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# PTEN Overexpression Cooperates With Lithium to Reduce the Malignancy and to Increase Cell Death by Apoptosis via PI3K/Akt Suppression in Colorectal Cancer Cells

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## ABSTRACT

Lithium is a well-established non-competitive inhibitor of glycogen synthase kinase-3β (GSK-3β), a kinase that is involved in several cellular processes related to cancer progression. GSK-3β is regulated upstream by PI3K/Akt, which is negatively modulated by PTEN. The role that lithium plays in cancer is controversial because lithium can activate or inhibit survival signaling pathways depending on the cell type. In this study, we analyzed the mechanisms by which lithium can modulate events related to colorectal cancer (CRC) progression and evaluated the role that survival signaling pathways such as PI3K/Akt and PTEN play in this context. We show that the administration of lithium decreased the proliferative potential of CRC cells in a GSK-3β-independent manner but induced the accumulation of cells in G2/M phase. Furthermore, high doses of lithium increased apoptosis, which was accompanied by decreased proteins levels of Akt and PTEN. Then, cells that were induced to overexpress PTEN were treated with lithium; we observed that low doses of lithium strongly increased apoptosis. Additionally, PTEN overexpression reduced proliferation, but this effect was minor compared with that in cells treated with lithium alone. Furthermore, we demonstrated that PTEN overexpression and lithium treatment separately reduced cell migration, colony formation, and invasion, and these effects were enhanced when lithium treatment and PTEN overexpression were combined. In conclusion, our findings indicate that PTEN overexpression and lithium treatment cooperate to reduce the malignancy of CRC cells and highlight lithium and PTEN as potential candidates for studies to identify new therapeutic approaches for CRC treatment. J. Cell. Biochem. 117: 458–469, 2016. 

**KEY WORDS:** COLORECTAL CANCER; PI3K/Akt; LITHIUM; PTEN

Colorectal cancer (CRC) is one of the principal causes of death worldwide. According to the *Instituto Nacional de Câncer*, in Brazil, CRC is the third most common cancer diagnosed among men and women and the third leading cause of cancer death [Ministério da Saúde, 2014]. Many critical molecular alterations involved in the development and progression of CRC have been identified in the past two decades. Most of the alterations (either genetic and/or epigenetic) have been implicated in the adenoma-carcinoma

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sequence, a model that explains the progression of CRC [Fearon, 2011]. However, genes specifically involved in promoting or suppressing CRC progression are still poorly understood. Thus, the identification of genes that play important roles during CRC progression would lead to an understanding of new strategies for CRC treatment. In this context, the Phosphatase and Tensin Homolog Deleted on Chromosome 10 (PTEN), a potent tumor-suppressor gene originally identified through its association with sporadic malignancies (such as prostate, breast, and brain), appears to be a potential regulator of CRC progression [Tamguney and Stokoe, 2007].

PTEN is a negative regulator of the PI3K/Akt signaling pathway, which can influence many tumorigenesis-related processes, such as cell proliferation, survival, migration, and invasion [Chalhoub and Baker, 2009]. We have previously demonstrated that inhibition of PI3K/Akt with LY294002 prevents the malignant phenotype of HCT-116 cells, suggesting that this pathway may represent a potential therapeutic target to inhibit CRC progression [de Araújo et al., 2010]. Altered PTEN levels have frequently been found in tumors, and studies focusing on PTEN downregulation or overexpression have shown the role that PTEN plays in tumor development and progression. For example, PTEN knockdown increases cell spreading, migration, and invasion and increases tumor size in nude mice [Langlois et al., 2010]. In contrast, PTEN overexpression in CRC cells results in the induction of G2/M cell cycle arrest and apoptosis [Saito et al., 2003]. PTEN overexpression causes cell growth suppression by upregulating p27 in breast cancer cells [Weng et al., 1999] and induces cyclin D1 downregulation in an Akt-dependent manner in glioblastoma cells [Radu et al., 2003]. In CRC patients, downregulation of PTEN and/or the presence of mutated PI3K induces resistance to treatment with cetuximab, an EGFR-targeting antibody; similar results have been observed in CRC cell lines [Frattini et al., 2007; Jhawer et al., 2008]. Together, these findings show the important role that PTEN plays in the control of multiple cellular processes related to tumorigenesis; however, the role of PTEN during disease progression in CRC remains unclear.

Lithium is an FDA-approved drug and an important therapeutic agent for the treatment of patients suffering from bipolar disorders [D′Souza et al., 2011]. Lithium is also a specific and non-competitive inhibitor of GSK-3β in vitro and in vivo and mimics the effects of Wnt/β-catenin signaling on gene expression [Ryves and Harwood, 2001]. Previous studies have shown that patients with mental disorders who have been treated with lithium carbonate display a decreased risk of cancer development compared with untreated patients [Cohen et al., 1998]. Furthermore, lithium administration to mice with a mutation in *adenomatous polyposis coli* (APC) does not increase the number of colonic tumors [Gould et al., 2003]. In this context, lithium would be considered an anti-cancer agent, due to these inhibitory effects on cell proliferation in a variety of tumors. For instance, lithium has been shown to induce G2/M arrest in R6T2 cells, a transformed rat fibroblast cell line, but this arrest is independent of β-catenin signaling [Tsui et al., 2012]. In hepatocellular cancer cells, lithium can inhibit cell growth through mechanisms involving downregulation of Akt and cyclin E, resulting in cell cycle arrest [Erdal et al., 2005]. Moreover, lithium inhibits the increase in cell proliferation and migration caused by epidermal growth factor (EGF) in colorectal cancer cells [Vidal et al., 2011]. On the other hand, lithium induces a neuroprotective effect and increases the activity of anti-apoptotic pathways, including the PI3K/Akt pathway [Rowe and Chuang, 2004]. In neuronal cells, lithium protects against glutamate-induced cytotoxicity in vitro by increasing PI3K/Akt pathway activity and inactivating GSK-3β [Liu et al., 2011]. Certainly, the response to treatment with lithium is completely different between neuronal and tumor cells due to differences in underlying genetic factors. Although the biochemical mechanisms by which lithium affects cells involve PI3K/Akt-PTEN signaling, the contribution of these mechanisms in CRC is unclear.

In the present study, we analyzed the mechanisms by which lithium can modulate events related to CRC progression, focusing on the role that survival signaling pathways (such as PI3K/Akt and PTEN) play in this context. We demonstrate that PTEN overexpression and lithium cooperate to reduce the malignancy of CRC cells. Therefore, our findings suggest that lithium and PTEN can be potential candidates for studies to identify new therapeutic approaches for CRC treatment.

## MATERIALS AND METHODS

### MATERIALS

Rabbit polyclonal anti-PTEN, rabbit polyclonal anti-α tubulin, rabbit monoclonal, anti-GSK-3β, anti-phospho-GSK-3β (pSer9), anti-Akt, and anti-phospho-Akt (ser473) were purchased from Cell Signaling Technology (Danvers, MA). Mouse anti-GAPDH (6C5) was purchased from Santa Cruz Biotechnology (Dallas, TX). The secondary antibodies peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Invitrogen Co. Lithium chloride (LiCl) and SB216763 (3 - (2,4-Dichlorophenyl) -4 (1-methyl-1H-indol-3-yl)-1H-spiro-2,5-dione) were purchased from Sigma–Aldrich Co. (St. Louis, MO).

### CELL CULTURE AND TREATMENTS

The human colon cancer cell lines HT-29 and HCT-116, which were obtained from ATCC (Rockville, MD), were used in this study. HT-29 cells (ATCC, HTB-38) are moderately differentiated and contain mutant forms of *P53* and *APC* [Flatmark et al., 2004]. HCT-116 cells (CCL-247) grow in multiple layers, are incapable of polarization, and contain mutant forms of *K-Ras* and β-*catenin* [Ahmed et al., 2013]. The cells were grown in Dulbecco′s Modified Eagle′s medium (DMEM) (Invitrogen Co.) supplemented with 10% fetal bovine serum, penicillin G (60 mg/l) and streptomycin (100 mg/l) at 37°C in a humidified atmosphere of 5% $CO_2$/air. The cells were treated with lithium chloride at concentrations of 20 mM or 50 mM and, depending on the assay performed, were incubated for 24, 48 or 72 h. For pharmacological inhibition of GSK-3β, the inhibitor SB216763 was administered at final concentrations of 5 μM or 10 μM for 24, 48, or 72 h in the proliferation assay.

### CELL LYSATES AND WESTERN BLOT ANALYSIS

Total cell lysates were obtained by incubating the cells in lysis buffer: 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 150 mM NaCl, 2 mM EDTA, 10 mM Hepes (pH 7.4), 20 mM NaF, 1 mM orthovanadate and a protease inhibitor cocktail (Sigma–Aldrich Co.)

(1:100 dilution) for 30 min at 4°C. After centrifugation at 10,000*g* for 10 min at 4°C, the supernatant was removed and stored at −20°C for subsequent analysis. Equal amounts of cell protein (30–40 μg/lane), quantified by the BCA protein assay kit (BioRad, Hercules, CA), were electrophoretically separated by SDS-PAGE in 10% gels and transferred to nitrocellulose membranes with a semi-dry transfer cell (BioRad) at 10 V for 60 min. The membranes were blocked for 1 h with TBS-T (20 mM Tris-HCl, pH 7.6; 137 mM NaCl; and 0.1% Tween-20) containing 5% low-fat dried milk or with 1% BSA (Sigma–Aldrich Co.) and incubated overnight with primary antibodies. After washing, the membranes were incubated for 1 h with the appropriate peroxidase-conjugated secondary antibodies. The proteins were visualized using an enhanced chemiluminescence kit (GE Healthcare). The optical densities of the band images from three independent experiments were quantified using Lab-Works 4.6 software (BioRad).

