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Research Article

Cancer Prevention Research

A Novel Mechanism of Indole-3-Carbinol Effects on Breast Carcinogenesis Involves Induction of Cdc25A Degradation

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Abstract

The natural compound indole-3-carbinol (I3C; found in vegetables of the genus *Brassica*) is a promising cancer prevention or therapy agent. The cell division cycle 25A (Cdc25A) phosphatase is overexpressed in a variety of human cancers and other diseases. In the present study, I3C induced degradation of Cdc25A, arrest of the G_1 cell cycle, and inhibition of the growth of breast cancer cells. We also showed that the Ser124 site of Cdc25A, which is related to cyclin-dependent kinase 2, is required for I3C-induced degradation of Cdc25A in breast cancer cells, and that interruption of the ATM-Chk2 pathway suppressed I3C-induced destruction of Cdc25A. Our *in vivo* studies of different mutated forms of Cdc25A found that the mutation $Cdc25A^{S124A}$ (Ser124 to Ala124), which confers resistance to I3C-induced degradation of Cdc25A, attenuated I3C inhibition of breast tumorigenesis in a mouse xenograft model. The present *in vitro* and *in vivo* studies together show that I3C-induced activation of the ATM-Chk2 pathway and degradation of Cdc25A represent a novel molecular mechanism of I3C in arresting the G_1 cell cycle and inhibiting the growth of breast cancer cells. The finding that I3C induces Cdc25A degradation underscores the potential use of this agent for preventing and treating cancers and other human diseases with Cdc25A overexpression. *Cancer Prev Res; 3(7); 818–28. ©2010 AACR.*

Introduction

Breast cancer accounts for the highest incidence of cancer and cancer-related deaths in women in both developed and developing countries (1). Epidemiologic studies have shown that a high dietary intake of fruits and vegetables protects against breast cancer (2). Among vegetables with anticarcinogenic properties, the cruciferous vegetable family including broccoli, cabbage, brussels sprouts, and cauliflower seems to be most effective at reducing the risk of cancers (3). Indole-3-carbinol (I3C), a common phytochemical in the human diet, is present in almost all members of the cruciferous vegetable family. There is growing evidence that the nutritional supplement factor I3C is effective in the treatment of intraepithelial neoplasia with few observed side effects (4).

In vitro, I3C has been shown to suppress the proliferation of various tumor cells including breast cancer (5, 6). This effect seems to result primarily from I3C induction of a G_1 cell cycle arrest of both estrogen-responsive and unresponsive human breast cancer cells (6–8). I3C has been shown to target multiple pathways in G_1-S phase, such as inhibiting cyclin-dependent kinase 6 (*Cdk6*) gene expression by downregulating its promoter activity, disrupting processing of cyclin E associated with the Cdk2 protein complex, and regulating p21 in a p53-dependent manner (7, 9, 10). Despite compelling evidence for the potent anticarcinogenic properties of this indole, the precise molecular mechanism(s) underlying the striking cell cycle effects of this phytochemical on neoplastic cells are still unclear.

Eukaryotic cellular growth relies on the activation of Cdk-cyclin protein complexes that function at specific stages of the cell cycle. Deregulated cell cycle progression is a hallmark of cancer (11). The cell division cycle 25A (Cdc25A) phosphatase functions as a critical regulator of cell cycle progression by activating Cdks (12–15). *Cdc25A* was identified as a potential human oncogene (16) and is overexpressed in a variety of human cancers (17–21). It was found that approximately 50% of breast cancer cases exhibit Cdc25A overexpression, which is indicative of a poor prognosis (19).

Degradation of Cdc25A in response to stress has been proposed as a major way of delaying cell cycle progression. Cdc25A is rapidly degraded in a proteasome-dependent manner in cells exposed to UV light, hydroxyurea, or ionizing radiation (12, 22, 23). Several residues have been identified as critical for mediating Cdc25A degradation depending on the type of DNA damage incurred, including Ser76, Ser82, and Ser124 (24–26). Phosphorylation of

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Ser124 has been reported to regulate Cdc25A stability after ionizing radiation exposure (26).

The crucial roles played by Cdks and checkpoints in the control of the cell cycle make them attractive pharmacologic targets. For several years, various strategies have been proposed to directly or indirectly inhibit the activity of Cdc25A in the development of antiproliferative cancer drugs (20, 27–29), but their application has been limited due to the safety of the reagents. In this study, we exploit a novel and uncovered connection between I3C and degradation of Cdc25A in breast cancer cells, and therefore hypothesized that Cdc25A degradation by I3C could be an important approach for the prevention and treatment of breast cancer and other human diseases related to overexpression of Cdc25A.

Materials and Methods

Cell culture and cell growth inhibition

The human breast cancer cell lines MCF7, MDA-MB-231, and MDA-MB-468 were obtained from the American Type Culture Collection and cultured in DMEM (Life Technologies, Inc.), supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 1 mmol/L glutamine (culture medium) in a 5% CO_2 atmosphere at 37°C. The cells were seeded at a density of 3×10^5 cells in a six-well culture dish. After 24 hours, MCF7, MDA-MB-231, and MDA-MB-468 cells were treated respectively with 200, 200, and 100 μmol/L I3C (LKT Laboratory) separately and then dissolved in DMSO; control cells were treated with 0.1% DMSO alone. The cells treated with I3C or DMSO for 1 to 5 days were harvested by trypsinization, stained with 0.4% trypan blue, and counted using a hemocytometer.

Plasmid, site-directed mutagenesis, and transfection

Full-length Cdc25A was amplified from MCF10A cells and cloned into pCDNA4/TO doxycycline (Dox)-inducible promoter (Nitrogen). The primer sequences containing the *Bam*HI and *Eco*RI sites were forward, 5′-TTAGGATCCATGGAACTGGGCCCGGAG-3′, and reverse, 5′-CCGAATTCTCAGAGCTTCTTCAGACGACTGT-3′. To generate phosphorylation-site mutants of Cdc25A, a site-directed mutagensis kit (Stratagene) was used with the following pairs of primers. For $Cdc25A^{S76A}$, 5′-GTAATCTGCAGAGAATGGGCGCCTCCGAGTCAACAGATTC-3′/5′-GAATCTGTTGACTCGGAGGCGCCCATTCTCTGCAGATTAC-3′; for $Cdc25A^{S82A}$, 5′-CTCCGAGTCAACAGATGCAGGTTTCTGTCTAGATTCTC-3/5′-GAGAATCTAGACAGAAACCTGCATCTGTTGACTCGGAG-3′; and for $Cdc25A^{S124A}$, 5′-CTCTGAAGAGGAGCCATGCTGATTCTCTTGACCATG-3′/5′-CATGGTCAAGAGAATCAGCATGGCTCCTCTTCAGAG-3′. Finally, the correct mutation derivatives were verified by DNA sequencing. To establish cell lines with inducible expression of Cdc25A and its mutation derivatives in MDA-MB-231 and MDA-MB-468, Tet-on cells were transfected using plasmid DNA (pCDNA4/TO-puro-Cdc25A) with Lipofectamine 2000 reagent according to the manufacturer's instruction (Invitrogen). The cells were selected with 1.5 μg/mL puromycin. To continually induce Cdc25A expression, the cells were cultured in DMEM with 10 ng/mL Dox.

