RNA Interference–Mediated Choline Kinase Suppression in Breast Cancer Cells Induces Differentiation and Reduces Proliferation

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Abstract

Choline kinase is overexpressed in breast cancer cells and activated by oncogenes and mitogenic signals, making it a potential target for cancer therapy. Here, we have examined, for the first time, the effects of RNA interference (RNAi)–mediated down-regulation of choline kinase in nonmalignant and malignant human breast epithelial cell lines using magnetic resonance spectroscopy (MRS) as well as molecular analyses of proliferation and differentiation markers. RNAi knockdown of choline kinase reduced proliferation, as detected by proliferating cell nuclear antigen and Ki-67 expression, and promoted differentiation, as detected by cytosolic lipid droplet formation and expression of galectin-3. The functional importance of RNAi-mediated choline kinase down-regulation on choline phospholipid metabolism was confirmed by the significant reduction of phosphocholine detected by MRS. These results strongly support the targeting of choline kinase in breast cancer cells with RNAi and show the potential ability of noninvasive MRS to detect and evaluate future treatments incorporating such strategies. (Cancer Res 2005; 65(23): 11034-43)

Introduction

Overexpression and increased activity of choline kinase in malignant cells and tumors (1–5) and oncogene-mediated increase in the activity and basal levels of choline kinase (6–10) have been reported in several studies. Both increased enzymatic activity as well as overexpression of choline kinase correlated strongly with histologic tumor grade and negative estrogen receptor status in breast carcinomas (1). Because choline kinase converts free choline to phosphocholine, magnetic resonance studies over the past decade have also consistently detected an elevation of phosphocholine and total choline-containing compounds in cancer cells in vivo and in vitro (11–18).

Ras oncogene transformation has been related to choline kinase stimulation and to an increase of phosphocholine levels (6, 7, 9, 19). The effect of ras signaling on choline kinase activity is mediated through a combination of its effectors Ras-GDP dissociation stimulator, a nucleotide exchange factor, and phosphatidylinositol 3-kinase, a lipid kinase, downstream of Ras (8). Transformation of murine NIH3T3 fibroblasts with other oncogenes, such as v-raf, v-mos, and src, were also found to increase basal choline kinase activity and cellular phosphocholine levels (20). Choline kinase was also activated by serum (21), hormones (22, 23), or other mitogenic/growth factors, such as insulin (21, 24), platelet-derived growth factor (PDGF; ref. 25), fibroblast growth factor (FGF; ref. 25), epidermal growth factor (24), pro lactin (26), and estrogens (22, 27). Expression of human choline kinase in fibroblasts increased the mitogenic potential of insulin, insulin-like growth factor-I, FGF, and PDGF (28). Stress induced chemically with carcinogens was also found to induce choline kinase expression (23, 29), possibly due to a xenobiotic responsive element in the putative promoter region of the chk-2 gene (29).

These observations have led to pharmacologic inhibitors of choline kinase being explored as potential antitumor agents (13, 18, 20). Strong antitumor activity of these inhibitors has been shown in vivo against breast, colon, and epidermoid cancer xenografts (3, 10, 30). Although the toxicity and side effects of these drugs have largely been minimized with newly designed chemical compounds (20), the long-term down-regulation of choline kinase and its effect on the tumor phenotype are as yet unknown. RNA interference (RNAi) provides a molecular approach to down-regulate the expression of specific target genes in mammalian cells (31–33), without the associated side effects of chemical agents, especially in long-term studies.

In this study, RNAi knockdown of choline kinase was investigated, for the first time, in a nonmalignant immortalized human mammary epithelial cell (HMEC) line and in malignant breast cancer cell lines. The effects of choline kinase down-regulation were assessed with transiently transfected small interfering RNA (siRNA) specific for choline kinase mRNA and in a breast cancer cell line stably expressing short hairpin RNA (shRNA) specific for choline kinase mRNA. Functional evaluation of RNAi with magnetic resonance spectroscopy (MRS) of cell extracts, together with molecular analyses of choline kinase expression and markers of proliferation and differentiation, showed that RNAi-mediated down-regulation of choline kinase had a profound effect on choline kinase expression, choline phospholipid metabolism, proliferation, and differentiation in breast cancer cells. These studies strongly support the use of specific targeting of choline kinase with RNAi for breast cancer treatment and show the feasibility of using MRS to detect such targeting in preclinical models in vivo and potentially in the clinical setting.

Materials and Methods

Cell lines. An immortalized nonmalignant human mammary epithelial cell line, MCF-12A, was used as model of normal human mammary epithelium (34). MCF-7, an estrogen-sensitive poorly metastatic breast cancer cell line (35), and MDA-MB-231 and MDA-MB-435, two metastatic human breast cancer cell lines, were used as models of malignant human breast cancer cells (17). All cell lines (American Type Culture Collection, Rockville, MD) were maintained as previously described (17, 34, 35).