### CELL PROLIFERATION ASSAY

HT-29 ($10^3$ cells/mL) and HCT-116 ($2 \times 10^4$ cells/mL) were cultured in 96-well plates in the presence or absence of LiCl for 24, 48, or 72 h. After these culture periods, the cells were fixed with ethanol for 10 min and incubated with a crystal violet solution (0.05% crystal violet and 20% methanol) for an additional 10 min. The cells were washed twice with water and then solubilized with methanol. Then, the absorbance at 595 nm was measured with a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA).

### WOUND-HEALING ASSAY

Cell monolayers were manually wounded by scraping with a pipette tip. For each plate, 3–5 wounds were generated, and three sites of regular wounds, verified under the microscope, were selected and marked. After treatment, the cells were permitted to migrate into the denuded area immediately after wounding (0 h) and at the end of the experiment (24 h). The wounds were photographed with an Axio Observer Z1 microscope (Carl Zeiss Inc., Jena, Germany). The distance of cell migration into the wounded area was quantified from three independent experiments and is represented as percentages in a graph of cell migration.

### FLOW CYTOMETRY

For cell cycle analyses, HT-29 and HCT-116 cells ($10^4$–$10^6$ cells/mL) were cultured in 6-well microtiter plates. After the different treatments, the cells were harvested by trypsinization and washed once with ice-cold PBS. The cells were then stained in the dark with 75 μM propidium iodide (Sigma–Aldrich Co.) for at least 30 min in the presence of 1% Triton X-100. The DNA content was evaluated by collecting 10,000 events for the cell cycle and sub-G1 analysis using a FACSCalibur flow cytometer and CellQuest software (BD Bioscience, San Jose, CA). For determination of apoptosis, the colon cancer cell lines were resuspended in 100 μL of Annexin V binding buffer (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) containing Annexin V-FITC and PI (1 μg/mL) for 15 min. FACS analysis was performed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA); the cells negative for both annexin V and PI were considered viable (survival).

### ANCHORAGE-DEPENDENT AND -INDEPENDENT COLONY FORMATION ASSAYS

For anchorage-dependent colony formation assays, the cells were seeded at a low density (500 cells/well) for 4 h in 12-well plates. After the different treatments, the ability of a single cell to form a colony was monitored for 10 days. Treatment with 20 mM lithium or doxycycline (to induce PTEN overexpression) was continuous during the 10 days. At the end of the experiment, the cells were fixed with 100% ethanol for 10 min and subsequently incubated with a solution containing 0.05% crystal violet in 20% ethanol for 10 min. After the cells were washed with distilled water, the crystal violet was eluted with 100% methanol and then spectrophotometrically (595 nm) quantified using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA). For anchorage-independent colony formation assays, the cells (500 cells/well) were seeded into a 12-well plate previously coated with 1 mL of a semi-solid medium containing 0.3% agarose. After 10 days of treatment, the colonies formed were observed and counted with an Axio Observer Z1 microscope.

### INVASION ASSAY

To analyze cell invasion, a transwell with a 6.5 mm polycarbonate filter and 8 μm pore size (Costar, NY) was coated with 20 μl Matrigel® (BD Biosciences) diluted with DMEM to a proportion of 1:10 and incubated at 37°C for 30 min. The transfected cells ($3 \times 10^4$), in 200 μl of serum-free medium, were seeded in the upper chamber of the transwell, and 10% FBS was added as a chemoattractant in the lower chamber. After 24 h of incubation, the upper surface of the membrane was scrubbed with a cotton swab. The invading cells on the lower membrane were fixed with ethanol and stained with crystal violet. The number of invading cells was expressed as the average of four random fields observed under the microscope. Cells transfected to overexpress PTEN were treated with doxycycline for 5 days before the experiment and throughout the course of the assay.

### ZYMOGRAPHY ASSAY

Equal numbers of transfected cells were seeded and left untreated or treated with doxycycline for 24 h in the presence of serum. After 24 h, the supernatants were collected, normalized for total protein concentration using the BCA protein assay kit (BioRad) and subjected to SDS-PAGE with 10% (v/v) gels containing 0.2% gelatin (Sigma–Aldrich Co.). After electrophoresis, the gels were washed twice (30 min each at room temperature) in 10 mM Tris/HCl, pH 8.8 containing 2.5% Triton X-100, then incubated in activation buffer (5 mM CaCl2, 0.02% $NaN_3$, and 50 mM Tris/HCl, pH 8.0) at 37°C overnight. The next day, the gels were stained with Coomassie Brilliant Blue R-250 and distained in 10% (v/v) acetic acid and 40% (v/v) methanol. The gelatinolytic activity of MMPs was detected as transparent bands on the blue background. The gels were scanned for quantification by optical density with the Lab-Works 4.6 software.

### PLASMID CONSTRUCTIONS, PRODUCTION OF RECOMBINANT LENTIVIRUS AND INFECTION OF HT-29 AND HCT-116 CELLS

To create the plasmid pLENTI-CMV/TO-PTEN, we first obtained the construct containing human PTEN cDNA from Addgene (plasmid

#10787, pBabe-puroLFlagHA-PTEN; Cambridge. MA 02139, USA). BamHI and EcoRI were used to subclone this construct into the pENTR4 plasmid (plasmid #17424, Addgene), which was later recombined using Gateway technology (Invitrogen) into the lentiviral tetracycline-responsive vector pLENTI-CMV/TO (plasmid #17293, Addgene), thus generating the construct pLENTI-CMV/TO-PTEN. The tetracycline repressor-expressing vector pLENTI-CMV-TetR-Blast (Plasmid #17492, Addgene) was used to repress PTEN expression from the pLENT-CMV/TO vector. HEK-293 cells were used as viral packaging cells by co-transfection with the packaging vectors pMDLg-RRE, pRSV-REV, and pMD2-G with pLENTI-CMV-TetR-Blast or pLENTI-CMV/TO-PTEN for lentiviral transduction by calcium phosphate precipitation for 24 h. The cell-free virus-containing supernatant was collected 48 h after transfection, mixed 1:1 with fresh medium, supplemented with 8 μg/mL polybrene (FLUKA Chemie, Buchs, Switzerland), and immediately used for spin-infection ($2 \times 45$ min at 400 g, room temperature) of $5 \times 10^4$ HT-29 or HCT-116 cells. The infected cells were incubated at 37°C for an additional 24 h, trypsinized, and used in subsequent experiments.

### CONSTRUCTION OF HT-29$^{CMV/TO\text{-}PTEN}$ AND HCT-116$^{CMV/TO\text{-}PTEN}$ CELLS

HT-29$^{CMV/TO\text{-}PTEN}$ and HCT-116$^{CMV/TO\text{-}PTEN}$ cells were generated by transducing HCT-116 and HT-29 wild-type cells with pLENT-CMV-TetR-Blast and selecting for blasticidin (5 μg/mL; Sigma-Aldrich Co.) resistance for at least 5 days. These cells were then transduced with pLENT-CMV/TO-PTEN and selected for puromycin (7.5 μg/mL; Sigma–Aldrich Co.) resistance for at least 5 days. Clones were isolated, and PTEN overexpression was confirmed by immunoblotting of the cultured cells in the presence of 2 μg/mL doxycycline (Sigma–Aldrich Co.).

### STATISTICAL ANALYSIS

Statistical analysis of three independent experiments was performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) using a one-way ANOVA followed by the Bonferroni post-test for comparisons between groups. The data were expressed as the mean ± SEM, and differences were considered significant at $P < 0.05$.

## RESULTS

### LITHIUM DECREASES CELL PROLIFERATION THROUGH A GSK-3β-INDEPENDENT MECHANISM

Glycogen synthase kinase-3β (GSK-3β), a multifunctional serine/threonine kinase that controls various cellular events including cell survival and proliferation, can be inhibited by lithium. To determine the role of GSK-3β in lithium-mediated cell proliferation in CRC, we initially treated two types of colorectal cancer cells (HT-29 and HCT-116) with lithium at concentrations of 20 and 50 mM for 24, 48, and 72 h. We observed that 20 mM lithium reduced cell proliferation in HT-29 and HCT-116 cells after 72 h of treatment, while 50 mM lithium completely inhibited cell growth. To determine whether the effects of lithium on cell proliferation were mediated by GSK-3β, we used another pharmacological inhibitor of this kinase, SB-216763. Treatment with this inhibitor did not alter HT-29 or HCT-116 cell proliferation (Fig. 1A). To confirm inhibition of GSK-3β by lithium, we analyzed the phosphorylation status of GSK-3β by Western blot using a phospho-ser9 GSK-3β antibody and observed that lithium induced the phosphorylation of GSK-3β in HT-29 and HCT-116 cells; this effect was more evident upon treatment of the cells with 50 mM lithium (Fig. 1B). Because lithium decreased cell proliferation and the GSK-3β inhibitor SB-216763 had no effect, the possibility exists that the effects of lithium are mediated by cell cycle alterations. To test this possibility, we analyzed cell cycle profiles by flow cytometric analysis of DNA content. As shown in Table I, after 24 h, 20 mM and 50 mM lithium treatment led to an accumulation of HT-29 cells in the G2/M phases of the cell cycle, with greater sensitivity observed upon treatment with the lower dose of lithium. In contrast, HCT-116 cells showed G2/M phase arrest only upon treatment with 50 mM lithium (Table II). Our finding that 50 mM lithium inhibits cell growth may suggest that high concentrations of lithium might induce apoptosis in these cell lines. To address this hypothesis, we analyzed the apoptotic response by annexin V/PI-based flow cytometric analysis. We found that only treatment with 50 mM lithium significantly increased apoptosis in both cell lines after 48 h and 72 h of treatment, whereas 20 mM lithium did not induce apoptosis (Fig. 2). Overall, these findings indicate that a low dose of lithium can reduce cell proliferation by arresting the cells in G2/M phase, while a high dose of lithium (50 mM) can induce cell death by apoptosis. Furthermore, the results suggest that these events are independent of GSK-3β inhibition.

