**Atheroma development in apolipoprotein E-null mice is not affected by partial inactivation of PTEN**

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## 1. ABSTRACT

PTEN is a dual-specificity phosphatase that has been shown to inhibit vascular smooth muscle cell (VSMC) proliferation and migration, two key events in the ethiopathogenesis of atherosclerosis. Adenovirus-mediated PTEN overexpression inhibited the formation of vascular obstructive lesions induced by mechanical injury of the vessel wall. In this study, we investigated whether PTEN protects against atheroma formation in apolipoprotein E-null mice *(apoE-/-)*, a widely used animal model characterized by the development of hypercholesterolemia and atherosclerosis. We examined atheroma development in the aorta of *apoE-/-* mice with an intact *Pten* gene and *apoE-/-* mice lacking one allele of *Pten (Pten+/-apoE-/-)* that were challenged for six weeks with an atherogenic diet. Compared with *apoE-/-* controls, Western blot analysis of arterial cell lysates from *Pten+/-apoE-/-* mice revealed a decrease in PTEN expression. This correlated with increased phosphorylation of AKT, thus demonstrating that *Pten* inactivation in *Pten+/-apoE-/-* mice has functional consequences. However, the extent of atherosclerosis was undistinguishable in both groups of fat-fed mice. Likewise, the atheroma of *Pten+/-apoE-/-* and *apoE-/-* mice displayed similar VSMC content, cellularity and rates of proliferation and apoptosis. Thus, in spite of the cytostatic and antimigratory activities of PTEN, and in contrast to previous studies demonstrating that *Pten* is haplo-insufficient for tumor suppression, our results demonstrate that atherosclerosis in hypercholesterolemic mice is not aggravated by partial inactivation of *Pten*.

## 2. INTRODUCTION

Atherosclerosis (from the Greek words *athero*: gruel or paste, and *sclerosis*: hardness) is a complex multifactorial disease of medium and large arteries that involves distinct cell types and molecular events, including both adaptative and innate immune mechanisms (1-6). Endothelial dysfunction induced by a variety of atherogenic stimuli promotes the adhesion and transendothelial migration of blood circulating leukocytes, which accumulate within the subendothelial space to form the so-called fatty streak, an early atheromatous lesion which contains mostly highly proliferative macrophages that avidly uptake lipoproteins to become lipid-laden foam cells (1,6). At homeostasis, VSMCs are primarily located in the arterial tunica media in a non-proliferative state. However, activated leukocytes in growing atheromas produce a plethora of inflammatory chemokines and cytokines that promote VSMC proliferation and migration from the tunica media towards the neointimal lesion, thus further contributing to plaque development (7,8). It has been demonstrated that hyperplastic growth and migration of adventitial myofibroblasts also contributes to neointima formation (9). In addition to cellular components, atheromatous lesions contain cholesterol and other fatty materials, and increased content of specific extracellular matrix components. Plaque rupture or erosion at advanced disease stages can lead to acute occlusion due to thrombus formation, resulting in myocardial infarction or stroke.

Animal and human studies have identified signaling networks and factors that play a key role in the regulation of VSMC proliferation and migration in vitro and in vivo (7,8). The tumor suppressor PTEN is a dual-specificity lipid and protein phosphatase that negatively regulates the PI3K/AKT and FAK signaling pathways (10-12). In addition to its role as tumor suppressor, mounting evidence strongly implicates PTEN in cardiovascular physiology and disease (13). PTEN can modulate cardiac myocyte hypertrophy and survival (14). Moreover, the PI3K/AKT pathway and PTEN are involved in the maintenance of endothelial cell (EC) and VSMC homeostasis (15-17). In vitro studies have shown that PTEN inhibits VSMC proliferation, migration and survival (18), and increased PTEN activity mediates perlecan-induced suppression of VSMC proliferation (19). In ECs, adenovirus-mediated overexpression of a dominant negative PTEN mutant enhanced VEGF-mediated cell survival, mitogenesis and migration, and these processes were strongly inhibited by overexpression of wild-type PTEN (20). Similarly, adenovirus-mediated expression of PTEN inhibited both basal and PDGF-mediated proliferation, migration and survival in VSMCs (18). More recently, it has been shown that PTEN induces G1 cell cycle arrest and inhibits MMP-9 expression via the regulation of NF-kappaB and AP-1 in VSMCs (21). In addition, adenovirus-mediated intraarterial delivery of PTEN inhibits neointimal hyperplasia and percent of stenosis in a rat model of balloon angioplasty (22). Finally, morpholino-mediated loss of endogenous PTEN induced a serum-independent growth phenotype in cultured serum-dependent VSMCs, and decreased activity of PTEN was associated with high in vivo VSMC growth rates (23).

