detailed molecular understanding of the PKB/Akt promotion of tumour formation and growth is needed for improved drug targeting. In this regard, single knockout mice of PKB/Akt proteins could be examined for for xenograft growth after tumour cell injection to see how tumour development is disrupted in the absence of PKB/Akt.

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Oral presentation: PKB/Akt ablation studies in mice reveal essential role in metabolism, growth and development.

LIST OF PUBLICATIONS

Zhong-zhou Yang and Brian A. Hemmings. (2001) The complexity of protein kinase B (PKB/Akt) signaling — implications for human cancer. In: Protein Modules in Cellular Signalling edited by Ludwig Heilmeyer and Peter Friedrich, pp29-40, *IOS*, Ohmsha.

Zhong-zhou Yang, Oliver Tschopp, Maja Hemmings-Mieszczak, Jianhua Feng, Daniela Brodbeck, Elias Perentes and Brian A. Hemmings. (2003) Protein Kinase Bα/Akt1 Regulates Placental Development and Fetal Growth. *J. Biol. Chem.* **278**, 32124-32131.

Zhong-zhou Yang, Oliver Tschopp, Anne Baudry, Bettina Duemmler, Deborah Hynx and Brian A. Hemmings. (2004) Physiolgical functions of PKB/Akt. *Biochem. Soc. Trans.* **32**: 350-354.

Derek Brazil, **Zhong-zhou Yang** and Brian A. Hemmings. (2004) New advances in Protein Kinase B signaling: *AKT*ion on multiple fronts. *Trends Biol. Sci*. **29**:233-242.

Zhong-zhou Yang, Oliver Tschopp, Elisabeth Bruder, Bettina Duemmler, Deborah Hynx and Brian A. Hemmings. PKBα/Akt1 is more important than PKBγ/Akt3 for mouse survival but both are required for mouse development. In preparation.

Oliver Tschopp, **Zhong-Zhou Yang**, Daniela Brodbeck, Maja Hemmings-Mieszczak, Bettina Duemmler, Takashi Watanabe, Thomas Michaelis, Jens Frahm and Brian A. Hemmings. Reduced brain size in protein kinase Bγ (PKBγ/Akt3) null mutant mice. In preparation.

Appendix 1

Early characterization of PKBα/Akt1 null ES cells, MEFs and mice

1. Generation of *Pkbα* knockout ES cells

At high concentration of G418, heterozygous ES cells of *Pkbα* were converted into homozygous ES cells. The ES clones were first characterized by Southern bloting with 2 different probes [Fig. 1A1) and 2)]. Western blotting using a PKBα specific antibody confirmed the absence of the protein [Fig. 1A3)]. Preliminary studies showed that the knockout ES cells grew comparably to wild-type (Fig. 1B)

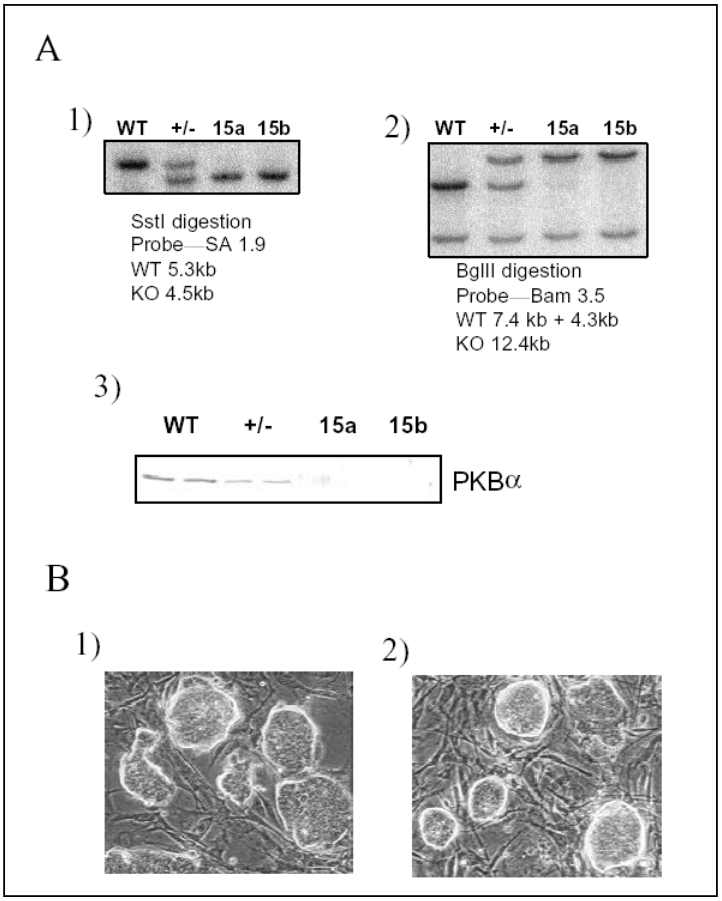


Fig. 1. Generation of PKBα null ES cells. A.1 and 2) Southern blotting analysis of the two ES mutants of 15a and 15b. 3) Western blotting confirms the absence of PKBα. **B.** 1) wild-type ES cells and 2) mutant ES cells.