### TREATMENT WITH LITHIUM RESULTS IN DECREASED LEVELS OF TOTAL AND PHOSPHORYLATED Akt

The PI3K/Akt pathway is frequently activated in human cancer to induce cell survival. Because lithium induced apoptosis in the colorectal cancer cells, we investigated whether this cell death correlated with the Akt status. As shown in Figure 3A-B, both HT-29 and HCT-116 cells displayed elevated levels of total and phosphorylated Akt, and treatment with lithium for 24 h caused a depletion of total Akt protein levels that was accompanied by decreased Akt phosphorylation in a dose-dependent manner. Considering that lithium induced apoptosis and decreased the total protein levels of Akt, the endogenous levels of PTEN may differ between the cells lines examined. This difference could explain the higher sensitivity of HT-29 cells to lithium. Therefore, we analyzed the levels of endogenous PTEN in HT-29 and HCT-116 cells lines by immunoblotting before and after lithium treatment. Our results demonstrate that HT-29 cells express higher levels of PTEN than HCT-116 cells and that lithium could induce a gradual decrease in PTEN levels after 24 h; this effect was more evident in HCT-116 cells (Fig. 3C). Taken together, these observations provide evidence that a reduction in Akt levels is implicated in lithium-induced growth control of colorectal cancer cells.

### INDUCED EXPRESSION OF PTEN AND LITHIUM TREATMENT INCREASED APOPTOSIS

A series of studies have suggested that PTEN inactivation plays an important role in various cancer types. Accordingly, PTEN over-expression could be able to enhance the anti-tumor effect of

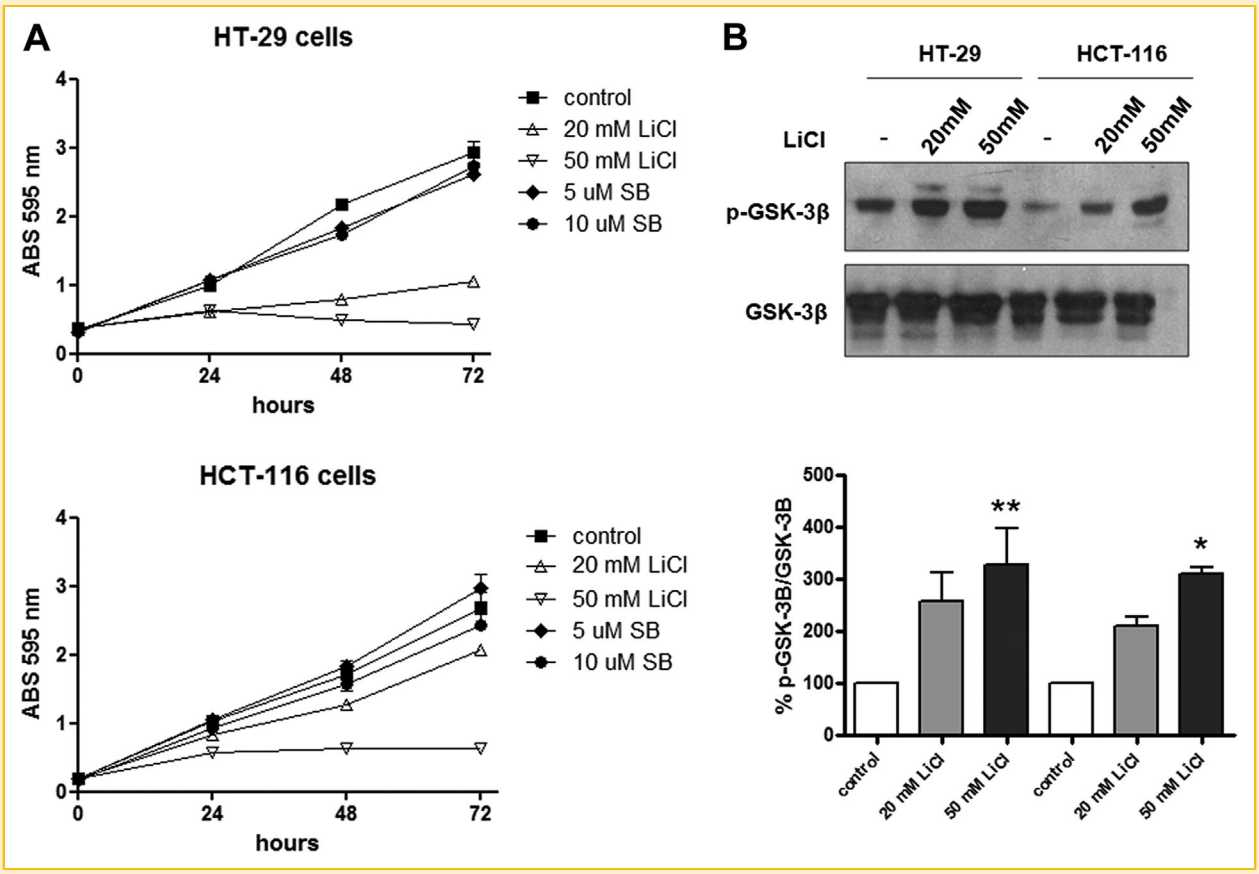


**Fig. 1.** Lithium inhibits proliferation in cancer colon cells. (A) Cell proliferation assay using the crystal violet technique. HT-29 and HCT-116 cells were untreated or treated with 20 mM or 50 mM of lithium and the GSK-3β inhibitor SB-216763 for 24, 48 and 72 h. Lithium treatment decreased cell proliferation in both cell lines after 72 h ($P < 0.05$), but SB-216763 treatment did not affect cell proliferation compared with the control. (B) Representative Western blotting and densitometric analyses of phospho-GSK-3β (ser9) in HT-29 and HCT-116 cells untreated or treated with lithium for 24 h. The results represent the mean ± SEM of three independent experiments performed in triplicate, ANOVA ($^{*}P < 0.05$, $^{**}P < 0.01$).

chemotherapy drugs. Here, we observed that 20 mM lithium does not induce apoptosis and that a reduction in PTEN levels can represent a mechanism for cell survival in our study. Therefore, to gain further mechanistic insights into the effects of lithium on cell death by apoptosis, we generated a tetracycline-inducible lentiviral system to overexpress PTEN in HT-29 and HCT-116 cells. Figure 4A shows that PTEN protein levels increased in the presence of doxycycline in both cell lines and that this increase appears to be time-dependent. To confirm that the increased expression level of PTEN obtained in the inducible system is functional, we verified the phosphorylation status of Akt, a downstream target of PI3K that is inactivated by PTEN activity. As shown in Figure 4B, a decrease in the level of p-Akt was observed when PTEN overexpression was induced with doxycycline for 72 h in both cell lines.

Next, we further investigated the relative importance of PTEN expression upon treatment with a low dose of lithium (20 mM). First, we observed that induced overexpression of PTEN alone decreased HT-29 and HCT-116 proliferation after 72 h. Transfected cells that were not exposed to doxycycline and treated with 20 mM lithium displayed decreased cell proliferation, and transfected cells that were treated with both doxycycline and lithium demonstrated a greater reduction in cell growth, which was more pronounced in HT-29 cells (Fig. 5A). We further investigated whether the decreased proliferation after treatment with low doses of lithium in cells overexpressing PTEN was due to the induction of cell death. By

TABLE I. Cell-Cycle Distribution Analysis in HT-29 Cell After Treatment With Lithium

| | | Time point | | |
|---|---|---|---|---|
| Treatment | Phase | 24 h | 48 h | 72 h |
| Control | $G_1$ | 47.4 ± 1.9 | 40.5 ± 4.3 | 53.4 ± 7.6 |
| | S | 27.0 ± 3.1 | 26.5 ± 1.5 | 23.4 ± 2.5 |
| | $G_2$-M | 30.0 ± 0.2 | 33.0 ± 2.5 | 27.5 ± 1.2 |
| 20 mM | $G_1$ | 42.8 ± 4.8 | 46.5 ± 7.7 | 46.0 ± 4.6 |
| | S | 20.0 ± 1.7$^{**}$ | 22.1 ± 3.4$^{*}$ | 10.3 ± 2.5$^{*}$ |
| | $G_2$-M | 42.8 ± 4.1$^{*}$ | 48.7 ± 3.6$^{**}$ | 40.9 ± 0.6$^{*}$ |
| 50 mM | $G_1$ | 26.5 ± 8.6$^{***}$ | 25.3 ± 6.2$^{**}$ | 35.5 ± 2.0$^{**}$ |
| | S | 44.8 ± 3.6$^{**}$ | 23.3 ± 4.0 | 40.5 ± 2.7$^{**}$ |
| | $G_2$-M | 37.3 ± 0,1$^{*}$ | 48.8 ± 2.4$^{**}$ | 43.6 ± 6.8$^{**}$ |

Results represent the average of a minimum of 3 replications (± SD).
$^{*}P < 0.05$.
$^{**}P < 0.01$.
$^{***}P < 0.001$.