More recent investigations have also correlated increased levels of PTEN with decreased lesion development or VSMCs proliferation. Cholesterol-fed rabbits treated with propylthiouracil (PTU) (a drug with hypothyroid effect) showed a marked reduction in VSMC/macrophage ratio in atherosclerotic plaque, and addition of PTU to cultured rat VSMCs led to increased PTEN expression and reduced cell proliferation (24). It has been also suggested that PPARgamma-mediated transcriptional activation of PTEN by Rosiglitazone and Lovastatin might contribute to the therapeutic effects of these drugs (25).

In view of the above results, we hypothesized that PTEN inactivation would enhance atheroma progression. To examine this possibility, we took advantage of the availability of the atherosclerosis-prone *apoE-/-* mouse (26,27), a widely used animal model that has allowed major advances in understanding the molecular basis of atherosclerosis (28). These mice spontaneously develop hypercholesterolemia and complex atherosclerotic lesions resembling to those observed in humans, a process that can be accelerated upon exposure to a high-fat cholesterol-rich diet. Since full inactivation of *Pten* causes embryonic lethality (29), the present study was designed to assess the effect of inactivating one allele of *Pten* on atherosclerosis in *apoE*-null mice. As expected, *Pten+/-apoE-/-* mice exhibit reduction expression of PTEN in aortic tissue and this correlated with augmented AKT phosphorylation compared to *apoE-/-* mice. However, atheroma size, VSMC content, cellularity and rates of proliferation and apoptosis were similar in both groups of mice.
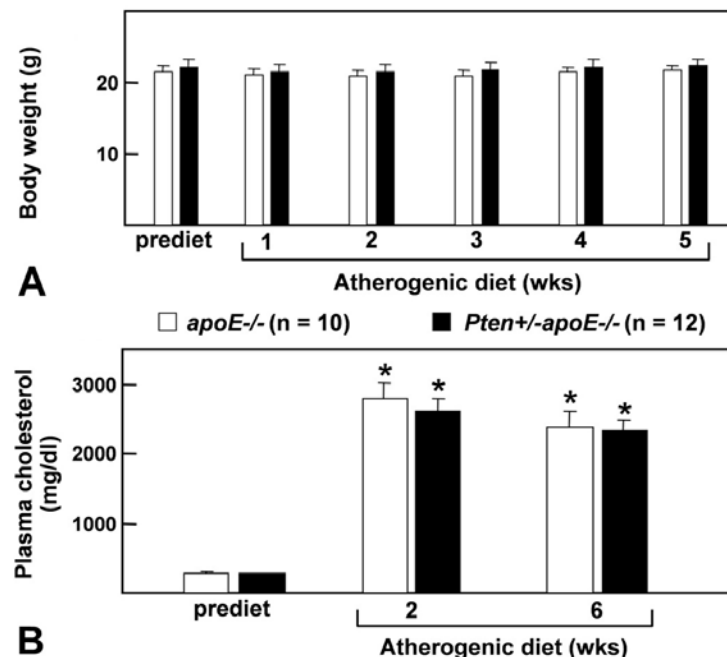
## 3. MATERIALS AND METHODS