RNA interference experiments. siRNA specific for choline kinase (siRNA-chk) was designed with the sequence 5’-CAUCGUUCCAGUGCUCC-3’ and 5’-GUAGCUGUGAAAGUGCUCC-3’.
purchased as a duplex (Dharmacon, Lafayette, CO). Approximately 10^6 cells at 40% to 50% confluence were transfected with 2.6 nmol of the annealed siRNA-chk (Dharmacon) using 772 µl of oligofectamine (Invitrogen, Carlsbad, CA) in Opti-MEM (Invitrogen). Depending upon the number of cells required, proportionately larger or smaller amounts of siRNA-chk, oligofectamine, and Opti-MEM were applied. Cells were treated with siRNA-chk for 48 hours, because this incubation period resulted in the most effective reduction of choline kinase levels as determined by reverse transcriptase-PCR (RT-PCR) and Western blotting.

**Generation of stable clones.** An oligonucleotide cDNA insert encoding a shRNA specific for down-regulating choline kinase mRNA was designed using the RNA Oligo Retriever software (http://katahdin.cshl.org/9331RNAi/ ref. 33). The oligonucleotides selected encoded the same region of the chkn gene used for synthetic siRNA transfection experiments. The oligonucleotides were synthesized, purified (DNA/RNA Synthesis Core Facility, Johns Hopkins University Bloomberg School of Public Health), annealed, and ligated into the pSHAG vector containing the U6 promoter for RNA Polymerase III catalyzed transcription (33). The U6 promoter plus shRNA sequence for choline kinase was cut from this vector and ligated into the multiple cloning site of a pCR3.1 vector (Invitrogen) devoid of any promoter. This final construct, referred to as U6-shRNA-chk vector, was transfected into malignant MDA-MB-231 breast cancer cells selected with G418 (Invitrogen). Forty-five stable clones expressing this vector were screened using quantitative real-time RT-PCR (qRT-PCR) analysis and Western blotting using choline kinase antibody. Selection of the two clones used in these studies was based on the significantly low amounts of choline kinase mRNA and protein levels in these two clones compared with parental MDA-MB-231 cells.

**Total RNA isolation, reverse transcriptase-PCR, and quantitative real-time reverse transcriptase-PCR analysis.** Total cellular RNA was isolated from 3 x 10^6 MDA-MB-231 or MCF-12A cells using the QiAshredder homogenizer spin columns (Qiagen, Inc., Valencia, CA) and RNasey Mini Kit (Qiagen), and its concentration, purity, and integrity was assessed according to the manufacturer’s protocol. Reverse transcription was done using SuperScript II (Invitrogen) according to the manufacturer’s protocol. One microliter of the resulting cDNA reaction mix was used for PCR. The human ribosomal protein 36B4 was used as internal loading control for conventional as well as quantitative RT-PCR. The PCR primers for amplifying 36B4 and choline kinase were as follows for both the conventional RT-PCR and qRT-PCR: 36B4 sense strand, 5’-GATGGGC-TACCCAATCTTGTCGA-3’; antisense strand, 5’-CAGGGCGACGCACCAAA-AAGG-3’; choline kinase sense strand, 5’-ATCCACCAAGAACAAGCAGC-3’; antisense strand, 5’-TGTTGAAAATGGCATTCAAGG-3’. Conventional PCR reactions were carried out using the Rapid Cycler (Idaho Technology, Salt Lake City, UT), and a buffer containing 50 mmol/L Tris (pH 8.3), 2.5 mg/mL bovine serum albumin (BSA), and 2 mmol/L magnesium chloride (Idaho buffer 10/C2 with BSA and 20 mmol/L magnesium chloride, Idaho Technology). The denaturation temperature was 94°C, the annealing temperature was 56°C, and the extension temperature was 74°C. Thirty amplification cycles were used. qRT-PCR was done using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and the iCycler (Bio-Rad). PCR reactions were also done on RNA samples without prior reverse transcription to ensure that DNA contamination of the samples had not occurred.

**Protein lysates, gel electrophoresis, and Western blotting.** Approximately 3 x 10^6 cells at 70% to 80% confluence were homogenized with lysis buffer containing a protease inhibitor cocktail (P8340, Sigma-Aldrich, St. Louis, MO) as previously described (36). Twenty micrograms of total protein, as determined by a modified Lowry assay (Bio-Rad), was loaded in each lane, and two lanes were loaded with molecular weight standards. Conventional PAGE and Western blotting were done as previously described (36). Membranes were incubated for 1 hour with an appropriate dilution of primary antibody followed by incubation with horseradish peroxidase–conjugated second-step antibody (Amersham, Arlington Heights, IL), and visualization using the Supersign West Pico chemiluminescent substrate kit (Pierce Biotechnology, Rockford, IL) recorded on Blue Bio film (Denville Scientific, Metuchen, NJ). The films were scanned and densitometry was done using the Gel-analysis tool in Image (NIH, Bethesda, MD).

**Choline kinase antibody.** The polyclonal choline kinase antibody (IgG) used here was raised against a hydrophilic synthetic peptide (Protein tech Group, Inc., Chicago, IL) specific for choline kinase. The peptide was designed by employing Hopp-Woods hydropathy plots of human choline kinase and visualized with the Swiss-Pdb Viewer (http://us.expasy.org/spdbv/text/download.htm) to assess surface exposure of choline kinase amino acids. The peptide representing the amino acids 73 to 90 of choline kinase was synthesized at 90% purity as verified by mass spectrometry, conjugated with keyhole limpet hemocyanin, and used to immunize two rabbits using a standard immunization protocol (Protein tech Group). Antiserum specificity was assessed by ELISA against the synthetic peptide. Antibodies were isolated and purified by affinity chromatography using the peptide as affinity ligand. Using this antibody, choline kinase was detected in Western blots at an apparent molecular weight of 48 kDa.