TABLE II. Cell-Cycle Distribution Analysis in HCT-116 Cell After Treatment With Lithium

| Treatment | Phase | Time point 24 h | 48 h | 72 h |
|---|---|---|---|---|
| Control | $G_1$ | 56.0 ± 6.8 | 59.8 ± 7.3 | 61.9 ± 5.6 |
| | S | 30.9 ± 3.3 | 25.7 ± 1.2 | 17.1 ± 3.3 |
| | $G_2$-M | 19.9 ± 2.5 | 21.8 ± 1.5 | 19.6 ± 3.8 |
| 20 mM | $G_1$ | 54.0 ± 4.5 | 58.9 ± 5.9 | 51.6 ± 3.8* |
| | S | 25.2 ± 2.9 | 15.9 ± 3.1 | 18.7 ± 3.1 |
| | $G_2$-M | 22.6 ± 2.2 | 27.4 ± 8.1 | 28.7 ± 3.2*** |
| 50 mM | $G_1$ | 49.4 ± 10.9 | 37.7 ± 12.6** | 33.4 ± 5.0*** |
| | S | 20.0 ± 4.8 | 20.6 ± 6.4 | 23.5 ± 1.0 |
| | $G_2$-M | 30.4 ± 6.2* | 41.5 ± 8.3** | 43.0 ± 4.0*** |

Results represent the average of a minimum of 3 replications (± SD).
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

performing annexin V/PI-based flow cytometric analysis, we observed that HT-29 transfected cells that were not exposed to doxycycline and treated with 20 mM lithium demonstrated an increase in apoptosis, but this increase was not significant. Conversely, transfected HT-29 cells cultured in the presence of doxycycline and treated with lithium displayed increased apoptosis during the 72 h of treatment (Fig. 5B). Importantly, apoptosis was evident in HCT-116 cells that overexpress PTEN only after 72 h of treatment with lithium (Fig. 5B). Collectively, these results suggest that a low dose of lithium induces apoptosis in cells that overexpress PTEN.

## INDUCED OVEREXPRESSION OF PTEN AND LITHIUM TREATMENT INHIBITS ANCHORAGE-DEPENDENT AND ANCHORAGE-INDEPENDENT COLONY FORMATION

Because the ability of tumor cells to form anchorage-dependent and anchorage-independent colonies constitutes an important parameter to measure tumorigenic potential in vitro, we evaluated the effects of induced PTEN overexpression and lithium treatment in HT-29 and HCT-116 cells on colony formation. Figure 6A shows that induced PTEN overexpression significantly reduced anchorage-dependent colony formation and that colony formation was abolished in both types of transfected cells in the absence or presence of doxycycline plus treatment with lithium. We next assessed the effect of induced PTEN overexpression on anchorage-independent growth using a soft agar colony formations assay. Doxycycline-induced PTEN overexpression significantly inhibited the colony growth of HT-29 and HCT-116 cells, and lithium treatment in cells that had not been exposed to doxycycline also reduced the number and size of the colonies. Additionally, the reduced colony number was more evident when the cells were treated with lithium in the presence of doxycycline (Fig. 6B). These results suggest that the combination of induced PTEN overexpression and treatment with lithium leads to a reduction in the tumorigenic potential of colorectal cancer cells.

## INDUCED OVEREXPRESSION OF PTEN AND LITHIUM TREATMENT INHIBIT CELL MIGRATION AND INVASIVENESS

Cell migration and invasion are well-known critical steps of cancer progression that facilitate metastasis. To further explore the roles that doxycycline-induced PTEN overexpression and lithium play in these events, we first assessed the migratory potential of the HT-29 and HCT-116 cells using a wound-healing assay. Cells treated with doxycycline (to overexpress PTEN) exhibited decreased cell migration compared to cells that had not been treated with doxycycline. Both transfected cells with no induced PTEN overexpression plus lithium and cells with induced PTEN overexpression plus lithium showed decreased cell migration in HT-29 and HCT-116 cells, indicating that the effect on cell migration was due to lithium treatment in this case (Fig. 7). Next, the effects of induced PTEN overexpression and lithium treatment on invasiveness were determined using invasion chambers coated with Matrigel. HT-29 and HCT-116 cells overexpressing PTEN clearly exhibited decreased invasive capacities compared to cells that did not overexpress PTEN. Furthermore, the reduction of invasiveness was greater in cells with

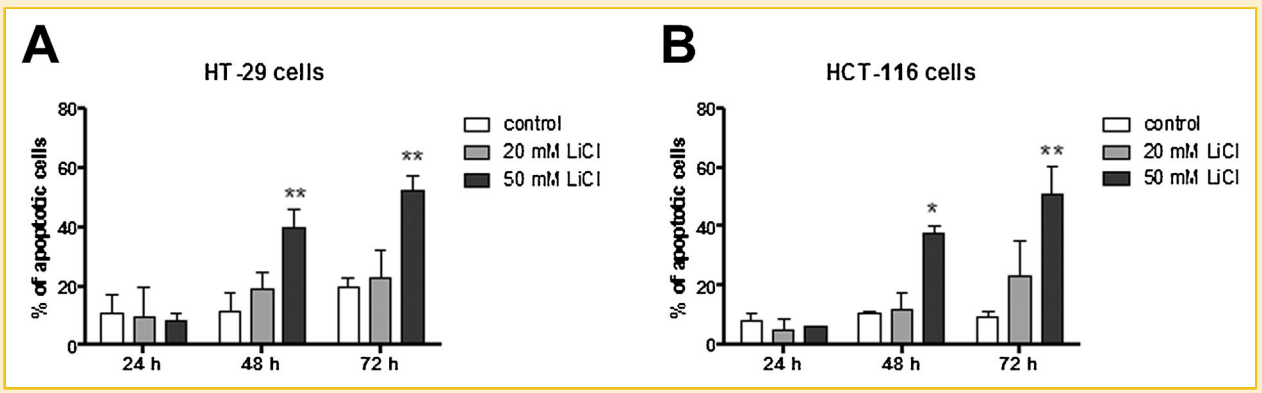


Fig. 2. Lithium induces apoptosis in colorectal cancer cells. HT-29 and HCT-116 cells were treated with 20 and 50 mM lithium for 24, 48, and 72 h. After treatment, apoptosis was detected by flow cytometry using Annexin V/PI staining. The number of apoptotic cells was expressed as a percentage. Lithium significantly induced apoptosis at a concentration of 50 mM in HT-29 and HCT-116 cells. The results represent the mean ± SEM of three independent experiments performed in triplicate, ANOVA (*$P < 0.05$, **$P < 0.01$).

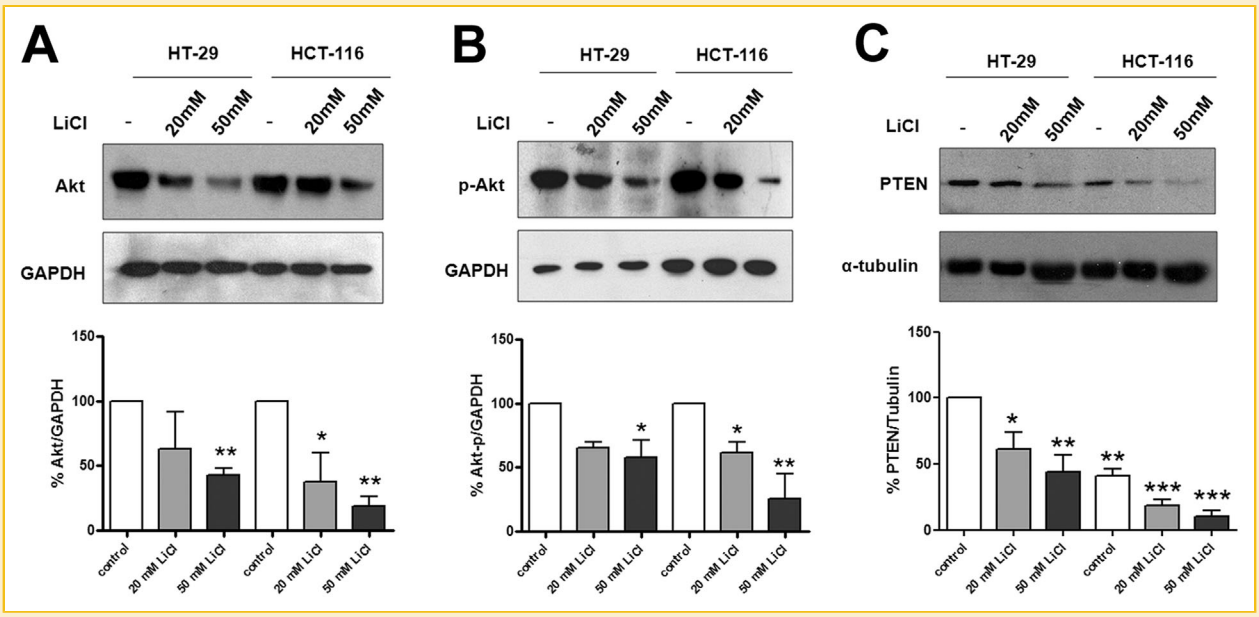


Fig. 3. Lithium decreases the protein levels of Akt and PTEN. HT-29 and HCT-116 cells were incubated with 20 and 50 mM lithium for 24 h. Cell lysates were prepared to Western blott with total anti-Akt (A), anti-p-Akt (ser473) (B), and anti-PTEN (C) antibodies. Lithium caused a decrease in the total levels of Akt, inhibited the activity of Akt, and decreased the PTEN levels in the cells. The graphic represents the densitometry of the levels of total Akt, p-Akt and PTEN as a percentage of the control values. The results represent the mean ± SEM of three independent experiments performed in triplicate, ANOVA ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). GAPDH and α-tubulin proteins were used as loading controls.

