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PTEN attenuates PIP₃/Akt signaling in the cochlea of the aging CBA/J mouse

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Abstract

We have previously reported the activation of cell death pathways in the sensory cells of the aging cochlea. Here we investigate age-associated changes in survival mechanisms focusing on phosphatidylinositol 3,4,5-trisphosphate (PIP₃)/Akt signaling. The animal model is the CBA/J mouse of 18 months of age prior to the onset of major functional loss (ABR thresholds, 26.8 dB SPL) which is compared to young animals of 3 months of age (ABR thresholds, 19.7 dB SPL). Immunostaining on cochlear cryosections revealed a wide-spread distribution of PIP₃ in the cochlea which was markedly attenuated in old animals in inner and outer hair cells, Deiters cells and pillar cells. Protein levels of the lipid phosphatase PTEN which regulates PIP₃ increased in those cells with aging while its mRNA did not, suggesting an age-related reduction of PTEN degradation. Furthermore, staining intensity of phosphorylated PTEN (ser380) and its nuclear localization increased. Consistent with a reduction of PIP₃, the phosphorylation of the downstream target Akt at threonine 308 significantly decreased in outer hair cells. The results suggest a decline of the survival capacity of aging outer hair cells due to a decrease in PIP₃/Akt signaling caused by an increase of PTEN.

Keywords

outer hair cells; PTEN; PIP₃; Akt; aging cochlea

Introduction

Phosphatidylinositol 3,4,5-trisphosphate (PIP₃), the product of the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP₂) by phosphoinositide 3-kinase (PI3-kinase), plays an important role in transducing signals from growth factors, hormones and other extracellular activators to intracellular pathways. PIP₃ signaling, in particular, is associated with the control of cell survival and cell death (Irvine, 2003; Parker, 2004). PIP₃ binds to and activates the phosphoinositide-dependent protein kinase-1 which, in turn, phosphorylates and activates the downstream target Akt, also known as protein kinase B (Denley A. et. al., 2009; Elghazi and Bernal-Mizrachi, 2009). Akt activation leads to phosphorylation of numerous downstream proteins and affects cell growth, cell survival, and cell differentiation. It is considered pivotal as an anti-apoptotic factor in many different cell death paradigms (Paez and Sellers 2003).

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Of the three mammalian isoforms of Akt, Akt1 is ubiquitously expressed at high levels, Akt2 expression is highest in insulin-sensitive tissues and Akt3 is restricted mainly to brain and testes (Woodgett, 2005; Franke, 2008). Although encoded by different genes, these isoforms share a high degree of homology and similar basic activation processes (Misra et al., 2008). Their structures include three functionally distinct regions: an NH₂-terminal pleckstrin homology domain which mediates binding to PIP₃, a central catalytic and a C-terminal hydrophobic domain which both contain phosphorylation sites (Woodgett, 2005). The phosphorylation of a specific threonine residue (thr308) in the catalytic domain is essential for Akt activation and executed by the 3-phosphoinositide-dependent protein kinase-1 while a second phosphorylation at a serine residue (ser473) in the C-terminal domain contributes to a maximal activation and is regulated by 3-phosphoinositide-dependent protein kinase-2 (Scheid and Woodgett, 2001).

The levels of the crucial signaling molecule PIP₃ are reciprocally controlled by phosphoinositide 3-kinase and the phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome ten). PTEN is a dual-function phosphatase capable of dephosphorylating specific protein and lipid substrates. As a lipid phosphatase, PTEN reverses the phosphorylation of PIP₃, returning it to PIP₂. As a consequence, an increased activity of PTEN antagonizes the function of PI3-kinase and inhibits signaling through Akt (Wu et al., 2003). Conversely, a PTEN-deficiency would boost the Akt pathway and deficient cells indeed show a significantly reduced sensitivity to agonist-induced apoptosis (Stambolic et al., 1998; Podsypanina et al., 1999).

PTEN activity is also subject to regulatory mechanisms. The PTEN protein contains three domains: an N-terminal catalytic phosphatase domain, and two domains with potential serine and threonine phosphorylation sites, namely a lipid-binding central C2 domain and a C-terminal tail (Wang and Jiang, 2008). Cysteine residues in the N-terminal region render it sensitive to oxidation and inactivation. Phosphorylation in general reduces the membrane-association of PTEN which is required for activity but also increases the stability of the protein and influences its cytoplasmic/nuclear translocation (Zhu et al., 2006).