However, there seems to be some difference between wild-type and the mutant ES cells in early embryoid body formation (Fig. 2). In the mutants, there are some detached cells which are rare in the wild-type.

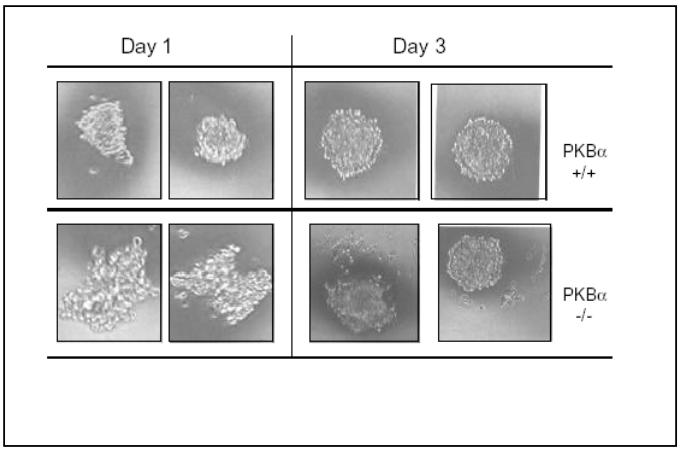


Fig. 2 Growth of wild-type and PKBα null embryoid body.

2. Early characterization of PKBα mutant mice

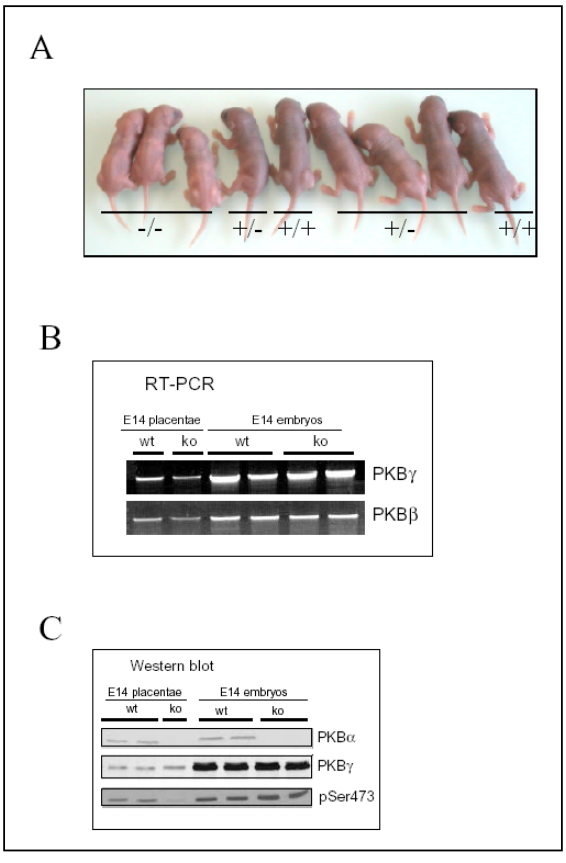
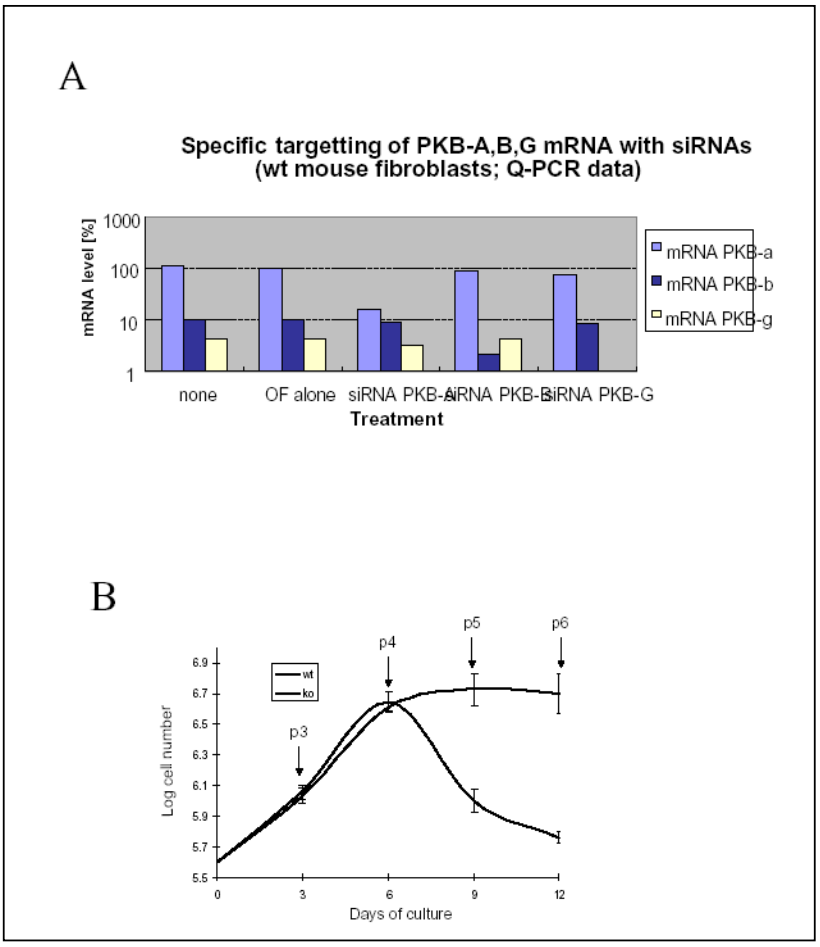


