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13. ABSTRACT (Maximum 200 Words)

Breast cancer is the most common malignancy in women. The recently identified tumor suppressor gene PTEN has turned out to be a promising candidate for mammary tumorigenesis. Mice heterozygous for Pten develops mammary tumors starting from 6 weeks. The goal of this project is to determine the role of AKT, a major downstream target of PI3K pathway, in PTEN mediated mammary tumor development.

To study the function of AKT in breast cancer development, we have deleted Akt gene and are studying its role both in vivo and in vitro. In cell culture, we demonstrated that AKT is not only responsible for the survival phenotype but also important for the cell proliferation phenotype of *Pten* null ES cells. In vivo analysis demonstrated that deletion of *Akt* on top of *Pten* resulted in both decreased tumor occurence and shorter tumor onset.

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INTRODUCTION

PTEN is a tumor suppressor gene frequently deleted in many human cancers, including mammary cancers [1, 2]. One of the unequivocal affect of deleting *Pten* is the activation of an oncogene AKT[3, 4]. The goal of this funded proposal is to study the function of AKT in tumorigenesis *in vitro* and in mammary cancer development *in vivo* and to evaluate whether deleting *Akt-1* can rescue or partially rescue the phenotype of *Pten* deletion both in vivo and in vitro.

Four specific tasks should be accomplished in this study:

I. To generate mouse embryonic stem (ES) cells and mouse embryonic fibroblasts (MEF) with deleted alleles of *Akt-1* via homologous recombination;
II. To evaluate tumorigenesis of ES cells carrying mutations in *Akt-1* gene, *Pten* gene, or both in nude mice.
III. To study the function of AKT-1 in vitro in wild type and *Pten* null ES and MEF cells by deleting one or both copies of *Akt-1* gene.
IV. To generate animal strains carrying the above mutations and to study the function of AKT-1 in animal development and tumorigenesis in vivo.

BODY: STUDIES AND RESULTS

I. To generate cells with deleted alleles of *Akt-1* (Accomplished in year 1, for detail, see attached publication)

I-1. To generate ES cells carrying deleted alleles of *Akt-1* via homologous recombination (accomplished before the beginning of the funding period).

I-2. To generate MEF cells with deleted alleles of *Akt-1* (not accomplished, addressed in year 1 report, for detail see attached publication)

II. The production of nude mice teratomas from the ES cells (Accomplished in year 1, for detail, see attached publication)

III. To study cell cycle profiles of the *Pten*$^{-/-}$; *Akt-1*$^{-/-}$ ES cells. (Accomplished in year 1, for detail, see attached publication)

IV. To generate animals carrying deletions of *Pten, Akt-1*, and both

IV-a. To generate mice carrying deletions of *Akt-1*

The *Akt-1*$^{+/-}$ ES cells generated in Aim I were injected into blastocysts and 7 chimeric mice were obtained (3 male and 4 female). These chimeric mice were backcrossed to C57/B6 and Balb/c mice to generate germline founder mice (Fig 1). Analysis of tail DNA from surviving F2 mice did not yield homozygous *Akt-1*$^{-/-}$ deleted (Table 1). However, the ratio of Akt+/-:Akt+/+ is 3:1

rather than the madalian distribution expected (2:1). Therefore, we will modify our strategy of genotyping to see if this is due to the false negative in genotyping. We have already designed primers for genotyping purpose. We will also test other probes for southern analysis.

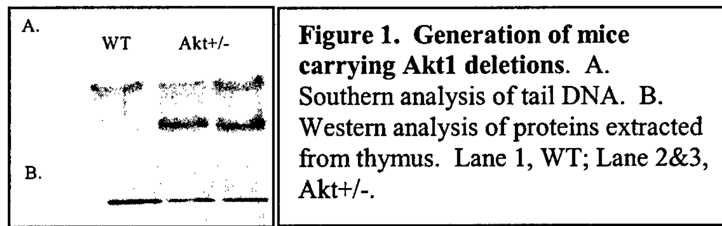

Figure 1. Generation of mice carrying Akt1 deletions. A. Southern analysis of tail DNA. B. Western analysis of proteins extracted from thymus. Lane 1, WT; Lane 2&3, Akt+/-.

Table 1. Distribution of AKT genotypes

genotypes	Number of mice
Akt-1$^{+/+}$	43
Akt-1$^{+/-}$	118
Akt-1$^{-/-}$	0

Several other groups also generated *Akt-1* knockout mice. The results from the different groups appeared to vary significantly. Birmbaum's group deleted exon 4-8 of *Akt-1*, which encodes the kinase domain [7]. Their homozygous knock-out mice suffered from early postnatal lethal with incomplete penetration. Chen *et al* deleted from exon 8-11 of *Akt-1*, which includes part of the kinase domain and the c-terminal [8]. Homozygous animals carrying this deletion were not lethal and were capable of living to 2-3 years old with smaller body size. A third group deleted exon 3-8, one exon extra than what Birmbaum's group did, and their result is embryonic lethal before plantation (Personal communication), similar to what we have observed when we deleted exon 2-3, the two exons composed the PH domain of AKT molecule. Our initial analysis showed that deleting these two exons results in embryonic lethality up to E7.5. However, until we answer the genotyping question, we cannot definitively conclude the embryonic lethality phenotype. We are continuing our effort on analyzing the embryonic phenotypes.

Based on the ES cell results and the information from the third group mentioned above, we hypothesize that we will not be able to find *Akt-1* null embryos. It is highly likely that homozygous deletion of *Akt-1* (when deletion included exon 3) is lethal to the ES cells' survival. It is not clear why deleting exon 3 is lethal, possibly because exon 3 contains critical components necessary for all AKTs to function. Deleting this domain may create a transient dominant negative AKT, which inhibits all AKTs resulting in complete knock down of AKT activity, thus resulting in embryonic lethality.

IV-b. <u>To generate mice carrying deletions of both *Pten* and *Akt-1*</u>

We have bred the *Pten*$^{+/-}$ mice with *Akt-1*$^{+/-}$ mice. The F2 generations are being analyzed for tumor formation phenotypes. We are using these animals to answer two of our questions: 1) Does deletion of *Akt* and *Pten* together rescues each other and results in non lethal phenotype. 2) Dose deletion of *Akt* and *Pten* diminishes and slows the tumorigenesis process of Pten deleted mice.
Animals carrying the following genotypes will be used: 1) *Pten*$^{+/+}$; *Akt-1*$^{+/+}$, 2)*Pten*$^{+/+}$; *Akt-1*$^{+/-}$, 3)*Pten*$^{+/-}$; *Akt-1*$^{+/+}$, 4)*Pten*$^{+/-}$;*Akt-1*$^{+/-}$, 5)*Pten*$^{+/-}$;*Akt-1*$^{-/-}$, 6)*Pten*$^{-/-}$; *Akt-1*$^{+/-}$, 7)*Pten*$^{-/-}$; *Akt-1*$^{-/-}$. Mice with the first four genotypes are being used to survey tumor formations and determine whether deleting *Akt-1* on top of *Pten* can decrease the mammary tumor formation observed in *Pten* deleted mice. It is not clear whether the last three genotypes can survive through embryogenesis. Preliminary analysis suggests that compound deletion of *Pten* and *Akt- 1*(group 7) may not be

able to rescue the embryonic lethality phenotype of deleting either of them alone. The litter size of the breeding is listed in Table 2. Double mutant mating did not seem to result in larger litter size than the single mutant crosses. This result suggesting that compound heterozygous deletion of *Pten* and *Akt* was not able to rescue the lethality phenotype of each other.

Table 2. Statistics on Akt+/- and Pten +/- mice breeding

Mating group	AktxPten	PtenxAkt	DMxDM
Litter size	4.6	8.6	4.8
m/f	0.79	1.3	1
Akt+/-:Akt+/+	1.2	1.1	4
Pten+/-:Pten+/+	0.76	0.96	0.69

Breeding groups: Akt, *Akt+/-;* Pten, *Pten+/-;* DM, *Akt+/-;Pten+/-*

The distribution of *Akt*+/- to *Akt*+/+, however, is nonetheless madalian distribution in the *Pten* and *Akt* breeding, possibly because we are not expecting any *Akt*$^{-/-}$. In the double mutant breeding, this distribution is similarly distorted as what was observed in the *Akt* mutant breeding. These results further suggesting that genotyping false negative might be the reason for the non-madalian distribution. The reason for the distorted distribution will be studied after the question about genotyping is answered.

Compound heterozygous mice were analyzed for tumorigenic phenotypes. Deletion of Akt on top of *Pten* resulted in delay of tumor onset (Figure 2A). The percentage of animals with tumor burden is also decreased (Figure 2B).

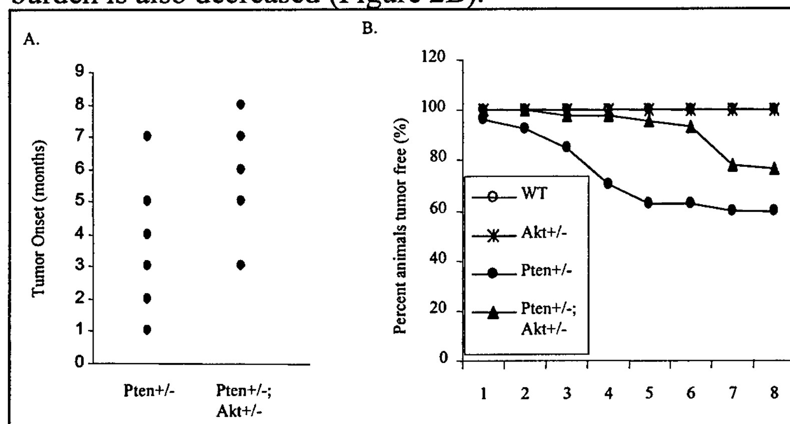

Figure 2. *Akt* deletion attenuate *Pten* deletion resulted tumorigenic phenotype. A. Tumor onset. B. Percent of animals tumor free.