### 3.1. Mice, genotyping and diet

*apoE-/-* mice (C57BL/6J, Charles River) and *Pten+/-* mice (29) (mixed 129/C57BL/6 genetic background) were mated and the double heterozygous F1 offspring were crossed with *apoE-/-* mice. The F2 offspring was genotyped by PCR analysis to identify *Pten+/-apoE-/-* and *apoE-/-* mice and brother-sister mating of mice of these genotypes was performed to obtain the two experimental groups (*apoE-/-* and *Pten+/-apoE-/-* mice). After weaning, mice were maintained on a low-fat standard diet (2.8% fat, Panlab, Barcelona, Spain). At 2 months of age, mice received for 6 weeks an atherogenic diet containing 15.8% fat, 1.25% cholesterol and 0.5% sodium cholate (S4892-S010, Ssniff, Germany).

### 3.2. Serum cholesterol, quantification of atherosclerosis, and immunohistochemistry

Blood was withdrawn before, and after the high-fat diet to measure plasma cholesterol levels using enzymatic procedures (Sigma, St. Louis, Missouri). To determine the extent of atherosclerosis in the aortic arch region, fat-fed mice were killed and their aorta was fixed in situ with 4% paraformaldehyde. Tissue was extracted and fixation continued for approximately 24h. Specimens were paraffin-embedded and mounted in a Microm microtome (Heidelberg, Germany) to quantify atherosclerosis essentially as previously described (30). Briefly, once the 3 valve cusps were reached, sections throughout the first ~2-mm of the ascending aorta were discarded. Then, ~25 consecutive sections (4 μm thickness) were taken from 2-3 regions of the aortic arch separated by ~60 μm. Three cross-sections from each region were stained with hematoxylin/eosin. Images were captured with a Sony DKC-CM30 camera (Tokyo, Japan) mounted on a Zeiss Axiolab stereomicroscope and the area occupied by atherosclerotic lesions (intima) and the area of the media was determined by computer-assisted quantitative morphometry to determine the intima-to-media ratio using Sigma Scan Pro v5.0 (Jandel Scientific, San Rafael, California). Blood cholesterol and atheroma size were measured by a researcher who was blinded to genotype. The extent of atherosclerosis for each animal was calculated by averaging the values obtained in 2-3 independent aortic arch regions. Differences in lesion area between males and females were not significant, so data from both sexes were included in the analyses.

To quantify lesion cellularity, the number of cells per $mm^2$ of plaque was determined by examining hematoxylin-stained arterial cross-sections. VSMCs were identified with alkaline phosphatase-conjugated anti-smooth muscle alpha-actin (SMalpha-actin) antibody (1/20, a-5691, Sigma). Alkaline phosphatase activity was detected with Fast Red (Sigma). VSMC content in atherosclerotic

**Figure 1.** Body weight and plasma cholesterol level in mice fed control chow or a cholesterol-rich diet. Data are shown as mean ± SEM of the indicated number of animals. Differences among groups were evaluated using ANOVA and Fisher's PLSD post hoc test. There were no differences in the body weight between both genotypes. Regardless of the genotype for *Pten*, 2 and 6 weeks of fat feeding produced a statistically significant increase in plasma cholesterol versus prediet level (*, p<0.0001). No differences were observed between 2 and 6 weeks of fat feeding.

lesions was determined morphometrically by dividing the SMalpha-actin-positive area by total plaque area. Apoptosis was measured using the ApopTag Peroxidase in situ Apoptosis Detection Kit according to the recommendations of the manufacturer (Serologicals Corporation, Norcross, GA). The enzymatic addition of deoxynucleotides to nicked ends of DNA was stopped with a Stop/Wash buffer, and the slides were incubated with the anti-digoxigenin conjugate. After incubation, slides were washed and then colour was developed by addition of the peroxidase substrate. Cell proliferation within atherosclerotic lesions was quantified using a monoclonal antibody against the proliferation marker Ki67 (clon SP6, Master Diagnostics). Before immunostaining, slides were boiled with 10 mM citrate buffer for 10 min for antigen retrieval and endogenous peroxidase was blocked with 0.3% $H_2O_2$. Detection was performed using a biotin-conjugated anti-rabbit secondary antibody and ABC kit system (Vectastin) using DAB as peroxidase substrate (Vector laboratories). Slides were counterstained with hematoxylin as before and immunoreactive cells per mm$^2$ of atheroma were counted.