**Dual-phase extraction, magnetic resonance data acquisition, and processing.** Approximately 10^6 cells were harvested and both lipid and water-soluble cell extract fractions were obtained using a dual-phase extraction method based on methanol/chloroform/water (1:1:1, v/v/v) as previously described (18, 37). Briefly, cells were harvested by trypsinization, and a small aliquot was counted for quantification. The cells were washed twice with 10 mL of saline and pooled into a glass centrifuge tube. Four milliliters of ice-cold methanol were added to the pellets, vigorously vortexed, and kept on ice for 10 minutes. Four milliliters of chloroform were added, vortexed, and kept on ice for 10 minutes. Finally, 4 mL of water were added, vortexed, and kept at 4°C overnight for phase separation. The samples were centrifuged for 30 minutes at 35,000 x g at 4°C, and the phases were carefully separated. The water/methanol phase containing the water-soluble cellular metabolites, such as choline, phosphocholine, and glycero-phosphocholine, was treated with 10 mg of chexel (Sigma-Aldrich) for 10 minutes on ice to remove divalent cations. The chexel beads were then removed. Methanol was removed by rotary evaporation, and the remaining water phase was lyophilized and stored at –20°C. The chloroform phase containing the cellular lipids, such as phosphatidylcholine, was dried in a stream of N2 and stored under N2 at –20°C. The samples were dissolved in deuterated solvents containing 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid (TSP; Sigma-Aldrich) in the case of water-soluble fractions or tetramethylsilane (TMS; Cambridge Isotope Laboratories, Andover, MA) in the case of lipid fractions to serve as concentration standards and chemical shift references. Fully relaxed 1H magnetic resonance spectra of the extracts were acquired on a Bruker Avance 500 spectrometer operating at 11.7 Tesla (Bruker BioSpin Corp., Billerica, MA) using a 5-mm HX inverse probe as previously described (18). Magnetic resonance spectra were analyzed using Bruker XWIN-NMR 3.5 software (Bruker BioSpin). Integris of the N-(13C2) signal of free choline at 3.209 ppm, phosphocholine at 3.227 ppm, and glycerophosphocholine at 3.236 ppm in the 1H magnetic resonance spectra of water-soluble metabolites, and of phosphatidylcholine at 3.220 ppm, and the methylene groups in fatty acids (Fmix) at 1.245 to 1.364 ppm in the 1H magnetic resonance spectra of lipids, were determined and normalized to cell size and number as previously described (17, 18). Briefly, the signal integrals of the N-(13C2) groups of choline, phosphocholine, glycero-phosphocholine, and the standard TSP in the water-soluble 1H magnetic resonance spectra, and phosphatidylcholine and the standard TMS in the lipid 1H magnetic resonance spectra were determined using XWIN-NMR 3.5. The concentration of each metabolite in mM was calculated according to the following equation:

\[
\text{[metabolite]} = \left( \frac{\text{I}_{\text{metabolite}} \times \text{[standard]}}{\text{I}_{\text{standard}} \times \text{cell number} \times \text{cell volume}} \right)
\]

In this equation, [metabolite] represents the intracellular concentration of the metabolite of interest expressed in mM/L, \( I_{\text{metabolite}} \) represents the signal integral of the metabolite of interest divided by the number of
proteins, and ([standard]) represents the amount of TSP (water-soluble metabolites) or TMS (lipids) used in mol divided by the number of protons. The number of cells in each sample (cell number) was counted before extraction. The cell volumes were determined as previously described (17).

**Differentiation assays:** Nile red staining, fluorescence microscopy, and marker proteins. Cells were grown on Permanox chamber slides (Nalge Nunc, Naperville, IL) to 60% to 70% confluence, treated as described above in the case of siRNA-chk experiments, washed with PBS, and fixed with 3% (w/v) paraformaldehyde. Cells were washed with PBS and incubated with a 1:1,000 dilution in PBS of a 1 mg/mL stock solution of Nile red (9-diethylamino-5H-benzo[a]phenoxazine-5-one; Sigma-Aldrich) in acetone for 10 minutes at room temperature. Nile red (Sigma-Aldrich) stains lipid droplets (38–40), which are a marker of differentiation in epithelial cells (41, 42). Cell nuclei were counterstained with Hoechst H-33342 (Molecular Probes, Eugene, OR). Cells were washed and mounted using Faramount aqueous mounting medium (DakoCyto- mation, Carpinteria, CA). Fluorescence microscopy was done with a Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss, Inc., Thornwood, NY) using a Plan-Apochromat 63×/1.4 oil immersion lens (Zeiss). Nile red and H-33342 were excited at 488 and 405 nm, respectively, and fluorescence emission was detected with photomultipliers using 530-nm thickness containing the maximal number of lipid droplets were imaged. Numbers of lipid droplets per cell were quantified using customized software that was developed in-house as previously described (36). Additionally, SDS-PAGE and immunoblotting using the differentiation marker protein galectin-3 (refs. 43, 44; ab2785, Abcam, Cambridge, MA) were done.