We will continue to analyze the tumorigenic phenotype of *Akt, Pten* and compound heterozygous mice. We will also start to analyze the tumor tissue samples collected in the last few month for immunohistology and biochemistry analysis.

KEY RESEARCH ACCOMPLISHMENTS

- Partially analyzed the phenotype of *Akt-1*$^{+/-}$ mice.
- Analyze the embryonic lethality phenotype of *Akt-1* mutant mice.
- Breed compound heterozygous mice carrying *Akt* and *Pten* mutations.
- Observe tumor formation on mice carrying *Akt* and *Pten* mutations and collect tumor samples for future analysis.

REPORTABLE OUTCOMES

- Publication:

Stiles, B., Gilman,V., Khnzenzon,N., Lesche, R., Li, A., Qiao,R., Liu, X., and Wu, H.(2002) *The Essential Role of AKT-1/Protein Kinase B in PTEN Controlled Tumorigenesis.* Mol Biol Cell, 22:3842-51.

Stiles, B., Gilman, V., Khnzenzon, N., Lesche, R., Li, A., Qiao, R., Liu, X., and Wu, H. (2003) AKT Deletion Attenuates the PTEN mutant phenotype. In: Ninty-Forth Annual Meeting of the American Association for Cancer Research, Washington DC, July 2003 (This presentation was awarded the Scholars-In-Training Award).

CONCLUSION

During the second funding period of this project, we have partially accomplished Aims IV. We have generated mice carrying deletions of Akt. We have performed initial characterization of these mice and suggested a potential embryonic lethality phenotype. The genotyping issue encountered during the analysis blurred this conclusion and is currently under investigation. We have bred the $Akt^{+/-}$ mice with the $Pten^{+/-}$ mice. We have obtained F2 progenies and are currently observing the tumor formation phenotype in 4 of the surviving genotype groups. Our initial observation suggested that deleting *Akt* on top of *Pten* can partially revert the tumorigenic phenotype of Pten deletion. Mice carrying compound heterozygous deletion was shown to have later tumor onset and occurrence than the *Pten* deleted mice only. The tumor spectrum awaits further immunohistology analysis.

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APPENDICES

Reprints of one recent publication

Stiles, B., Gilman,V., Khnzenzon,N., Lesche, R., Li, A., Qiao,R., Liu, X., and Wu, H.(2002) *The Essential Role of AKT-1/Protein Kinase B in PTEN Controlled Tumorigenesis.* Mol Biol Cell, 22:3842-51

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Stiles, B., Gilman, V., Khnzenzon, N., Lesche, R., Li, A., Qiao, R., Liu, X., and Wu, H. (2003) AKT Deletion Attenuates the PTEN mutant phenotype. In: Ninty-Forth Annual Meeting of the American Association for Cancer Research, Washington DC, July 2003

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Essential Role of AKT-1/Protein Kinase Bα in PTEN-Controlled Tumorigenesis

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***PTEN* is mutated at high frequency in many primary human cancers and several familial cancer predisposition disorders. Activation of AKT is a common event in tumors in which the *PTEN* gene has been inactivated. We previously showed that deletion of the murine *Pten* gene in embryonic stem (ES) cells led to increased phosphatidylinositol triphosphate (PIP_3) accumulation, enhanced entry into S phase, and better cell survival. Since PIP_3 controls multiple signaling molecules, it was not clear to what degree the observed phenotypes were due to deregulated AKT activity. In this study, we mutated *Akt-1* in *Pten*$^{-/-}$ ES cells to directly assess the role of AKT-1 in PTEN-controlled cellular processes, such as cell proliferation, cell survival, and tumorigenesis in nude mice. We showed that AKT-1 is one of the major downstream effectors of PTEN in ES cells and that activation of AKT-1 is required for both the cell survival and cell proliferation phenotypes observed in *Pten*$^{-/-}$ ES cells. Deletion of *Akt-1* partially reverses the aggressive growth of *Pten*$^{-/-}$ ES cells in vivo, suggesting that AKT-1 plays an essential role in PTEN-controlled tumorigenesis.**

The *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) tumor suppressor gene is the first phosphatase identified as being frequently mutated or deleted somatically in various human cancers (22, 23, 39). One of the primary targets of PTEN is lipid phosphatidylinositol triphosphate (PIP_3), a direct product of phosphatidylinositol (PI) 3-kinase (27, 40). Loss of PTEN function, either in murine embryonic stem (ES) cells or in human cancer cell lines, results in accumulation of PIP_3 and activation of its downstream effectors, such as AKT/protein kinase B (15, 36) and Rac-1/Cdc42 (24). Activation of AKT stimulates cell cycle progression by down-regulation of p27, a major inhibitor for G_1 cyclin-dependent kinases (36). Activated AKT/protein kinase B is also a well-characterized survival factor in vitro and prevents cells from undergoing apoptosis by inhibiting the proapoptotic factors BAD (12, 36) and caspase 9 (8) as well as the nuclear translocation of Forkhead transcription factors (20, 38).

The cellular proto-oncogene c-*Akt* was first identified as a homologue of the viral oncogene v-*akt*, which is capable of transforming mink lung cells (CCL64) in culture and causing T-cell lymphomas when injected into newborn mice (34, 35). The *Akt* gene encodes a serine/threonine protein kinase that is an essential downstream target of PI 3-kinase and propagates the cell survival signals of growth factors (14). It also exerts its function in glucose metabolism and other cellular processes. Amplification of the *AKT* gene or increase of its expression was shown previously to be associated with a number of malignant diseases, including adenocarcinomas of the breast, prostate, and intestines (9, 29, 34). Frequent chromosomal alterations in the *AKT* region have also been observed in patients with lymphomas and leukemia (5). In addition, activation of AKT was observed in many primary human tumor samples or cell lines carrying *PTEN* mutations and in cancers developed in *Pten*$^{+/-}$ mice (16, 37). Therefore, up-regulation of AKT appears to be a common event in cancers, especially in PTEN-controlled tumorigenesis.

AKT activity is largely regulated at the posttranslational level and is dependent on its pleckstrin homology (PH) domain (4). Binding of the lipid second-messenger PIP_3 to the PH domain results in translocation of the AKT molecule to the vicinity of the membrane and its subsequent phosphorylation by the PIP_3-dependent protein kinases, PDK1 and PDK2 (3). Phosphorylation of AKT is required for its function. Thus, AKT activity is positively regulated by PI 3-kinase, which phosphorylates PIP_2 to produce PIP_3 (15). On the other hand, PTEN exerts its function by dephosphorylating PIP_3 at position 3, negatively regulating AKT activity (27). To date, three mammalian *Akt* genes have been identified, *Akt-1*, *Akt-2*, and *Akt-3*, or *PKBα*, *PKBβ*, and *PKBγ* (10). The three *Akt* genes share high homology in both the nucleic acid and peptide sequences. *Akt-1* is ubiquitously expressed in every tissue, and *Akt-2* expression is high in the skeletal muscles, the β-islet cells of the pancreas, and the brown fat, while the expression of *Akt-3* is more restricted, to the heart and placenta, with low expression in a limited number of other tissues (2, 28).

To investigate the molecular basis for the tumor suppressor function of PTEN, we previously deleted the murine *Pten* gene in ES cells (36). We demonstrated that PTEN acts as a nega-

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tive regulator for the PI 3-kinase/AKT signaling pathway, which controls and coordinates two major cellular processes, cell cycle progression and cell death. In this study, we further delineated the PTEN-regulated pathways by deleting one of the *Akt* genes, *Akt-1*, from *Pten*-null ES cells. Through this genetic approach, we demonstrated that AKT-1 up-regulation is essential for the tumorigenic phenotype observed for *Pten*-null ES cells.

MATERIALS AND METHODS

Generation of *Pten*$^{-/-}$;*Akt*$^{-/-}$ ES cell lines. Genomic DNA clones corresponding to the *Akt-1* locus were isolated from an isogenic 129(J1) genomic library. The targeting vector contains the PGKpuropA cassette flanked by an 8.0-kb *Not*I-*Bam*HI fragment (5′ arm) and a 2.1-kb *Xho*I fragment (3′ arm) in the backbone of pBluescript vector. Linearized targeting plasmid (50 μg) was electroporated into 2×10^7 *Pten*-null ES cells. After 3 days of puromycin selection (1.5 μg/ml), drug-resistant ES clones were isolated and expanded. Genomic DNAs were prepared for Southern blot analysis with an external probe. Recombinant clones were further confirmed with an internal probe and a 5′-arm probe. To obtain ES clones homozygous for the *Akt-1* deletion, heterozygous ES clones were subjected to a higher-level puromycin selection (70 μg/ml) for 3 days. At the end of selection, new feeder layers were replated and the puromycin concentration was reduced to 4 μg/ml to allow surviving ES clones to recover. The optimal concentration and the time course of puromycin selection were predetermined experimentally to allow efficient killing. Homozygous deletion was confirmed by Southern blot analysis as well as Western blot analysis.

Cells and tissue culture. ES cells were cultured on irradiated fibroblast feeder layers as described previously (36). Briefly, feeder cells were plated 1 day prior to the seeding of ES cells. ES cells were cultured in knockout Dulbecco modified Eagle medium (Gibco BRL) containing 15% fetal calf serum supplemented with nonessential amino acids, glutamic acids (100 mM), leukemia inhibitory factor (1,000 U/ml), and β-mercaptoethanol. All cell lines were cultured under this growth condition.