Recently, we have reported on the activation of cell death pathways in the aging cochlea (Sha et al., 2009). We now hypothesize that, in addition, decreasing survival signaling contributes to the imbalance in cellular homeostasis leading to age-related hair cell loss. We use CBA/J mice which have been well characterized in regard to their inner ear pathology as a model for studying sensorineural presbycusis (Sha et al., 2008). The aging CBA/J mouse slowly develops elevated auditory thresholds at high frequencies beginning at an age of about 12 months, affecting some individuals more than others. For this study, we selected mice of 18 months of age that have retained relatively good thresholds in order to compare the regulation of cell survival pathways to that in young mice of 3 months of age. The PIP₃/Akt pathway was chosen as our focus because of its central importance in cell survival and because we have previously demonstrated the involvement of this pathway in mediating aminoglycoside-induced hair cell death (Jiang et al., 2006).

Materials and Methods

Animals

Male CBA/J mice were purchased from Harlan Sprague-Dawley Co. (Indianapolis, IN) through the National Institute on Aging two weeks prior to the intended age for the experiments of 3 and 18 months, respectively. The animals were kept at 22 °C under a 12h:12h light-dark cycle, and had free access to water and a regular mouse diet (Purina 5025, St. Louis, MO). Animal care was supervised by the University of Michigan's Unit for Laboratory Animal Medicine

and all experimental protocols were approved by the University of Michigan Committee on Use and Care of Animals.

Materials

ECL (enhanced chemiluminescence) reaction kits for antibody detection on Western blots were purchased from Amersham Pharmacia Biotech (Piscataway, NJ); Mouse monoclonal antibody for PTEN was obtained from Chemicon International (Temecula, CA); rabbit polyclonal anti-phospho-Akt1, Akt1/2 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); Rabbit anti p-Akt (ser473), p-Akt (thr308) and p-PTEN (ser380) from Cell Signaling Technology; Mouse anti PIP₃ IgM from Echelon Biosciences Inc. Secondary antibodies for Western blotting were purchased from Jackson ImmunoResearch (West Grove, PA) and secondary fluorescence antibodies (Alexa 488 and Alexa 546), rhodamine phalloidin, Hoechst 33342 and propidium iodide (PI) from Molecular Probes (Eugene, OR). Complete™ mini EDTA-free protease inhibitor cocktail tablets were purchased from Roche Diagnostic (Mannheim, Germany). BenchMark™ Protein ladders, TRIzol® reagent and SuperScript^{III} First-Strand Synthesis Kit from Invitrogen (Carlsbad, CA); Taqman probes from AB applied Biosystems (Foster City, CA); and Master Mixture from Qiogene (Carlsbad, CA). All other reagents were obtained from Sigma Chemical (St. Louis, MO).

Extraction of total proteins

Cochleae were rapidly removed and dissected in ice-cold 10 mM phosphate-buffered saline (PBS) at pH 7.4. In order to extract total protein, tissue from one mouse cochleae was homogenized in ice-cold RIPA lysis buffer (Upstate, Waltham, MA, cat# 20-188) by using a glass/glass micro Tissue Grind pestle and vessel for 30 sec. After 30 min on ice, tissue debris was removed by centrifugation at $12,000 \times g$ at 4°C for 10 min and the supernatants were retained as the total protein fraction. Protein concentrations were determined using the Bio-Rad Protein Assay dye reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as a protein standard.