**Statistical analysis of experiments.** An unpaired two-tailed t test (z = 0.05) was used to detect significant differences between experimental groups using the JMP software package (Brooks/Cole-Thomson Learning, Belmont, CA). Ps < 0.05 were considered significant.

### Results

The custom-made choline kinase antibody consistently detected an immunoreactive band at 48 kDa, the anticipated molecular weight of choline kinase (29), which increased with increasing malignancy of the HMECs (Fig. 1). Nonmalignant MCF-12A cells had almost undetectable levels of choline kinase, which increased for the poorly metastatic malignant MCF-7 breast cancer cell line and were highest for the metastatic malignant MDA-MB-231 and MDA-MB-435 breast cancer cell lines (Fig. 1). Densitometry of the Western blots showed that choline kinase levels were significantly higher in all three breast cancer cell lines (P < 0.01, n = 3) compared with the nonmalignant MCF-12A HMEC line (Fig. 1B).

To evaluate the role of choline kinase in breast cancer, cellular choline kinase mRNA levels were knocked down in nonmalignant MCF-12A cells and in malignant MDA-MB-231 breast cancer cells by transiently transfecting these cells with siRNA capable of down-regulating choline kinase mRNA (siRNA-chk). RT-PCR analysis of choline kinase mRNA was done following siRNA-chk treatment at 24, 48, and 72 hours after transfection. The ribosomal protein 36B4 mRNA was amplified as an internal loading control. A significant reduction in choline kinase mRNA was detected within 48 hours of treatment with siRNA-chk, as shown in Fig. 2A. Also evident were larger basal choline kinase mRNA levels (Fig. 2A) and choline kinase protein levels (Fig. 2B) in malignant MDA-MB-231 cells compared with nonmalignant MCF-12A cells (Fig. 2A). The corresponding choline kinase protein levels were determined by immunoblotting using choline kinase antibody as shown in Fig. 2B. Nonmalignant MCF-12A cells and malignant MDA-MB-231 cells were treated for 48 hours with oligofectamine alone (control oligo), or siRNA-chk premixed with oligofectamine (Fig. 2B). Choline kinase protein levels were significantly (P < 0.05, n = 4) lower in siRNA-chk-treated nonmalignant MCF-12A cells and malignant MDA-MB-231 cells compared with the controls, which were treated with oligofectamine alone (Fig. 2C). Fully relaxed 1H magnetic resonance spectra showed that phosphocholine levels, the product of choline kinase enzyme activity, as well as total choline-containing compound levels were significantly lower in control nonmalignant MCF-12A cells compared with control malignant MDA-MB-231 cells (Fig. 2D and E). No significant changes in cellular phosphocholine, total choline-containing compound, glycerophosphocholine, choline, and phosphatidylcholine levels were detected following siRNA-chk treatment for 48 hours in nonmalignant MCF-12A cells (Fig. 2D and E). By comparison, phosphocholine and total choline-containing compounds were significantly (P < 0.01 and P < 0.05, respectively, n = 6) lower in the malignant MDA-MB-231 breast cancer cells, following choline kinase knockdown (Fig. 2D and E). Glycerophosphocholine levels increased slightly following siRNA-chk treatment in malignant MDA-MB-231 cells (Fig. 2D and E), whereas choline (Fig. 2D and E), and lipid phosphatidylcholine levels (data not shown) remained constant. In summary, choline kinase mRNA, choline kinase protein, phosphocholine, and total choline-containing compound levels significantly decreased following transient choline kinase knockdown in malignant MDA-MB-231 breast cancer cells, whereas nonmalignant MCF-12A cells were hardly affected because of their low basal choline kinase and phosphocholine levels.
Stable transfection of malignant MDA-MB-231 breast cancer cells was done with a construct expressing a shRNA, analogous to the siRNA-chk used in the transient transfection experiments, under the control of a U6 promoter (U6-shRNA-chk vector). Stable expression of the U6-shRNA-chk vector in malignant MDA-MB-231 breast cancer cells significantly reduced choline kinase mRNA levels in these cells compared with empty vector control, as displayed in Fig. 3A. Quantitative RT-PCR revealed that choline kinase mRNA decreased in excess of 10 cycles in U6-shRNA-chk vector expressing cells compared with empty vector control cells. Immunoblotting with choline kinase antibody showed that the corresponding choline kinase protein levels were significantly ($P < 0.01, n = 6$; Fig. 3B and C) reduced in U6-shRNA-chk vector expressing MDA-MB-231 cells compared with vector control (Fig. 3C). Stably knocking down choline kinase expression significantly reduced cellular phosphocholine ($P < 0.01, n = 5$) and total choline-containing compound ($P < 0.05, n = 5$) levels in $^1$H magnetic resonance spectra of MDA-MB-231 U6-shRNA-chk vector cells compared with empty vector cells, as shown in Fig. 3D and E. Lipid phosphatidylcholine levels were slightly lower in malignant MDA-MB-231 breast cancer cells with stably knocked down choline kinase compared with vector controls (Fig. 3E). Glycerophosphocholine and choline levels remained constant (Fig. 3E). Thus, similar to the transient transfection, the stable expression of U6-shRNA-chk in malignant MDA-MB-231 breast cancer cells resulted in a significant reduction of choline kinase mRNA, choline kinase protein, phosphocholine, and total choline-containing compound levels. Low expression of choline kinase mRNA and protein, and $^1$H MRS-detectable phosphocholine and total choline-containing compound levels remained stable in the clones, which were maintained in cell culture for up to 21 passages, over a period of 155 days. Changes in phospholipid metabolite levels were comparable with transient and stable choline kinase down-regulation in MDA-MB-231 cells as evident in Fig. 2E and Fig. 3E.