PTEN overexpression and treated with lithium compared to cells without PTEN overexpression and treated with lithium (Fig. 8A). Invasion is a consequence of not only the breakdown of cell-cell junctions and increased migration of tumor cells but also focal proteolysis of the extracellular matrix. Because induced PTEN overexpression decreased the invasive potential of the cells, we next analyzed whether PTEN overexpression could also regulate MMP activity. We performed a zymography assay and observed that the

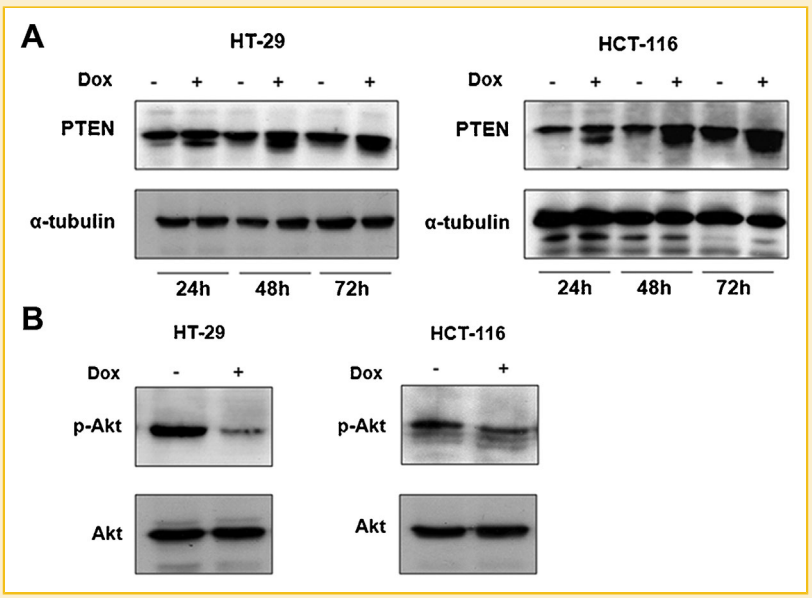


Fig. 4. PTEN overexpression in HT-29 and HCT-116 cells and effect on Akt phosphorylation. (A) HT-29 and HCT-116 cells were co-transfected with lentivirus containing the tetracycline-dependent repressor of the tetracycline operator (pLENT-CMV-TetR-Balst) and lentivirus containing wild-type PTEN (pLENT-CMV/TO-PTEN). The cells were grown in medium with or without doxycycline for 24, 48 or 72 h, and PTEN expression was analyzed by Western blot. (B) Representative Western blot analysis showing that PTEN overexpression induced a decrease in phospho-Akt. The α-tubulin protein was used as a loading control.

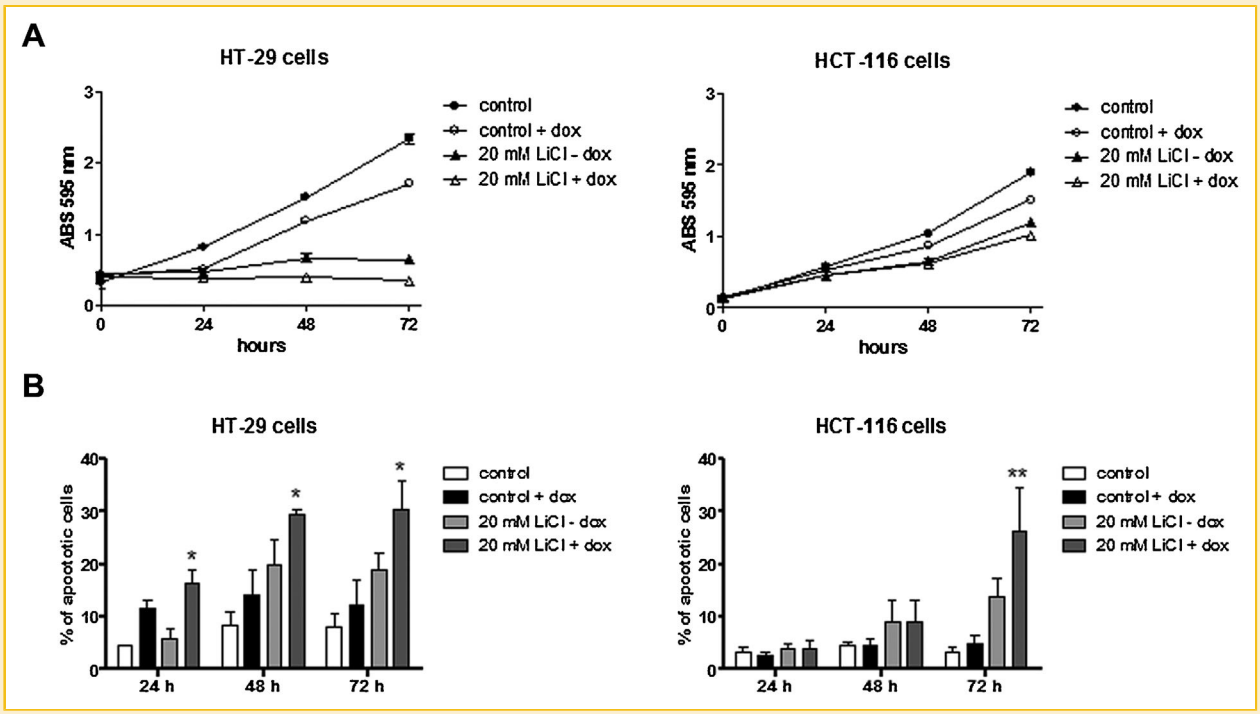


Fig. 5. Lithium in combination with PTEN overexpression inhibits proliferation and potentiates cell death. (A) Cells were treated as indicated, and cell proliferation was determined by the crystal violet assay. PTEN overexpression and lithium treatment alone decreased proliferation, and this effect was most pronounced with both treatments after 72 h ($P < 0.05$). The results represent three independent experiments performed in triplicate. (B) Quantification of apoptosis in HT-29 and HCT-116 cells. Cells were treated as indicated, and apoptosis was assessed by flow cytometry. Note that induced expression of PTEN increased apoptosis in cells treated with 20 mM lithium. The number of apoptotic cells was expressed as a % of the total apoptotic cells. The results represent the mean ± SEM of three independent experiments performed in triplicate, ANOVA ($^{*}P < 0.05$, $^{**}P < 0.01$).

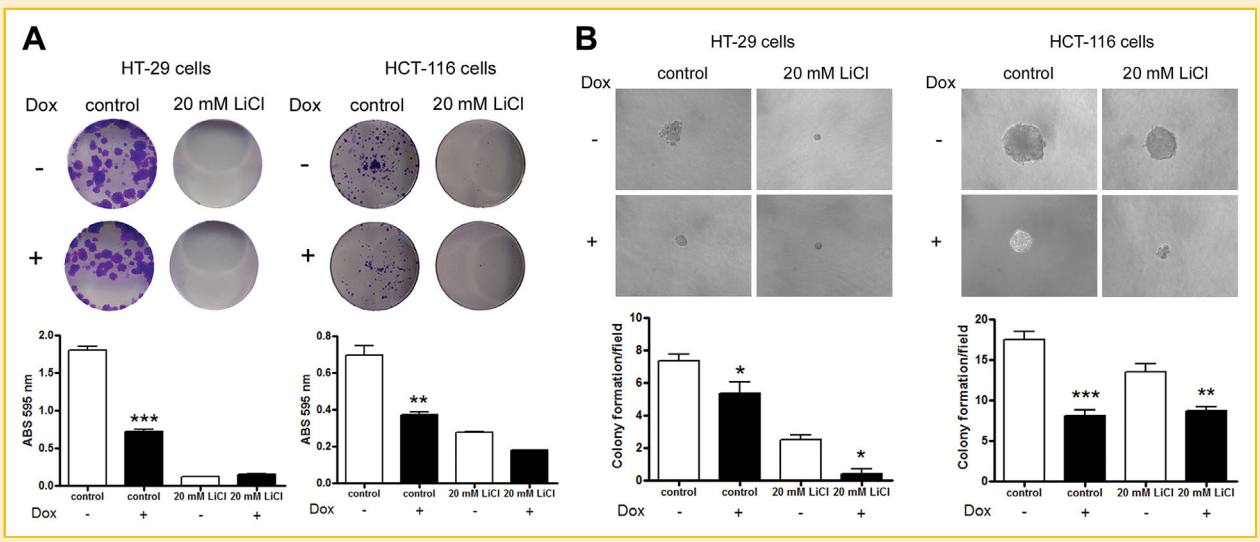


Fig. 6. The impact of PTEN overexpression and lithium treatment on anchorage-dependent and anchorage-independent colony formation. (A) Representative images and quantification of anchorage-dependent colonies from cells overexpressing PTEN and treated with lithium that were stained with crystal violet. The bar graphs show the means of the relative cell numbers as determined by the resultant absorbance at 595 nm. (B) Anchorage-independent colony formation from a soft agar colony assay. The bar graphs corresponding to cells overexpressing PTEN and treated with lithium show the means of the numbers of colonies that were quantified in the selected fields by optical microscopy. The error bars indicate the mean ± SEM (n = 3); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, as determined by an ANOVA test.