### 3.3. Statistical analysis

Results are reported as mean ± SEM. In experiments with 2 groups, differences were evaluated using a 2-tail, unpaired Student t-test. Analyses involving more than 2 groups were done using ANOVA and Fisher's post-hoc test (Statview, SAS institute, Cary, North Carolina).
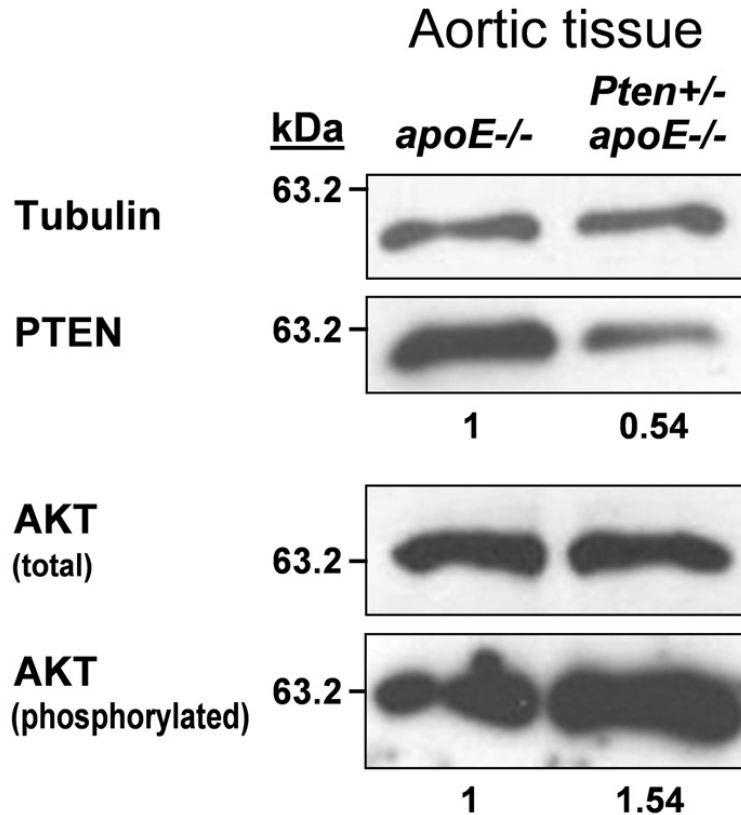
### 3.4. Western blot analysis

Snap-frozen arteries from three fat-fed mice of each genotype were pooled and lysed in ice-cold 50mM

Tris-HCl buffer (pH 7.5) containing 1% Triton X-100, 150mM NaCl, 1mM DTT and protease inhibitor Complete Mini cocktail (Roche, Mannheim, Germany) using an Ultraturrax T25 basic (IKA Labortechnik, Staufen, Germany). Western blot analysis was performed using the following primary antibodies: rabbit polyclonal anti-PTEN (1/500, NeoMarkers RB-072-PO), rabbit polyclonal anti-phospho-Ser473-AKT (1/250, Cell Signalling 9271S), goat polyclonal anti-AKT (1/1000, Santa Cruz sc-1619) and mouse monoclonal anti-tubulin (1/100, Santa Cruz sc-3035). Immunocomplexes were detected using an ECL detection kit according to the recommendations of the manufacturer (Amersham Biosciences). The relative intensity of protein bands was determined by densitometry.