A significant increase in the mixed fatty acid signal ($F_{\text{mix}}$) at 1.3 ppm was observed in transiently siRNA-chk-transfected nonmalignant MCF-12A cells and malignant MDA-MB-231 breast cancer cells compared with control cells, whereas the phosphatidylycholine signal at 3.22 ppm remained constant in $^1$H magnetic resonance spectra of the cellular lipid fractions (Fig. 4A).
the *F*\textsubscript{mix}/phosphatidylcholine signal integral ratio was significantly higher in siRNA-chk-treated cells versus control cells for both nonmalignant MCF-12A cells (*P* < 0.05, *n* = 3; Fig. 4B) and malignant MDA-MB-231 cells (*P* < 0.05, *n* = 3, Fig. 4B), indicating an increase in cellular triacylglycerol. These data were verified by two-dimensional scalar-correlated spectroscopy (ref. 45; data not shown). Similarly, a significant (*P* < 0.001, *n* = 5) increase in triacylglycerol was detected in MDA-MB-231 breast cancer cells stably expressing U6-shRNA-chk vector compared with empty vector control cells, as quantified from the *F*\textsubscript{mix}/phosphatidylcholine ratio (Fig. 4C). The increase of the *F*\textsubscript{mix}/phosphatidylcholine ratio relative to controls was comparable in MDA-MB-231 breast cancer cells with transient or stable choline kinase down-regulation (Fig 4B and C).

The increase in triacylglycerol correlated with an increase in cellular lipid droplets in transiently siRNA-chk-transfected nonmalignant MCF-12A cells and malignant MDA-MB-231 cells, as evident in the confocal fluorescence images shown in Fig. 5A. An accumulation of cellular lipid droplets containing mobile triacylglycerol indicates differentiation in human breast epithelial cells (41, 42). Results from the analyses of lipid droplets in 20 to 22 cells from four independent siRNA-chk experiments each are summarized in Fig. 5B. Treatment with siRNA-chk resulted in a significant increase in the number of lipid droplets compared with nonmalignant MCF-12A cells (*P* < 0.001, *n* = 20) and malignant MDA-MB-231 cells (*P* < 0.001, *n* = 22). In addition, malignant MDA-MB-231 breast cancer cells were found to contain significantly (*P* < 0.01, *n* = 20-22) fewer lipid droplets compared with nonmalignant MCF-12A HMECs (Fig. 5B). In addition, as shown in Fig. 5C, in the stable clones, U6-shRNA-chk vector expressing MDA-MB-231 cells displayed significantly more lipid droplets compared with wild-type MDA-MB-231 cells (*P* < 0.001, *n* = 21) or empty vector expressing MDA-MB-231 cells (*P* < 0.001, *n* = 20). This increase in lipid droplets was more dramatic in transient choline kinase down-regulation (Fig. 5B and C).

Baseline levels of *F*\textsubscript{mix}/phosphatidylcholine did not differ significantly between nonmalignant MCF-12A cells and malignant MDA-MB-231 cells, although the number of lipid droplets was slightly higher in nonmalignant MCF-12A cells. This small difference was most likely not detected by the *F*\textsubscript{mix}/phosphatidylcholine ratio due to poor sensitivity of this variable to subtle changes. The more pronounced changes in lipid droplets following transient or stable choline kinase down-regulation were detected by the *F*\textsubscript{mix}/phosphatidylcholine ratio (Fig. 4B-C and Fig. 5B-C).

Proliferation was significantly (*P* < 0.001, *n* = 5) slower in MDA-MB-231 breast cancer cells stably expressing U6-shRNA-chk compared with empty vector controls (Fig. 6A). The cell doubling time of U6-shRNA-chk expressing MDA-MB-231 cells was significantly longer (34.7 hours) than the doubling time for MDA-MB-231 cells with the control vector (28.8 hours). Expression levels of both proliferation markers, proliferating cell nuclear antigen (PCNA; refs. 46, 47; Fig. 6B) and Ki-67 (ref. 48; Fig. 6F), were significantly (*P* < 0.01 and *P* < 0.05, respectively, *n* = 3; Fig. 6D and G) higher in the two metastatic malignant breast cancer cell lines MDA-MB-231 and MDA-MB-435 compared with the poorly metastatic malignant MCF-7 human breast cancer cell line and the nonmalignant MCF-12A HMEC line (Fig. 6). Both PCNA (Fig. 6C) and Ki-67 (Fig. 6F) levels were significantly (*P* < 0.01, *n* = 3; Fig. 6D and G) lower in MDA-MB-231 cells stably expressing U6-shRNA-chk compared with empty vector MDA-MB-231 cells. PCNA and Ki-67 protein levels in Figure 3. A conventional RT-PCR of choline kinase (Chk) mRNA levels in U6-shRNA-chk expressing MDA-MB-231 cells compared with MDA-MB-231 cells stably expressing the control vector. 36B4 mRNA levels served as an internal control. B, choline kinase Western blot of wild-type, control vector, and U6-shRNA-chk vector expressing MDA-MB-231 cells. Actin was probed as a loading control. C, densitometry of Western blots showed semiquantitative changes relative to the respective control cells (*n* = 6). Relative fold changes in choline kinase were normalized to the choline kinase immunoreactive band in MDA-MB-231 wild type cells, which was set to 1. Columns, mean; bars, SD. **, *P* < 0.01, compared with wild type; #, *P* < 0.01, compared with control vector. D, expanded regions of 1H magnetic resonance spectra and (E) quantitation of these 1H magnetic resonance spectra (*n* = 5) from cell extracts of U6-shRNA-chk expressing MDA-MB-231 compared with control vector expressing MDA-MB-231 cells. Abbreviations: Cho, free choline; GPC, glycerophosphocholine; PC, phosphocholine; tCho, total choline-containing compounds; ns, not significant. Columns, mean; bars, ± SE.* *, *P* < 0.05; **, *P* < 0.01.
malignant MDA-MB-231 cells expressing U6-shRNA-chk were comparable with the expression levels in nonmalignant MCF-12A cells (Fig. 6B-G).

