Interaction of E-cadherin and PTEN Regulates Morphogenesis and Growth Arrest in Human Mammary Epithelial Cells

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Abstract

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a dual-function phosphatase with tumor suppressor function compromised in a wide spectrum of cancers. Because tissue polarity and architecture are crucial modulators of normal and malignant behavior, we postulated that PTEN may play a role in maintenance of tissue integrity. We used two nonmalignant human mammary epithelial cell lines that form polarized, growth-arrested structures (acini) when cultured in three-dimensional laminin-rich extracellular matrix gels (lrECM). As acini begin to form, PTEN accumulates both in the cytoplasm and at cell-cell contacts where it colocalizes with the E-cadherin/β-catenin complex. Reduction of PTEN levels by shRNA in lrECM prevents formation of organized breast acini and disrupts growth arrest. Importantly, disruption of acinar polarity and cell-cell contact by E-cadherin function-blocking antibodies reduces endogenous PTEN protein levels and inhibits its accumulation at cell-cell contacts. Conversely, in Skbr-3 breast cancer cells lacking endogenous E-cadherin expression, exogenous introduction of E-cadherin gene causes induction of PTEN expression and its accumulation at sites of cell interactions. These studies provide evidence that E-cadherin regulates both the PTEN protein levels and its recruitment to cell-cell junctions in three-dimensional lrECM, indicating a dynamic reciprocity between architectural integrity and the levels and localization of PTEN. This interaction thus seems to be a critical integrator of proliferative and morphogenetic signaling in breast epithelial cells. [Cancer Res 2009;69(10):4545–52]

Introduction

The dual-function phosphatase and tensin homologue deleted on chromosome 10 (PTEN; MMAC/TEP-1) is one of the most common targets of mutations in human cancers (1–4). PTEN is primarily found in normal cells and directly correlates with the tumor stage and grade. For example, complete loss of PTEN is more frequent in metastatic cancer than in primary tumors. The loss of one copy of PTEN increases the risk that a tumor will develop, and the level of expression of PTEN dramatically affects the initiation and progression of tumors in mouse models (5). Low expression of PTEN correlates also with unresponsiveness to breast cancer therapies such as trastuzumab (Herceptin; ref. 10), tamoxifen (11), and gefitinib (12). Thus, an understanding of how PTEN levels and its subcellular localization are regulated is important for understanding the mechanism by which PTEN may be involved in protecting breast cells from proceeding to malignancy.

PTEN acts as a tumor suppressor, in part, by regulating or attenuating the activity of the oncogene PI3K. There is some evidence that it does so by catalyzing the degradation of a critical second messenger, phosphotidylinositol-(3,4,5)-triphosphate (PIP3), generated by PI3K (7). PTEN activity negatively influences multiple PI3K downstream targets, most notably protein kinase B/Akt (13–15), and exogenous addition of PTEN suppresses the growth of tumor cells (16–18) by up-regulation of p27kip1 and down-regulation of cyclin D1 in an Akt-dependent manner (19, 20). PTEN functions also in regulating dynamic cell surface interactions that involve integrins, focal adhesion kinase, cell migration, and the cytoskeleton (21–24). Moreover, it has been shown that PTEN interacts with cell adhesion molecules such as β-catenin and E-cadherin through MAGI-2 protein to inhibit migration and proliferation (25–28). In all these studies, however, the involvement of PTEN has been studied on two-dimensional tissue cultures. We hypothesized that the multiple consequences of loss of PTEN function observed in breast cancer could be related to a crucial but epigenetic role of PTEN in the tissue-specific integration of form and function.

Recently, several reports have indicated that PTEN shuttles between the nuclear and cytoplasmic compartments. Nuclear PTEN is primarily found in normal cells and directly correlates with cell differentiation (29). A short sequence in the NH2-terminal region of PTEN has been identified to localize PTEN in the cytoplasm (30). Neither the cause(s) nor the consequences of these differences in PTEN subcellular localization have been studied in a physiologically relevant model.

The phosphorylation state of PTEN contributes to the regulation of PTEN subcellular localization and function. PTEN phosphorylation at Ser380 and Thr382/383 within its COOH-terminal tail strongly influences PTEN protein stability and its localization to the cell membrane. Several kinases, including casein kinase 2, LKB1, RhoA-associated kinase, the microtubule-associated kinase MAT205, and glycogen synthase kinase 3β, have been reported to phosphorylate PTEN (29).