Fig. 3 .**Early characterization of PKBα mutant placentas and embryos. A**. a litter of newborns from *Pkbα*$^{+/-}$ intercross. Note that the mutant mice are smaller than their littermates. **B**. RT-PCR analysis of E14 placentas and embryos. There is no up-regulation of these two genes in mutant placentas and embryos. **C**. There is no up-regulation of PKBβ and γ proteins shown by Western blotting. However, the level of phospho-PKB in mutant placenta is significantly reduced.

3. PKBα null murine embryonic fibroblasts (MEFs)

We generated MEFs from PKBα mutant embryos. We found that after several passages of culture, the mutant cells grew slower that wild-type and showed morphology of senescence (Fig.4B). In addition, the mutant cells are sensitive to UV irradiation.



We also designed specific oligos to knock down the the three PKB isoforms in wild-type MEFs (with help from Maja Hemmings). Figure 4 A shows that PKBα can be efficiently knocked down.

Appendix 2

PKBα is dispensable for lymphocyte development

In collaboration with prof. Antonius Rolink of Department of Immunology, University of Basel-Pharmacen

PKBα expression is abundant in thymus and spleen

PKBα protein levels were examined in several tissues from wildtype mice by Western blot using an isoform-specific antibody (figure 1). Three adult mice were analysed and gave similar results. Compared with the other tissues, thymus and spleen have relatively higher levels of PKBα. In addition, it was found that the expression of PKBα is more abundant in thymus than in spleen, which was confirmed by quantitative PCR (data not shown).

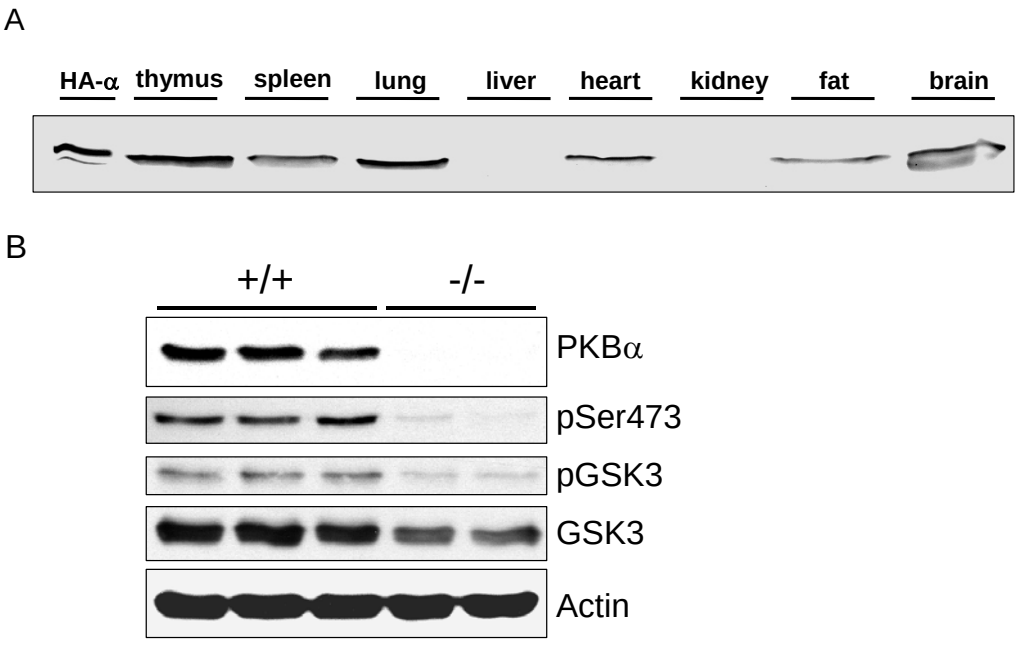


Fig.1 PKBα is abundant in thymus and spleen. A. Tissue distribution of PKBα shown by Western blot. **B**. Phospho-PKB level is reduced in PKBα mutant thymus.