Protein extraction and Western blot analysis

Cells were washed twice with ice-cold PBS and lysed by sonication in lysis buffer [50 mmol/L HEPES-KOH (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 2.5 mmol/L EGTA, 1 mmol/L DTT, 10 mmol/L β-glycerophosphate, 1 mmol/L NaF, 0.1 mmol/L sodium orthovanadate, 0.2 mmol/L phenylmethylsulfonyl fluoride, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 1 μg/mL pepstatin A, 10 μg/mL soybean trypsin inhibitor, 10% glycerol, and 1% NP40 or Triton X-100]. Total protein extracts dissolved in SDS sample buffer were separated on 5% to 15% gradient SDS gel and transferred onto polyvinylidene difluoride membranes (Millpore). Blots were incubated with primary antibodies to Chk2, phospho-Chk2 (Thr68) (Cell Signaling Technology), Cdk2, phospho-Cdk2 (Thr14, Tyr15), Cdk4, Cdk6, ataxia telangiectasia mutated (ATM), phospho-ATM (Ser1981), and β-actin (Santa Cruz Biotechnology) and Cdc25A monoclonal antibody (mAb) Ab-3 (NeoMarkers). After incubation of each membrane with primary antibodies, they were washed with TTBS [20 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, and 0.1% Tween 20], incubated with secondary antibodies conjugated with peroxidase, and visualized by a chemiluminescent detection system (Pierce).

Flow cytometric analyses of DNA content

The breast cell lines were plated onto six-well tissue culture dishes (BD Biosciences). Cells treated with I3C or DMSO for 48 hours were harvested by trypsinization, washed twice with PBS, and fixed in 70% ethanol. Cells were treated with 100 units/mL RNase A for 20 minutes at 37°C, then resuspended and hypotonically lysed in 1 mL of PI buffer (0.5 mg/mL propidium iodide, 0.1% sodium citrate, 0.05% Triton X-100). The percentages of cells in the G_1, S, and G_2-M phases of the cell cycle were determined by analysis with the Multicycle computer program provided by Phoenix Flow Systems.

Immunofluorescent detection of Cdc25A

MDA-MD-231 or MDA-MB-468 cells were cultured on chamber slides for 24 hours. Cdc25A expression was then continuously induced with Dox (10 ng/mL), followed by treatment with I3C for 48 hours. Cells were fixed with 10% formalin and then rinsed with PBS supplemented with 0.1% Triton X-100. The cells were incubated with Cdc25A (1:500) mAb in PBST containing 1% bovine serum albumin at 25°C for 1 hour. After being washed with PBS, cells were incubated with fluorescein-conjugated anti-mouse immunoglobulin G antibody in PBS containing 1% bovine serum albumin at 25°C for 30 minutes. The slides were washed with PBS, then coverslips with cell-side down were mounted onto the glass with antifade mounting medium (Invitrogen) and sealed with nail polish.

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Human breast carcinoma xenograft study

Six-week-old BALB/c female athymic (nu/nu) mice were inoculated s.c. in the lateral flanks with 0.1 mL of PBS solution containing 1×10^6 MDA-MB-231 or MDA-MB-468 human breast cancer cells.

The mice were divided into four groups for each tested cell line: without Dox induction and I3C treatment (Dox^-I3c^-), group 1 ($n = 3 \times 10$); without I3C treatment but with Dox induction (Dox^+I3C^-), group 2 ($n = 3 \times 10$); without Dox but with I3C treatment (Dox^-I3C^+), group 3 ($n = 3 \times 10$); and with Dox induction and I3C treatment (Dox^+I3C^+), group 4 ($n = 3 \times 10$). Sesame seed oil was used to facilitate gavage and safely avoid irritation of the esophagus, as previously reported by other studies (30). When the implants began to appear as a tumor approximately 1 week after the inoculation, the mice in the intervention group were given I3C daily (1 mg/d per mouse) by oral gavage for 6 weeks. The control mice received only sesame seed oil without I3C or Dox induction. Dox (2 mg/mL) was added into daily feeder water as soon as the cells were inoculated in the flanks of mice (data not shown; ref 31). Fresh water was replaced twice weekly.

The palpable tumor diameters were measured twice per week. Tumor volumes were calculated as $ab^2/2$ (where a is the longest diameter and b is the shortest diameter; ref. 32). The mice were sacrificed 7 to 8 weeks after cell injection. On sacrifice, tumor tissue from each mouse was harvested and cut into two pieces: one part was frozen for molecular analysis and the other part was fixed in formalin and embedded in paraffin for immunohistochemical staining.

Statistical analysis

The statistical significance of differential findings between the experimental and control groups was determined by Student's t test as implemented by Excel 2000 (Microsoft Corp.), and $P < 0.05$ was considered significant.

Results

I3C inhibits cell proliferation of breast cancer cells through a G_1 cell cycle arrest

To dissect the connection between I3C treatment and G_1 cell cycle progression in breast cancer cells, we assayed levels of the G_1 Cdks and monitored cell cycle arrest in the MCF7, MDA-MB-231, and MDA-MB-468 breast cancer cell lines. In tumorigenic MCF7 breast cancer cells, we found that I3C inhibits the expression of Cdk6 (Fig. 1A) and induces a G_1 cell cycle arrest of MCF7 cells and cell growth inhibition (Fig. 1D and G), consistent with the previous report that I3C decreased the levels of Cdk6 (7). In MDA-MB-231 and MDA-MB-468 cells, either treated with I3C or untreated, the protein levels of Cdk6 are not altered (Fig. 1B and C), and fluorescence-activated cell sorting analysis and assay for cell growth showed that I3C still induces a G_1 cell cycle arrest of these breast cancer cells (Fig. 1E and F) and inhibits cell growth (Fig. 1H and I). It has been shown that Cdk6 activity positively connects with the levels of Cdk6 protein in the breast cancer cells (6, 7, 33); Cdk6 plays an important role in cell cycle by titration of binding Cdk inhibitors (tumor suppressors) without considering its activity (34). This difference in the change in Cdk6 protein level in different breast cancer cell lines indicated that the I3C inhibition of G_1 cell cycle progression and the proliferation of breast cancer cells may be mediated by an as yet undiscovered molecular mechanism, at least in the aggressive breast cancer MDA-MB-231 and MDA-MB-468 cell lines.