One of the earliest manifestations of breast cancer is the loss of cellular organization within a tissue. This loss of function can be mimicked in three-dimensional culture systems where its causes can be readily studied. In three-dimensional lrECM, nonmalignant
human mammary epithelial cells (HMEC) form polarized, self-organizing structures that closely resemble structures observed in the mammary gland in vivo, whereas malignant cells form structures that remain disorganized (31). We have shown that signaling pathways respond to microenvironmental cues analogous to those that occur in mammary acini in vivo (32). Recently, we showed additional utility of three-dimensional lrECM cultures of nonmalignant HMEC for identification of molecular signatures predictive of clinical outcome in breast cancer (33). The expression patterns of genes significantly down-modulated during acinar morphogenesis in three-dimensional cultures could be used to distinguish groups of patients with poor versus good prognosis. Thus, three-dimensional lrECM cultures of HMEC offer an opportunity to study the regulation of endogenous PTEN in a manipulatable and physiologically relevant context.

Here, we used the three-dimensional lrECM cultures of nonmalignant HMEC to investigate possible new roles for PTEN and how its level is regulated by microenvironment cues. We found that PTEN is indeed involved in acini formation and growth arrest. We also show that its level and localization is modulated by E-cadherin.

Materials and Methods

Cell culture. Immortalized nonmalignant HMT-3522 S1 (S1) HMEC (34) were cultured in H1 medium (DMEM/F-12 containing 250 ng/mL insulin, 10 µg/mL transferrin, 2.6 ng/mL sodium selenite, 10–10 mol/L estradiol, 1.4 × 10⁻⁶ mol/L hydrocortisone, 10 ng/mL epidermal growth factor, and 5 µg/mL prolactin). Finite life span 184 HMEC were grown in serum-free MCDB 170 medium (MEGM; Clonetics Division of BioWhittaker) as described previously (35). Skbr-3 cells were obtained from American Type Culture Collection and cultured in DMEM/F-12 and supplemented with insulin (5 µg/mL; Sigma Chemical) and 5% (v/v) FCS. Both S1 and 184 cells (1 × 10⁶/mL) were cultured in three-dimensional lrECM (Matrigel; BD Biosciences) as previously described (36). Mouse anti–E-cadherin (clone SHE78-7; Zymed Laboratories, Inc.) was added to lrECM to a final concentration of 5 µg/mL before cell seeding for function-blocking experiments. Colonies were isolated from the lrECM in ice-cold PBS/5 mmol/L EDTA for 60 min at 4°C after 3, 5, 7, and 15 d. For immunoblot analysis, the colonies were lysed in buffer containing 150 mmol/L NaCl, 1% NP40, 50 mmol/L Tris (pH 8.0). Nuclear and cytoplasmic cell extracts were prepared using NE-PER nuclear and cytoplasmic extract reagent kit (Pierce) according to the manufacturer's instructions.

mRNA profiling. The full microarray results were published in a previous study (33) and data can be retrieved at the public database links GEO Series GSE8096 and ArrayExpress E-MEXP-1006. In short, cell samples were harvested in duplicate at 3, 5, and 7 d post seeding in lrECM. Purified total cellular RNA was biotin labeled and hybridized to human oligonucleotide microarrays (Affymetrix HG-U133A) as previously described (33). Experiments with Affymetrix-present P-call rates of >30% were included in the analysis. Signal values from each of the 22,283 probe sets were calculated by means of robust multiarray analysis (37) and genes were normalized to the mean of the 3-d time point for each cell type. Transcripts exhibiting ≥2-fold differences in two independent microarray experiments were analyzed by one-way ANOVA as a function of time in three-dimensional cultures. Genes that were significantly different (P < 0.05) were selected. From these, we identified genes that were modulated early in the time course (by our definition, on day 5) in both cell types. Three probe sets on Affymetrix HG-U133A GeneChips uniquely match PTEN, including 204054_at, 204053_x_at, and 211711_s_at (GeneCards/GeneTide data).

Results represent the mean ± SE of the three probes.