For biochemical analysis, ES cells were passaged twice without feeder cells at a density of 10^7 cells/10-cm-diameter plate. Two days later, these cultures were further passaged at a one-to-three without feeders to minimize feeder contamination and continuously cultured for 9 to 10 h, at which point cells were either treated with Colcemid (Sigma) for cell cycle studies or lysed for Western blot analysis. For insulin-like growth factor 1 (IGF-1) and serum stimulation, cells were washed and split continuously cultured in serum-free medium (in the presence of leukemia inhibitory factor) for 16 h and then stimulated with a final concentration of 1 μg of IGF-1 (a generous gift from Amgen)/ml or 15% serum for 10 or 30 min before protein analysis.

For cell cycle analysis, ES cells were preshaken, washed, and then treated with Colcemid (0.06 g/ml) for 4 h as described previously (33, 36). After mitotic shake-off, ES cells were cultured under normal growth conditions without feeders to allow reentry into the cell cycle. Cells were collected at each time point, and cell cycle profiles were studied by fluorescence-activated cell sorting (FACS) analysis. Briefly, cells were trypsinized, washed two times with cold phosphate-buffered saline (PBS), and then permeabilized overnight with 50% ethyl alcohol in PBS. ES cells were then washed with PBS, and DNA was stained with propidium iodine (40 μg/ml) in PBS containing RNase A (100 μg/ml) for 30 min at 37°C. The cells were then stored at 4°C and later analyzed by FACS.

Growth of ES cells under reduced-serum conditions. ES cells (5×10^4 cells/well) were plated on top of feeder layers in 24-well tissue culture plates with medium containing 15% serum. One day later, cells were washed and changed to serially reduced serum conditions. Medium was changed daily, and cell numbers were counted 4 days after the initiation of serum starvation. For caspase inhibitor treatment, 4 μM caspase inhibitor cocktail (set III in dimethyl sulfoxide; Calbiochem) was added to ES cell cultures 1 day after serum starvation, and cell numbers were counted 4 days after the initiation of serum starvation. As a control, ES cells were treated with carrier dimethyl sulfoxide for the same period of time.

Growth competition assay. Double mutant (*Pten*$^{-/-}$;*Akt*$^{-/-}$) ES cells (2×10^5) were cocultivated in a six-well plate with equal numbers of wild-type (WT) or *Pten*$^{-/-}$ ES cells. When they reached 80 to 90% confluency, cultures were passaged at the original density (4×10^5 cells) as passage 2. Cultures were continuously cultured for a total of six passages, and genomic DNAs were prepared from each passage. Southern analysis was then performed to determine the contributions from each cell type.

Generation of teratomas with ES cells. Different ES clones were expanded and grafted onto immunoincompetent nude mice. Briefly, 5×10^5 ES cells were injected subcutaneously onto the backs of nude mice. Tumor formation was then observed at different time points. Tumor volume was obtained by measuring the parameter and the height of the tumors with three independent measurements. Before reaching 1.5 cm in diameter (according to National Institutes of Health guidelines), tumors were harvested and analyzed histologically.

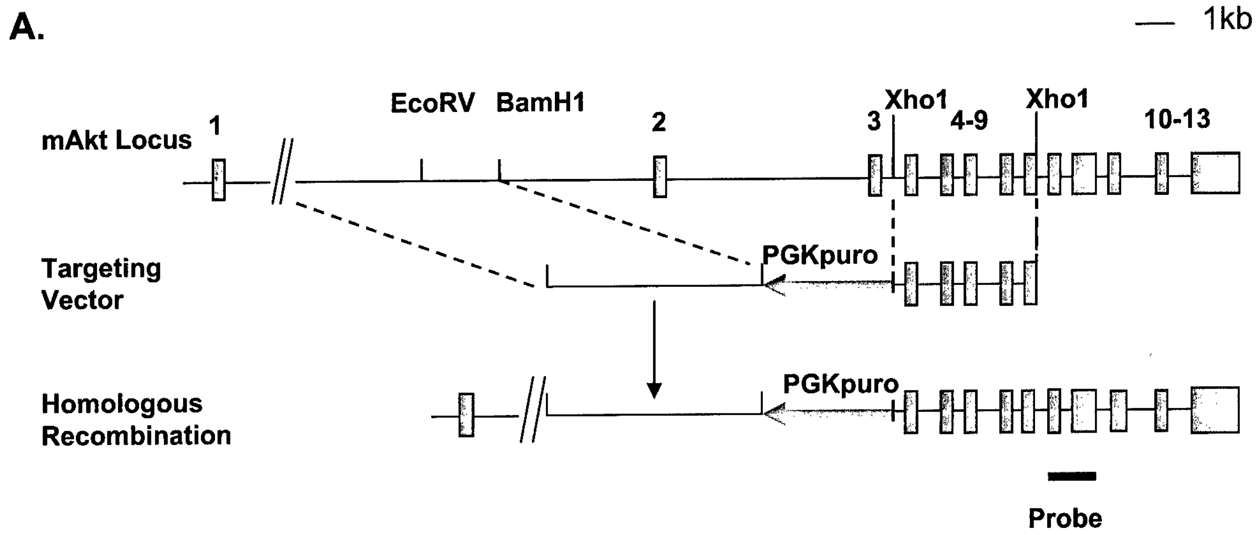
Antibodies and Western blot analysis. Cell lysate preparation and Western blot analysis were carried out as described previously (36). Antibody to the AKT-1 PH domain was purchased from Upstate Biotechnology. Three additional antibodies to the AKT C-terminal peptide were purchased from Upstate Biotechnology, Cell Signaling, and Santa Cruz Biotechnology. Antibodies specific for ERK, p27^{KIP1} (sc-528), cyclin D1 (R-124), p21$^{CIP1/WAF1}$ (sc-397), cyclin A, and mouse cyclin E (sc-481) were obtained from Santa Cruz Biotechnology. Antibodies for PTEN and phosphospecific antibodies for AKT, FKHR/FKHRL1, BAD, S6 ribosomal protein, and GSK-3α/β were from Cell Signaling. Antibodies for actin and vinculin were provided by Sigma.

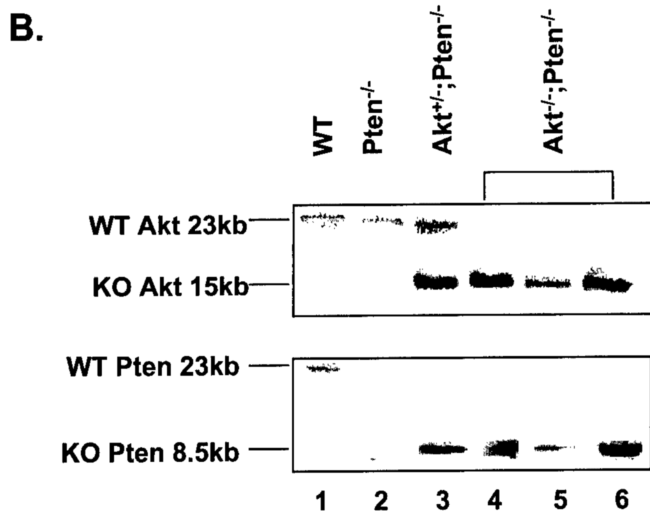
Statistical analysis. Analysis of variance was applied to all data subjected to statistical analysis, and Fisher's least significant difference test was used to determine the statistical differences among the groups. The paired Student t test was used on teratoma data to determine the differences between different groups. A P value of ≤ 0.05 was considered statistically significant.

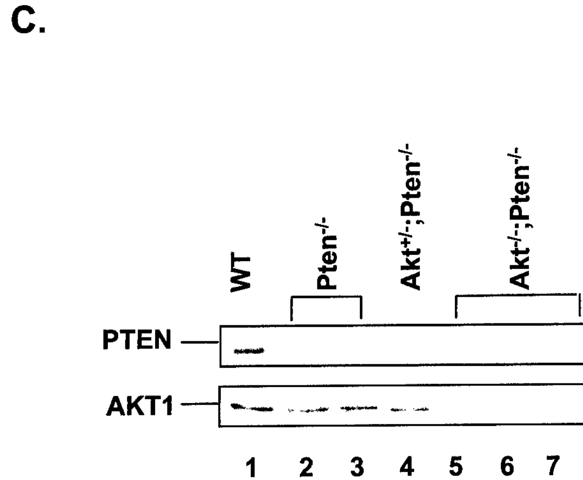
RESULTS

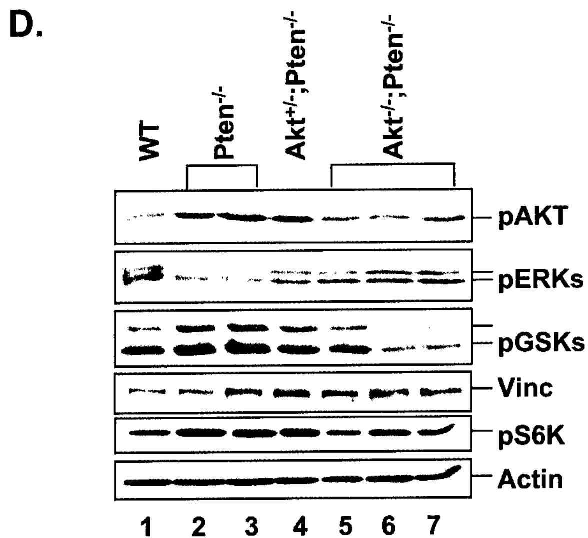
Targeted deletion of *Akt-1* via homologous recombination in WT and *Pten*-null ES cells. To delete the *Akt-1* gene, a targeting vector was constructed to delete the entire PH domain of the AKT-1 molecule (Fig. 1A). Since AKT membrane recruitment and subsequent activation depend on its PH domain, this construct will result in inactivation of AKT-1. The knockout construct carries a puromycin-selective cassette so that *Akt-1* targeting events can be selected when introduced into neomycin-resistant *Pten*$^{-/-}$ ES cells. Three out of 400 Puro^r colonies were identified as homologous recombinants by Southern analyses, and homozygous clones were later obtained by culturing heterozygous ES cells in a higher concentration of puromycin. Using this strategy, we obtained clones carrying the following genotypes: WT, *Pten*$^{-/-}$, *Akt*$^{+/-}$;*Pten*$^{-/-}$, and *Akt*$^{-/-}$;*Pten*$^{-/-}$. All clones were genotyped by Southern blot analysis and confirmed by Western blot analysis using antibodies for PTEN and AKT-1 PH domain (Fig. 1B and C). Furthermore, no proteins could be detected by using three independent antibodies to the C-terminal domains of the AKT-1 molecule, suggesting that no stable truncated protein could be produced from the targeted alleles (Fig. 1C). Two independent *Pten*$^{-/-}$ clones and three independent *Pten*$^{-/-}$;*Akt*$^{-/-}$ clones were used for further analysis.