Western Blot Analysis

Protein samples (50 µg) were separated by SDS-PAGE. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Pierce, Rockford, IL) and blocked with 5% nonfat dry milk in PBS with 0.1% Tween 20 (PBS-T). The membranes were incubated with primary antibodies, anti-PTEN, anti-phospho-Akt or anti-Akt1/2 at a dilution of 1:500 for overnight at 4°C, and then washed three times (10 min each) with PBS-T. Membranes were incubated with an appropriate secondary antibody at a dilution of 1:10,000 for 1 hr. Following extensive washing of the membrane, the immunoreactive bands were visualized by enhanced chemiluminescence using FluorChem SP instrumentation (Alpha Innotech, San Leandro, CA). Western blots were analyzed using AlphaEase software SpotDenso tool (Alpha Innotech, San Leandro, CA). Briefly, the band densities were analyzed after subtraction of the background. The relative densities of samples from different age groups normalized to the control values were assessed from four individual assays. Resulting ratios were averaged and calculated for percent changes. Band densities were tested for statistical significance with either a one- or two-tailed one-sample t-test using GraphPad Software (GraphPad Software, San Diego, CA).

Immunohistochemistry for cryosections

Following removal of the temporal bones, cochleae were fixed immediately in 4% paraformaldehyde overnight at 4°C. Cryostat sections of 8 µm were incubated in 0.5% Triton X-100 in PBS for 15 min at room temperature. The sections were then washed three times with PBS and blocked with 10% goat serum for 30 min at room temperature, followed by incubation with the primary antibodies of PTEN at a dilution of 1:100, p-PTEN (ser380) or PIP₃ at a

dilution of 1:50 for 48 hr at 4°C. After 3 rinses in PBS, the sections were incubated with the secondary antibody (Alexa 488 or Alexa 546 conjugated; 1:500) at 4°C overnight in darkness. After 3 washes with PBS, the sections were incubated with propidium iodide (2 µg/ml in PBS) or Hoechst 33342 (2 µg/ml in PBS) for 30 min at room temperature. After a final wash with PBS, the slides were mounted with GEL/MOUNT™. Control incubations were routinely processed without primary antibody. Preparations were observed with a Zeiss Axioplan microscope and imaged with a Zeiss laser confocal microscope (Zeiss LSM 510; Carl Zeiss Microimaging, Thornwood, NY).

Immunocytochemistry for surface preparations of the organ of Corti

Temporal bones were removed immediately after euthanasia of the mice and fixed with cold fixative containing 4% paraformaldehyde in PBS, and kept in this medium overnight at 4°C. Cochleae were then rinsed in PBS and decalcified in 4% sodium EDTA (adjusted with HCl to pH 7.4) for 48 h at 4°C. Following decalcification, the softened otic capsule, stria vascularis, Reissner's membrane, and tectorial membrane were removed. The epithelium of the organ of Corti (base to apex) was lifted off the modiolus in three sections, corresponding to apical, middle and basal portions of the cochlea. The hook region was generally not recovered. The epithelium of the organ of Corti was then washed three times and incubated in 0.5% Triton X-100 in PBS for 30 min at room temperature. The specimens were then washed three times with PBS and blocked with 10% goat serum for 30 min at room temperature, followed by incubation with the primary antibodies for PIP₃, p-Akt (thr308), p-Akt (ser473) at a concentration of 1:50 for 48 hr at 4°C. After 3 rinses in PBS, the specimens were incubated with the secondary antibody Alexa 488 at a 1:500 dilution overnight at 4°C in darkness. After 3 washes with PBS, the specimens were incubated with rhodamine phalloidin at 1:100 dilution 1h and Hoechst 33342 (1 µg/ml in PBS) for 30 min at room temperature. After a final wash with PBS, the slides were mounted. Preparations were observed with a Zeiss Axioplan microscope and imaged with a Zeiss laser confocal microscope (Zeiss LSM 510; Carl Zeiss Microimaging, Thornwood, NY).

Immunostaining of phospho-Akt (thr308), PIP₃ on surface preparation was quantified from confocal images taken under identical conditions and equal setting parameters using ImageJ software (National Institute of Health, Bethesda, MD). For each confocal session, tissues from young and old images were paired and the subsequent analyses were carried out by an observer blinded to the conditions of the experiments. The fluorescence intensity of the nuclei and cuticular levels of the outer hair cells was measured in 0.212-mm segments of the basal portion of the cochlea from a surface preparation containing about 70 outer hair cells. The "region of the interest" was outlined with a circle covering approximately 85% of the area (20 µm² area per outer hair cell). The average fluorescence intensity per nucleus or cell was then calculated, and the relative fluorescence intensity was quantified by normalizing the ratio of average fluorescence intensity of cells in old animals to the average fluorescence intensity of cells in young animals. Individual preparations from three animals per condition were examined and data were statistically analyzed by t-test using Primer of Biostatistics software (McGraw-Hill Software, New York, NY).