Indirect immunofluorescence and image acquisition. Acinar structures were fixed on four-well chamber glass slides in methanol/acetone (1:1) at −20°C for 10 min and air-dried. A primary block was done in immunofluorescence buffer (130 mmol/L NaCl, 7 mmol/L Na₂HPO₄, 3.5 mmol/L NaH₂PO₄, 7.7 mmol/L Na₂CO₃, 0.1% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween 20) and 10% goat serum (Sigma) for 1 h at room temperature. A secondary block was done in immunofluorescence buffer + 10% goat serum + 20 µg/mL goat anti-mouse F(ab')2 fragment (Jackson ImmunoResearch) for 30 min. The primary antibodies, rat anti–α-catenin (clone NC20-GoH3; Chemicon International), rabbit anti-Ki67 (Sigma), mouse anti-PTEN (26H9), rabbit anti–phosphorylated PTEN at Ser380 (Cell Signaling), rabbit anti–MAGI-2 (H-60; Santa Cruz), mouse anti–β-catenin, and mouse anti–E-cadherin (BD Transduction Laboratories), were diluted 1:50 in the latter blocking buffer and incubated overnight (15–18 h) at 4°C. Secondary antibodies conjugated with fluorescein or Texas red (Sigma) were diluted 1:100 in blocking buffer and incubated for 1 h at room temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Immunofluorescent images were acquired using a Nikon (DIAPHOT 300) inverted microscope equipped with a digital camera and SPOT software. Confocal analysis was done using a Zeiss 410 confocal microscope system and software. The images presented are representative of two or more independent experiments.

Flow cytometry. Acini were dispersed into single-cell suspensions by treatment with 0.25% trypsin at 37°C for exactly 10 min. The dispersed cells

Figure 1. Induction of PTEN during HMEC acinar morphogenesis. A, PTEN RNA expression levels were investigated by microarray analysis during acinar formation in S1 and 184 HMEC cultured in three-dimensional lrECM. Three probe sets on Affymetrix HG-U133A GeneChips uniquely match PTEN, including 204054_at, 204053_x_at, and 211711_s_at (GeneCards/GeneTide data). Points, mean of the three probes; bars, SE. The P values (t test comparing day 3 versus days 5 and 7) were 0.0025 and 0.015 for S1 and 184 cells, respectively. B, S1, an immortal nonmalignant HMEC line, was seeded in three-dimensional lrECM, and cytoplasmic (c) and nuclear (n) cell extracts were obtained on days (d) 3, 5, and 7 postseeding. Proteins were separated by SDS-PAGE and detected by immunoblotting. Representative of two independent experiments. Results of cell cycle analysis at days 3, 5, and 7 are shown below.
were washed thrice in PBS and fixed in 40% ethanol at 4°C overnight. They were then incubated with 500 μg/mL RNase A in PBS at 37°C for 30 min and stained at room temperature for 30 min with a 69 μmol/L propidium iodide solution prepared in PBS. The DNA content was determined by flow cytometry using a FACScan (Becton Dickinson), and the data were analyzed with CellQuest software (Becton Dickinson).

Immunoblot and immunoprecipitation. Cell lysates (20–100 μg protein) were separated by SDS-PAGE gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore). After blocking in 10% nonfat dry milk for 90 min at room temperature, the membranes were incubated with primary antibodies at 1:500 to 1:1,000 dilution in 2% nonfat dry milk for 2 h at room temperature or overnight at 4°C. The blots were then incubated with horseradish peroxidase–conjugated sheep anti-mouse IgG or anti-rabbit IgG at 1:2,000 in 2% nonfat dry milk for 90 min at room temperature. The blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Immunoprecipitation studies were carried out as previously reported (36), with minor modifications, using rabbit anti–h-catenin (Santa Cruz Biotechnology) or rabbit IgG at a dilution of 1:100 to 1:300. Signal was captured with the FluorChem 8900 analysis system (Alpha Innotech).

