Physiological Functions of Pten in Mouse Tissues

Hiroyuki Kishimoto1, Koichi Hamada1, Mary Saunders2, Stephanie Backman2, Takehiko Sasaki3, Toru Nakano4, Tak Wah Mak2, and Akira Suzuki*

1Department of Biochemistry, Akita University School of Medicine, Akita 010-8543, Japan, 2Advanced Medical Discovery Institute and Department of Medical Biophysics, University of Toronto, Toronto, Ontario, M5G 2C1 Canada, 3Department of Pharmacology, Tokyo Metropolitan Institute of Medical Science and PRESTO, Japan Science and Technology Corporation (JST), Tokyo 113-8613, Japan, and 4Department of Molecular Cell Biology, Research Institute for Microbial Disease, Osaka University, Osaka 565-0871, Japan

ABSTRACT. PTEN is a tumor suppressor gene mutated in many human sporadic cancers and in hereditary cancer syndromes such as Cowden disease, Bannayan-Zonana syndrome and Lhermitte-Duclos disease. The major substrate of PTEN is PIP3, a second messenger molecule produced following PI3K activation induced by variety of stimuli. PIP3 activates the serine-threonine kinase PKB/Akt which is involved in anti-apoptosis, proliferation and oncogenesis. In mice, heterozygosity for a null mutation of Pten (Pten+/− mice) frequently leads to the development of a variety of cancers and autoimmune disease. Homozygosity for the null mutation (Pten−/− mice) results in early embryonic lethality, precluding the functional analysis of Pten in various organs. To investigate the physiological functions of Pten in viable mice, various tissue-specific Pten mutations have been generated using the Cre-loxP system. This review will summarize the phenotypes of conditional mutant mice lacking Pten function in specific tissues, and discuss how these phenotypes relate to the physiological roles of Pten in various organ systems.

Key words: Pten/Cre-loxP system/tissue-specific mutant mice

PTEN (also called MMAC1 or TEP1) (Li et al., 1997) is a tumor suppressor gene which is mutated in human sporadic cancers such as glioblastoma and endometrial and prostatic cancers, as well as in hereditary disorders such as Cowden disease, Bannayan-Zonana syndrome and Lhermitte-Duclos disease (LDD). These latter syndromes are characterized by multiple hamartomas and an increased risk of cancer (Liaw et al., 1997; Marsh et al., 1997). PTEN is a multifunctional phosphatase whose lipid phosphatase activity is associated with tumor suppression (Myers et al., 1998). The major substrate of PTEN is phosphatidylinositol-3,4,5-triphosphate (PIP3) (Maehama and Dixon, 1998), a lipid molecule generated by the action of phosphoinositide-3-kinases (PI3Ks) (Fig. 1). The PI3Ks constitute a family of evolutionarily conserved lipid kinases (Toker and Cantley, 1997). To date, eight distinct PI3K isoforms have been reported in mammals, four of which belong to the class I PI3Ks that are activated following the stimulation of transmembrane surface receptors. PI3Kα, β, δ constitute the Class IA PI3Ks that are activated by receptor tyrosine kinase engagement, while the single Class IB PI3K, PI3Kγ, is activated by the βγ subunit of G-proteins and acts downstream of G protein-coupled receptors (GPCRs) (Stoyanov et al., 1995; Stephens et al., 1997), PI3K activation occurs in response to T cell receptor (TCR) and B cell receptor (BCR) engagement as well as signaling induced by epidermal growth factor (EGF) (Hu et al., 1992), hepatocyte growth factor (HGF) (Ponzetto et al., 1993), fibroblast growth factors (FGFs) (Raffioni and Bradshaw, 1992) insulin-like growth factor (IGF-1) (Yamamoto et al., 1992), and insulin (Hadari et al., 1992). These molecules are all associated with intracellular signaling pathways that promote cell proliferation and survival, increase resistance to apoptosis, and enhance adhesion and migration (Toker and Cantley, 1997). PIP3 in turn activates the serine-threonine kinase PKB/Akt which is involved in anti-apoptosis, proliferation and oncogenesis. By dephosphorylating
the D3 position of PIP3, PTEN negatively regulates the PI3K pathway and PKB/Akt activation and thus tumorigenesis.

To investigate the functions of PTEN in vivo, we and others generated null mutations of Pten in mice (Suzuki et al., 1998; Di Cristofano et al., 1998; Stambolic et al., 1998; Podsypanina et al., 1999). Animals heterozygous for these mutations (Pten<sup>+/−</sup> mice) developed a broad range of tumors, including cancers of the breast, thyroid, endometrium and prostate as well as T cell lymphomas. This spectrum of neoplasias closely resembles that in humans with PTEN mutations. Pten<sup>−−</sup> mice also developed signs of autoimmune dis-
ease (Di Cristofano et al., 1999). Homozygosity for the null Pten mutation in mice resulted in early embryonic lethality (~E9.5), precluding the functional analysis of Pten in adult organs. We and others therefore turned to conditional mutant mice generated via the Cre-loxP system (Pten\textsuperscript{flox/} mice) to analyze the functions of Pten in T cells, B cells, brain cells, cardiomyocytes, mammary gland cells and keratinocytes. In this review, we summarize our work and that of others in generating and analyzing the phenotypes of animals lacking Pten function specifically in these tissues. We then relate these phenotypes to the physiological functions of Pten in various organs.