I3C induces a decrease in Cdc25A protein in human breast cancer cells

To elucidate the potential mechanism of the antiproliferative activity of I3C on breast cancer during the G_1 phase of the cell cycle, we next investigated its effect on the expression levels of proteins involved in the regulation of G_1 phase. Western blot analyses revealed that I3C treatment did not alter the levels of Cdks and their inhibitors such as p21, p27, and Ink4a/arf (data not shown), but dramatically reduced the level of Cdc25A protein in MDA-MD-231 and MDA-MD-468 breast cancer cells (Fig. 2A and B). Immunofluorescence staining with anti-Cdc25A mAb confirmed that I3C led to a reduction in Cdc25A protein (Fig. 4A). It was also observed that I3C treatment results in the reduced expression of Cdc25A in the MCF7 cell line (Fig. 2C) and in other breast cancer cells (data not shown).

Cdc25A mainly activates the Cdk2-cyclin E and Cdk2-cyclin A complexes through dephosphorylation of the Thr14 and Tyr15 residues within the ATP binding loop of Cdk2 (35). Western blotting confirmed that the reduced protein levels of Cdc25A attenuated the dephosphorylation of its downstream Cdk2 and thus reduced Cdk2 activity in the breast cancer cells without altering its expression (Fig. 2A-C).

Ser124 of Cdc25A is required for I3C-induced Cdc25A degradation

As an important cell cycle regulator, Cdc25A has been linked to oncogenic transformation and approximately 50% of human breast cancer cases (19, 20, 36). To explore the mechanism by which I3C targets Cdc25A, we used real-time PCR and Northern blotting to determine the levels of Cdc25A mRNA in the breast cancer cells with and without I3C treatment. Our results showed insignificant changes in the Cdc25A mRNA level between the I3C-treated and untreated breast cancer cells (data not shown). Thus, we hypothesized that the reduced level of Cdc25A protein in the I3C-treated breast cancer cells was due to degradation of Cdc25A protein, a mechanism that may play an important role in the antitumorigenic effects of I3C on human breast cancer.

Cdc25A is regulated by dual-mode degradation: Skp1-Cullin-β-TrCP and Apc^{Cdh1} (24, 37). We initially investigated any change in the response of the above factors to I3C that might lead to alteration of the Cdc25A protein levels. We performed Western blotting with each antibody

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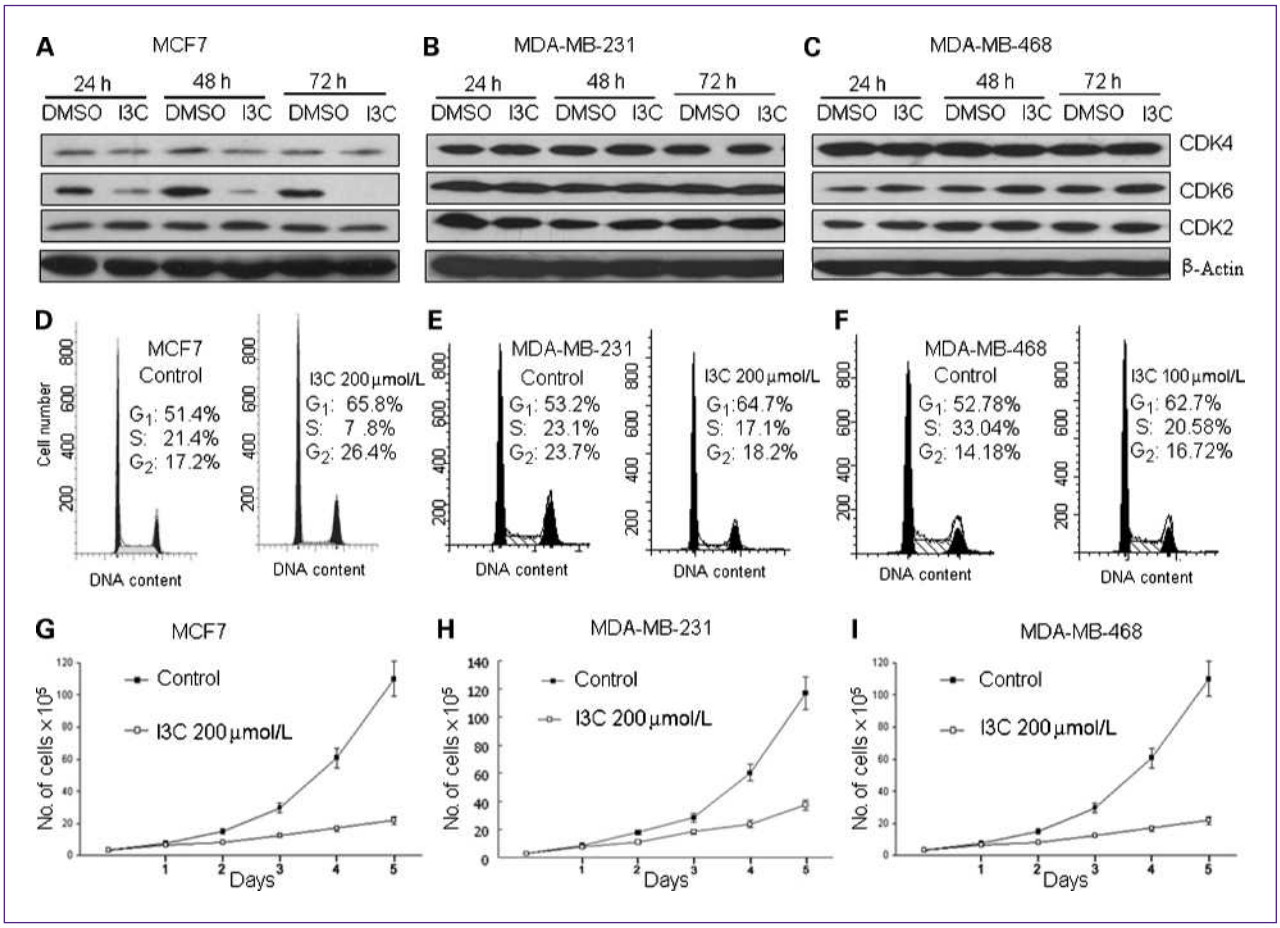