Retroviral vectors and virus production. The retroviral vectors containing GFP and PTEN shRNA were obtained from Open Biosystems,

Figure 2. Colocalization of h-catenin/E-cadherin with PTEN and MAGI-2. A, S1 cells were cultured in three-dimensional lECM for 7 d, and subcellular localization of endogenous PTEN-Ser380 or MAGI-2 (red) and h-catenin or E-cadherin (green) was recorded by confocal microscopy. Representative images of two independent experiments. Bar, 25 μm (left); 10 μm (right). B, cell lysates from S1 cells obtained at indicated intervals postseeding were immunoprecipitated with a polyclonal rabbit h-catenin antibody, followed by immunoblotting with monoclonal mouse anti–total PTEN, anti–E-cadherin, and anti–h-catenin antibodies. Representative results from one of two independent experiments.
whereas the LXSN retroviral vector encoding full-length E-cadherin cDNA was obtained from Dr. John Muschler. Retroviruses were prepared by transient cotransfection of 293 cells with retroviral constructs and packaging plasmids. Retrovirus-containing supernatants were collected at 24 and 48 h following transfection and stored at \(-80^\circ\text{C}\). HMEC were plated at 5 \(\times\) 10^5 per 60-mm dish. After 24 h, parallel cultures were transduced with experimental or control viruses. Stable populations were obtained by selection in 1 \(\mu\)g/mL puromycin for 7 to 10 d. Skbr-3 cells were infected with LXSN-E-cadherin or LXSN viruses and selected with 500 \(\mu\)g/mL G418 (Genetecin; Invitrogen Corp.). Experiments involved pooled populations of selected cells.

**Results**

**PTEN becomes bound to \(\beta\)-catenin/E-cadherin complexes during HMEC acinar morphogenesis.** Profiling of global mRNA expression as a function of time in three-dimensional lrECM indicated that PTEN expression increased significantly \((P < 0.05)\) in two nonmalignant HMEC undergoing acinar morphogenesis in three-dimensional lrECM cultures (Fig. 1A; ref. 33). To confirm the microarray results and analyze the distribution of endogenous PTEN protein in nonmalignant HMEC undergoing acinar morphogenesis, we isolated cytoplasmic and nuclear extracts from S1 cells cultured for 3, 5, and 7 days in three-dimensional lrECM. Consistent with microarray results, we observed that both total PTEN and phospho-PTEN (Ser380) protein levels were elevated in S1 three-dimensional cultures as a function of time. The bulk of endogenous PTEN and its Ser380 phosphorylated form (PTEN-Ser380) were localized to the cytoplasm (Fig. 1B) but PTEN-Ser380 levels in the nucleus increased with time (Fig. 1B). We chose to focus further analysis on PTEN-Ser380 because it displayed the greatest relative increase in cytoplasm and nuclear extracts. We detected increases in the levels of FOXO3a, a protein downstream of PTEN that translocates to the nucleus and up-regulates a series of target genes, including p27, that promote cell cycle arrest (38). Not surprisingly, p27 protein increased over time during three-dimensional acini formation, consistent with results from the microarray data and with its function as a promoter of cell cycle arrest (38). Parallel samples were collected and the percentage of cells in S phase of the cell cycle was determined by propidium iodide staining and flow cytometry. Cell cycle analysis showed a 64% decrease in the number of cells in S phase between days 3 and 7, consistent with cultures undergoing cell cycle arrest (Fig. 1B).

We previously showed that both PI3K and PIP3 were polarized to the basal surface of mammary acini (39). To determine whether PTEN-Ser380 is colocalized to this surface in growth-arrested acini as well, we performed indirect immunofluorescence and confocal microscopy. We observed that PTEN-Ser380 indeed was enriched...
at cell-cell contacts where E-cadherin, β-catenin, and MAGI-2 were localized (Fig. 2A). This suggested that PTEN may be part of the E-cadherin/β-catenin complex at cell-cell junctions. To explore this possibility, we performed coimmunoprecipitation studies with a polyclonal antibody against β-catenin. We observed increased ratio of PTEN protein complexed with β-catenin/E-cadherin in cells incubated in IrECM compared with conventional adherent (two-dimensional) conditions (PTEN/β-catenin ratio: 0.4 in three-dimensional versus 0.1 in two-dimensional condition; Fig. 2B).