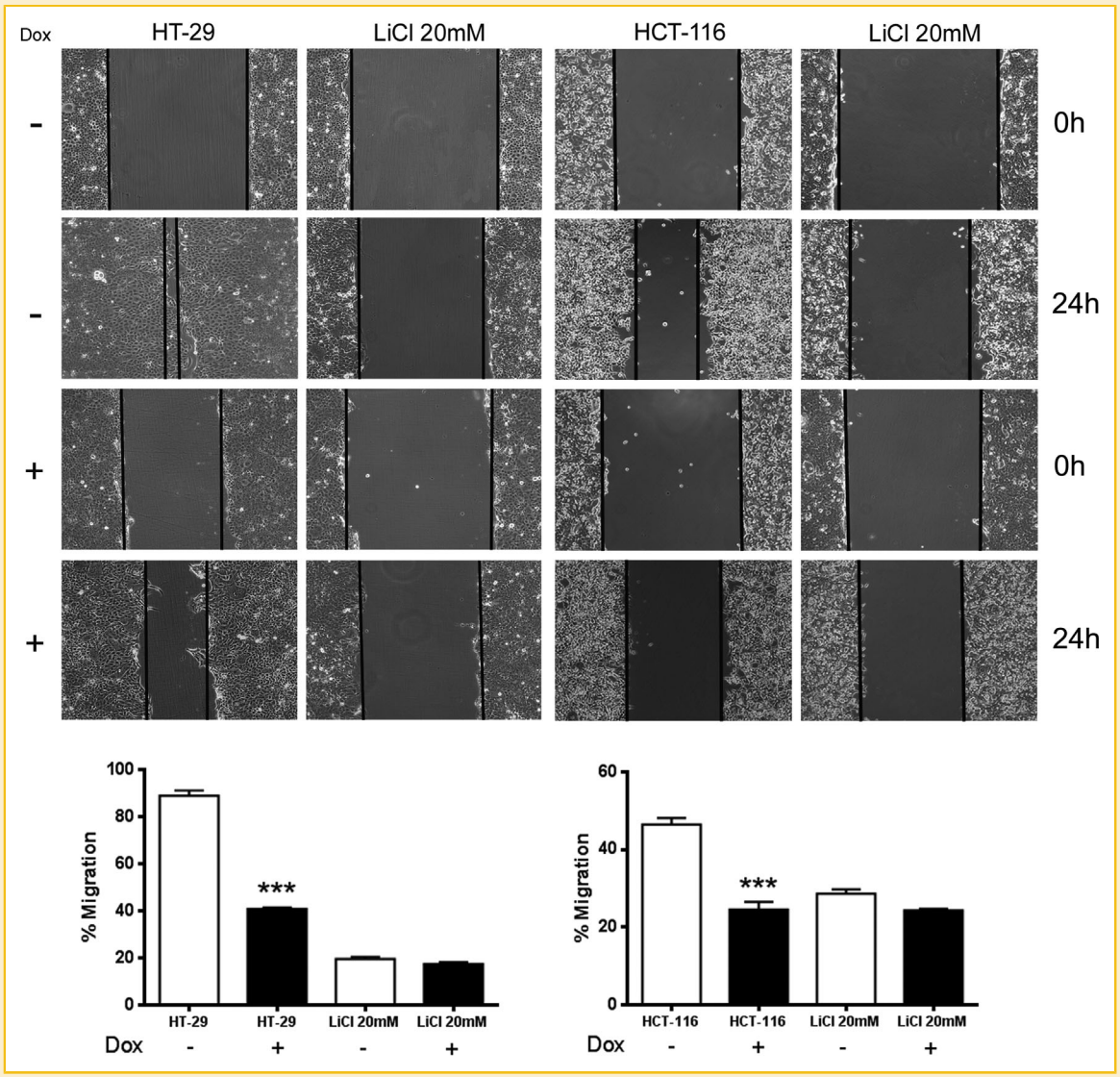


**Fig. 7.** PTEN and lithium decreased cell migration in colorectal cancer cells. Cell migration was evaluated using the wound healing assay. HT-29 and HCT-116 cells overexpressing or not overexpressing PTEN and treated with lithium were grown until they were confluent. Then, the cells were scratched, and the migration of cells into the scratched regions was monitored after 24 h. PTEN overexpression decreased migration, and the same effect was observed when these cells were treated with lithium. Bar = 100 μm. The results represent the mean ± SEM of three independent experiments performed in triplicate (***$P < 0.01$).

induced overexpression of PTEN decreased the levels of secreted MMP-2 and MMP-9 (Fig. 8B). These results indicate that induced PTEN overexpression inhibits cell migration and invasion of colorectal cancer cells and suggests that the loss of PTEN could be involved in metastasis.

## DISCUSSION

In cancer, lithium acts as an anti-cancer agent alone [Matsebatlela et al., 2012] or auxiliary to chemotherapy and radiotherapy [Gupta et al., 2014]. Lithium is a reversible inhibitor of GSK-3β with an IC50 of 2 mM and acts by both directly competing with Mg2+ to reduce the Mg2+ ATP-dependent kinase activity of GSK-3β and indirectly reducing phosphatase activity to increase the inactive form of GSK-3-β [Gupta et al., 2012]. GSK-3β is a pluripotent serine-threonine kinase with numerous target molecules and has been implicated in the regulation of metabolism, proliferation, and gene transcription [Beurel et al., 2015]. In this context, the inactivation of GSK-3β and the subsequent deregulation of β-catenin have been implicated in CRC tumorigenesis, but the molecular mechanisms underlying the effects of lithium during CRC progression have not been defined.

Our results demonstrate that lithium inhibits cell proliferation in HT-29 and HCT-116 at a low dose (20 mM) but completely blocks cell growth at a high dose (50 mM). The observed changes in cell cycle profiles (accumulation of cells in G2/M phase) when the cells were exposed to lithium are likely the primary reason for the decreased cell proliferation. Similar results have been observed in other cancer cell

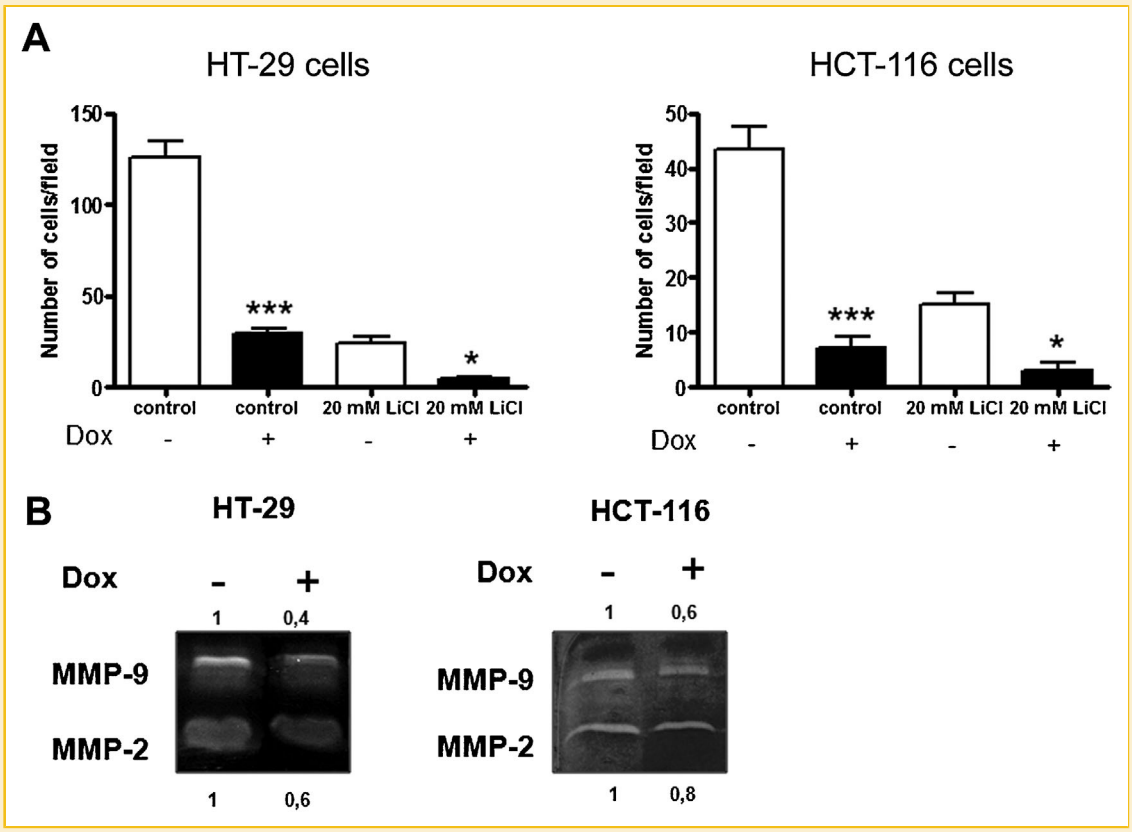


**Fig. 8.** Effects of PTEN overexpression and lithium on the invasiveness of colorectal cancer cells. (A) HT-29 and HCT-116 cells overexpressing PTEN were seeded on a Matrigel invasion chamber. After 24 h, the invading cells were quantified as described in the Material and Methods section. The results represent the mean ± SEM of three independent experiments performed in triplicate, ANOVA test ($^{*}P < 0.05$, $^{***}P < 0.01$). (B) The supernatants were collected from HT-29 and HCT-116 cells overexpressing PTEN, and MMP-2 and MMP-9 activity was evaluated by a zymography assay.