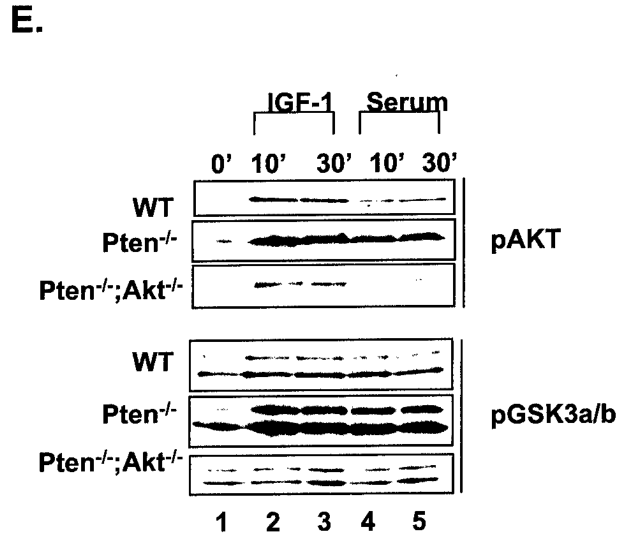
As *Akt-2* and *Akt-3* may still compensate for the loss of *Akt-1*, we checked overall AKT activity with a phosphospecific antibody which can recognize all three phosphorylated forms of AKT. Our results indicated that, by deleting *Akt-1* plus *Pten* (Fig. 1D, lanes 5 to 7), we were able to bring up-regulated AKT activity in *Pten*$^{-/-}$ cells (Fig. 1D, lanes 2 and 3) back to near-WT levels (Fig. 1D, lanes 5 to 7 versus lane 1). We also evaluated mitogen-activated protein kinase (Fig. 1D) and Jun kinase and β-catenin (data not shown) activities and were not able to find significant and consistent differences among different genotypes. On the other hand, as a direct consequence of changes in AKT activity, the phosphorylation status of GSK-3α and -3β, two of the substrates of AKT serine/threonine kinases, was affected in parallel, i.e., high in *Pten*$^{-/-}$ cells (Fig. 1D, lanes 2 and 3) but reduced in *Pten*$^{-/-}$;*Akt*$^{-/-}$ clones (Fig. 1D, lane 1 versus lanes 5 to 7). In addition, AKT-1


A.
1kb
mAkt Locus
1
EcoRV
BamH1
2
3
Xho1
4-9
Xho1
10-13
Targeting Vector
PGKpuro
Homologous Recombination
PGKpuro
Probe


B.
WT
Pten-/-
Akt+/-;Pten-/-
Akt-/-;Pten-/-
WT Akt 23kb
KO Akt 15kb
WT Pten 23kb
KO Pten 8.5kb
1 2 3 4 5 6


C.
WT
Pten-/-
Akt+/-;Pten-/-
Akt-/-;Pten-/-
PTEN
AKT1
1 2 3 4 5 6 7


D.
WT
Pten-/-
Akt+/-;Pten-/-
Akt-/-;Pten-/-
pAKT
pERKs
pGSKs
Vinc
pS6K
Actin
1 2 3 4 5 6 7


E.
IGF-1
Serum
0' 10' 30' 10' 30'
WT
Pten-/-
Pten-/-;Akt-/-
pAKT
WT
Pten-/-
Pten-/-;Akt-/-
pGSK3a/b
1 2 3 4 5

deletion also appeared to diminish the enzymatic activity of p70S6 kinase as measured by the phosphorylation status of its product, S6 ribosomal protein (Fig. 1D). The changes in the phosphorylation of S6 ribosomal protein paralleled the phosphorylation status of AKT: S6 ribosomal protein was hyperphosphorylated in the *Pten*$^{-/-}$ (Fig. 1D, lanes 2 and 3) and *Pten*$^{-/-}$;*Akt*$^{+/-}$ (lane 4) cell lines, while the phosphorylation was diminished in the *Pten*$^{-/-}$;*Akt*$^{-/-}$ clones (Fig. 1D, lanes 5 to 7). Thus, AKT-1 seems to play an important role in GSK-3α Ser21 and GSK-3β Ser9 phosphorylation as well as in the activation of a further downstream target, p70S6 kinase in the ES cells, suggesting that AKT-1 may be essential, among the three AKT members, in controlling the PI 3-kinase/AKT axis in ES cells.

We then analyzed whether AKT and GSK-3 kinases could be further activated by growth factor stimulation in the absence of AKT-1. As shown in Fig. 1E, the effects of serum on AKT phosphorylation were significantly less than those of IGF-1 in all cell lines. AKT was hyperphosphorylated in *Pten*$^{-/-}$ cells, even under serum starvation conditions (0′), which could be further induced by IGF-1 (lanes 3 and 4) or serum stimulation (lanes 4 and 5). WT cells did respond to IGF-1 stimulation and to a lesser extent to serum treatment (Fig. 1E, top panel). *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells were almost unresponsive to serum treatment, and the remaining phosphorylation observed after IGF-1 stimulation could be due to AKT-2 and AKT-3. Similarly, IGF-1 and serum stimulation induced hyperphosphorylation of GSK-3α/β in *Pten*$^{-/-}$ cells but had less effect on WT cells and almost no effect on *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells (Fig. 1E, lower panels). These results indicated that both AKT phosphorylation and activation of the downstream target of AKT are severely impaired in *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells.

Akt-1 deletion reverses cell survival phenotype of *Pten*-null ES cells. In order to assess the role of AKT-1 in PTEN/PI 3-kinase-mediated cell survival, ES clones were cultured with reduced serum concentrations. Consistent with our previous observations, *Pten*$^{-/-}$ cells demonstrated significantly enhanced cell survival compared to the WT cells under serum-starved conditions (Fig. 2A, upper and middle left panels). Deletion of *Akt-1* as well as *Pten* completely reversed the survival phenotype seen for *Pten*$^{-/-}$ cells (Fig. 2A, lower left panel). Interestingly, *Pten*$^{-/-}$;*Akt*$^{-/-}$ ES cells were even more sensitive to serum concentrations than were the WT cells: significantly more cell death was observed for *Pten*$^{-/-}$;*Akt*$^{-/-}$ cultures than for WT cell cultures when serum concentrations were reduced to less than 3.8% (Fig. 2B).

In order to determine whether the observed cell death was due to apoptosis, we treated the different ES cell lines with a cocktail of caspase inhibitors in the absence of serum. As shown in Fig. 2C, such treatment led to increased cell survival of WT and *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells to levels similar to those for *Pten*$^{-/-}$ cells.

The cell survival phenotype prompted us to study three of the downstream targets of AKT, BAD and two Forkhead transcriptional factors, FKHR and FKHRL1, which were known to regulate apoptosis through different mechanisms. FKHRL1 and FKHR phosphorylation were up-regulated in *Pten*$^{-/-}$ cells but reduced in *Pten*$^{-/-}$;*Akt*$^{-/-}$ ES cells (Fig. 2D). Similarly, phosphorylation of BAD was returned to the WT level by deleting *Akt-1* as well as *Pten* (Fig. 2D), suggesting that AKT-1 plays a major role in the PI 3-kinase-mediated cell survival pathway. In addition, IGF-1- and serum-induced FKHRL1 and FKHR phosphorylation was diminished in the *Akt-1*-deletion cell lines compared to that in WT and *Pten*$^{-/-}$ cells (Fig. 2E). Interestingly, IGF-1 and serum had similar effects on FKHR phosphorylation, which is quite different from their effects on AKT phosphorylation (Fig. 1E), suggesting that FKHR phosphorylation can be regulated by both AKT-dependent and AKT-independent pathways.

Akt-1 deletion reverses the cell growth phenotype of *Pten*-null ES cells. In addition to cell survival, we also noticed differences in cell growth. The growth rate of *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells appeared to be lower than that of WT and *Pten*$^{-/-}$ ES cells (Fig. 3A). In order to determine changes in the growth properties of *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells, we cocultivated *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells with equal numbers of either WT or *Pten*$^{-/-}$ ES cells. As shown in Fig. 3B, middle panel, *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells grew slower than did the WT cells, and upon passage 4, most of the culture was taken over by the WT cells (as judged by the ratio of WT band to mutant band). This is even more apparent when *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells were cocultured with *Pten*$^{-/-}$ cells (Fig. 3B, right panel). The ratio of WT band to mutant band increased significantly on passage 2, and *Pten*$^{-/-}$ cells became the dominant cell type in the cocultures between passages 2 and 3. Thus, deletion of *Akt-1* not only reverses the growth advantage seen in *Pten*$^{-/-}$ cells but further decreases the cell proliferation rate to a level lower than that of the WT cells.