Reduced PTEN levels during mammary acini formation abrogate IrECM-induced growth arrest. To critically test the contribution of increased PTEN expression to the IrECM-mediated growth arrest and self-organization of nonmalignant HMEC in three-dimensional cultures, we stably expressed PTEN shRNA in 184 HMEC using retroviruses. Immunoblotting of total cell extracts confirmed that PTEN protein levels were inhibited by almost 50% in cells expressing PTEN shRNA compared with cells expressing a control scrambled shRNA (Fig. 3A). We found that shRNA-mediated reduction of PTEN expression led to the formation of large disorganized structures composed of abundant cells expressing the proliferation marker Ki67, compared with the well-organized, largely Ki67(-) structures present in parallel control cultures (Fig. 3B). The numbers of DAPI-stained nuclei per acinus cross section were calculated, and acinar structures were classified by size as 1 to 4, 5 to 8, 9 to 12, or >12 cells per acinus cross section. Nearly twice as many of the structures contained >12 cells in the PTEN shRNA colonies in cross section compared with control cultures (Fig. 3C). These results indicate that PTEN expression is required for both growth arrest and formation of organized structure in three-dimensional IrECM cultures of breast epithelial cells. In concordance to our shRNA studies, conditional loss of PTEN in the mouse mammary gland showed a hyperproliferative phenotype (40).

E-cadherin function regulates acinar polarity, cellular proliferation, and PTEN levels. Given the increased association among PTEN, β-catenin, and E-cadherin during HMEC acinar morphogenesis, we hypothesized that E-cadherin may directly modulate the levels and subcellular localization of PTEN. E-cadherin plays a key role in the structural organization of cell-cell interactions, as evidenced by the alterations in cell polarity and proliferation in response to function-blocking antibody against E-cadherin (Fig. 4).
adhesion and serves as a widely recognized suppressor of invasion and proliferation of epithelial cancers. In addition, its functional elimination represents a key step in the acquisition of the invasive phenotype for many tumors, including breast cancers. Consistent with previous reports (36, 41, 42), we observed that blocking E-cadherin–dependent cell-cell contact using neutralizing antibodies disrupted the cell cycle arrest and polarity of HMEC cultured in three-dimensional lrECM. Cultures treated with E-cadherin blocking antibodies lost basal-restricted staining for α6 integrin (Fig. 4A) and had strong nuclear staining for Ki67 (Fig. 4B). Moreover, the number of cells per acinus cross section was increased in cultures treated with E-cadherin blocking antibody compared with those treated with control IgG antibody (Fig. 4C). Immunoblot analysis showed that the levels of cytoplasmic PTEN-Ser380 in E-cadherin blocking antibody–treated cultures were reduced by ~30% compared with those in control IgG–treated cultures (Fig. 5A). Total PTEN protein levels were also reduced as a result of neutralizing E-cadherin function (Fig. 5B). Conversely, we observed robust induction of cyclin E in cytoplasmic and nuclear extracts of HMEC in the same cultures. In both S1 (p53 negative) and 184 (p53 wild type) HMEC cultures treated with E-cadherin blocking antibody, we observed a reduction of PTEN levels at cell-cell interactions (Fig. 5C). These results indicate that blocking E-cadherin function in HMEC in three-dimensional lrECM has profound consequences for PTEN activation, acinar polarity, and loss of growth control.

Restoration of E-cadherin function in the malignant breast cancer cell line Skbr-3 restores PTEN expression. Having shown that E-cadherin function is important for maintaining PTEN expression levels, we examined whether reexpression of E-cadherin could restore PTEN-Ser380 protein expression patterns in the breast cancer cell line Skbr-3, which lacks E-cadherin. Expression of vector control or E-cadherin gene in Skbr-3 cells was confirmed by immunofluorescent analysis (Fig. 6; Supplementary Fig. S1). Figure 6 shows that in Skbr-3 cells expressing E-cadherin, PTEN-Ser380 immunofluorescence was prominent at sites of cell-cell contact where it colocalized with E-cadherin (arrowhead). In contrast, cells negative for E-cadherin expression (small arrow) displayed reduced levels of only diffuse PTEN-Ser380 immunofluorescence.