**Role of Pten in T cells**

Mice in which Pten was disrupted only in T cells (\(\text{Pten}^{\text{flox/fox}}\) mice) were generated by crossing Lck-Cre transgenic mice to Pten\textsuperscript{flox/} mice (Suzuki et al., 2001). Total numbers of double negative (CD4 + CD8\textsuperscript{+}), double positive (CD4\textsuperscript{+}CD8\textsuperscript{+}) and CD8\textsuperscript{+} single positive cells were increased in the mutant thymus compared to controls. In the periphery, the total numbers of mature CD4\textsuperscript{+} and B220\textsuperscript{+} cells were increased but the number of mature CD8\textsuperscript{+} cells was approximately normal. Tumor formation in \(\text{Pten}^{\text{flox/}}\) mice was observed from 10 weeks of age and all these animals died of T cell tumors in malignant T cell lymphomas within 17 weeks. Most of the T cell tumors in \(\text{Pten}^{\text{flox/}}\) mice could be classified as CD4\textsuperscript{+} SP T cell lymphomas, or a mixture of CD4\textsuperscript{+} SP and DP lymphomas. CD8\textsuperscript{+} SP T cell lymphomas were not observed.

To investigate the role of Pten in central tolerance, we crossed the \(\text{Pten}^{\text{flox/}}\) mutation into HY TCR transgenic (Tg) animals (C57BL/6J) (Kisielow et al., 1988) to generate HY\(\text{Pten}^{\text{flox/}}\) mice. The total number of thymic DP cells which expressed the Tg TCR was more than 10-fold greater in male HY\(\text{Pten}^{\text{flox/}}\) mice than in male HY\(\text{Pten}^{+/+}\) mice, indicating that HY\(\text{Pten}^{\text{flox/}}\) mice had a defect in thymic negative selection. Histological comparison showed a marked infiltration of small, typical lymphoid cells into both perivascular areas and some alveolar septa of the lungs of all male HY\(\text{Pten}^{\text{flox/}}\) mice examined. These observations suggested the occurrence of “lymphoid intestinal pneumonia”, a disorder thought to be caused by an autoimmune mechanism. An approximately 2-fold decrease in the number of transgenic CD8\textsuperscript{+} SP cells was observed in female HY\(\text{Pten}^{\text{flox/}}\) mice compared to female HY\(\text{Pten}^{+/+}\) mice, while the number of transgenic CD4\textsuperscript{+} SP cells was increased 1.5-fold. These results imply that HY\(\text{Pten}^{\text{flox/}}\) mice are impaired in CD4/CD8 lineage commitment and that DP precursor cells may be preferentially developing into CD4\textsuperscript{+} cells.

CD4/CD8 lineage commitment is influenced by ERK signaling. Development of CD4\textsuperscript{+} cells is increased when ERK activity is increased, while that of CD8\textsuperscript{+} cells is increased when ERK is decreased (Sharp and Hedrick, 1999). Indeed, ERK phosphorylation was consistently increased in our \(\text{Pten}^{\text{flox/}}\) mice. ERK plays a prominent role in signaling downstream of the T cell activation molecule Itk. Itk-deficient mice display a markedly reduced in the CD4\textsuperscript{+} subset (Liao and Littrman, 1995), and Itk-deficient T cells show decreased proliferation in response to TCR engagement. Interestingly, Shan et al. have demonstrated that Pten deficiency results in constitutive tyrosine phosphorylation of Itk (Shan et al., 2000). It is possible that a lack of Pten in T cells enhances Itk and thus ERK signaling, which in turn biases T cell differentiation towards CD4\textsuperscript{+} and away from CD8\textsuperscript{+}.

The effect of Pten deficiency on peripheral tolerance was investigated using deletion of peripheral T cells induced by the intraperitoneal injection of the superantigen Staphylococcal enterotoxin B (SEB). SEB specifically recognizes TCRs containing V\(\text{BJ}\) (Kawabe and Ochi, 1991). V\(\text{BJ}^{+}\) T cells from \(\text{Pten}^{\text{flox/}}\) mice expanded to almost the same degree as those from \(\text{Pten}^{+/+}\) mice but were resistant to deletion. T cells in \(\text{Pten}^{\text{flox/}}\) mice were autoreactive and levels of serum anti-ssDNA Ab in \(\text{Pten}^{\text{flox/}}\) mice were higher than those in controls. In vitro, loss of Pten in thymocytes conferred resistance to apoptosis induced by the specific adenosine receptor agonists ADAC and CGS. Interestingly, loss of Pten in peripheral T cells conferred resistance to anti-Fas Ab, γ-irradiation, UV-irradiation, or IL-2 withdrawal. This resistance to apoptosis correlated with elevations in the phosphorylation of PKB/Akt and Bcl-X\(_L\) in the mutant cells. Thus, loss of Pten both impairs peripheral tolerance and prevents T cells from initiating apoptosis in response to several stimuli, including some known to be mutagenic. T cells lacking Pten also showed enhanced proliferation in response to various stimuli, and increased production of both Th1 and Th2 cytokines. Elevated IL-10 and IL-4 production may at least partially account for the enhanced level of serum IgG1 and the increased B cell number observed in \(\text{Pten}^{\text{flox/}}\) mice (Briere et al., 1994; Esser and Radbruch, 1989).