Fig. 1. Analysis of I3C-induced profile of Cdks in different breast cancer cells, G1 cell cycle arrest, and cell growth inhibition. A to C, profiles of the I3C-induced G_1 Cdks. MCF7, MDA-MB-231, and MDA-MB-468 breast cancer cells were treated with 0.1% DMSO alone or with 100 to 200 μmol/L I3C for 24, 48, and 72 h. Cell lysates were prepared and Western blot analysis was done with the indicated antibodies. Representative data from one experiment are shown ($n = 5$). D to F, analysis of cell cycle of the breast cancer cells. The cells, treated with either 0.1% DMSO alone or with I3C, were harvested in PBS and stained with a hypotonic solution containing propidium iodide. Stained nuclei were subjected to flow cytometry analysis. G to I, cell growth inhibition by I3C. The breast cancer cells were treated with 100 to 200 μmol/L I3C and control cells with 0.1% DMSO and then harvested by trypsinization; cell numbers were determined. The number of living cells was plotted versus the days of I3C exposure. Points, mean ($n = 5$); bars, SEM. Differences between solvent control and treatment are significant ($P < 0.05$).

for Skp1-Cullin-β-TrCP, Apc^{Cdh1}, and ubiquitin and did not find any insignificant change in the above protein levels in treatments with and without I3C. Thus, we further investigated Cdc25A itself. Regulation of Cdc25A levels through the cell cycle requires phosphorylation at multiple sites by different kinases and the presence of intact recognition motifs on Cdc25A including Ser76, Ser82, and Ser124 (24–26), which are related to the degradation pathways mediated by Chk1, Smad3, and Chk2, respectively (Fig. 3A). To investigate the response of different phosphorylation sites and motifs in Cdc25A to I3C, we developed a Tet-on system with Dox-inducible expression of either human $Cdc25A^{WT}$ or the mutation derivatives $Cdc25A^{S76A}$, $Cdc25A^{S82A}$, or $Cdc25A^{S124A}$ in the breast cancer cells (Fig. 3A and B).

We next assayed the protein stability of $Cdc25A^{WT}$ and its derivatives $Cdc25A^{S76A}$, $Cdc25A^{S82A}$, and $Cdc25A^{S124A}$ in the breast cancer cells with or without I3C treatment. Western blot showed that the $Cdc25A^{S124A}$ mutant was resistant to I3C-induced degradation in MDA-MD-231 cells (Fig. 3C). Conversely, the $Cdc25A^{WT}$ and the mutant $Cdc25A^{S82A}$ (related to Samd3) proteins in the I3C-treated cells were rapidly degraded (Fig. 3C). It was noted that the mutant $Cdc25A^{S76A}$ (related to Chk1) was degraded by I3C, but the degraded fraction is much lower compared with that of $Cdc25A^{WT}$ and mutant $Cdc25A^{S82A}$ (Fig. 3C). Similar results were observed for p53-deficient MDA-MB-468 cells (Fig. 3D). The levels of $Cdc25A^{WT}$ and its mutation derivatives in I3C-treated and untreated cells were examined by direct immunofluorescence staining with Cdc25A antibody. As shown in Fig. 4, without I3C treatment, most cells with $Cdc25A^{WT}$ or its derivatives had high levels of Cdc25A; in contrast, $Cdc25A^{WT}$ and $Cdc25A^{S82A}$ in the cells with I3C treatment were absent (less stained),

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but Cdc25A^{S124A} is stable in the treated cells (Fig. 4D). The breast cancer cells with the mutation Cdc25A^{S124A} did not show I3C-induced G_1 cell cycle arrest or inhibition of cell growth (data not shown). These results suggest that the Ser124 site of Cdc25A (related to Chk2) is required for I3C-induced Cdc25A degradation, and that cell cycle arrest mediated by Chk2-induced degradation of Cdc25A represents an important mechanism of response to I3C treatment."

I3C activates the ATM-Chk2-Cdc25A signaling pathway

It has been previously reported that Cdc25A is phosphorylated on Ser124 by Chk2 kinase in response to ionizing radiation (26). This modification induces a rapid, proteasome-dependent degradation of Cdc25A and subsequent silencing of Cdk2-cyclin A and Cdk2-cyclin E kinase activity (12, 13, 15). Considering that Cdc25A is a key target of negative regulation by various checkpoint and stress pathways and that phosphorylation of Ser124 is important to the stability of the protein, we wanted to test whether the upstream signaling pathway of Chk2 mediates the destabilization of Cdc25A observed with I3C treatment. Western blotting was done on extracts from MDA-MB-231 cells treated with I3C and 0.1% DMSO for 24, 48, or 72 hours. I3C treatment was found to significantly induce phosphorylation of ATM at Ser1981 (Fig. 5), a residue that has been shown to regulate ATM activity (38). This activated form of ATM then activates downstream Chk2 kinase by phosphorylation of its Thr68 (Fig. 5; ref. 39). To confirm that ATM phosphorylation is related to Chk2-Cdc25A in I3C-regulated G_1-S phase arrest, we examined the effects of ATM inhibition on Cdc25A protein stability in the breast cancer cells. Cdc25A protein was degraded efficiently by treatment with I3C (Figs. 2 and 5). However, addition of the ATM inhibitor KU55933, which blocks the activity of ATM and its downstream Chk2, prevents Cdc25A from being degraded efficiently (Fig. 5). Similar results were observed using MDA-MB-468 breast cancer cells (data not shown).

These studies indicate that Chk2-mediated control over the basal turnover of Cdc25A is ATM dependent, and that the ATM-Chk2-Cdc25A pathway is required for the accelerated destruction of Cdc25A with I3C treatment (Figs. 3–5).