To test for differentiation of the different breast epithelial cell lines and the choline kinase knockdown clones, we probed for galectin-3 protein expression by SDS-PAGE followed by immunoblotting. Galectin-3 is involved in several biological processes (49), including cell adhesion, migration, cell growth, differentiation, apoptosis, tumor progression, and metastasis (43, 44, 50), in human breast epithelial cells. Figure 7A shows that nonmalignant MCF-12A cells expressed almost undetectable levels of galectin-3 compared with malignant MCF-7, MDA-MB-231, and MDA-MB-435 breast cancer cells, where progressively increasing galectin-3 levels matched the decreasing differentiation of the cell lines. Galectin-3 protein expression levels were significantly ($P < 0.05, n = 3$) higher in all three malignant breast cancer cell lines compared with the nonmalignant MCF-12A HMEC line (Fig. 7A and C). Consistent with the transient transfection studies, galectin-3 expression was significantly lower ($P < 0.05, n = 3$; Fig. 7B-C), approaching levels in nonmalignant MCF-12A cells, in MDA-MB-231 cells stably expressing U6-shRNA-chk compared with empty vector control cells (Fig. 7B-C).

**Discussion**

Here we have shown, for the first time, that the use of siRNA specific for choline kinase provides a valuable strategy to specifically and efficiently target choline kinase in breast cancer cells. At least three isoforms of choline kinase are known to exist in mammalian cells, which are encoded by two separate genes: ck-$\alpha$ and ck-$\beta$ (29). The two functional isoforms CK-$\alpha$1 and CK-$\alpha$2 are derived from the same gene ck-$\alpha$ by alternative splicing (29) with homodimeric or heterodimeric forms of choline kinase conferring enzymatic activity (29). Choline kinase can be

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**Figure 5.** A, number of lipid droplets visualized by Nile red staining (red) following siRNA-chk treatment in nonmalignant MCF-12A cells and malignant MDA-MB-231 breast cancer cells. Nuclei (green). Bar, 10 $\mu$m. B, quantitation of the number of lipid droplets from 20 to 22 cells obtained from four independent experiments following siRNA-chk treatment in nonmalignant MCF-12A cells and malignant MDA-MB-231 cells. C, quantitation of the number of lipid droplets obtained from MDA-MB-231 cells with stably knocked down choline kinase compared with wild-type and empty-vector control cells. ##, $P < 0.01$, compared with MCF-12A; ###, $P < 0.001$, compared with wild-type MDA-MB-231; ***, $P < 0.001$, compared with control vector.

**Figure 4.** A, representative $^1$H magnetic resonance spectra and (B) quantitation of the Fmix/phosphatidylcholine ratio from these $^1$H magnetic resonance spectra ($n = 3$) obtained from lipid cell extracts of nonmalignant MCF-12A cells and malignant MDA-MB-231 breast cancer cells treated with siRNA-chk. C, Fmix/phosphatidylcholine ratio from $^1$H magnetic resonance spectra ($n = 3$) of MDA-MB-231 cells with stably knocked down choline kinase (Chk) compared with empty vector control. Abbreviation: PtdCho, phosphatidylcholine. Columns, mean; bars, $\pm$ SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 

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down-regulated efficiently by RNAi, because the mRNA sequences transcribed from the chk-α and chk-β genes allow for the design of a single siRNA capable of down-regulating both of these choline kinase mRNAs, as was done in our studies. The importance of choline kinase in malignant transformation and progression is apparent from RNAi knockdown of choline kinase increasing differentiation and reducing proliferation of breast cancer cells. These results are also consistent with data from pharmacologic inhibition of choline kinase in breast cancer cells, which resulted in decreased proliferation and tumor xenograft growth (3).