A comparison of \(\text{Pten}^{\text{flox/}}\) mice with several mutants in which elements of the PI3K pathway are altered suggests that the most of the abnormalities in \(\text{Pten}^{\text{flox/}}\) mice are likely due to an accumulation of PI(3)P. We have generated mice deficient for the p110γ catalytic subunit of PI3Kγ (Sasaki et al., 2000), and p110γ deficiency and Pten deficiency appear to have opposing effects in T cells. p110γ\textsuperscript{−/−} mice showed decreased numbers of splenic CD4\textsuperscript{+} SP cells. Proliferation of p110γ\textsuperscript{−/−} T cells is reduced in response to a variety of stimuli and p110γ\textsuperscript{−/−} thymocytes are less susceptible to apoptotic stimuli such as ADAC and CGS. Interestingly, the deficiency for the p85α, a regulatory subunit of the class IA PI3Ks, leads to a partial block in early B cell development but T cell development is not impaired (Furu-man et al., 1999; Suzuki et al., 1999). A p65\textsuperscript{ERK} transgenic mouse has been described (Bolrado et al., 2000) that expresses a constitutively active truncated form of p85α in T cells and exhibits phenotypes very similar to those of...
Role of Pten in B cells

PTEN mutations also occur in human B cell malignancies (Gronback et al., 1998; Hyun et al., 2000; Nakahara et al., 1998). We generated B cell-specific Pten-deficient mice (bPten<sup>flox/flox</sup> mice) by crossing Pten<sup>flox</sup> mice to CD19-Cre transgenic mice (Suzuki et al., in revision). Although there were no obvious differences in either the total number of pro-B cells or pre-B cells in bone marrow, B1a cells were increased 24-fold in the peritoneal cavity and 11-fold in the spleen. Among mature B2 cell populations, the total number of splenic marginal zone B (MZB) cells was dramatically increased in bPten<sup>flox/flox</sup> mice, while that of follicular B (FOB) cells was correspondingly decreased. Marked reductions in the serum levels of most IgG subclasses and IgA were also observed in bPten<sup>flox/flox</sup> mice. In contrast, serum IgM levels were elevated 4-fold over normal. Production of antigen-specific IgG in response to the thymus-independent (TI) antigen NP-CG (nitro-phenyl-acetyl-chicken γ-globulin) was dramatically decreased in bPten<sup>flox/flox</sup> mice as was germinal center formation. Production of antigen-specific IgG in response to the thymus-independent antigen (TI) type II NP-Ficoll was also severely impaired in the absence of Pten. The reduction of germinal center formation in the bPten<sup>flox/flox</sup> mice was apparent even in the presence of strong activation signals delivered via IgM and CD40, and even though the activation of intracellular signaling pathways mediated by PKB/Akt and Btk was intact. FOB cells are required to form germinal centers, and the reduction in this process in bPten<sup>flox/flox</sup> mice may partially account for the observed defect.

The altered serum Ig profile observed in bPten<sup>flox/flox</sup> mice led us to examine class switch recombination (CSR). The production of IgG1 and IgG3 was reduced in bPten<sup>flox/flox</sup> B cells after stimulation by LPS with or without IL-4 as measured by flow cytometry, ELISA, RT-PCR and DC-PCR. These results imply that Pten deficiency in B cells leads to a defect in CSR, consistent with several other lines of evidence. First, MZB and B1 cells are important for TI responses (Fagarasan and Honjo, 2000; Guinamard et al., 2000), but even though these populations were elevated in bPten<sup>flox/flox</sup> mice, the production of antigen-specific IgG in response to TI-II antigen was profoundly decreased. Secondly, both bPten<sup>flox/flox</sup> MZB and FOB cells showed defective CSR at the cellular level. CSR depends in part on the activity of AID, a member of the RNA-editing cytidine deaminase family. AID was recently reported to regulate CSR (Muramatsu et al., 2000) and is activated by LPS in vitro as well as by antigens in vivo. In bPten<sup>flox/flox</sup> mice, AID expression was markedly reduced after stimulation by LPS, suggesting that Pten is indispensable for CSR because it regulates the induction of AID expression.