As a result of *Akt-1* deletion, the levels of several cyclins and G_1/S cell cycle inhibitors were altered. As reported in a previous paper (36), the level of p27 is down-regulated in *Pten*$^{-/-}$ cells but returned to near-WT level in *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells (Fig. 3C, top panel). Cyclin A levels were changed inversely (Fig. 3C, middle panel). Levels of cyclin A were elevated in *Pten*$^{-/-}$ cells and yet decreased when *Akt* was deleted in addition to *Pten*. The cyclin D1 level, on the other hand, was not affected by deleting *Pten* or *Akt-1* (Fig. 3C, top two panels). Cyclin E levels were similar between *Pten*$^{-/-}$ and WT cells, and yet *Akt-1* deletion resulted in a diminished cyclin E protein

FIG. 1. Inactivation of mouse *Akt-1* gene. (A) A restriction map of the mouse *Akt-1* locus is shown in the top panel with exons depicted. The middle panel shows the targeting vector with exons 2 and 3 deleted and replaced with a PGKpuro cassette. The bottom panel is the predicted recombinant harboring the deletions with the position of the 3′ external probe indicated below it. (B) Southern blot analysis of the *Pten* and *Akt-1* locus. (C) Western analysis of PTEN and AKT-1 levels in the indicated ES cell clones. Cell lysates (20 μg) were run on a polyacrylamide gel and Western blotted with antibodies for PTEN (top) and AKT-1 (bottom). (D) Western blot analyses of phospho-AKT, ERK, GSK-3α/β, and p70S6 kinase status in indicated ES clones. Cell lysates (20 μg) were run on a polyacrylamide gel and Western blotted with antibodies for the different molecules. The same blots were reblotted with vinculin (Vinc) or actin as loading controls. (E) Serum-starved ES cells were treated with either 1 μg of IGF-1/ml or 15% fetal calf serum for 10 or 30 min. Cell lysates were analyzed for phospho-AKT (upper panels) and GSK-3 (lower panels) status. The same blots were also reblotted with actin as loading controls (data not shown). pGSK3a/b, pGSK3α/β.

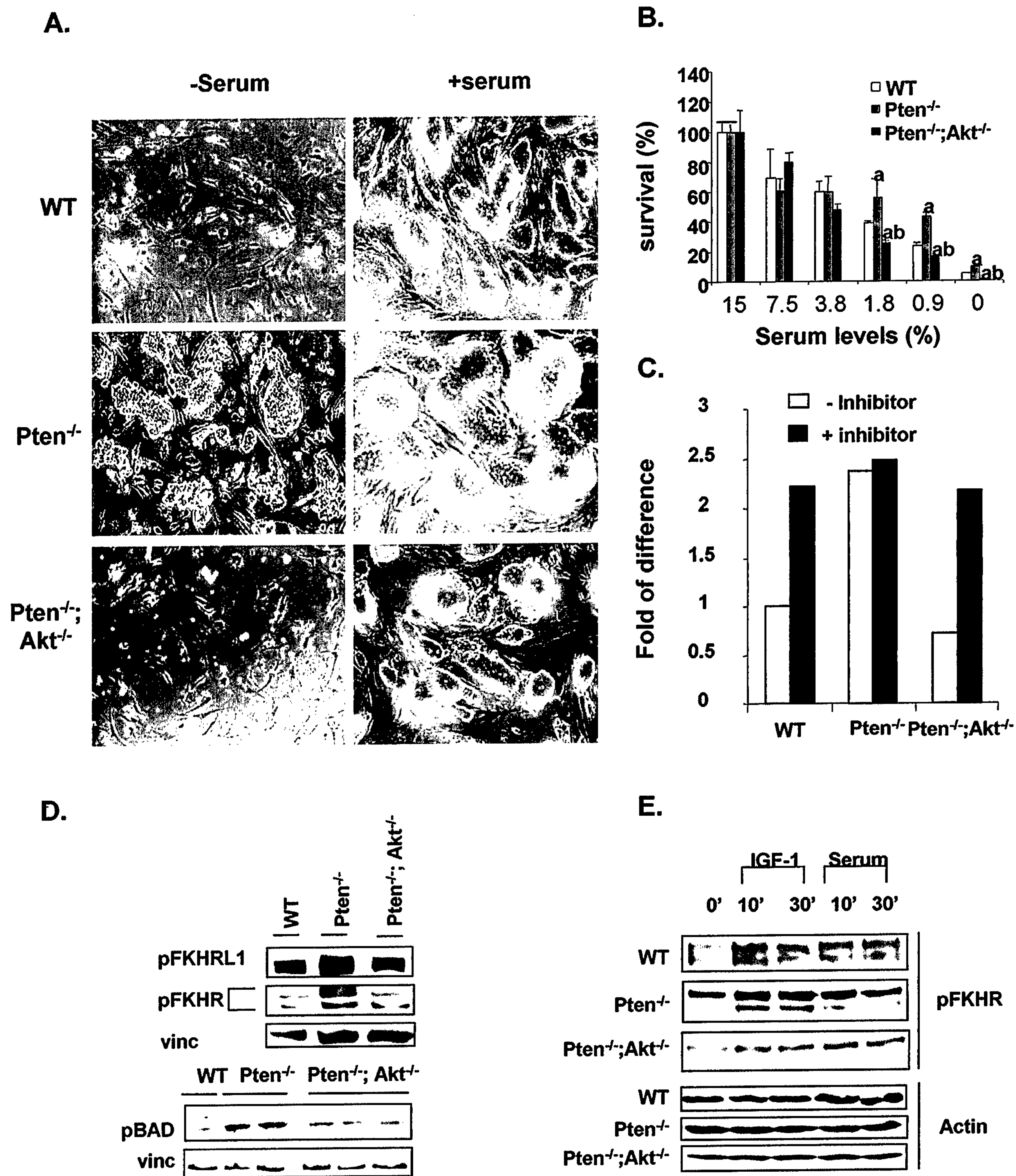


FIG. 2. Cell survival potential and apoptotic signals in ES clones carrying *Pten/Akt* deletions. (A) Survival of ES cells in response to serum starvation. ES cells (5×10^4) were seeded the day before serum withdrawal. Cells were cultured without serum for 4 days. Cell survival was observed under a light microscope and quantified by counting the number of surviving cells under each culture condition. Photographs are representative of three independent experiments. Left panels, cells grown under serum-free condition. Right panels, cells grown under normal growth conditions (15% serum). (B) Quantification of cell numbers at different serum concentrations. Data presented are means ± standard errors of the means of $n = 3$. Bars designated with the letter "a" are statistically significantly different from the WT bars at the same serum concentration ($P \leq 0.05$). Bars designated with the letter "b" are statistically significantly different from the $Pten^{-/-}$ bars at the same serum concentration ($P \leq 0.05$). (C) Quantification of cell numbers with or without caspase inhibitor cocktail. Data are presented as fold increases over cell numbers in the WT cultures in the absence of the inhibitor cocktail. (D) Western blot analyses of the phosphorylation status of FKHRL1, FKHR, and BAD under normal culture conditions. Total cell lysates (20 μg) were run on a polyacrylamide gel and Western blotted with antibodies for phosphor-FKHR/FKHRL1 and BAD. The same blots were also blotted with vinculin (vinc) as controls. (E) Serum-starved ES cells were treated with either 1 μg of IGF-1/ml or 15% fetal calf serum for 10 or 30 min. Cell lysates were then analyzed for phospho-FKHR status by Western blotting (upper panels). The same blots were also blotted with actin as controls (lower panels).