Characterization of a panel of HMEC lines representing nonmalignant MCF-12A cells and malignant MCF-7, MDA-MB-231, and MDA-MB-435 breast cancer cells revealed that changes in choline kinase levels matched the changes in proliferation and differentiation markers in these cells. First, consistent with previous studies (2), we detected increased choline kinase protein expression levels in malignant MCF-7, MDA-MB-231, and MDA-MB-435 breast cancer cell lines compared with the nonmalignant MCF-12A HMEC line. Choline kinase expression levels increased with progressively increasing malignancy in these human breast epithelial cell lines. Second, the expression levels of proliferation markers, such as PCNA (46, 47) and Ki-67 (48), also matched choline kinase expression levels, suggesting a causal relationship between choline kinase levels and proliferation. These data are in excellent agreement with previous studies showing that activation of choline kinase is a critical requirement for proliferation of HMECs (3). Choline kinase was shown to play an essential role in the proliferation of primary HMECs by stimulating DNA synthesis and promoting G1 → S transition of the cell cycle (3). In malignant MCF-7 breast cancer cells, choline kinase activation was required during growth factor stimulation with heregulin to promote DNA synthesis (3). We also showed, for the first time, that choline kinase expression levels matched galectin-3 expression levels in the nonmalignant MCF-12A cell line and malignant MCF-7, MDA-MB-231, and MDA-MB-435 breast cancer cell lines representing

Figure 6.

A, proliferation rate of MDA-MB-231 cells containing stably knocked down choline kinase (Chk) compared with vector control. Points, mean; bars, ± SE. For comparison, PCNA immunoblotting was done in a panel of human breast epithelial cell lines exhibiting different degrees of malignancy. C, PCNA immunoblotting in MDA-MB-231 cells stably expressing U6-shRNA-chk compared with control vector cells. D, densitometry of Western blots showed semiquantitative fold changes relative to nonmalignant MCF-12A cells (n = 3). Relative fold changes in PCNA were normalized to the PCNA immunoreactive band in nonmalignant MCF-12A cells, which was set to 1. Columns, mean; bars, SD. E, Ki-67 immunoblotting of the nonmalignant MCF-12A HMEC line, the poorly invasive malignant MCF-7 breast cancer cell line, compared with the highly invasive malignant breast cancer cell lines MDA-MB-231 and MDA-MB-435. F, Ki-67 protein expression levels in MDA-MB-231 cells stably expressing U6-shRNA-chk compared with control vector. Actin was served as a loading control in all blots. G, densitometry of Western blots showed semiquantitative changes relative to nonmalignant MCF-12A cells (n = 3). Relative fold changes in Ki-67 were normalized to the Ki-67 immunoreactive band in MCF-12A cells, which was set to 1. Columns, mean; bars, SD. *, P < 0.05; **, P < 0.01. Abbreviation: ns, not significant compared with MCF-12A.
mediated choline kinase down-regulation on choline kinase, several growth factors (28, 51). The negligible effect of siRNA-chk be a second messenger or mediator for the mitogenic activity of Phosphocholine itself has been reported to be mitogenic and may with transient and stable down-regulation of choline kinase. The functional outcome of this down-regulation was evident breast cancer cells but not in the nonmalignant MCF-12A HMEC kinase mRNA and protein levels in malignant MDA-MB-231 expression by RNAi resulted in significantly reduced choline and a reduction of proliferation, were detected following RNAi-mediated down-regulation of choline kinase. Both transient and stable knockdown of choline kinase resulted in an accumulation of triacylglycerol-containing intracellular lipid droplets in malignant MDA-MB-231 cells and nonmalignant MCF-12A cells. This increase in the number of intracellular lipid droplets was more pronounced following transient down-regulation of choline kinase levels in MDA-MB-231 cells using siRNA-chk treatment compared with sustained down-regulation in MDA-MB-231 cells stably expressing the U6-shRNA-chk vector and may be due to cells adapting to sustained choline kinase down-regulation. Cytosolic triacylglycerol-containing lipid droplets are used by the mammary gland for the production of milk (54) and are therefore a marker of differentiation in human breast epithelial cells (41, 42). They are the precursors for milk fat globules, which are a major component of milk secreted by HMECs during lactation (54, 55). Down-regulation of choline kinase increases the production of these triacylglycerols, which are stored in intracellular lipid droplets in breast cancer cells and HMECs. Upon choline kinase down-regulation, 1,2-diaclyglycerol may be used in the Kennedy pathway to a much lesser extent because of low choline kinase and phosphocholine levels. This excess 1,2-diaclyglycerol in choline kinase knockdown clones may be used for the synthesis of triacylglycerol by acyl-CoA/diaclyglycerol acyltransferase (EC 2.3.1.20; refs. 56, 57) and stored in the form of lipid droplets to potentially detoxify cells from excess 1,2-diaclyglycerol. Tetraphosphonium-based cationic lipophilic chemotherapeutic agents were also shown to increase lipid droplet formation in breast cancer cells (58–60), and a strong correlation was observed between magnetic resonance–detectable triacylglycerol accumulation and cytoplasmic lipid droplets (58–60). Another possible explanation of our data may be that triacylglycerol-containing lipid droplets increased following RNAi-mediated choline kinase knockdown to provide cells with a potential reservoir of energy (60). The changes in lipid droplet formation are also consistent with the significant increase in the mixed fatty acid signal (Fmix) at 1.3 ppm following RNAi of choline kinase. Because it is possible to detect signal from mixed fatty acids in proton spectra obtained noninvasively in vivo, monitoring an increase of this mixed fatty acid signal in vivo may be yet another potential approach to detect choline kinase down-regulation. The differentiation of malignant breast cancer cells following RNAi-mediated down-regulation of choline kinase was additionally supported by the reduction of galectin-3 levels in malignant MDA-MB-231 cells following RNAi.