Total RNA isolation and quantitative RT-PCR (Q-PCR)

Total RNA was isolated from a pool of 16 cochleae (3 pools of each age group) by using TRIzol® Reagent, and subjected to real-time quantitative PCR using a TaqMan Probe. Total RNA (1 µg) was reverse transcribed to cDNA by using SuperScript III reverse transcriptase (Invitrogen) following the protocol from the manufacturer. The first strand cDNA was diluted (1:10) with DEPC-H₂O and stored as aliquots at -20 °C. TaqMan primer and probes were obtained from the 'Assays by Design' (PTEN) or 'Assays on Demand' services (S16) (ABI, Foster City, CA). A 96-well plate was used for the PCR reactions. Triplicate PCR reactions

were run for each RNA sample. A housekeeping gene, S16, was also determined on each plate. The C_T value (the number of cycles at which the PCR reaction reaches an arbitrary threshold value) was calculated for each reaction (Livak and Schmittgen, 2001). All expression levels were normalized to S16 and evaluated for significance ($p < 0.05$) by ANOVA analysis using Primer of Biostatistics software (McGraw-Hill Software, New York, NY). They were tabulated as the x-fold increase for each sample.

Results

ABR thresholds

Mice were chosen from a large cohort that had previously been characterized for their age-related hearing loss and pathology (Sha et al., 2008). The ages of the animals used in the current study were 3 months for “young” animals and 18 months for “old” animals. The criteria for selection were individual ABR thresholds below 40 dB SPL to insure that no major functional loss had occurred to the cochlea. The mean ABR thresholds at 24 kHz were 19.7 s.d. ($n = 16$) and 26.8 s.d. ($n = 13$) dB SPL for animals of 3 and 18 months of age, respectively.

PIP₃ decreases in the aging cochlea

Immunostaining on cochlear cryosections revealed a wide-spread distribution of PIP₃ in the cochlea (figure 1A). In 18-months old animals staining was markedly attenuated with obvious decreases in inner and outer hair cells, Deiters cells and pillar cells.

PIP₃ staining on surface preparations confirmed the presence of PIP₃ in outer hair cells and Deiters cells. A reduced intensity of PIP₃ labeling was apparent in outer hair cells and phalangeal processes of Deiters cells at 18 months both at the level of the cuticular plate (figure 1B) and the nucleus (figure 1C). The decrease of PIP₃ staining in outer hair cells was statistically significant for both regions (figure 1D).

PTEN increases in the aging cochlea

To investigate possible reasons for the decrease of PIP₃ in the organ of Corti, we analyzed the expression of PTEN. Western blots using cochlear homogenates showed a trend towards an increase of PTEN at the age of 12 months and a significant elevation of more than 50% at the age of 18 months (figure 2A). In contrast to the protein levels of PTEN, the mRNA for PTEN remained unchanged from 3 to 18 months, as determined by real-time quantitative PCR (figure 2B).

Immunostaining of total PTEN showed that PTEN was present in essentially all cell types of cells in the organ of Corti. It increased with aging, with especially strong staining evident in outer hair cells, the top of the pillar cells and the phalangeal processes of Deiters cells at 18 months of age (figure 2C). Phosphorylated PTEN (ser380) had a similarly widespread appearance and distinct changes in old animals. The most prominent age-related increases were found in outer hair cells and in the nuclei of all cell types of the organ of Corti (figure 2D).

Phosphorylation of Akt decreases in outer hair cells

Akt is a downstream target of the PIP₃ signaling pathway and is activated by phosphorylation of specific serine and threonine residues. Western blotting of cochlear protein revealed reduced levels of the phosphorylated isoforms Akt1 and Akt2 at 18 months (figure 3A). In order to further characterize the phosphorylation and the cellular localization of p-Akt, we stained cochlear surface preparations with antibodies to phosphorylated serine 473 or threonine 308 residues, the two phosphatidylinositol-3 kinase (PI3K)-dependent phosphorylation sites involved in stimulus-dependent activation of Akt. p-Akt (ser473) did not change (figure 3B) while the staining of p-Akt (thr308) decreased in outer hair cells with aging at both the level

of the cuticular plate (figure 3C) and the nucleus (figure 3D). Quantitative analysis of relative fluorescence intensity confirmed a significant decrease of p-Akt (thr308) in outer hair cells with aging (figure 3E).