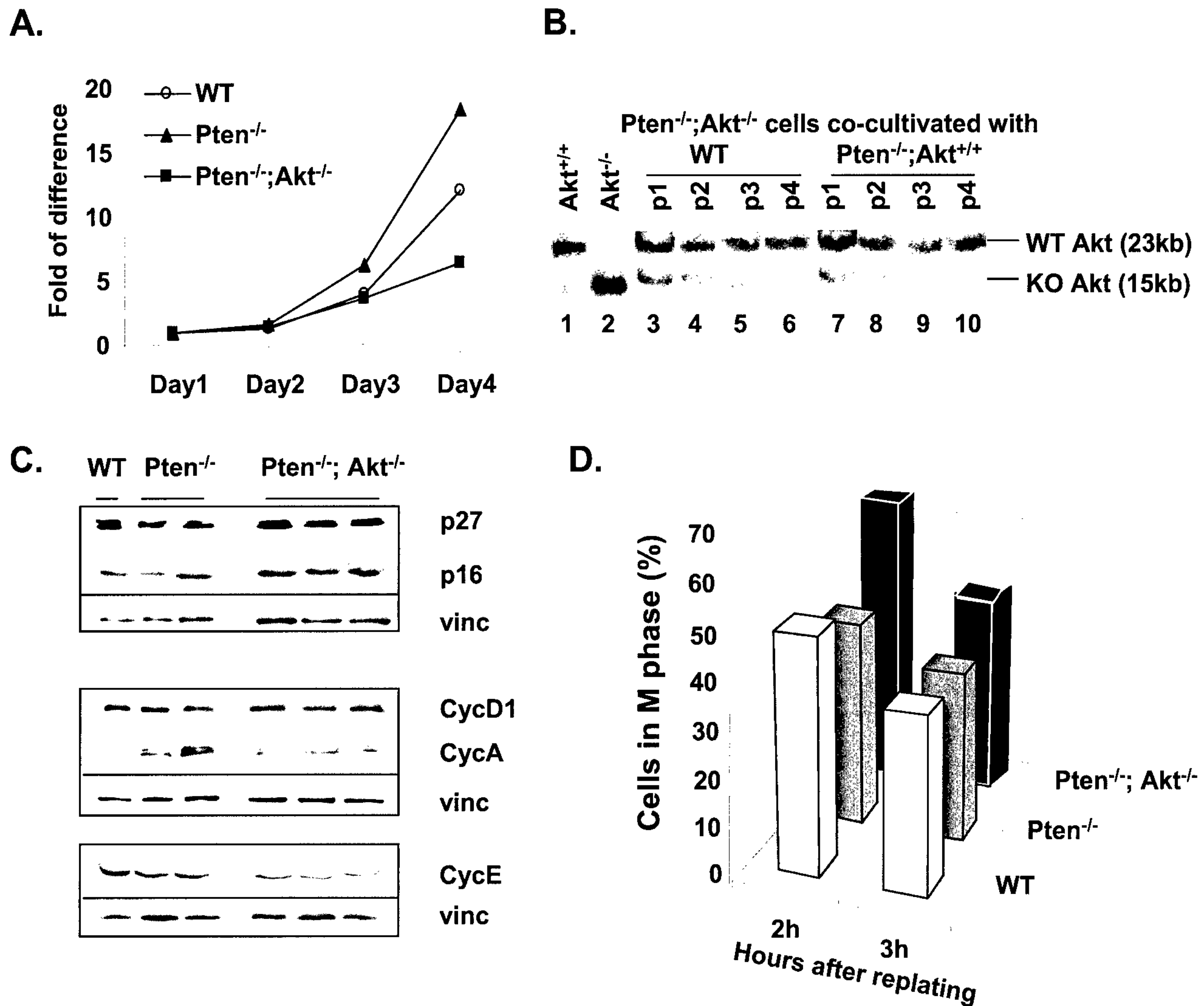


FIG. 3. Growth potential, cell cycle profile, and analysis of cell cycle regulators in *Pten/Akt* clones. (A) Growth curve of indicated ES clones. (B) Growth competition assay. *Pten*$^{-/-}$;*Akt*$^{-/-}$ ES cells were cocultivated with equal numbers of WT or *Pten*$^{-/-}$ cells. DNA was extracted from the cocultures after each passage and was analyzed with Southern analysis to determine the ratio of WT *Akt* allele (23 kb) to mutant Akt allele (15 kb). Lanes 1 and 2, WT and *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells cultured alone. Lanes 3 to 6, *Pten*$^{-/-}$;*Akt*$^{-/-}$ ES cells cocultured with WT cells at different passages (p1 to p4). Lanes 7 to 10, *Pten*$^{-/-}$;*Akt*$^{-/-}$ ES cells cocultured with *Pten*$^{-/-}$ cells at different passages (p1 to p4). KO, knockout. (C) Western blot analysis of cell cycle regulators. Total cell lysates (20 μg) were separated on sodium dodecyl sulfate-polyacrylamide gels and blotted with cell cycle inhibitors p27^{KIP1} and p16^{INK1} as well as cyclins A, D1, and E. Vinculin (vinc) was used on each blot as a loading control. (D) Cell cycle analysis. ES cells were synchronized at M phase with Colcemid treatment followed by replating in Colcemid-free medium to allow reentry into the cell cycle. FACS analysis was performed on each clone at different time points (2 and 3 h), and the percentages of cells remaining in M phase are shown here.

level (Fig. 3C, bottom lane). Levels of other cell cycle inhibitors were either not detectable (p15 and p21) or not changed (p16).

Akt-1 deletion delays M-phase exit in *Pten*$^{-/-}$;*Akt*$^{-/-}$ ES cells. Overexpression of *Drosophila melanogaster PTEN* (*dPTEN*) inhibits cell cycle progression at mitosis and promotes cell death during eye development (17). Overexpression of *PTEN* in mammalian cells, on the other hand, induces G_1/S cell cycle arrest and cell death (21). Our previous studies also suggested that deletion of *Pten* in the ES cells leads to an accelerated G_1/S cell cycle transition (36). To address the possible role of PTEN and PI 3-kinase/AKT in other phases of the cell cycle, we tested the rates of exit of ES cells from mitotic block. ES cells with different genotypes were treated with the M-phase blocker Colcemid, and synchronized mitotic cells were collected after mitotic shake-off (36). Cells were then cultured in Colcemid-free fresh medium, and their cell cycle status was analyzed by FACS 2 and 3 h after the release, corresponding to the beginning of S phase in ES cells (36). Approximately 45 and 38% of cells remained in M phase at 2 and 3 h, respectively, when WT ES cells were released following mitotic arrest (Fig. 3D, open bars). In contrast, *Pten*$^{-/-}$ cells showed an increased M-phase exit rate (40 and 35% at 2 and 3 h, respectively). To our surprise, the *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells remained in M phase much longer than did WT cells. Almost 70 and 50% of the *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells remained in M phase

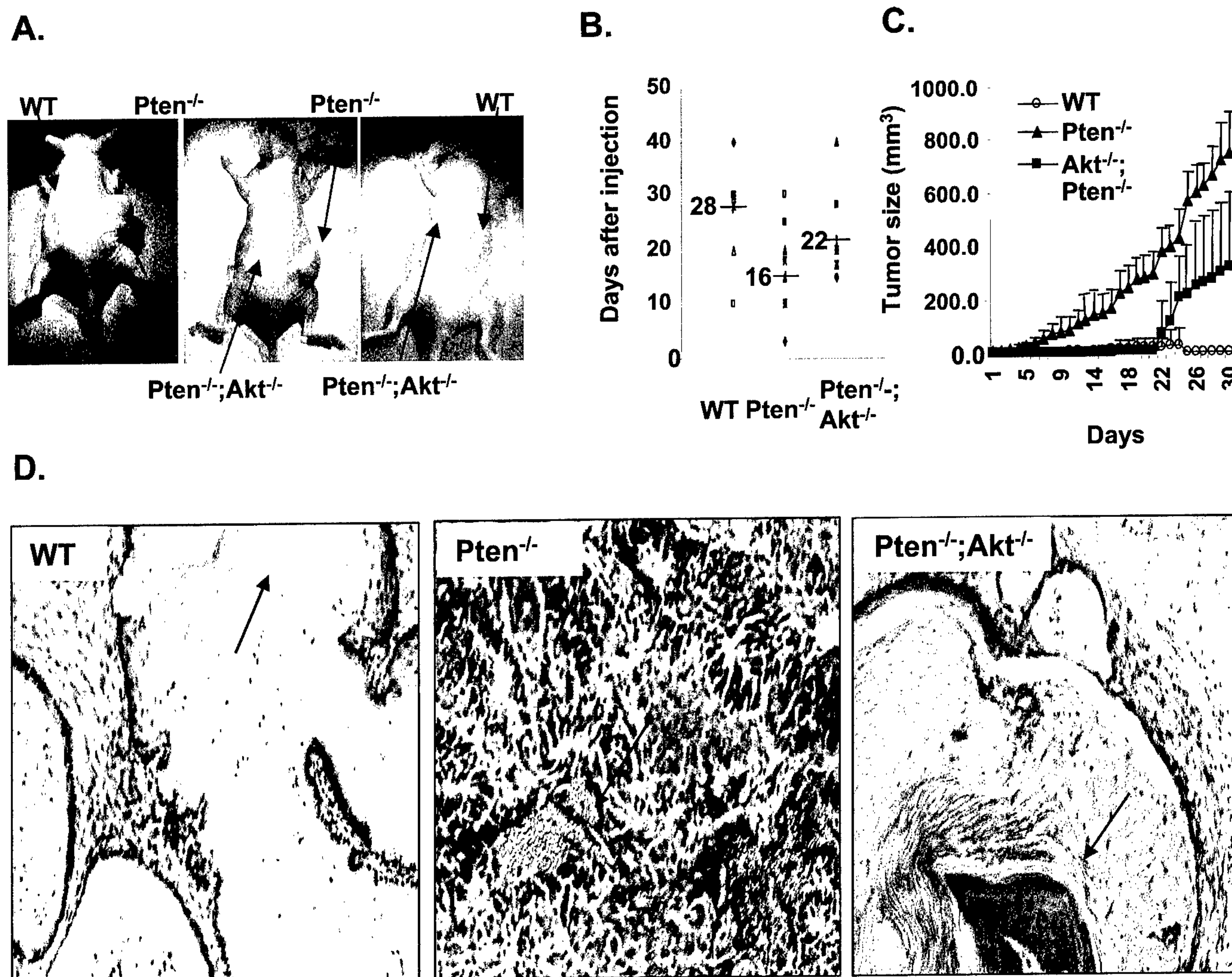


FIG. 4. Deleting *Akt-1* in addition to *Pten* delays the onset of teratoma formation and decreases the sizes of tumors formed by $Pten^{-/-}$ ES cells. WT, $Pten^{-/-}$;$Akt^{+/+}$, and $Pten^{-/-}$;$Akt^{-/-}$ cells (5×10^6) were injected subcutaneously onto the backs of immunoincompetent nude mice. Each mouse was injected bilaterally with cells carrying a different genotype. Teratoma formations were observed and recorded at indicated time points. (A) Examples of teratomas growing on the backs of nude mice. (B) Onset of teratomas formed from different ES clones. Each dot represents one animal; $n = 9$ in each group. The average time for tumor appearance is indicated as a line with the actual number beside it. (C) Progression of teratomas formed by different ES clones. The day of tumor detection is set as day 0. Data are presented as means ± standard errors of the means of $n = 5$. (D) Histology analysis of teratomas harvested from the nude mice. Left panel, tumors formed by injecting WT ES cells showed well-differentiated tissues with skin characteristics (arrow); middle panel, teratomas formed by $Pten^{-/-}$ ES cells are highly proliferative, less differentiated, and well vascularized (arrow); right panel, tumors formed by injection of $Pten^{-/-}$;$Akt^{-/-}$ ES cells. Deletion of *Akt-1* in addition to *Pten* partially reverses the aggressive growth of $Pten^{-/-}$ teratomas. The arrow indicates the more differentiated keratinized skin, similar to tumors generated by WT ES cells (left panel).