![Figure 5](image.png)

**Figure 5.** E-cadherin function regulates PTEN protein levels. S1 cells were cultured in three-dimensional lrECM for 10 d in the presence of 5 μg/mL function-blocking antibody against E-cadherin or IgG control. Parallel cultures were grown to obtain cytoplasmic (c) and nuclear (n) cell extracts (A) and total cell extract (B). Levels of indicated proteins were determined by immunoblotting. Ponceau was used for loading control. C, S1 and 184 cells were grown in three-dimensional lrECM in the presence of isotype IgG or E-cadherin function–blocking antibody for 10 d. The treated cells were fixed as described in Materials and Methods and immunostained for PTEN-Ser380 (red). Representative images of three independent experiments. Bar, 25 μm.
E-cadherin Regulates PTEN in Breast Cells

Discussion

Our results indicate that growth arrest in a physiologically relevant model of acinar morphogenesis is dependent on E-cadherin–mediated up-regulation and accumulation of PTEN. Although PTEN has been previously shown to interact with cell adhesion complexes and to stabilize intercellular junctions, allowing a reduction of invasiveness in a range of cancer cells (43), regulation of endogenous PTEN in nonmalignant cells and its ability to contribute to the formation or blockage of intercellular junctions have not been previously shown. Our work ties together two tumor suppressor pathways, and suggests that PTEN may constitute a key internal node at which extracellular signals for growth arrest are integrated. Aberrations in E-cadherin expression or function may be the proximal cause of loss of PTEN expression in cancers, such as those that frequently occur in breast, where PTEN expression is lost without identifiable mutations in the PTEN gene itself. Our work has shown that even partial abrogation of PTEN expression is sufficient to cause continued proliferation and to impede the formation of organized acinar structures in the three-dimensional IrECM model of HMEC morphogenesis. The surprising sensitivity of mammary cells to the PTEN regulatory node may explain why it is so low in a variety of solid tumors where tissue structure is compromised.

Previous work done in nonmalignant HMEC cultured in three-dimensional IrECM has indicated that signaling pathways influenced by PTEN play key roles during mammary acini formation. For example, constitutive Akt activation has been shown to disrupt the organization and maintain proliferation of MCF-10A cells in three-dimensional IrECM (44). Conversely, inhibition of PI3K signaling was shown to cause cell cycle arrest and restoration of baso-apical polarity in breast cancer cell lines in three-dimensional IrECM (39). Interestingly, PI3K and its lipid product, PIP3, are relocalized to the basal surfaces, away from regions of cell-cell contact and PTEN-Ser380 accumulation, in organized structures formed when malignant HMEC are reverted in IrECM (39). This suggests that the recruitment of PTEN-Ser380 to regions of cell-cell contact is part of a larger reorganization of cytoplasmic components orchestrated by the formation of adherens junctions between adjacent epithelial cells, and that a primary consequence of localized PTEN may be to allow compartmentalization of PI3K activity as well as other signaling molecules.

PTEN-dependent changes in PI3K signaling may operate in conjunction with other E-cadherin–dependent processes to cause cessation of cell growth. In the canonical model of E-cadherin–mediated growth suppression, E-cadherin sequesters β-catenin in cell adhesion complexes where the latter plays an essential role in the structural organization and function of cadherins by linking them to the actin cytoskeleton. In the presence of stimulatory signals, dissociation of cadherin-catenin complexes is mediated by activation of receptor tyrosine kinases and cytoplasmic tyrosine kinases. The free β-catenin is then available to bind to T-cell factor/lymphoid enhancer factor transcription factors and to initiate events such as cyclin D expression and cell proliferation (45). PTEN can indirectly inhibit this process through stimulation of glycogen synthase kinase-3–mediated phosphorylation and subsequent degradation of β-catenin (46).

Although other studies have indicated that phosphorylated PTEN has decreased affinity for cell membranes (47), our study indicates that in three-dimensional cultures of HMEC, PTEN-Ser380 is recruited to regions of cell-cell contacts where it may be stabilized by interactions with E-cadherin/β-catenin complexes. PTEN phosphorylation at serine residues has been associated with an increase in PTEN protein stability (48, 49). Additional studies will be required to determine the mechanism(s) by which PTEN mRNA and protein are up-regulated by E-cadherin, as well as the role of PTEN phosphorylation in the model of IrECM-mediated morphogenesis.

Disclosure of Potential Conflicts of Interest

M.V. Fournier: Employment, GlaxoSmithKline. The other authors disclosed no potential conflicts of interest.

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