Discussion

The lipid PIP₃ and the protein kinase Akt are crucial components of a signaling pathway that regulates metabolic activity, protein synthesis, cell proliferation and survival via both gene expression and post-transcriptional mechanisms. The salient finding of this study is that the PIP₃/Akt pathway is compromised in aging cochlear outer hair cells, thus potentially contributing to age-related loss of hair cells and hearing. Furthermore, we can postulate that the down-regulation of this pathway is due to an increase in the activity of the lipid phosphatase PTEN.

Akt is the hub of many anti-apoptotic pathways affected by growth factors and other molecular signals (Scheid and Woodgett, 2001). Akt also plays a critical role in the cochlea. Following ototoxic treatment of mice with kanamycin, PIP₃ and activated Akt decrease in outer hair cells, consistent with the high susceptibility of these cells to aminoglycoside damage (Jiang et al., 2006). The activation of Akt in response to homeostatic messengers is controlled by membrane translocation and phosphorylation at specific threonine (thr308) and serine (ser473) residues (Chan and Tsichlis, 2001). Mutation of thr308 to an alanine eliminates growth factor-mediated activation of Akt while a mutation at ser473 only partially attenuates it, illustrating the absolute requirement for phospho-thr308 in the activation process.

Phosphorylation of Akt (thr308) is a phosphatidylinositol-3 kinase (PI3K)-dependent process, making PIP₃ an essential upstream regulator of the entire pathway. The decrease in PIP₃ observed in the aging cochlea should therefore result in an impaired activation of Akt which is indeed the case. Specifically, the reduction of the critical phosphorylation at thr308 suggests that the PIP₃/Akt pathway becomes unresponsive to homeostatic signals and will exert less anti-apoptotic influence. This down-regulation of Akt is a compounding failure of homeostatic maintenance in the aging cochlea at a time when cell death pathways become activated. Oxidative stress in presbycusis CBA/J mice enhances cytochrome C release and caspase 9 as well as the JNK and p38 pathways (Sha et al., 2009), all potentially contributing to apoptosis. An intact Akt pathway would have the ability to alleviate these apoptotic influences (Berra et al., 1998; Cerezo et al., 1998) while a compromised pathway would leave aging hair cells without this protection.

While homeostatic signals activate the Akt pathway, the lipid phosphatase PTEN is a negative regulator as it dephosphorylates PIP₃. PTEN has mostly been studied as a tumor suppressor in various cancers due to its ability for shutting down Akt-mediated cell proliferation (Ramaswamy et al., 1999; Leslie et al., 2003). It is mutated in many human tumors with a frequency second only to p53 (Sansal and Sellers, 2004), leading to an accumulation of PIP₃ and constitutive activation of Akt. As an antagonist to Akt activators, it is also involved in developmental regulation, for example in neuronal differentiation and synaptogenesis (Perandones et al., 2004). Furthermore, PTEN plays a crucial role in apoptosis: knockdown of PTEN protected hippocampal cells from staurosporine-induced mitochondria-dependent apoptotic damage (Zhu et al., 2006). Conversely, an increase in PTEN reduces the levels of PIP₃ and blunts the anti-apoptotic activity of the Akt pathway.

We can invoke the latter scenario in the aging cochlea. The reduced activity of the Akt pathway is well correlated with reduced levels of PIP₃ and an increased level of PTEN. The regulation of PTEN itself is complex and not completely understood but includes transcriptional regulation, post-translational modifications, lipid and protein interactions and subcellular

translocation (Gericke et al., 2006; Tamguney and Stokoe, 2007; Wang and Jiang, 2008). The increased protein levels of PTEN in the cochlea in the face of unchanged mRNA expression suggest a reduced rate of degradation. The control of protein degradation via ubiquitination and proteasomes is a universal mechanism to assure cell homeostasis (Jung et al., 2009) and a potential regulatory step for PTEN (Gericke et al., 2006). Proteasomal activity in general decreases in aging cells (Goto et al., 2001) and can lead to cell death in neurons (Keller and Markesbery, 2000). Interestingly, the modes of cell death induced by proteasomal failure include caspase activation (Qiu et al., 2000) which we have observed in the aging cochlea (Sha et al., 2009). Impaired degradation, therefore, appears a rational explanation for the age-related changes in PTEN.