Pkbα$^{-/-}$ mice have reduced peripheral lymphocytes

We studied PKBα deficient mice for its function in maintain lymphocyte development. Two months old wild-type and *Pkbα*$^{-/-}$ mice (each genotype including 3 males and 3 females) were sacrificed for blood collection. Hematological analysis showed that the total white blood cell number in peripheral blood was reduced in the *Pkbα*$^{-/-}$ mice (wild type, 8.1± 1.7 x 10^3/mm^3 and *Pkbα*$^{-/-}$, 4.98±0.95 x 10^3/mm^3. P<0.01. *Pkbα*$^{-/-}$ /wild type=61%. Table1). Further analysis indicates that this reduction resulted from the decreased lymphocytes (wild-type, 6.94±1.68 x 10^3/mm^3 and *Pkbα*$^{-/-}$, 3.74±0.84 x 10^3/mm^3. P<0.05. *Pkbα*$^{-/-}$/wild type=54%. Table 1). Thus, peripheral lymphocytes in *Pkbα*$^{-/-}$ mice were reduced.

Table 1. Peripheral blood parameters of wild type and *Pkbα*$^{-/-}$ mice

	wild type	*Pkbα*$^{-/-}$	P value
Total WBC (x10^3/mm^3)	8.18± 1.70	4.98± 0.95	<0.01
Lymphocytes (x10^3/mm^3)	6.94± 1.68	3.74± 0.84	<0.05

In each group, 6 mice (3 males and 3 females) of 2 months were analyzed.

Lymphocyte development is normal in *Pkbα*$^{-/-}$ mice

The reduced peripheral lymphocytes suggested that the development of lymphocytes is abnormal. To test this, mice of 2 weeks, 4 weeks and 8 weeks old were sacrificed for dissection of thymus, spleen, bone marrow and lympho-node. Single cell suspensions were prepared from these lymphoid tissues for flow cytometry using a wide series of cell-specific markers, such as B220, CD19, IgM, IgD, CD4, CD8, H57, CD62, CD23,

493, CD21, γ1δ, NK1, CD3, CD25, CD44, c-kit, TAC. Cells isolated from spleen and bone marrow were analysed for B cell development and cells from thymus for T cell status. The presence of both T, B cells at different stages in lymphonode were also examined. The data indicated the existence of T, B cells of various developmental stages in *Pkbα* $^{-/-}$ mice. The relative ratios of these cells are comparable between wild type and *Pkbα* $^{-/-}$ mice. In vitro proliferation of mutant lymphocytes is also normal (Fig. 2).

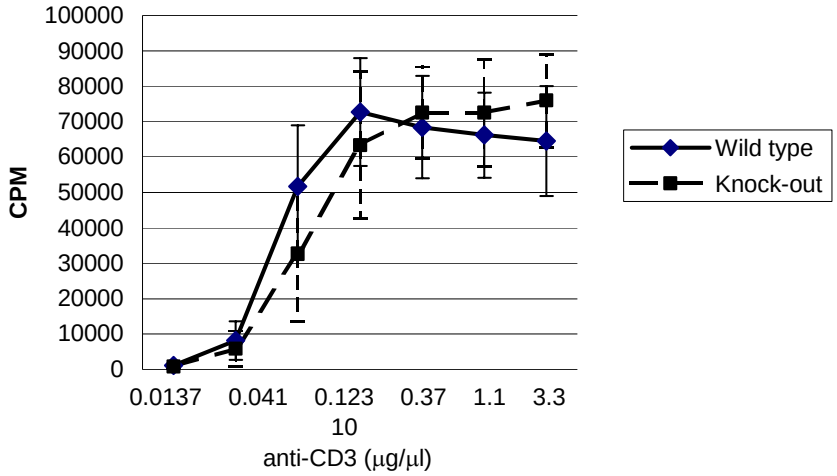


Fig. 2. In vitro proliferation assay.

Decreased phosphorylation of total PKB and GSK3 in *Pkbα* $^{-/-}$ thymus

We examined the phosphorylation levels of PKB in wild type and mutant thymus by use of an antibody which recognizes all three phosphorylated isoforms. Thymus lacking PKBα displayed significant reduction of total phosphorylated PKB compared with wild type. In addition, we found that the phosphorylation of GSK3, one of the best recognized substrates, decreased greatly. This indicated that loss of PKBαsubstantially changed total PKB activity in thymus (Fig.1).

Appendix 3

Copies of published papers