## 4. RESULTS

### 4.1. Aortic expression of PTEN and quantification of atherosclerosis in fat-fed *apoE-/-* and *Pten+/-apoE-/-* mice

We intercrossed *apoE*-null mice and mice deficient for one allele of *Pten* to generate *apoE*-null mice with an intact *Pten* gene (*apoE-/-*) and with one allele of *Pten* disrupted (*Pten+/-apoE-/-*). After weaning, mice were maintained on a low-fat standard diet. At two months of age, blood was collected and mice were switched to a high-fat cholesterol-rich diet for six weeks. As shown in Figure 1A, body weight before the onset of the atherogenic diet and throughout fat-feeding was undistinguishable when comparing *apoE-/-* and *Pten+/-apoE-/-* mice. Likewise, fat-feeding caused similar level of hypercholesterolemia in both groups of

## Aortic tissue



**Figure 2.** Expression of PTEN and AKT in the aorta of fat-fed *apoE-/-* and *Pten+/-apoE-/-* mice. Aortic lysates were subjected to Western blot analysis using the indicated antibodies. For AKT expression, we used antibodies directed against total AKT or AKT phosphorylated in Ser473. Relative protein abundance was estimated by densitometric analysis of two independent blots. Aortic PTEN expression in *Pten+/-apoE-/-* mice was reduced to $0.51 \pm 0.02$ versus the level in *apoE-/-* (set as 1, normalized by tubulin content). By contrary, the level of phosphorylated AKT in the aorta of *Pten+/-apoE-/-* mice was increased to $1.48 \pm 0.06$ as compared to the level in *apoE-/-* (set as 1, normalized by total AKT).

mice (Figure 1B). Because *Pten* inactivation has been shown to alter peripheral B lymphocyte fate (31), we analyzed by flow cytometry the presence of B-cells in the blood. These studies revealed similar B lymphocyte counts in *apoE-/-* and *Pten+/-apoE-/-* mice, both when analyzed before and after fat-feeding (data not shown).
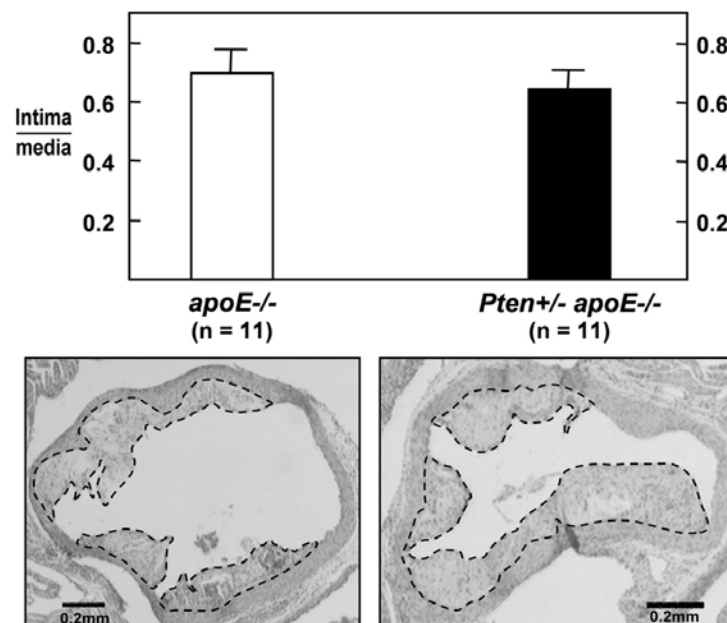
To assess whether partial genetic inactivation of *Pten* led to reduced aortic PTEN expression, we analyzed by Western blot aortic cell lysates. Densitometric analysis of two independent blots revealed an average ~50% decrease of PTEN protein expression in the aorta of *Pten+/-apoE-/-* as compared to *apoE-/-* mice (Figure 2). Importantly, the level of AKT phosphorylation, a parameter that is negatively regulated by PTEN (32), was increased in the aorta of *Pten+/-apoE-/-* mice (Figure 2), demonstrating that diminished PTEN expression in these mice has functional consequences.