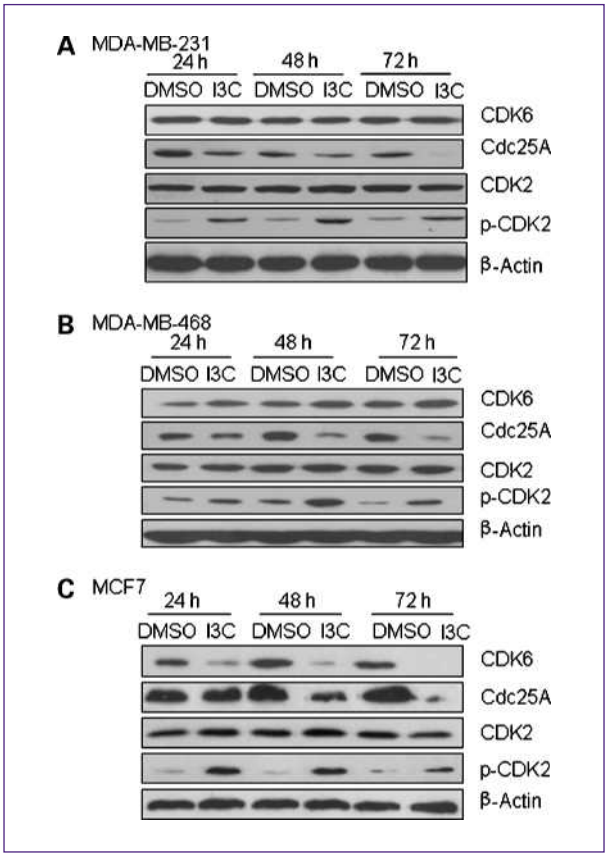

Fig. 2. I3C reduces the level of Cdc25A protein in breast cancer cells. MDA-MB-231 (A), MDA-MB-468 (B), and MCF7 (C) breast cancer cells were treated with 0.1% DMSO alone or with 100 to 200 μmol/L I3C for 24, 48, and 72 h. Cell lysates were prepared and Western blot analysis was done with the indicated antibodies. Cdk6 was reduced only in the I3C-treated MCF7 cells, whereas reduced levels of Cdc25A were observed in all cell types with I3C treatment, which attenuated the dephosphorylation of its downstream Cdk2. Representative data from one experiment are shown (n = 3).

Mutation Cdc25A^{S124A} confers resistance to the antitumorigenic effect of I3C on breast cancer cells in a xenograft model

The above cell studies revealed that mutation of Cdc25A at Ser124 (abolishing the Chk2-Cdc25A regulatory pathway) blocked I3C-induced Cdc25A degradation. To determine whether the deficiency in the degradation mechanism would affect the tumorigenesis of the transplanted breast cancer cells *in vivo*, we further investigated the effect of abolishing the Chk2-Cdc25A pathway on the growth and development of breast cancer cells transplanted into BALB/c female athymic mice at 6 weeks of age. The mice were then divided into four groups for each of the tested cell lines: without Dox induction or I3C treatment (Dox$^-$I3c$^-$), group 1; with Dox induction but without I3C treatment (Dox$^+$I3C$^-$), group 2; without Dox induction but with I3C treatment (Dox$^-$I3c$^+$), group 3; and with Dox induction and I3C treatment (Dox$^+$I3c$^+$), group 4 (Fig. 6; Table 1). The previous studies showed that breast cancer cells with Cdc25A^{WT} and its derivatives Cdc25A^{S76A}, Cdc25A^{S82A}, and Cdc25A^{S124A} transplanted in the flanks of mice could be effectively induced by addition of 2 mg/mL Dox dissolved in daily feeder water for the mice (our data not shown; ref. 31). The majority of implants appeared as tumor 1 week after the mice were injected with 1×10^6 different cells into the flanks. The mice in the intervention group were further given I3C (1 mg/d per mouse) daily by oral gavage as described in Materials and Methods. The palpable tumor diameters were measured twice per week. Tumor volumes were calculated as $ab^2/2$ (where a is the longest diameter and b is the shortest diameter; ref. 32).

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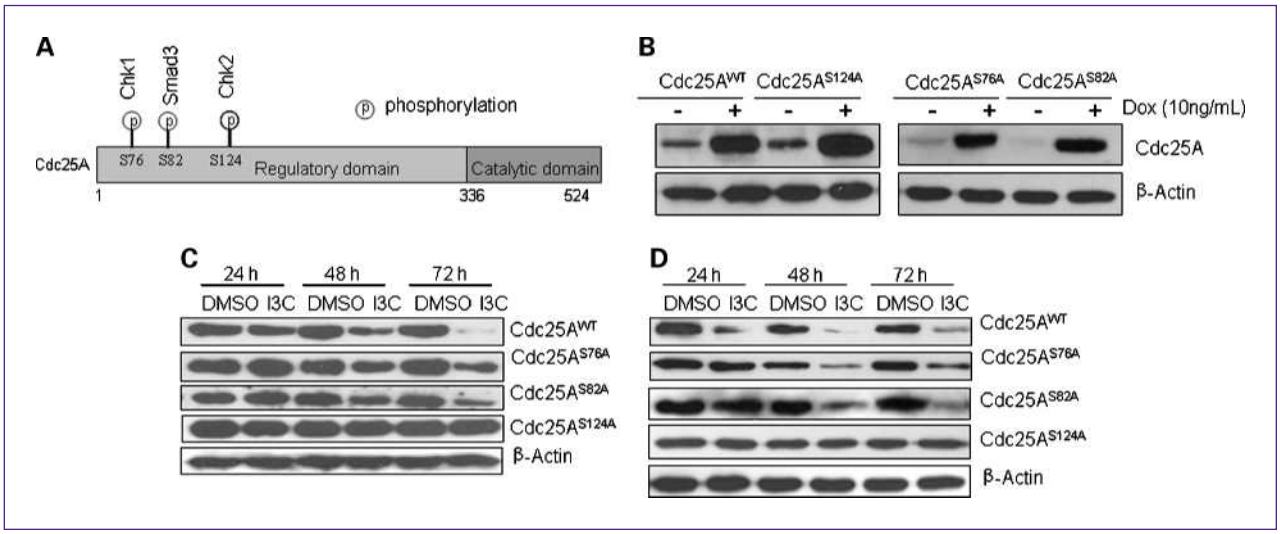

Fig. 3. The Ser124 site of Cdc25A is required for Cdc25A degradation in response to I3C induction. A, a sketch for multiple pathways related to Chk1, Smad3, and Chk2 regulating Cdc25A degradation (23–25). B, Dox-induced expression of Cdc25A and its derivatives. The wild-type Cdc25A cDNA from MCF10A cells was cloned into the pCDNA4/TO vector with Tet-on system, and each mutated derivative, $Cdc25A^{S76A}$, $Cdc25A^{S82A}$, and $Cdc25A^{S124A}$, in the vectors was constructed according to the procedures described in Materials and Methods. In cultured medium with 10 ng/mL Dox, Cdc25A and its derivatives were effectively induced. C and D, response of the mutant Cdc25A derivatives to I3C treatment in MDA-MB-231 breast cancer cells (C) and MDA-MB-468 breast cancer cells (D). Cell lysates were prepared and Western blot analysis was done with the indicated Cdc25A and control antibodies. Representative data from one experiment are shown ($n = 3$).