2 and 3 h after release from mitotic block, respectively. Thus, AKT may play a critical role in cell cycle progression at M phase. The downstream targets of AKT at M phase are not yet defined. However, the recent identification of Myt1 as one of the AKT substrates suggested that AKT may be a crucial kinase involved in the phosphorylation-dephosphorylation cascades occurring though G_2/M phase of the cell cycle (31).

Akt-1 deletion partially reverses the tumorigenesis phenotype of *Pten*-null ES cells. To validate the functional significance of our findings, we injected the $Pten^{-/-}$;$Akt^{-/-}$ ES clones into nude mice to directly assess their in vivo tumorigenic potential. ES cells are known to produce benign, usually well-differentiated teratomas in this setting. However, teratomas generated from $Pten^{-/-}$ cells were highly proliferative, less differentiated, and well vascularized (Fig. 4D, middle panel). In contrast, deletion of *Akt-1* as well as *Pten* significantly delayed the onset, as well as decreased the aggressive growth rate, of $Pten^{-/-}$ teratomas (Fig. 4B and C). The $Pten^{-/-}$; $Akt^{-/-}$ tumors were also significantly smaller than the $Pten^{-/-}$ tumors (Fig. 4C). Histological analysis indicated that $Pten^{-/-}$; $Akt^{-/-}$ tumors were more differentiated and less vascularized than $Pten^{-/-}$ tumors, similar to tumors generated by WT ES cells (Fig. 4D, left and right panels). Thus, deletion of *Akt-1* can partially reverse the tumorigenic phenotype of $Pten^{-/-}$ ES cells to a level at least intermediate between those of $Pten^{-/-}$ and WT tumors.

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DISCUSSION

This study focuses on the PTEN/AKT pathway, which selectively targets the serine/threonine protein kinase AKT-1. Using a genetic approach, we studied the function of AKT-1 in an isogenic system. We showed that AKT-1 is the main downstream effector of PTEN and is required for both the cell survival and cell proliferation phenotype observed for the *Pten*-knockout ES cells.

Two interesting observations were made during our initial study of *Pten*$^{-/-}$ ES cells (36). First, the *Pten*$^{-/-}$ cells were more resistant to serum starvation-induced cell death than were the WT cells. Second, the *Pten*$^{-/-}$ ES cells had shorter doubling times and earlier S-phase entry than the WT cells, accompanied by up-regulation of AKT and down-regulation of G_1 cell cycle inhibitor p27. In this study, we demonstrated that deleting *Akt-1* alters both the cell survival and growth advantage of *Pten*$^{-/-}$ cells and that AKT-1 modulates the levels of proapoptotic factor BAD, transcriptional factor FHKR, and cell cycle inhibitor p27, as well as cyclins A and E.

Previous in vitro studies indicated that AKT activation might play an essential role in tumorigenesis, especially in PTEN mutation-associated tumor formation. However, most of these studies were conducted with human tumor cell lines which may carry other mutations or abnormalities (1, 6, 18, 26, 32). Using a genetic approach, we generated isogenic cell lines so that the function of AKT in PTEN-controlled signaling pathways and tumorigenesis could be directly assessed. Our data indicated that AKT plays a significant role in regulating PTEN-mediated cell growth and cell survival. First, we demonstrated that deleting *Akt-1* reversed the cell survival phenotype in *Pten*$^{-/-}$ cells. Furthermore, deleting both alleles of *Akt-1* appeared to have additional effects, and mutated cells were more sensitive to serum starvation-induced cell death than were the WT cells. Second, we showed that the *Akt-1* knockout was able to reverse the growth advantage of *Pten*$^{-/-}$ cells. Not only did the *Pten*$^{-/-}$; *Akt*$^{-/-}$ clones lose the ability to compete with *Pten*$^{-/-}$ cells, but they also failed to compete with the WT cells in growth competition assays, though to a lesser extent. This could be due to the presence of AKT-1 in the WT cells, which could respond to growth factor stimulation. The fact that overall AKT phosphorylation in response to IGF-1 or serum treatment is significantly diminished in the *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells, compared to that in WT cells, supported this idea. Therefore, the cell survival and cell proliferation signals are propagated more properly in the WT cells than in the *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells. It is also possible that pathways other than PI 3-kinase-dependent signals may also regulate AKT and be responsible for the additional effects seen with double-knockout cells.

Evidence from in vivo teratoma formation supported the in vitro data. ES cells can produce benign teratomas in nude mice regardless of *Pten* deletion. However, the onset as well as the progression of tumor formation is much earlier and more aggressive in *Pten*-null teratomas than in WT teratomas. By deleting *Akt-1*, we were able not only to reduce the size of *Pten*$^{-/-}$ teratomas but also to delay the onset of tumor formation. However, unlike the in vitro cell survival and cell growth experiments, deletion of *Akt-1* as well as *Pten* did not completely reverse the aggressive growth phenotype of *Pten*$^{-/-}$ cells. Indeed, both the tumor size and onset of tumor development in *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells are intermediate between those of *Pten*$^{-/-}$ and WT cells, suggesting that other molecules could have taken over once the tumor development reached a certain stage. Teratomas contain many different cell types. Even though our data suggested that AKT-1 is crucial for cell survival and cell growth in ES cells, it is not surprising that the growth of the teratoma could escape the control of AKT-1, e.g., by being compensated for by AKT-2 and AKT-3 since the three AKTs were differentially regulated in different cell types (30). Together, the in vitro and in vivo characteristics of *Pten*$^{-/-}$; *Akt*$^{-/-}$ ES clones strongly implied that AKT-1 is the main downstream effector of PTEN, although other targets may also play roles in PTEN-mediated tumorigenesis.

We demonstrated that overall phosphorylation of AKT appeared to be diminished to the WT level when *Akt-1* is deleted, suggesting that possible compensation by *Akt-2* and *Akt-3* is minimal and is responsible only for baseline AKT phosphorylation when PTEN is intact in ES cells. This was further confirmed with treatment with IGF-1 and serum, which failed to induce an overall AKT phosphorylation in *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells similar to that in the WT or *Pten*$^{-/-}$ cells. This is not surprising, since the different AKTs are differentially activated by growth factors in a cell-type-dependent manner (30). The diminished phosphorylation of GSK-3 and S6R in the *Akt-1*-deletion cells further implicated AKT-1 as a general regulator for the PI 3-kinase pathway and a primary target of PTEN regulation.

Activation of AKT leads to the phosphorylation of several downstream targets. One of the unequivocal consequences of deleting *Akt-1* is diminished phosphorylation of GSK-3α/β, which by itself may play a significant role in cell growth as well as other cellular processes (19). We demonstrated in this paper that deleting Akt-1 resulted in significantly diminished phosphorylation levels of GSK-3α/β in the *Pten*$^{-/-}$;*Akt*$^{-/-}$ cells compared to those in the *Pten*$^{-/-}$ cells. Deletion of *Akt-1* as well as *Pten* also changes the activities of several cell-death-related molecules, such as BAD and FKHR. BAD is the proapoptotic factor (12) which associates with the antiapoptosis molecule Bcl-2 to prevent Bcl-2-mediated antiapoptosis function. Phosphorylation of BAD by AKT prevents this association and shifts the apoptosis signal to antiapoptosis, thereby promoting cell survival (11). We demonstrated that BAD phosphorylation is mainly dependent on AKT-1. When *Akt-1* is deleted from *Pten*$^{-/-}$ ES cells, BAD phosphorylation is brought down to the basal level. Among the other AKT targets are the Forkhead transcriptional factors, such as FKHR/FKHRL1 and AFX (7, 13, 20). Recent studies of both *Drosophila* and mammalian cells have firmly established Forkhead family members as substrates of AKT. FKHR/FKHRL1 phosphorylation is increased in *Pten*$^{-/-}$ ES cells, but to a much lesser extent than that of BAD. When Akt-1 is deleted, all of these downstream targets of AKT are affected in parallel, including their response to growth factor stimulation, which appears to be highly dependent on the presence of AKT-1 in the cells. Although each downstream target responded to a different extent, all of them, including the ribosomal protein kinase S6K, demonstrated reduced stimulation by IGF-1 or serum compared to that for the *Pten*$^{-/-}$ cells.