Greater amounts of anti-ssDNA IgM Ab in both absolute and relative terms were observed in bPten<sup>flox/flox</sup> mice, possibly due to the increase in B1a cells. However, the absolute amount of anti-ssDNA IgG Ab was not significantly higher in bPten<sup>flox/flox</sup> mice, suggesting that the CSR defect may partially mitigate the elevation of IgG autoantibodies in bPten<sup>flox/flox</sup> mice. To our surprise, bPten<sup>flox/flox</sup> mice had a normal lifespan free of autoimmune disease or B cell malignancy.

bPten<sup>flox/flox</sup> B cells treated in vitro with immobilized anti-IgM were more resistant to apoptosis and showed enhanced proliferation compared to the WT in response to stimuli such as anti-IgM, anti-CD40, LPS or PDBu plus ionomycin. Phosphorylated PKB/Akt was significantly elevated in the mutant B cells compared to WT B cells following stimulation with anti-IgM. Moreover, phosphorylation was completely abolished in both WT and mutant B cells by the addition of PI3K inhibitors. The migration of Pten-deficient B cells, especially FOB cells, was greater than that of controls against SDF-1α as determined by the Transwell™ assay. Dammers et al. have reported that MZB cells are derived from a subset of FOB cells by migration (Dammers et al., 1999). Knockout studies have shown that Pyk2 (Guinamard et al., 2000), Lsc (Girkontaite et al., 2001) and DOCK2 (Fukui et al., 2001), all molecules involved in cell motility, are indispensable for MZB formation. MZB cells are also decreased in mutant mice lacking p110δ, a catalytic subunit of PI3Kδ (Clayton et al., 2002; Okkenhaug et al., 2002), while B1 cells are decreased in these animals as well as in p85α-deficient mice (Fruman et al., 1999; Suzuki et al., 1999). Pten deficiency thus alters B1a, MZB and FOB populations in mice, most likely because dysregulated activation of the PI3K pathway disrupts apoptotic resistance, proliferation and cell migration.

Role of Pten in the brain

Since Pten is ubiquitously expressed in the central nervous system, and Pten mutations have been noted in glioblastomas and in Cowden and LDD patients, Pten was expected to have the important roles in brain development.
and oncogenesis. Pten<sup>flx/flx</sup> mice were crossed with GFAP-Cre transgenic mice to generate brain-specific Pten-deficient mice (GFAP-CrePten<sup>flx/flx</sup> mice) (Backman et al., 2001, Kwon et al., 2001). Loss of Pten expression was observed in granule cells of the cerebellum and dentate gyrus (DG), as well as in some cortical neurons. These sites are consistent with the pattern of Cre-mediated recombination seen in reporter assays of the GFAP-Cre parental line (Kwon et al., 2001).

Starting at 9 weeks of age, GFAP-CrePten<sup>flx/flx</sup> mice developed seizures and ataxia characterized by front paw tremors and hind leg spasms. All GFAP-CrePten<sup>flx/flx</sup> mice died suddenly at 29 weeks of age. The brains of GFAP-CrePten<sup>flx/flx</sup> mice were substantially larger than those of age-matched controls, and hydrocephalus of the lateral ventricles was frequently observed. Histologically, heterotopic clusters of dysplastic and disorganized cerebellar granule cells persisted at the pial surface and were scattered throughout the molecular layer (ML) of the cerebellum. Extensive astrogliosis and ML thickening were often seen at the sites of the heterotopic lesions. The ML exhibited elevated myelination and a partial loss of Purkinje cells. The remaining Purkinje cells were atrophic or dysplastic with dendritic coarsening and axonal swelling.

GFAP-CrePten<sup>flx/flx</sup> mice also underwent extensive loss of Pten in DG granule cells. Symptomatic animals showed striking disorganization of the DG characterized by marked undulation of the DG granule cell layer. Intercellular spaces between granule cell bodies were expanded in GFAP-CrePten<sup>flx/flx</sup> mice and contained thick neuronal dendrites and astrocytes. The prominent astrogliosis in the hippocampus and subpial surface and a reduction in pyramidal cell density of the cornu ammonis in the mutant mice was reminiscent of the mesial temporal sclerosis detected in some epilepsy patients (Babb, 1987).

Cerebellar granules from GFAP-CrePten<sup>flx/flx</sup> mice were approximately 2-fold larger in size than the control and had increased levels of phosphorylated PKB/Akt. Null mutations of Pten in the Drosophila eye also result in enlarged mutant cells (Goberdhan et al., 1999, Huang et al., 1999), suggesting that Pten regulates cell size by antagonizing PI3K signaling via PKB/Akt (Huang et al., 1999; Goberdhan et al., 1999; Scanga et al., 2000). The neuronal expansion and macroencephaly observed in brain-specific Pten-deficient mice may also be due to an increase in the size of individual cells caused by dysregulation of the PI3K-PKB/Akt pathway (possibly via the activation of S6K (Valentinis et al., 2000), a downstream effector of PKB/Akt). Significantly, the phenotypes of GFAP-CrePten<sup>flx/flx</sup> mice closely resemble the hallmark symptoms of LDD (Wiestler et al., 1999).