We next examined the extent of diet-induced atherosclerosis in aortic tissue. Consistent with numerous studies in *apoE-/-* mice, atherosclerosis prevailed within the aortic arch in both groups of mice. Thus, we quantified by computer-assisted planimetry the area of atheroma in aortic
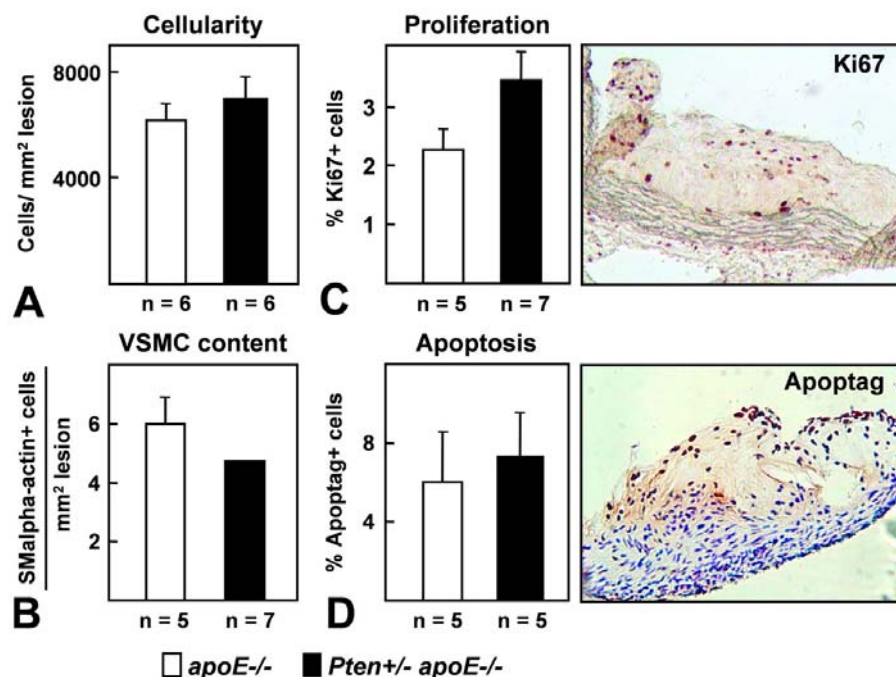
arch cross-sections stained with hematoxylin/eosin (Figure 3). This analysis disclosed no statistical differences in the intima-to-media ratio when comparing *apoE-/-* and *Pten+/-apoE-/-* mice ($0.70 \pm 0.09$ and $0.64 \pm 0.07$, respectively; n=11, p>0.05). Collectively, these results demonstrate that reduced PTEN expression does not affect aortic atherosclerosis in fat-fed hypercholesterolemic *apoE-/-* mice.

### 4.2. Immunohistochemical characterization of atherosclerotic lesions

We next carried out immunohistochemical analysis to characterize the atheroma in fat-fed mice. Both the total number of cells per mm$^2$ of atheroma (Figure 4A) and the area of atheroma occupied by VSMCs, as determined by SMalpha-actin immunoreactivity (Figure 4B), were statistically undistinguishable when comparing *apoE-/-* and *Pten+/-apoE-/-* mice (cellularity: 6168±637 versus 6971±806 cells per mm$^2$ atheroma, respectively, p>0.05; VSMC content: 6±3 versus 4.7±0.9 SMalpha-actin-positive cells/mm$^2$ atheroma, respectively, p>0.05). Similarly, the percentage of proliferating cells, as determined by Ki67 immunoreactivity (Figure 4C) and apoptotic cells, as determined by the Apoptag kit (Figure 4D), were comparable in the atheroma of both groups of

**Figure 3.** Reduced PTEN expression in the artery wall of *Pten+/-apoE-/-* does not affect the size of aortic atherosclerotic plaques. Mice with the indicated genotypes were challenged with a high-fat diet for six weeks. The graph represents the intima-to-media ratio in cross-sections of the aortic arch, which did not show statistical differences between the two experimental groups (Student's t-test, $p>0.05$). Representative examples of cross-sections stained with hematoxylin and eosin are shown at the bottom. The edge of the atherosclerotic plaque is drawn with a discontinuous line.