Growing evidence has drawn a connection between AKT and cell cycle progression, and yet the molecular mechanisms

underlying this connection were not completely elucidated. Using mouse ES cells lacking both alleles of the *Pten* gene, we showed that the level of G_1 cell cycle inhibitor p27 was down-regulated. These ES cells also have increased AKT activity and a selective growth advantage compared to the heterozygous and WT cells (36). In addition, deletion of another PIP_3 phosphatase, *Ship*, was also shown previously to result in activation of AKT (25). Thus, AKT activation is a key phenomenon when PIP_3 levels are elevated in both *Pten*- and *Ship*-null cells. In either event, deletions of *Pten* or *Ship* generated hyperplasia and tumor phenotype (25, 36). Recent discoveries of the AKT/Forkhead pathway also indicate that AKT may regulate cell growth by regulating the key cell cycle inhibitor p27. Therefore, we investigated the role of AKT-1 at the G_1/S transition, especially its role in regulating p27 and cyclin E/Cdk2. The level of change in p27 is correlated with phosphorylation of AKT in the different ES clones. Deleting *Akt-1* as well as *Pten* was able to reverse the diminished p27 levels in $Pten^{-/-}$ clones. In addition, we also observed a decrease in the level of cyclin A, which controls S-phase, G_2/M, and early M-phase progressions. Furthermore, when subjected to cell cycle analysis after mitotic arrest, the $Pten^{-/-}$;$Akt^{-/-}$ ES cells demonstrated significant delays in exiting mitosis compared to the $Pten^{-/-}$ or WT cells. This evidence brings up another level of regulation by which AKT may exert its function dependently or independently of PTEN and deserves future investigation. The G_2/M cell cycle progression depends on the dephosphorylation and activation of cyclin B-cdc2. A number of phosphorylation-dephosphorylation events happen which lead to the final dephosphorylation of cyclin B-cdc2 and M-phase progression and eventually M-phase exit. One of the kinases in this cascade, Myt1, a Wee family member, was recently identified as a substrate for AKT (31). This study may provide mechanistic support for our observation that deleting AKT-1 results in delayed M-phase progression-exit, though the exact target responsible for this phenotype needs further investigation.

In summary, two important processes in tumorigenesis, apoptosis and cell cycle control, are regulated by AKT. Deletion of *Akt-1* as well as *Pten* was able to at least partially reverse the *Pten*-deletion-associated phenotypes observed in the ES cells. We demonstrated that deleting *Akt*-1 had a wide spectrum of impacts on *Pten*-knockout cells even though the deletion was not able to completely reverse the effect of the *Pten* knockout, partially due to other AKTs or parallel pathways which might be active during the growth of ES cells. Importantly, AKT-1 appears to regulate not only apoptotic pathways but also a number of checkpoints during the cell cycle, including G_1/S transition and S-phase progression, as well as exit from mitosis.

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#3946 The role of TIMP-3 in the regulation of inflammation. David Stephen Smookler, Waseem Kalair, and Rama Khokha. *Ontario Cancer Institute, Toronto, Ontario, Canada.*

Chronic inflammation can contribute to the development of abnormal growth, ultimately leading to neoplasia. Tissue inhibitor of metalloproteinases 3 (TIMP-3) inhibits a number of proteases involved in inflammation, including matrix metalloproteinases (MMPs), sheddases (ADAM-10, ADAM-17) and aggrecanases (ADAM TS4, ADAM TS5). Although well-studied in vitro, little is known about the in vivo role of this inhibitor. We postulate that TIMP-3 has an important role in both local and systemic inflammation. Using timp-3 null mice developed in our lab, we have found a significant increase in sensitivity of these mice to intra-peritoneal injections of lipopolysaccharide (LPS), a model of acute systemic inflammation. A dose of 200 micro-grams of LPS is sub-lethal in the wild-type animals, but almost 100 percent lethal to the mutants within five days. Concurrent with this response, we observed a significant increase of TNFα release in serum levels of the timp-3 null mice 1.5 hours post LPS injection. The increased mortality of the TIMP-3 animals was rescued in the presence of a broad protease inhibitor, signifying that this is a metalloproteinase-mediated effect. Furthermore when the timp-3 null animals are crossed with tnfr1-/- animals, a strain lacking an intact TNFα signalling pathway, the animals survive, indicating that TNFα signalling is required for this effect to occur. A model of more localized and chronic inflammation, dinitrofluorobenzene (DNFB)-induced contact hypersensitivity, has also revealed a heightened inflammatory response in the null mice, as measured by local swelling. These findings are the first evidence that the absence of TIMP-3 enhances inflammation at both a local and systemic level. In summary, the data demonstrate that TIMP-3 modulates cytokine bio-activity in vivo.

#3948 Mast cells express EphB4 and ephrinB2 and are activated after EphB4 tyrosine phosphorylation. Darya Soto, Gabriele Maier, and George H. Caughey. *University of California, San Francisco, San Francisco, CA.*

Several lines of evidence suggest that mast cells promote tumor angiogenesis. The receptor tyrosine kinase EphB4 and its co-receptor ephrinB2 are implicated as key regulators of angiogenesis during development. These receptors are also expressed in adult mouse vascular tissue in normal and tumor angiogenic vessels, apparently also playing a role in pathogenic angiogenesis. The present work shows for the first time that human mast cells (HMC-1) express the gene transcripts of several B-class Eph and ephrin family members implicated in angiogenesis. Furthermore, HMC-1 cells express surface EphB4 tyrosine kinase, which is tyrosine-phosphorylated in response to soluble ephrinB2. Incubation with ephrinB2 alters the HMC-1 cell transcriptional profile as demonstrated by multiplex, real-time PCR. We also show expression of EphB4 and ephrinB2 in mast cells cultured from mouse bone marrow. By immunohistochemistry, mast cells expressing either EphB4 or ephrinB2 are present near tumor-associated vessels in mouse models of squamous cell carcinoma of the skin (K14-HPV16) and bronchoalveolar carcinoma (CC10-SV40Tag). Thus we show for the first time the expression and activation of a new class of potential angiogenic modulators in mast cells.

#3949 Adhesion receptor crosstalk in carcinoma cells: Activation of intregrin by P-selectin. Elsa Merit Reyes-Reyes and Steven K. Akiyama. *NIEHS, Durham, NC.*

Metastasis involves invasion of tumor cells through basement membrane into the blood or lymphatic system, arrest at a distant site, extravasation, and subsequent proliferation at the metastatic site. It has been postulated that adhesion and migration of tumor cells on and through the vascular endothelium could share some molecular steps with that of leukocyte extravasation, a process initiated by cell rolling on the blood vessel wall followed by arrest and transendothelial migration. Leukocyte rolling is mediated by selectins, while the two subsequent steps require integrins. Because certain cancer cells express selectin ligands, we have tested the hypothesis that P-selectin binding to tumor cells can result in activation of specific integrins, we examined the adhesive characteristics of Colo-320 human colon carcinoma cells that were stimulated with P-selectin-IgG chimeric protein. We found P-selectin-IgG binding to these cells can specifically stimulate a 2-fold increase of the adhesion of COLO-320 cells to fibronectin in a time- and dose-dependent manner without affecting adhesion to collagen type I, collagen type IV, or laminin. P-selectin-induced adhesion to fibronectin occurs through the $\alpha5\beta1$ integrin. P-selectin binding also induced tyrosine phosphorylation of a number of proteins and specific activation of p38 MAPK and phosphoinositol 3-Kinase (PI 3-K). The general tyrosine kinase inhibitor, genestein, and specific PI 3-K inhibitor, LY294002, blocked selectin-mediated integrin activation. However, two specific p38 MAPK inhibitors, SB203580 and SB202190, had no inhibitory effect on cell adhesion. We also found p38 MAPK and PI 3-Kinase can form a complex in a manner that is dependent to P-selectin stimulation. These results suggest that P-selectin ligand and $\alpha5\beta1$ integrin interact via a PI-3-kinase mediated pathway that may also involve as yet unidentified tyrosine kinases and implicate P-selectin ligands as potential signaling molecules important the regulation of tumor cell adhesion.

#3951 Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. Julia Lesley Wilson, Hagen Kulbe, and Frances Balkwill. *Cancer Research UK - Translational Oncology, London, UK and Cancer Research UK -Translational Oncology, London, UK.*

We investigated the role of the chemokine receptor CXCR4 and its ligand CXCL12 in ovarian cancer. Of 14 chemokine receptors investigated, only CXCR4 was expressed on ovarian cancer cells. CXCL12 was found in ovarian cancer ascites and in the cytoplasm of cells in ovarian tumour biopsies, but neither the receptor or ligand were expressed by normal ovarian epithelium. CXCL12 stimulated tumor cell proliferation in vitro and migration/invasion towards a CXCL12 gradient. Stimulation of cells with CXCL12 resulted in sustained activation of Akt/PKB, biphasic phosphorylation of p44/42 MAPK and induced mRNA and protein for the pro-inflammatory cytokine TNF-α, a cytokine that is expressed by tumour cells in ovarian cancer biopsies. In contrast to other ovarian cancer cell lines, the SKOV line did not express CXCR4. We therefore transfected CXCR4 into this cell line. Stimulation of SKOV-CXCR4 with CXCL12 resulted in increased proliferation in response to CXCL12, migration and invasion towards a CXCL12 gradient and increased adhesion to fibronectin compared to mock transfected SKOV cells. Stimulation of SKOV-CXCR4 also upregulated mRNA for CXCR4, CXCL12 and TNF-α. Expression of CXCR4 in SKOV cells altered their pattern of growth in agar with the usual 300-400 mm diameter spheroids being replaced with smaller diffuse colonies although the in vitro growth rate in monolayer culture was unchanged. In a mouse xenograft model, where SKOV-CXCR4 cells were injected intra-peritoneally, tumor development appeared slower than SKOV-mock. However, the SKOV-CXCR4 tumors were more widely dispersed with tumors predominantly found on distant organs, suggesting that CXCR4-CXCL12 interactions may play a role in tumor dissemination in the peritoneum. Gene array analysis demonstrated that $\beta1$-integrin, TGF-$\beta1$ and uPAR were up-regulated by stimulation of ovarian cancer cells with CXCL12 with preliminary evidence that TNF-α may act as an intermediary in gene induction. IFN-$\beta1$ and ICAM-1 were down-regulated. Thus, the chemokine CXCL12 may have multiple biological effects on ovarian cancer cells, stimulating invasion through extracellular matrix but also facilitating DNA synthesis and establishment of a cytokine network in situations that are sub-optimal for tumour cell growth.