Other groups have used different neuron-specific promoters to generate mice lacking Pten in the brain. Groszer et al. crossed Pten<sup>flx</sup> mice with Nestin-Cre transgenic mice (Groszer et al., 2001). Nestin is expressed in CNS stem/progenitor cells such that the Pten gene is deleted in almost all brain cells by midgestation. These mutant mice died soon after birth. Histological examination revealed that the mutant brains showed a proportional increase in overall brain structures with the loss of nuclei in the brainstem. In addition, the laminar patterns in the cortex, hippocampus and cerebellum were severely disturbed and disorganized. Individual cell size was increased. Numbers of neuronal cells (TuJ-1<sup>+</sup> cells) and glial cells (GFAP<sup>+</sup> cells) were not significantly different, suggesting that cell fate determination was not overtly disturbed in the absence of Pten. Although Backman et al. reported no differences between the control and Pten-deficient brain cells in BrdU and TUNEL staining patterns (Backman et al., 2001), Groszer et al. showed significant increases in the numbers of BrdU positive cells in the ventricular zone and of TUNEL positive cells in the telencephalon (Groszer et al., 2001). It is likely that these differences are due to differences in the extent of Pten deletion in the brain.

Using the neurosphere system, Groszer et al. found that both the total number and size of spheres were increased in Nestin-CrePten<sup>flx/flx</sup> mice. Moreover, BrdU incorporation and CFSE “wash-out” were elevated in the mutant spheres, indicating that the number of CNS stem/progenitor cells was increased in the absence of Pten. Thus, mutant progenitor cells showed enhanced proliferation/cell division.

Marino et al. generated mice specifically expressing the Pten mutation in the medial region of the cerebellum by crossing Pten mutant mice to engrailed 2 (En2)Cre transgenic mice. All precursor cells in these animals were of enlarged size, resulting in cerebellar anlage of increased size, impaired Purkinje cell migration, and abnormal cerebellar architecture. While dysplastic cellular changes occurred in these mutant mice, no neoplastic lesions were detected during the 28 week observation period (Marino et al., 2002). Thus, inactivation of Pten is not sufficient to elicit neoplastic transformation in the brain.

**Role of Pten in the heart**

The function of Pten in cardiomyocytes was analyzed in mckCrePten<sup>flx/flx</sup> mice generated by crossing Pten<sup>flx</sup> mice with muscle-specific mck-Cre transgenic mice (Crackower et al., 2002). Newborn mckCrePten<sup>flx/flx</sup> mice showed increased heart size due to cardiac hypertrophy. The size of individual cardiomyocytes was significantly increased, consistent with a previous report in which adenovirus-mediated expression of dominant-negative Pten in rat cardiomyocytes resulted in cardiac hypertrophy in vitro (Schwartzbauer and Robbins, 2001). Interestingly the cardiac hypertrophy in mckCrePten<sup>flx/flx</sup> mice did not lead to fibroblastic or structural changes even at age 1 year, indicating that Pten deficiency does not cause cardiac decompensation or dilated cardiomyopathy. As well as cardiac hypertrophy, mckCrePten<sup>flx/flx</sup> mice showed a
marked reduction in cardiac contractility. However, there were no differences in heart rate, dilation, wall thinning or tissue fibrosis even in aged mice.

Overexpression of constitutively active PI3Kα (p110α) also results in cardiac hypertrophy, whereas overexpression of dominant-negative PI3Kα (DN-PI3Kα) leads to the formation of abnormally small hearts of normal function (Shioi et al., 2002). To analyze the interaction of Pten and PI3Kα in the heart, Crackower et al. generated muscle-specific Pten/DN-PI3Kα double mutant mice (Crackower et al., 2002). The cardiac hypertrophy and activation of PKB/Akt observed in mice deficient for Pten alone was rescued by the transgenic expression of the DN-PI3Kα. However, cardiac contractility remained impaired in the double mutant mice. In mice lacking PI3Kγ (p110γ), heart size, heart rate and heart structure (including cardiac fibrosis) were normal but contractility was increased (Crackower et al., 2002). Pten/PI3Kγ double mutant mice showed hypercontractility to the same degree as PI3Kγ single mutant mice. However, the cardiac hypertrophy and hyper-phosphorylation of PKB/Akt observed in Pten single mutant mice were not rescued by deficiency of PI3Kγ. Thus, cardiac hypertrophy can be genetically uncoupled from cardiac contractility, and cardiac contractility depends on PI3Kγ signaling. Crackower et al. also showed that the Pten-PI3Kγ signaling pathway regulates cardiac muscle contractility at the single cell level. The G protein-coupled β2-adrenergic receptors (β2-AR) are linked to both Gzs and Gαi G-proteins that can either increase (Gαs) or decrease (Gαi) cAMP levels. In cardiomyocytes, increased cAMP levels result in enhanced contractility via activation of protein kinase A and subsequent phosphorylation of phosphorolamban (PLB) in the sarcoplasmic reticulum. A marked increase in cAMP production and enhanced phosphorylation of PLB were observed in both unstimulated and β2-AR-stimulated cardiomyocytes lacking PI3Kγ compared with wild type cells. cAMP production in response to Gαs-coupled β1-AR stimulation was comparable in knock-out and wild type cells, suggesting that stimulated PI3Kγ may negatively regulate β2-AR-cAMP signaling by modulating the activity of Gαi. The precise molecular mechanisms underlying the regulation of β2-AR-Gαi signaling by Pten and PI3Kγ remain to be investigated.