As expected, at termination of the study after 7 weeks, the tumor size observed with I3C treatment (group 3, Dox^-I3C^+) was significantly reduced (Fig. 6A and B) compared with the controls without I3C treatment (group 1, Dox^-I3c^-). No exogenous Cdc25A or $Cdc25A^{S76A}$, $Cdc25A^{S82A}$, or $Cdc25A^{S124A}$ was induced in the absence of Dox in group 1 or in group 3, and thus they did not contribute to differences in tumor formation and size between the different cell lines for those two groups (data not shown). Without I3C treatment, the tumor size from the cells in group 2 (Dox^+I3C^-) with Dox induction was slightly bigger compared with tumors from the cells with endogenous Cdc25A (group 1, Dox^-I3C^-) in the absence of Dox. In contrast, I3C can significantly reduce the tumor size from cancer cells with endogenous Cdc25A (group 2, Dox^-I3C^+) and from cells expressing exogenous $Cdc25A^{WT}$ and $Cdc25A^{S82A}$ (group 4, Dox^+I3C^+; $P < 0.005$), but not from cells with $Cdc25A^{S124A}$ (Fig. 6A and B; Table 1). The final average tumor volume for the cells with $Cdc25A^{S124A}$ (Dox^+I3C^+) with I3C treatment is similar to that of the breast cancer cells without I3C treatment (group 2, Dox^+I3C^-) and is much higher ($1{,}137 \pm 156$ mm^3) than that of the $Cdc25A^{WT}$ (415 ± 103 mm^3) or $Cdc25A^{S82A}$ (376 ± 125 mm^3) controls in the Dox^+I3C^+ group (Fig. 6A and B). These data show that Cdc25A degradation, regulated by Chk2 at Ser124, contributes to a significant reduction in tumor size (G and I versus J, 64% and 67%, respectively; $P < 0.005$; Fig. 6B; Table 1). It is notable that there is a reduction in tumor size (about 27.7%) even for the mutant $Cdc25A^{S124A}$ cell line with I3C treatment (J versus E, Fig. 6B and Table 1), indicating the contribution of other potential pathways or mechanisms.

Additional results from Western blotting of the lysates and immunohistochemical staining with anti-Cdc25A mAb for the different tumors showed that the level of $Cdc25A^{S124A}$ in the tumor is much higher even when I3C treated, whereas the protein levels of the controls $Cdc25A^{WT}$ and $Cdc25A^{S82A}$ are much lower in response to I3C treatment (data not shown).

These significant differences were also observed when breast cancer MDA-MB-468 cells expressing $Cdc25A^{WT}$ or its derivatives, $Cdc25A^{S76A}$, $Cdc25A^{S82A}$, or $Cdc25A^{S124A}$, were transplanted and treated with I3C. I3C treatment led to an average of 70% decrease in tumor volume for all cells, with the exception of the $Cdc25A^{S124A}$ cells, compared with the untreated controls ($P < 0.05$; data not shown).

These results show that the mutant form $Cdc25A^{S124A}$ confers resistance to I3C-induced degradation and attenuates the effect of I3C on breast cancer cells in a rodent xenograft model. Thus, Ser124 of Cdc25A (related to Chk2) plays an important role in controlling Cdc25A protein degradation and inhibiting breast cancer cell growth *in vivo*.

Discussion

I3C, a naturally occurring component of *Brassica* vegetables such as cabbage, broccoli, and brussels sprouts, was one of the few natural products tested positive as a chemopreventive agent in a panel of short-term bioassays relevant

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to carcinogen-induced DNA damage, tumor initiation, and promotion (6–8, 40). Most of these effects seem to occur because I3C induces a G_1 cell cycle arrest (6–8) and has been shown to target multiple pathways to accomplish this, such as inhibiting *Cdk6* gene expression by downregulating its promoter activity, disrupting the processing of cyclin E associated with the Cdk2 protein complex, and regulating p21 in a p53-dependent manner (7, 9, 10). However, the molecular mechanism described above may only be applicable for some special individual mammary epithelial cells or breast cancer cell lines, such as MCF7, as it was found that I3C treatment does not alter the levels of Cdk6 protein in breast cancer MDA-MB-231 or p53-deficient MDA-MB-468 cells, but still induces a G_1 arrest of these breast cancer cells and inhibits cell growth (Fig. 1A-H). The results prompt us to investigate other as yet unknown molecular mechanisms that mediate the effect of I3C on G_1 cell cycle and proliferation of breast cancer cells.

It was further revealed that I3C induced degradation of phosphatase Cdc25A, a critical regulator of cell cycle progression and checkpoint response (26, 38), whose overexpression is found in approximately 50% of breast cancer patients and is associated with a poor prognosis (19). Cdc25A activates Cdk2 by dephosphorylating the Thr14 and Tyr15 residues within its ATP binding loop of Cdk2 (35). By accelerating the destruction of Cdc25A and thus attenuating its downstream dephosphorylation of Cdk2, I3C reduces Cdk2 activity in breast cancer cells (Fig. 2). Notably, a number of studies show the antitumor effect of reducing Cdk2 activity (but not altering total Cdk2 protein level; ref. 41), which may produce different effects than with the knockdown or knockout of total Cdk2 (34, 42). In addition, Cdk4/Cdk6 have regions of high homology to the motif GXGXY[15]GXVXKAR found in the ATP binding loop of Cdk2. Cdc25A might dephosphorylate Tyr residues of Cdk4/Cdk6