models, such as hepatocellular carcinoma [Erdal et al., 2005] and esophageal cancer [Wang, 2008], where 20 mM lithium induced G2/M phase arrest. A recent study using the colon cancer cell line SW480 has shown that the inhibition of proliferation caused by lithium induces the accumulation of reactive oxygen species and reduced expression of NF-κB [Li et al., 2014]. Lithium also inhibits cell proliferation caused by EGF treatment by arresting cells in G2/M phase in Caco-2 colorectal cancer cells [Vidal et al., 2011]. However, lithium has been shown to stimulate cell proliferation of human breast cancer cells, but a low therapeutic concentration of lithium (1-5 mM) was used in that study [Suganthi et al., 2012]. Indeed, differences in the genetic factors underlying the different cell lines and the tested concentrations of the drug could explain the contradictory effects on cell proliferation after lithium treatment. Lithium is well-known to induce GSK-3β inactivation and subsequent stabilization of nuclear β-catenin, which can regulate the cyclin-dependent kinase Cdc2/cyclin B1 complex that controls passage through the G2/M transition. Thus, our result demonstrating that the inhibitor of GSK-3β, SB216763, does not affect cell proliferation (Fig. 1A) strongly suggests that the effect on cell cycle arrest observed here is specific to lithium and independent of GSK-3β.

Although low dose of lithium reduced cell proliferation in both cell lines, our results show that HT-29 cells are more sensitive to lithium than HCT-116 cells (Fig. 1A). One possible explanation for the differential effect of lithium is differences in the genetic features of these two cell lines, such as altered cell signaling pathways or mutations in *P53, APC,* and *K-Ras*. Lithium treatment has been shown to induce apoptosis in various human malignant tumors, but the mechanism by which this occurs has not been resolved. Similarly, other studies have shown that high doses of lithium (50 and 100 mM) increases apoptosis through activation of caspase-2 and -7 in caspase-3-deficient MCF-7 cells [Suganthi et al., 2012]. Furthermore, lithium has been shown to inhibit cellular proliferation in combination with the regulation of the expression of genes involved not only with the apoptosis signaling pathway [Zhang et al., 2005] but also with the production of inflammatory cytokines [Matsebatlela et al., 2012]. In CRC, lithium induced apoptosis through a mechanism that is independent of p53 activity with increased production of TNF-α and FasL, two death-receptor ligands that activate the extrinsic apoptosis pathway [Kaufmann et al., 2011]. Whether this mechanism occurs in our models remains to be elucidated.

Survival signaling pathways, such as PI3K/Akt, are also targets of lithium. Here, we observed that lithium decreased Akt protein levels, which was accompanied by decreased levels of phosphorylation of Ser473 (Fig. 3). Decreased Akt levels after lithium treatment has also been described in hepatocellular carcinoma cells, but the mechanism

by which the protein is degraded was not addressed in that study [Erdal et al., 2005]. Additionally, treatment with other drugs, such as Hsp90 inhibitors [Basso et al., 2002], a VEGF receptor inhibitor [Riesterer et al., 2004], and TNF-α [Medina et al., 2005], has also been shown to induce apoptosis by depletion of Akt.

We observed that GSK-3β undergoes inhibition of site-specific phosphorylation at the serine 9 residue in response to lithium treatment (Fig. 1B). GSK-3β is known to be an Akt substrate and can be inhibited by phosphorylation. Surprisingly, in our study, we observed increased phosphorylation of GSK-3β at ser9 despite decreased protein levels and Akt phosphorylation. We speculate that the observed increase in GSK-3β phosphorylation probably results from inhibition of a phosphatase protein that normally actives GSK-3β by removing a phosphate from the regulatory serine, as reported by Mora et al., 2002. Additionally, lithium treatment of neuronal cells has been shown to induce Akt activation, which is essential for cell survival. However, the differential and tissue-specific expression of the three Akt isoform (Akt1, Akt2, and Akt3) are important to note and may be factors that are responsible for different responses [Hers et al., 2011]. For example, Akt1 displays a wide tissue distribution and is implicated in cell growth and survival [Dummler and Hemmings, 2007], whereas Akt3 is more tissue-restricted (with expression primarily in the brain) and can exert protective functions [Easton et al., 2005].

Here, the inducible overexpression of PTEN decreases the levels of Akt phosphorylation and inhibits cell growth in colorectal cancer cells. Surprisingly, the anti-proliferative effect of lithium on cells that overexpress or do not overexpress PTEN was high compared with those that overexpress PTEN alone. PTEN overexpression by adenovirus has been shown to result in the induction of G2 cell cycle arrest and apoptosis in colorectal cancer cells but not in normal colon fibroblast cells [Saito et al., 2003]. In addition, the mechanism by which PTEN suppresses cell growth has been shown to depend on phosphatase activity to downregulate signaling through the Akt [Fang et al., 2007]. Therefore, because PTEN overexpression decreases Akt phosphorylation, the inhibition of cell growth observed in our study may occur via PTEN phosphatase activity. PTEN downregulation after lithium treatment has also been observed in ovarian cancer cells and involves the activation of β-catenin to regulate the transcription factor of PTEN, Erg1 [Lau et al., 2011]. Further studies are necessary to address whether this transcription factor is involved in the results observed in the present study.

We further showed that induced PTEN overexpression inhibited colony formation and displayed a synergetic inhibitory effect in combination with lithium on the formation and number of colonies, strongly suggesting a reduced tumorigenic capacity of HT-29 and HCT-116 cells. Recently, a study has shown that PTEN overexpression leads to the inhibition of anchorage-independent growth in lung cancer cells, in which reduced transcriptional activity of NF-κB was observed [Akca et al., 2011]. We also showed that induced PTEN overexpression combined with lithium inhibited cell migration and invasion of HT-29 and HCT-116 cells. A previous study has shown that lithium inhibits the motility and invasion of glioma cells [Fu et al., 2014]. Additionally, lithium drastically inhibits cell motility and compromises the invasive phenotype of v-Src-transformed cells independent of v-Src tyrosine kinase inhibition or Wnt signaling [Néel et al., 2009]. The mechanism by which lithium in combination with PTEN overexpression exerts these effects in colorectal cancer cells requires further elucidation.

Invasion is a critical step in the metastatic process, and PTEN may control this event because PTEN knockdown has been shown to increase the invasion of human colorectal cancer cells [Danielsen et al., 2015]. We demonstrated that inducible PTEN overexpression decreased the invasion of HT-29 and HCT-116 cells, which may be a consequence of the low MMP-2 and MMP-9 activity in cells overexpressing PTEN, as showed in figure 8. Recent studies have suggested that the loss of PTEN expression (83%) is more prevalent in advanced stage colorectal cancer, particularly in metastatic disease [Rychahou et al., 2008]. Although this relationship remains to be elucidated, the loss of PTEN expression may correlate with poor prognosis in colorectal cancer patients due to the increased potential for metastases.

In conclusion, we have shown that lithium has important effects on events related to the progression of colorectal cancer cells. Our study suggests that lithium treatment induces cell death at high doses but requires PTEN overexpression at low doses. Lithium also downregulated and inactivated the pro-survival Akt signaling pathway, which may be one possible cause of the increased apoptosis in colon cancer cells. Finally, we demonstrate that induced PTEN overexpression is an important contributor to controlling the migration and invasion of cancer cells and that the combination of PTEN overexpression with lithium treatment has cooperative effects. Taken together, our results suggest that lithium and PTEN may be strong candidates for further studies of anti-invasive and anti-proliferative agents for new therapeutic approaches in CRC.

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## REFERENCES

Ahmed D,Eide PW, Eilertsen IA, Danielsen SA, Eknæs M, Hektoen M, Lind GE, Lothe RA. 2013. Epigenetic and genetic features of 24 colon cancer cell lines. Oncogenesis 2:e71.

Akca H, Demiray A, Tokgun O, Yokota J. 2011. Invasiveness and anchorage independent growth ability augmented by PTEN inactivation through the PI3K/AKT/NFkB pathway in lung cancer cells. Lung Cancer 73:302–309.

Basso AD, Solit DB, Chiosis G, Giri B, Tsichlis P, Rosen N. 2002. Akt forms an intracellular complex with Heat Shock Protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 Function. J Biol Chem 277:39858–39866.

Beurel E, Grieco SF, Jope RS. 2015. Glycogen synthase kinase-3 (GSK3): Regulation, actions, and diseases. Pharmacol Ther 148:114–131.

Chalhoub N, Baker SJ. 2009. PTEN and the PI3-Kinase Pathway in Cancer. Annu Rev Pathol Mech Dis 4:127–150.

Cohen Y, Chetrit A, Cohen Y, Sirota P, Modan B. 1998. Cancer morbidity in psychiatric patients: influence of lithium carbonate treatment. Med Oncol 15:32–36.

Danielsen SA, Eide PW, Nesbakken A, Guren T, Leithe E, Lothe RA. 2015. Portrait of the PI3K/AKT pathway in colorectal cancer. Biochim Biophys Acta BBA—Rev. Cancer 1855:104–121.