Although it has been reported that PI3Kγ (p110γ) is upregulated in pressure overload cardiac hypertrophy (Naga Prasad et al., 2000), PI3Kγ levels during cardiac failure have not been examined as yet. Drugs inhibiting PI3Kγ signaling thus may be excellent candidates for improving cardiac function in heart failure patients.

### Role of Pten in glucose metabolism

The mckCrePTENfloxflox mice cited above also exhibited deletion of Pten in the majority of skeletal muscles (Crackower et al., 2002). However, no gross abnormalities, changes in cell size, or alterations to serum glucose levels were observed. Butler et al. reported that specific inhibition of Pten by in vivo administration of antisense oligonucleotide improves the hyperglycemia in diabetic mice (Butler et al., 2002). The generation of mutant mice specifically lacking Pten in hepatocytes or adipocytes will be required to clarify the function of Pten in glucose metabolism.

### Role of Pten in the mammary gland

Pten mutations or loss of heterozygosity (LOH) have been frequently observed in human sporadic breast cancers, and breast cancer is one of the most common malignancies associated with Cowden disease. Pten+/– mice develop breast cancer (Stambolic et al., 2000), and the crossing of Pten+/– mice to MMTV-Wnt-1 transgenic mice induces the development of mammary tumors earlier than in the parental strains (Li et al., 2001). Li et al. generated mammary gland-specific Pten-deficient mice by mating Ptenfloxflox mice with MMTV-Cre transgenic mice (Li et al., 2002). MMTV-CrePtenfloxflox mice were fertile, had normal-sized litters and were able to nurse their pups. However, female MMTV-Cre Ptenfloxflox mice developed tumors as early as 2 months of age, and the mean latency period prior to tumor appearance was 9–10 months. Histologically the tumors ranged from benign fibroadenomas to pleiomorphic adenocarcinomas.

In virgin female MMTV-CrePtenfloxflox mice, the mammary gland ducts grew faster during puberty, showed excessive side branches or small protrusions at 6 weeks of age, and contained numerous lobulo-alveolar buds. These phenomena are normally observed in WT mice only in early pregnancy or during hormonal stimulation. The precocious alveolar development was accompanied by functional differentiation since mammary glands in the mutant virgins contained both α- and β-casein (milk-specific proteins) as early as 10 weeks after birth, a situation never observed in WT mice. Similar abnormalities in breast development have been reported in Cowden’s disease patients.

Terminal end buds in the mammary glands of MMTV-CrePtenfloxflox mice showed increased numbers of BrdU-labeled mammary epithelial cells. When transplanted into a WT fat pad, Pten-deficient mammary epithelium displayed increased side-branching, suggesting that the enhanced proliferation and differentiation observed in the mutant mice might be caused in a cell-autonomous manner. In mutant mice that were allowed to bear litters, the involution of mammary tissue and the sustained expression of β-casein by the mammary gland were observed. Butler et al. reported that specific inhibition of Pten by in vivo administration of antisense oligonucleotide improves the hyperglycemia in diabetic mice (Butler et al., 2002). The generation of mutant mice specifically lacking Pten in hepatocytes or adipocytes will be required to clarify the function of Pten in glucose metabolism.

### Role of Pten in the mammary gland

Pten mutations or loss of heterozygosity (LOH) have been frequently observed in human sporadic breast cancers, and breast cancer is one of the most common malignancies associated with Cowden disease. Pten+/– mice develop breast cancer (Stambolic et al., 2000), and the crossing of Pten+/– mice to MMTV-Wnt-1 transgenic mice induces the development of mammary tumors earlier than in the parental strains (Li et al., 2001). Li et al. generated mammary gland-specific Pten-deficient mice by mating Ptenfloxflox mice with MMTV-Cre transgenic mice (Li et al., 2002). MMTV-CrePtenfloxflox mice were fertile, had normal-sized litters and were able to nurse their pups. However, female MMTV-Cre Ptenfloxflox mice developed tumors as early as 2 months of age, and the mean latency period prior to tumor appearance was 9–10 months. Histologically the tumors ranged from benign fibroadenomas to pleiomorphic adenocarcinomas.