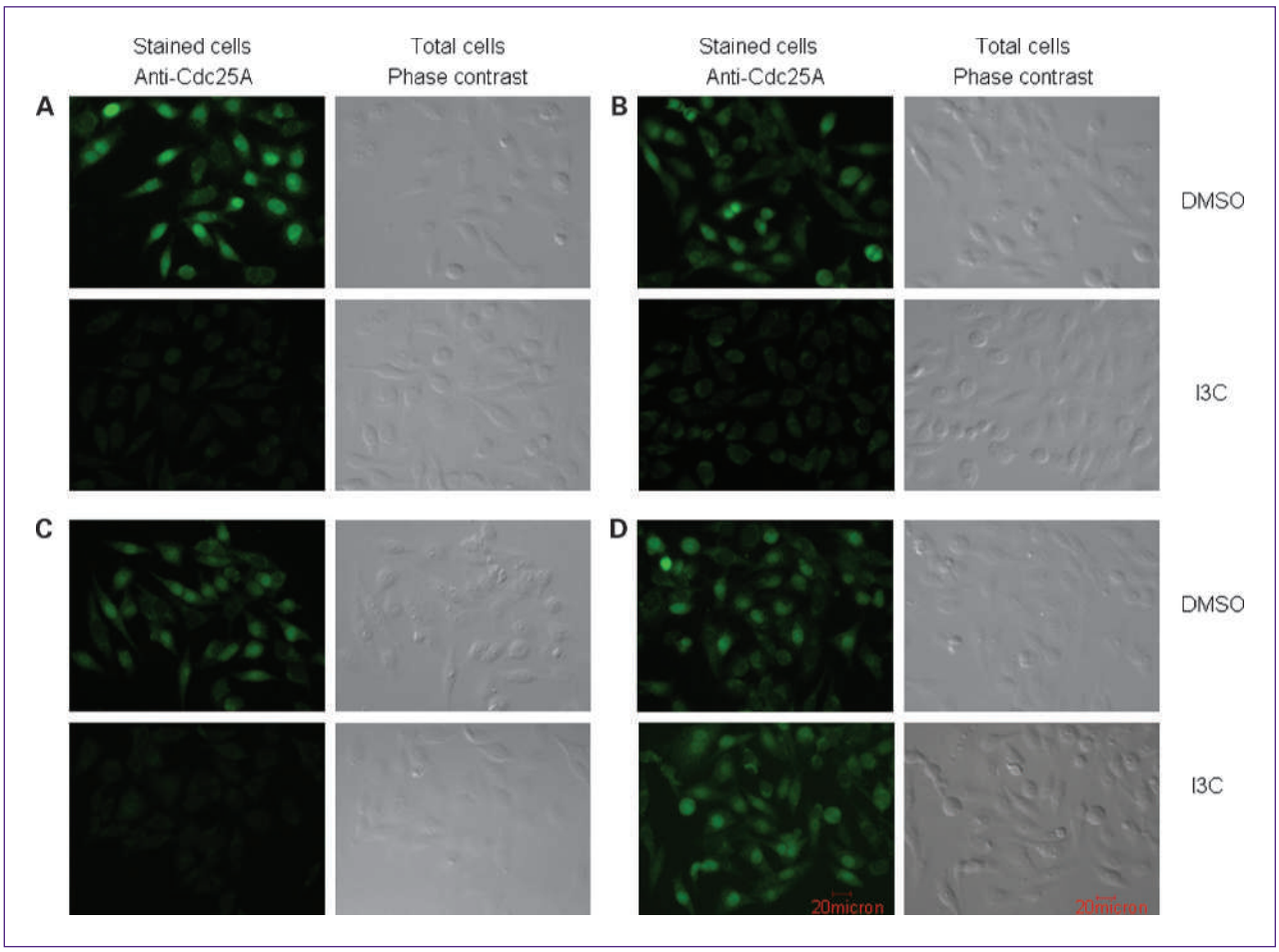

Fig. 4. Detection of protein levels of $Cdc25A^{WT}$ and its mutation derivatives in breast cancer cells with I3C treatment. $Cdc25A^{WT}$ and its derivatives continued to be induced in MDA-MD-231 breast cancer cells with 10 ng/mL Dox while the cells were treated with either 0.1% DMSO alone or with 200 μmol/L I3C for 48 h. The cells were then stained with anti-Cdc25A mAb. Signals were visualized by incubation with fluorescein-conjugated anti-mouse immunoglobulin G antibody, followed by analysis with a fluorescence microscope. The cells maintain normal cell shape (phase-contrast images) and 4′,6-diamidino-2-phenylindole staining also shows normal nuclear shape (data not shown). Results are representative of three independent sets of experiments. A, $Cdc25A^{WT}$; B, $Cdc25A^{S76A}$; C, $Cdc25A^{S82A}$; D, $Cdc25A^{S124A}$.

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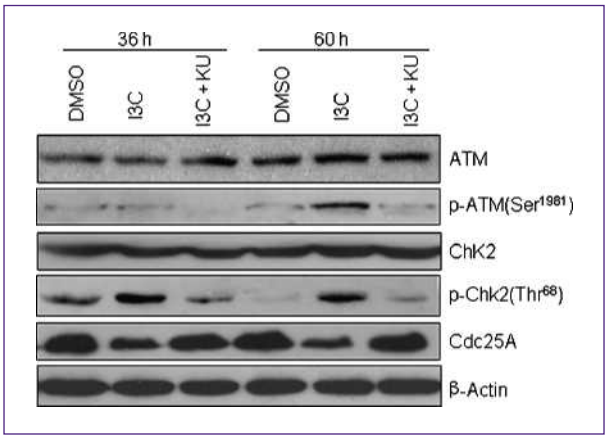

Fig. 5. I3C activates the ATM-Chk2-Cdc25A pathway in human breast cancer cells. Breast cancer MAD-MB-231 cells were treated with 0.1% DMSO alone, with 200 μmol/L I3C, or with 200 μmol/L I3C and 10 μmol/L of ATM inhibitor KU55933 (KU) for 36 and 60 h, respectively. Cell lysates were then prepared and Western blot analysis was done with the indicated antibodies. The inhibitor KU55933 can effectively block the I3C-induced phosphorylation of ATM at residue 1981, the activity of downstream Chk2 by phosphorylation at Tyr68, and the degradation of downstream Cdc25A. Representative data from one experiment are shown (n = 3).

(Tyr16 in Cdk4, Tyr24 in Cdk6) similar to Tyr15 in Cdk2. This possibility can be further assessed when phospho-specific antibodies for the Tyr of Cdk4/Cdk6 are effectively developed in the future.

Interestingly, it has been reported that I3C activates the ATM signaling pathway and induces p21 in a p53-dependent manner, and that p53 is required for the I3C-induced arrest of the immortalized but nontransformed MCF10A cells (10). However, our studies showed that I3C-induced G_1 cell cycle arrest and inhibition of cancer cell growth by the ATM-Chk2-Cdc25A pathway can be p53 independent, as shown by the results from the p53-deficient breast cancer MDA-MB-468 cells (Figs. 1–3). There may be different response mechanisms to I3C between breast cancer cells and nontransformed cells.

Several studies have shown an interaction between the indole compound and plasmid DNA (43). Through gel-retardation and fluorescence quenching experimental methods (43), our results showed that I3C could bind to DNA generated by random PCR of total genomic DNA template from breast cancer cells (data not shown). It is notable that I3C induces ATM but does not cause DNA damage (10). Although we anticipate specific *in vivo* experiments on the interaction between I3C and DNA, current studies have led us to believe that I3C binds to DNA in the cells, triggers the activity of ATM and downstream Chk2, and results in phosphorylation of Cdc25A at Ser124, and thus causes Cdc25A degradation, which leads to G_1 cycle arrest and inhibition of breast cancer cell growth independently of p53.