In addition to changes in PTEN levels, we observe alterations in its phosphorylation which can decrease its enzymatic activity but also influence its subcellular distribution. Intriguingly, oxidative stress which is present in the aging mouse cochlea (Jiang et al., 2007) specifically contributes to the nuclear retention of p-PTEN phosphorylated at ser380, the species that we find in aging cochlear hair cells. The nuclear functions are a largely unexplored new chapter in the many complex facets of PTEN activities. They appear to be independent of its phosphatase activity and may involve chromosome stability and DNA repair (Shen et al., 2007) and, relevant in the context of aging hair cells, relate to increased cell death (Chang et al., 2008).

In summary, our data suggest that a decrease in the PIP₃/Akt survival pathway with aging is due to an elevation of PTEN. Since the PIP₃/Akt cascade plays a key role in maintaining cell homeostasis, an attenuation of this pathway will shift the balance between cell survival and cell death, and thus contribute to age-related hearing loss.

Acknowledgments

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Abbreviations

PI	propidium iodide
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PIP ₃	phosphatidylinositol 3,4,5-trisphosphate
PTEN	phosphatase and tensin homologue deleted on chromosome ten
PBS	phosphate-buffered saline

References

- Berra E, Diaz-Meco MT, Moscat J. The activation of p38 and apoptosis by the inhibition of Erk is antagonized by the phosphoinositide 3-kinase/Akt pathway. *J Biol Chem* 1998;273:10792–7. [PubMed: 9553146]
- Cerezo A, Martinez AC, Lanzarot D, Fischer S, Franke TF, Rebollo A. Role of Akt and c-Jun N-terminal kinase 2 in apoptosis induced by interleukin-4 deprivation. *Mol Biol Cell* 1998;9:3107–18. [PubMed: 9802900]
- Chan TO, Tsichlis PN. PDK2: a complex tail in one Akt. *Sci STKE* 2001 2001:PE1.
- Chang CJ, Mulholland DJ, Valamehr B, Mosessian S, Sellers WR, Wu H. PTEN nuclear localization is regulated by oxidative stress and mediates p53-dependent tumor suppression. *Mol Cell Biol* 2008;28:3281–9. [PubMed: 18332125]