In virgin female MMTV-CrePtenfloxflox mice, the mammary gland ducts grew faster during puberty, showed excessive side branches or small protrusions at 6 weeks of age, and contained numerous lobulo-alveolar buds. These phenomena are normally observed in WT mice only in early pregnancy or during hormonal stimulation. The precocious alveolar development was accompanied by functional differentiation since mammary glands in the mutant virgins contained both α- and β-casein (milk-specific proteins) as early as 10 weeks after birth, a situation never observed in WT mice. Similar abnormalities in breast development have been reported in Cowden’s disease patients.

Terminal end buds in the mammary glands of MMTV-CrePtenfloxflox mice showed increased numbers of BrdU-labeled mammary epithelial cells. When transplanted into a WT fat pad, Pten-deficient mammary epithelium displayed increased side-branching, suggesting that the enhanced proliferation and differentiation observed in the mutant mice might be caused in a cell-autonomous manner. In mutant mice that were allowed to bear litters, the involution of mammary tissue and the sustained expression of β-casein by the mammary gland were observed. Butler et al. reported that specific inhibition of Pten by in vivo administration of antisense oligonucleotide improves the hyperglycemia in diabetic mice (Butler et al., 2002). The generation of mutant mice specifically lacking Pten in hepatocytes or adipocytes will be required to clarify the function of Pten in glucose metabolism.
the mammary gland led to involution defects similar to those observed in MMTV-Cre Pten\textsuperscript{flox/flox} mice. However, no significant differences were observed in ductal growth or epithelial differentiation. Moreover, spontaneous tumor formation was not observed in the MMTV-PKB/Akt transgenic mice. This discrepancy may be due to insufficient PKB/Akt expression levels or compensation by other downstream effectors. Like MMTV-Cre Pten\textsuperscript{flox/flox} mice, MMTV-Wnt\textsubscript{1} transgenic mice exhibit increased mammary gland side-branching, a feathery morphology, and precocious lobulo-alveolar development (Tsukamoto \textit{et al.}, 1988). Further study will be required to determine the effect of the Wnt/β-catenin pathway on Pten-deficient mammary glands, as Pten deficiency caused skin hyperplasia, hyperkeratosis and tumor formation. Transgenic animals with trichilemmomas in the face skin papules, papillomatosis of the acral region of the skin (Hildenbrand \textit{et al.}, 2001). The association of cutaneous squamous cell carcinomas with Cowden disease has also been reported (Camisa \textit{et al.}, 1984; Nuss \textit{et al.}, 1978).

The importance of epidermal growth factor receptor (EGFR), IGF-1 and Ha-ras signaling in morphogenesis and carcinogenesis is clearly evident in skin (Rho \textit{et al.}, 1996; Vassar \textit{et al.}, 1992; Yamamoto \textit{et al.}, 1986). Activation of the TGFα/EGFR pathway (Dominey \textit{et al.}, 1993; Sibilia \textit{et al.}, 2000; Vassar and Fuchs, 1991), IGF-1 (DiGiovanni \textit{et al.}, 2000; Rho \textit{et al.}, 1996) or v-Ha-ras (Greenhalgh \textit{et al.}, 1993) in the epidermis of transgenic mice leads to skin hyperplasia, hyperkeratosis and tumor formation. Transgenic animals overexpressing IGF-1 also show accelerated hair growth (Bol \textit{et al.}, 1997), and mice lacking IGF-1R exhibit hypoplastic skin (Liu \textit{et al.}, 1993). Since each of EGFR, IGF-R and Ras triggers PI3K signaling, and decreased Pten activity contributes to the malignant conversion stage in chemically-induced mouse skin cancers (Segrelles \textit{et al.}, 2002), we generated keratinocyte-specific Pten-deficient mice (k5CrePten\textsuperscript{flox/flox} mice) by crossing Pten\textsuperscript{flox} mice to K5-Cre transgenic mice (Suzuki \textit{et al.}, in revision).

The skin of k5CrePten\textsuperscript{flox/flox} mice 3 days of age or older was noticeably wrinkled due to hyperplasia. Most mutant mice were significantly smaller than their WT littermates from 3–5 days of age and their hair coats were abnormally ruffled and shaggy. Surprisingly, more than 90% of k5Pten\textsuperscript{flox/flox} mice died due to malnutrition during the lactation period, possibly due to esophageal dysfunction caused by hyperkeratosis in the esophagus. However, animals that survived past 2 months of age had normal lifespans without severe malnutrition. Examination of the hair of k5Pten\textsuperscript{flox/flox} mice showed that the cuticles, which normally cover the hair shafts, were almost completely detached from the hair shafts.