**Figure 4.** Immunohistochemical analysis of atherosclerotic lesions. Analysis was performed in cross-sections from the aortic arch and all parameters were quantified in the atheroma: lesion cellularity in hematoxylin-stained specimens (A), VSMC content as determined by SMalpha-actin immunoreactivity (B), proliferative cells as determined by Ki67 immunoreactivity (C), and apoptotic cells as revealed using the Apoptag kit (D). In all cases, differences between *apoE-/-* and *Pten+/-apoE-/-* mice were not statistically significant (Student's t-test, $p>0.05$).

mice (Ki67-positive cells: 2.3±0.3% versus 3.5±0.5%, p>0.05; Apoptag-positive cells: 6.03±2.54% versus 7.26±2.31%, p>0.05, in *apoE-/-* and *Pten+/-apoE-/-* mice, respectively).

## 5. DISCUSSION

Previous studies have conclusively demonstrated that PTEN inhibits VSMC proliferation and migration in vitro and reduces neointimal thickening in the rat carotid artery model of balloon angioplasty (18,19,21,22). To our knowledge, however, the role of PTEN on atheroma development has not been investigated. Therefore, we sought to examine the consequences of genetically inactivating *Pten* on diet-induced atherosclerosis using the *apoE*-null mouse model, a well characterized animal model of atherosclerosis that recapitulates important features of the human disease (26,27). Since global *Pten* inactivation in the mouse causes lethality during embryogenesis, (29) we analyzed fat-fed *apoE-/-* and *Pten+/-apoE-/-* mice. Inactivation of one allele of *Pten* led to a 50% reduction in PTEN protein expression in the aorta of *Pten+/-apoE-/-* compared to *apoE-/-* mice. Importantly, PTEN inactivation had functional consequences, since phosphorylation of its target AKT/PKB in aortic tissue was increased by approximately 50% without changes in total AKT expression. However, we found no differences in aortic atherosclerotic lesion between *apoE-/-* and *Pten+/-apoE-/-* mice. Likewise, analysis of the atheromatous lesions in these animals disclosed no differences in several histopathological parameters, including the area occupied by VSMCs, cellularity, proliferation and apoptosis.

It has been previously shown that *Pten+/-* mice spontaneously developed germ cell, gonadostromal, thyroid and colon tumours (29), demonstrating that *Pten* is haplo-insufficient for tumor suppression. Of note in this regard, adenovirus-mediated intraarterial delivery of *Pten* after balloon injury in the rat carotid artery inhibits neointimal lesion development (22), a pathological process characterized by abnormally high hyperplastic growth of VSMCs (7,33,34). Thus, alterations in PTEN expression have a major impact on the course of highly proliferative disorders, such as cancer and neointimal hyperplasia induced by mechanical injury of the vessel wall. In contrast, we show here that atheroma development in *apoE-/-* mice is not affected by partial inactivation of *Pten*. Although vascular cell hyperplasia is also a feature of atherosclerosis, this disease involves additional processes that might not be regulated by PTEN (e. g., arterial lipid accumulation, neointimal foam cell formation, abundant extracellular matrix formation by arterial cells, VSMC dedifferentiation) (1-6). On the other hand, Shen et al. have recently suggested that PTEN expression may contribute to cardiovascular diseases by causing p38 MAPK stress signal-induced inhibition of insulin-signaling and eNOS activation (35). Thus, alterations in PTEN expression may promote both pro- and anti-atherogenic effects. Finally, we cannot rule out the possibility that the 2-fold reduction in aortic PTEN expression in *Pten+/-apoE-/-* mice (cf. Figure 2) might not be sufficient to aggravate atheroma development compared to *apoE-/-* mice. Since global *Pten*

inactivation causes embryonic lethality (29), addressing whether total *Pten* gene inactivation in the artery wall might indeed exacerbate atheroma progression will require the generation of *apoE-/-* mice with *Pten* disruption targeted to cell types known to participate in atherosclerosis (e. g., EC, VSMC, macrophage).

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