- Denley A, Gymnopoulos M, Kang S, Mitchell C, Vogt PK. Requirement of phosphatidylinositol(3,4,5) trisphosphate in phosphatidylinositol 3-kinase-induced oncogenic transformation. *Mol Cancer Res* 2009;7:1132–8. [PubMed: 19584261]
- Elghazi L, Bernal-Mizrachi E. Akt and PTEN: [beta]-cell mass and pancreas plasticity. *Trends in Endocrinology & Metabolism* 2009;20:243–251. [PubMed: 19541499]
- Franke TF. PI3K/Akt: getting it right matters. *Oncogene* 2008;27:6473–88. [PubMed: 18955974]
- Gericke A, Munson M, Ross AH. Regulation of the PTEN phosphatase. *Gene* 2006;374:1–9. [PubMed: 16675164]
- Goto S, Takahashi R, Kumiyama AA, Radak Z, Hayashi T, Takenouchi M, Abe R. Implications of protein degradation in aging. *Ann N Y Acad Sci* 2001;928:54–64. [PubMed: 11795528]
- Irvine RF. Nuclear lipid signalling. *Nat Rev Mol Cell Biol* 2003;4:349–60. [PubMed: 12728269]
- Jiang H, Sha SH, Schacht J. Kanamycin alters cytoplasmic and nuclear phosphoinositide signaling in the organ of Corti in vivo. *J Neurochem* 2006;99:269–76. [PubMed: 16903869]
- Jiang H, Talaska AE, Schacht J, Sha SH. Oxidative imbalance in the aging inner ear. *Neurobiol Aging* 2007;28:1605–12. [PubMed: 16920227]
- Jung T, Catalgol B, Grune T. The proteasomal system. *Mol Aspects Med* 2009;30:191–296. [PubMed: 19371762]
- Keller JN, Markesbery WR. Proteasome inhibition results in increased poly-ADP-ribosylation: implications for neuron death. *J Neurosci Res* 2000;61:436–42. [PubMed: 10931530]
- Leslie NR, Bennett D, Lindsay YE, Stewart H, Gray A, Downes CP. Redox regulation of PI 3-kinase signalling via inactivation of PTEN. *EMBO J* 2003;22:5501–10. [PubMed: 14532122]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–8. [PubMed: 11846609]
- Misra UK, Kaczowka S, Pizzo SV. The cAMP-activated GTP exchange factor, Epac1 upregulates plasma membrane and nuclear Akt kinase activities in 8-CPT-2-O-Me-cAMP-stimulated macrophages: Gene silencing of the cAMP-activated GTP exchange Epac1 prevents 8-CPT-2-O-Me-cAMP activation of Akt activity in macrophages. *Cell Signal* 2008;20:1459–70. [PubMed: 18495429]
- Paez J, Sellers WR. PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling. *Cancer Treat Res* 2003;115:145–67. [PubMed: 12613196]
- Parker RJ, Auld VJ. Signaling in glial development: differentiation migration and axon guidance. *Biochem Cell Biol* 2004;82:694–707. [PubMed: 15674437]
- Perandones C, Costanzo RV, Kowaljow V, Pivetta OH, Carminatti H, Radrizzani M. Correlation between synaptogenesis and the PTEN phosphatase expression in dendrites during postnatal brain development. *Brain Res Mol Brain Res* 2004;128:8–19. [PubMed: 15337313]
- Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM, Cordon-Cardo C, Catoretti G, Fisher PE, Parsons R. Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. *Proc Natl Acad Sci U S A* 1999;96:1563–8. [PubMed: 9990064]
- Qiu JH, Asai A, Chi S, Saito N, Hamada H, Kirino T. Proteasome inhibitors induce cytochrome c-caspase-3-like protease-mediated apoptosis in cultured cortical neurons. *J Neurosci* 2000;20:259–65. [PubMed: 10627603]
- Ramaswamy S, Nakamura N, Vazquez F, Batt DB, Perera S, Roberts TM, Sellers WR. Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *Proc Natl Acad Sci U S A* 1999;96:2110–5. [PubMed: 10051603]
- Sansal I, Sellers WR. The biology and clinical relevance of the PTEN tumor suppressor pathway. *J Clin Oncol* 2004;22:2954–63. [PubMed: 15254063]
- Scheid MP, Woodgett JR. PKB/AKT: functional insights from genetic models. *Nat Rev Mol Cell Biol* 2001;2:760–8. [PubMed: 11584303]
- Sha SH, Kanicki A, Dootz G, Talaska AE, Halsey K, Dolan D, Altschuler R, Schacht J. Age-related auditory pathology in the CBA/J mouse. *Hear Res* 2008;243:87–94. [PubMed: 18573325]
- Sha SH, Chen FQ, Schacht J. Activation of cell death pathways in the inner ear of the aging CBA/J mouse. *Hear Res* 2009;254:92–9. [PubMed: 19422898]
- Shen WH, Balajee AS, Wang J, Wu H, Eng C, Pandolfi PP, Yin Y. Essential role for nuclear PTEN in maintaining chromosomal integrity. *Cell* 2007;128:157–70. [PubMed: 17218262]

- Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* 1998;95:29–39. [PubMed: 9778245]
- Tamguney T, Stokoe D. New insights into PTEN. *J Cell Sci* 2007;120:4071–9. [PubMed: 18032782]
- Wang X, Jiang X. PTEN: a default gate-keeping tumor suppressor with a versatile tail. *Cell Res* 18:807–816. [PubMed: 18626510]
- Woodgett JR. Recent advances in the protein kinase B signaling pathway. *Curr Opin Cell Biol* 2005;17:150–7. [PubMed: 15780591]
- Wu H, Goel V, Haluska FG. PTEN signaling pathways in melanoma. *Oncogene* 2003;22:3113–22. [PubMed: 12789288]
- Zhu Y, Hoell P, Ahlemeyer B, Krieglstein J. PTEN: a crucial mediator of mitochondria-dependent apoptosis. *Apoptosis* 2006;11:197–207. [PubMed: 16502258]