Breast cancer cell proliferation was significantly slower upon down-regulation of choline kinase in malignant MDA-MB-231 cells, which was further supported by the reduction of proliferation.

different degrees of malignancy. Galectin-3 is involved, among other pathways, in cell adhesion, migration, differentiation, tumor progression, and metastasis (43, 44, 49, 50) in human breast epithelial cells. Collectively, these data support a strong correlation between malignancy and choline kinase expression in breast cancer cells.

Both transient and stable down-regulation of choline kinase expression by RNAi resulted in significantly reduced choline kinase mRNA and protein levels in malignant MDA-MB-231 breast cancer cells but not in the nonmalignant MCF-12A HMEC line. The functional outcome of this down-regulation was evident in the low phosphocholine and total choline-containing compound signals observed in magnetic resonance spectra of cells with transient and stable down-regulation of choline kinase. Phosphocholine itself has been reported to be mitogenic and may be a second messenger or mediator for the mitogenic activity of several growth factors (28, 51). The negligible effect of siRNA-chk-mediated choline kinase down-regulation on choline kinase, phosphocholine, and total choline-containing compound levels in nonmalignant MCF-12A HMECs was most likely due to the low basal choline kinase expression and consequently the low phosphocholine levels in these cells. Changes in phosphocholine were detected in high-resolution extract 1H magnetic resonance spectra, which may not be possible to detect in vivo; total choline-containing compounds on the other hand are easily detected in vivo (52). The reduction in total choline-containing compounds in our in vitro study suggests that 1H MRS imaging techniques (53) can potentially be used to detect the effect of choline kinase inhibition in vivo at the preclinical and clinical level.

Two major phenotypic changes, an increase in differentiation and a reduction of proliferation, were detected following RNAi-mediated down-regulation of choline kinase. Both transient and stable knockdown of choline kinase resulted in an accumulation of triacylglycerol-containing intracellular lipid droplets in malignant MDA-MB-231 cells and nonmalignant MCF-12A cells. This increase in the number of intracellular lipid droplets was more pronounced following transient down-regulation of choline kinase levels in MDA-MB-231 cells using siRNA-chk treatment compared with sustained down-regulation in MDA-MB-231 cells stably expressing the U6-shRNA-chk vector and may be due to cells adapting to sustained choline kinase down-regulation. Cytosolic triacylglycerol-containing lipid droplets are used by the mammary gland for the production of milk (54) and are therefore a marker of differentiation in human breast epithelial cells (41, 42). They are the precursors for milk fat globules, which are a major component of milk secreted by HMECs during lactation (54, 55). Down-regulation of choline kinase increases the production of these triacylglycerols, which are stored in intracellular lipid droplets in breast cancer cells and HMECs. Upon choline kinase down-regulation, 1,2-diaclyglycerol may be used in the Kennedy pathway to a much lesser extent because of low choline kinase and phosphocholine levels. This excess 1,2-diaclyglycerol in choline kinase knockdown clones may be used for the synthesis of triacylglycerol by acyl-CoA/diaclyglycerol acyltransferase (EC 2.3.1.20; refs. 56, 57) and stored in the form of lipid droplets to potentially detoxify cells from excess 1,2-diaclyglycerol. Tetraphosphonium-based cationic lipophilic chemotherapeutic agents were also shown to increase lipid droplet formation in breast cancer cells (58–60), and a strong correlation was observed between magnetic resonance–detectable triacylglycerol accumulation and cytoplasmic lipid droplets (58–60). Another possible explanation of our data may be that triacylglycerol-containing lipid droplets increased following RNAi-mediated choline kinase knockdown to provide cells with a potential reservoir of energy (60). The changes in lipid droplet formation are also consistent with the significant increase in the mixed fatty acid signal (Fmix) at 1.3 ppm following RNAi of choline kinase. Because it is possible to detect signal from mixed fatty acids in proton spectra obtained noninvasively in vivo, monitoring an increase of this mixed fatty acid signal in vivo may be yet another potential approach to detect choline kinase down-regulation. The differentiation of malignant breast cancer cells following RNAi-mediated down-regulation of choline kinase was additionally supported by the reduction of galectin-3 levels in malignant MDA-MB-231 cells following RNAi.

Breast cancer cell proliferation was significantly slower upon down-regulation of choline kinase in malignant MDA-MB-231 cells, which was further supported by the reduction of proliferation.
markers following RNAi. These results also compare well with recent findings that choline kinase is required for breast tumor progression, and that pharmacologic inhibition of choline kinase reduced tumor growth in an animal xenograft model of breast cancer.

In conclusion, specific down-regulation of choline kinase in malignant breast cancer cells induced profound alterations in proliferation and differentiation. The increase in differentiation and decrease of proliferation strongly support the development of therapeutic RNAi strategies targeting choline kinase (31) in breast cancer. Finally and equally importantly, such a therapeutic strategy would lend itself easily to imaging and characterization by magnetic resonance techniques in cells, in preclinical models in vivo, and, potentially, in the clinical setting.

Acknowledgments

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