Histologically, newborn k5CrePten\textsuperscript{flox/flox} mice exhibited hyperkeratosis, hypergranulosis and epidermal hyperplasia. Increased density of hair follicles, reduced interfollicular epidermis, and advanced sebaceous glands indicated that precocious skin development occurred in the absence of Pten. Analysis of the phases of hair development showed that skin formation was accelerated in k5Pten\textsuperscript{flox/flox} mice. The wnt/β-catenin/Lef-1 pathway has been associated with the acceleration of developmental morphogenesis in the skin (Gat \textit{et al.}, 1998). Pten has been shown to negatively regulate the β-catenin/Lef-1 pathway by inhibiting the nuclear accumulation of β-catenin and activation of Lef-1 in a prostatic cell line (Persad \textit{et al.}, 2001). However, we observed no definite differences in the subcellular distribution of β-catenin in k5Pten\textsuperscript{flox/flox} cells.

Surprisingly, 23% of k5Pten\textsuperscript{flox/+} and 100% of k5Pten\textsuperscript{flox/flox} mice developed spontaneous tumors by 9 months of age. Most of these spontaneous tumors were squamous papillomas that occurred on the face and palms of the front paws. However, many papillomas went on to become squamous cell carcinomas with nuclear atypia and increased mitosis. We also observed sebaceous carcinomas and adenocarcinomas of the sweat gland. The loss of the WT Pten allele was observed in all squamous carcinomas obtained from k5Pten\textsuperscript{flox/+} mice. When k5Pten\textsuperscript{flox/+} mice were treated with the carcinogen DMBA (dimethylbenzanthracene) plus tetradecanoyl phorbol acetate (TPA), 100% of them developed 5–15 skin papillomas in the treated area within 5 weeks of the initial DMBA treatment. No tumors developed on the skin of either k5Pten\textsuperscript{flox/+} or WT mice subjected to the same protocol.

Pten-deficient keratinocytes showed enhanced proliferation in response to EGFR treatment in vitro. In addition, the mutant keratinocytes were more resistant than WT keratinocytes to apoptosis induced by either high dose UV- or γ-irradiation. The phosphorylation of PKB/Akt and MAPK was significantly elevated in EGF-treated k5Pten\textsuperscript{flox/flox} keratinocytes compared to controls, consistent with a scenario in which enhanced proliferation and apoptotic resistance result from the loss of Pten-mediated regulation of PKB/Akt and MAPK activation.

The phenotypes observed in k5Pten\textsuperscript{flox/flox} mice are reminiscent of those of Ha-ras (Greenhalgh \textit{et al.}, 1993), TGF-α (Vassar and Fuchs, 1991), SOS (Sibilia \textit{et al.}, 2000) and IGF-1 (Bol \textit{et al.}, 1997; DiGiovanni \textit{et al.}, 2000) transgenic mice. However, the onset of skin tumor formation in mice requires EGFR and PKB/Akt signaling in addition to SOS/Ras/ERK signaling (Sibilia \textit{et al.}, 2000). Indeed, MAPK/
ERK has been reported to act in synergy with the PI3K pathway to stimulate CycD1 transcription in NIH3T3 cells (Gille and Downward, 1999). Other evidence suggests that PKB/Akt may be a key molecule regulating the onset of skin carcinogenesis in mice. The transplantation of keratinocytes overexpressing PKB/Akt results in highly aggressive skin tumors, and PKB/Akt activation is one of the first events in the chemical induction of skin tumors (Segrelles et al., 2002). Thus, the onset of tumors in k5Pten<sup>flx/flx</sup> mice may be caused primarily by cellular hyperproliferation and/or apoptotic resistance induced by PI3K and PKB/Akt hyperactivation, with a contribution by deregulated ERK activation. Studies to examine PTEN expression in human keratinocyte malignancies are ongoing.

**Conclusions**

The involvement of PTEN in cellular signal transduction pathways and the physiological role of Pten have been intensively studied. In this review, we have summarized what is known about the in vivo functions of Pten in various murine organs (Fig. 2). Pten function has now been implicated in the regulation of cell proliferation and apoptosis, and the control of cytoskeletal dynamics underlying cell migration and cell size. Without Pten function, T cells, B cells, brain cells, cardiomyocytes, mammary gland cells and keratinocytes (and perhaps other cell types still to be analyzed) show profoundly dysregulated behaviours that eventually impair the development and function of the relevant organ system. The resulting disorders may include autoimmune disease and/or tumorigenesis. Pten is therefore an essential regulator of normal homeostasis and oncogenesis in many organs. Accordingly, the inhibition of the PI3K-PIP3-PKB/Akt pathway may be an attractive approach for treating a wide variety of malignancies. It is our hope that tissue-specific mouse mutants lacking Pten will become
Physiological Functions of Pten

useful tools for the further analysis of intracellular signaling pathways, and may help in the search for new therapeutic drugs to treat patients with PTEN insufficiency.

Acknowledgments. We are grateful to all of our collaborators for their generous contributions to these projects. We also thank Dr. Tetsuo Noda (Tohoku University) for helpful discussions, Seiko Toyosawa and Miki Sato Suzuki for their valuable assistance.

References


Physiological Functions of Pten

mediated by extracellular signal-related kinase mitogen-activated pro-


(Received for publication, December 16, 2002 and accepted, December 17, 2002)