De Araújo WM, Vidal FCB, de Souza WF, de Freitas Junior JCM, de Souza W, Morgado-Diaz JA. 2010. PI3K/Akt and GSK-3ú prevents in a differential fashion the malignant phenotype of colorectal cancer cells. J Cancer Res Clin. Oncol 136:1773–1782.

D'Souza R, Rajji TK, Mulsant BH, Pollock BG. 2011. Use of lithium in the treatment of Bipolar Disorder in late-life. Curr Psychiatry Rep 13:488–492.

Dummler B, Hemmings BA. 2007. Physiological roles of PKB/Akt isoforms in development and disease. Biochem Soc Trans 35:231.

Easton RM, Cho H, Roovers K, Shineman DW, Mizrahi M, Forman MS, Lee VM-Y, Szabolcs M, de Jong R, Oltersdorf T, Ludwig T, Efstratiadis A, Birnbaum MJ. 2005. Role for Akt3/Protein Kinase Bγ in Attainment of Normal Brain Size. Mol Cell Biol 25:1869–1878.

Erdal E, Ozturk N, Cagatay T, Eksioglu-Demiralp E, Ozturk M. 2005. Lithium-mediated downregulation of PKB/Akt and cyclin E with growth inhibition in hepatocellular carcinoma cells. Int. J. Cancer 115:903–910.

Fang J, Ding M, Yang L, Liu L-Z, Jiang B-H. 2007. PI3K/PTEN/AKT signaling regulates prostate tumor angiogenesis. Cell Signal 19:2487–2497.

Fearon ER. 2011. Molecular Genetics of Colorectal Cancer. Annu Rev Pathol Mech Dis 6:479–507.

Flatmark K, Mælandsmo GM, Martinsen M, Rasmussen H, Fodstad Ø. 2004. Twelve colorectal cancer cell lines exhibit highly variable growth and metastatic capacities in an orthotopic model in nude mice. Eur J Cancer 40:1593–1598.

Frattini M, Saletti P, Romagnani E, Martin V, Molinari F, Ghisletta M, Camponovo A, Etienne LL, Cavalli F, Mazzucchelli L. 2007. PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients. Br J Cancer 97:1139–1145.

Fu Y, Zheng Y, Chan K-G, Liang A, Hu F. 2014. Lithium chloride decreases proliferation and migration of C6 glioma cells harboring isocitrate dehydrogenase 2 mutant via GSK-3β. Mol Biol Rep 41:3907–3913.

Gould T, Gould TD, Gray NA, Manji HK. 2003. Effects of a glycogen synthase kinase-3 inhibitor, lithium, in adenomatous polyposis coli mutant mice. Pharmacol Res DOI: 10.1016/S1043-6618(03)00096-3.

Gupta A, Schulze TG, Nagarajan V, Akula N, Corona W, Jiang X-, Hunter N, McMahon FJ, Detera-Wadleigh SD. 2012. Interaction networks of lithium and valproate molecular targets reveal a striking enrichment of apoptosis functional clusters and neurotrophin signaling. Pharmacogenomics J 12:328–341.

Gupta C, Kaur J, Tikoo K. 2014. Regulation of MDA-MB-231 cell proliferation by GSK-3β involves epigenetic modifications under high glucose conditions. Exp Cell Res 324:75–83.

Hers I, Vincent EE, Tavaré JM. 2011. Akt signalling in health and disease. Cell Signal 23:1515–1527.

Jhawer M, Goel S, Wilson AJ, Montagna C, Ling Y-H, Byun D-S, Nasser S, Arango D, Shin J, Klampfer L. others 2008. PIK3CA mutation/PTEN expression status predicts response of colon cancer cells to the epidermal growth factor receptor inhibitor cetuximab. Cancer Res 68:1953–1961.

Kaufmann L, Marinescu G, Nazarenko I, Thiele W, Oberle C, Sleeman J, Blattner C. 2011. LiCl induces TNF-a and FasL production, thereby stimulating apoptosis in cancer cells. Cell Commun Signal 9:15–15.

Langlois M-J, Bergeron S, Bernatchez G, Boudreau F, Saucier C, Perreault N, Carrier JC, Rivard N. 2010. The PTEN Phosphatase Controls Intestinal Epithelial Cell Polarity and Barrier Function: Role in Colorectal Cancer Progression. PLoS ONE 5:e15742.

Lau M-T, Klausen C, Leung PCK. 2011. E-cadherin inhibits tumor cell growth by suppressing PI3K/Akt signaling via β-catenin-Egr1-mediated PTEN expression. Oncogene 30:2753–2766.

Li H, Huang K, Liu X, Liu J, Lu X, Tao K, Wang G, Wang J. 2014. Lithium Chloride Suppresses Colorectal Cancer Cell Survival and Proliferation through ROS/GSK-3β/NF-κB Signaling Pathway. Oxid Med Cell Longev 2014:1–8.

Liu K-J, Lee Y-L, Yang Y-Y, Shih N-Y, Ho C-C, Wu Y-C, Huang T-S, Huang M-C, Liu H-C, Shen WW, Leu S-J. 2011. Modulation of the development of human monocyte-derived dendritic cells by lithium chloride. J Cell Physiol 226:424–433.

Matsebatlela T, Gallicchio V, Becker R. 2012. Lithium Modulates Cancer Cell Growth, Apoptosis, Gene Expression and Cytokine Production in HL-60 Promyelocytic Leukaemia Cells and Their Drug-Resistant Sub-clones. Biol Trace Elem Res 149:323–330.

Medina EA, Afsari RR, Ravid T, Castillo SS, Erickson KL, Goldkorn T. 2005. Tumor Necrosis Factor-α Decreases Akt Protein Levels in 3T3-L1 Adipocytes via the Caspase-Dependent Ubiquitination of Akt. Endocrinology 146:2726–2735.

Ministério da Saúde. 2014. Estimativa 2014: incidência de câncer no Brasil. Brazil: Rio de Janeiro.

Mora A, Sabio G, Risco AM, Cuenda A, Alonso JC, Soler G, Centeno F. 2002. Lithium blocks the PKB and GSK3 dephosphorylation induced by ceramide through protein phosphatase-2A. Cell Signal 14:557–562.

Néel BD, Lopez J, Chabadel A, Gillet G. 2009. Lithium suppresses motility and invasivity of v-src-transformed cells by glutathione-dependent activation of phosphotyrosine phosphatases. Oncogene 28:3246–3260.

Radu A, Neubauer V, Akagi T, Hanafusa H, Georgescu M-M. 2003. PTEN induces cell cycle arrest by decreasing the level and Nuclear Localization of CyclinD1. Mol Cell Biol 23:6139–6149.

Riesterer O, Zingg D, Hummerjohann J, Bodis S, Pruschy M. 2004. Degradation of PKB/Akt protein by inhibition of the VEGF receptor/mTOR pathway in endothelial cells. Oncogene 23:4624–4635.

Rowe MK, Chuang D-M. 2004. Lithium neuroprotection: Molecular mechanisms and clinical implications. Expert Rev Mol Med 6:1–18.

Rychahou PG, Kang J, Gulhati P, Doan HQ, Chen LA, Xiao S-Y, Chung DH, Evers BM. 2008. Akt2 overexpression plays a critical role in the establishment of colorectal cancer metastasis. Proc Natl Acad Sci 105:20315–20320.

Ryves WJ, Harwood AJ. 2001. Lithium Inhibits Glycogen Synthase Kinase-3 by Competition for Magnesium. Biochem Biophys Res Commun 280:720–725.

Saito Y, Swanson X, Mhashilkar AM, Oida Y, Schrock R, Branch CD, Chada S, Zumstein L, Ramesh R. 2003. Adenovirus-mediated transfer of the PTEN gene inhibits human colorectal cancer growth in vitro and in vivo. Gene Ther 10:1961–1969.

Suganthi M, Sangeetha G, Gayathri G, Ravi Sankar B. 2012. Biphasic Dose-Dependent Effect of Lithium Chloride on Survival of Human Hormone-Dependent Breast Cancer Cells (MCF-7). Biol Trace Elem Res 150:477–486.

Tamguney T, Stokoe D. 2007. New insights into PTEN. J Cell Sci 120:4071–4079.

Tsui MMK, Tai WCS, Wong WY, Hsiao WLW. 2012. Selective G2/M arrest in a p53Val135-transformed cell line induced by lithium is mediated through an intricate network of MAPK and β-catenin signaling pathways. Life Sci 91:312–321.

Vidal FCB, de Araújo WM, Cruz ALS, Tanaka MN, Viola JPB, Morgado-Diaz JA. 2011. Lithium reduces tumorigenic potential in response to EGF signaling in human colorectal cancer cells. Int J Oncol 38:DOI:10.3892/ijo.2011.955.

Wang J-S. 2008. Lithium inhibits proliferation of human esophageal cancer cell line Eca-109 by inducing a G2/M cell cycle arrest. World J Gastroenterol 14:3982.

Weng L-P, Smith WM, Dahia PLM, Ziebold U, Gil E, Lees JA, Eng C. 1999. PTEN Suppresses Breast Cancer Cell Growth by Phosphatase Activity-dependent G1 Arrest followed by Cell Death. Cancer Res 59:5808–5814.

Zhang WV, Jüllig M, Connolly AR, Stott NS. 2005. Early gene response in lithium chloride induced apoptosis. Apoptosis 10:75–90.