Figure 1. Levels of PIP₃ decrease in the aging cochleae

Cochleae were prepared as described in 'Methods'. Figures 1A, B and C are representative images from the basal turn of the organ of Corti with $n = 3$ for each age group. Scale bars = 10 μm .

1A: Immunostaining on cryosections showed PIP₃ (green) decreased in all cell types including outer and inner hair cells, Deiters cells and pillar cells in the organ of Corti from the age of 3 months to 18 months. PI (red) is propidium iodide counterstaining for nuclei.

1B: Staining of surface preparations showed that PIP₃ decreased at the cuticular level of outer hair cells, and the phalangeal processes of the Deiters cells. Rhodamine phalloidin (red) is counterstaining for actin outlining the region of the cuticular plate.

1C: Staining of the surface preparations showed that PIP₃ decreased at the nuclear level of outer hair cells. Hoechst 33342I (blue) is counterstaining for nuclei.

1D: Analysis of relative fluorescence intensity in outer hair cells both at the cuticular and nuclear level (as described in 'Methods') confirmed a statistically significant decrease of PIP₃ in outer hair cells. * $p < 0.05$, ** $p < 0.01$, $n = 3$ for each age group.

Figure 2. Protein levels of PTEN but not PTEN mRNA increase in the aging cochlea

2A. The level of total PTEN (50 kDa) on Western blots of cochlear homogenates showed a significant increase at the age of 18 months compared to 3 months ($n = 3$, $*P < 0.05$).

2B. Relative PTEN mRNA levels obtained by qT-PCR from cochlear homogenates showed no difference between age groups. The data come from pools of 16 cochleae (3 pools per age group).

2C. Immunostaining localized PTEN in all cell types in the organ of Corti. The intensity of the staining increased in outer hair cells (area in rectangle), inner hair cells, supporting cells and the top of pillar cells (arrow) at an age of 18 months compared to 3 months. Control incubations without primary antibody showed no staining (data not shown). Hoechst 33342I (blue) is counterstaining for nuclei. The panels are representative images from the basal turn of the organ of Corti ($n = 5$ for each age group). Scale bar = 10 μ m.

2D. Immunostaining of phospho-PTEN (ser380) increased in both cytoplasm and nuclei of outer hair cells at the age of 18 months compared to 3 months. Control incubations without primary antibody showed no staining (data not shown). PI (red) is propidium iodide

counterstaining for nuclei. The panels are representative images from the basal turn of the organ of Corti (n = 3 for each age group). Scale bar = 10 μ m.

Figure 3. p-Akt decreases in outer hair cells with aging

3A: Western blotting of total cochlear protein revealed reduced levels of phosphorylated Akt1/2 at 18 months of age as compared to 3 months ($n = 3$, $*p < 0.05$).

3B: Immunostaining (green) for p-Akt (ser473) on surface preparations showed no change between 3 and 18 months. Rhodamine phalloidin (red) is counterstaining for actin outlining the region of the cuticular plate. Hoechst 33342 (blue) is counterstaining for nuclei. The panels are representative images from the basal turn of the cochlea ($n = 3$ for each age group). Scale bar = 10 μm .

3C and D: Immunostaining (green) for p-Akt (thr308) showed a decrease in outer hair cells both at the cuticular and nuclear level at 18 months. Rhodamine phalloidin (red) is

counterstaining for actin outlining the region of the cuticular plate. Hoechst 33342I (blue) is counterstaining for nuclei. Panels are representative images from a total of 3 images per age group. Scale bar = 10 μ m.

3E: Quantitative analysis of relative fluorescence intensity (as described in 'Methods') show that p-Akt (thr308) significantly decreased at 18 months in outer hair cells at both the cuticular and nuclear level ($n = 3$, $**p < 0.01$).