During the assay for ATM inhibition, we observed that the ATM inhibitor wortmannin also induces Cdc25A

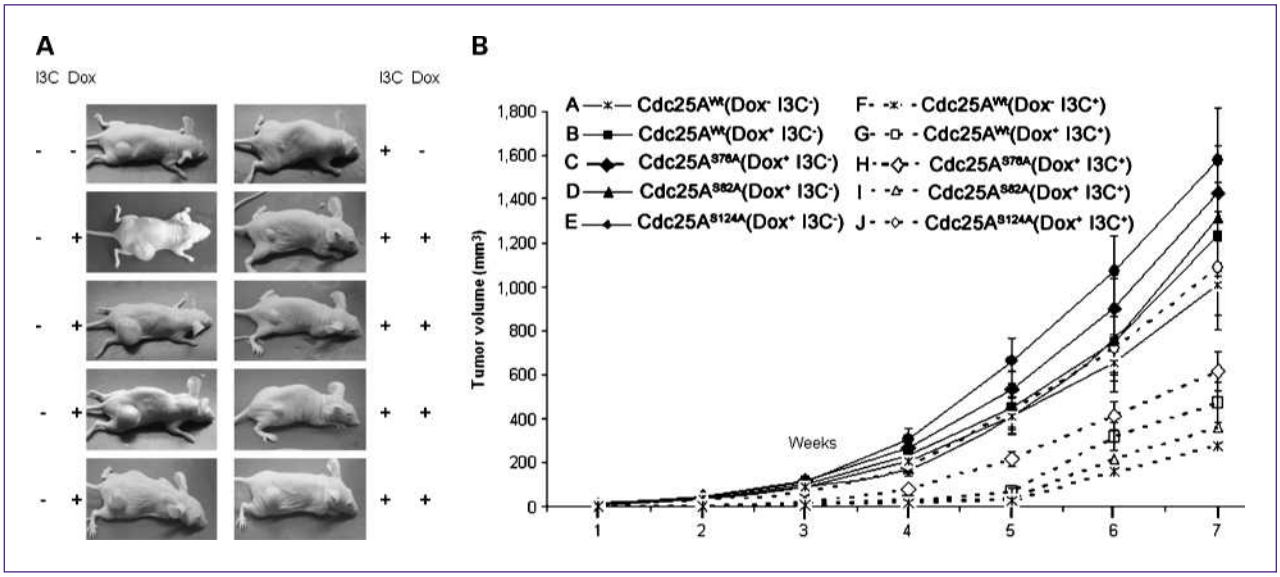

Fig. 6. The mutant Cdc25A^{S124A} is resistant to the effect of I3C on breast cancer in a mouse xenograft model. A, representative images of mice from each cell line and group, photographed at time of sacrifice. B, statistical analysis for tumor volume from each cell line and treatment group at different time points. The mice were inoculated s.c. in the lateral flanks with 0.1 mL of PBS solution containing 1 × 10^6 MDA-MB-231 human breast cancer cells. The mice in the intervention group were given I3C (1 mg/d per mouse) by oral gavage everyday for 6 wk as described in Materials and Methods. The control mice received only sesame seed oil without I3C. For Dox induction, Dox (2 mg/mL) was added into daily feeder water as soon as the cells were inoculated in the flanks of mice. Fresh water was replaced twice weekly. The mice were divided into four groups for each of the tested cell lines: without Dox induction or I3C treatment, group 1 (Dox$^-$I3C$^-$); group 2 (Dox$^+$I3C$^-$); group 3 (Dox$^-$I3C$^+$); and group 4 (Dox$^+$I3C$^+$). Each group contained 10 mice and 3 repeats. The cells and treatment methods used in each case are indicated in the figure. The palpable tumor diameters were measured and volumes were calculated twice per week. Under the experimental conditions, the mutation Cdc25A^{S124A} significantly inhibited the effect of I3C on breast cancer cell tumorigenesis in nude mice ($P < 0.05$).

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Table 1. Effects of I3C on breast cancer cells in nude mice

Group	No. of mice	Compound*	Subgroup	Cancer cells injected	%Mice with tumor	Tumor size, mm^3 (mean ± SD)	Reduction in tumor multiplicity (%)†	*P*†
1	3 × 10 each	Dox^-I3c^-	—	WT	97	1,001 ± 138	—	—
			A	$Cdc25A^{WT}$		996 ± 140	—	—
			—	$Cdc25A^{S76A}$		1,100 ± 152	—	—
			—	$Cdc25A^{S82A}$		998 ± 135	—	—
			—	$Cdc25A^{S124A}$		1,085 ± 151	—	—
2	3 × 10 each	Dox^+I3C^-	—	WT	97	1,105 ± 135	−2.8	>0.05
			B	$Cdc25A^{WT}$		1,187 ± 145	+4.4	>0.05
			C	$Cdc25A^{S76A}$		1,273 ± 162	+11.8	>0.05
			D	$Cdc25A^{S82A}$		1,234 ± 146	+8.5	>0.05
			E	$Cdc25A^{S124A}$		1,453 ± 186	+27.7	>0.05
3	3 × 10 each	Dox^-I3c^+	—	WT	94	301 ± 65	—	—
			F	$Cdc25A^{WT}$		258 ± 57	—	—
			—	$Cdc25A^{S76A}$		392 ± 71	—	—
			—	$Cdc25A^{S82A}$		318 ± 70	—	—
			—	$Cdc25A^{S124A}$		329 ± 76	—	—
4	3 × 1 0 each	Dox^+I3c^+	—	WT	97	338 ± 75	−70.2	<0.005
			G	$Cdc25A^{WT}$		415 ± 103	−64	<0.005
			F	$Cdc25A^{S76A}$		507 ± 132	−55.4	<0.005
			I	$Cdc25A^{S82A}$		376 ± 125	−67	<0.005
			J	$Cdc25A^{S124A}$		1,137 ± 156	—	—
CK	3 × 5	None	—	None	0	—	—	—

*I3C concentration is 1 mg/d per mouse; Dox (2 mg/mL) was added into daily feeder water. WT, breast cancer without exogenous Cdc25A as control. The mice were euthanized 7 wk after injecting breast cancer cells. The detailed experimental procedures are described in Materials and Methods.

†Compared with J in group 4.

degradation, similar to LY294002 (data not shown). It may be because wortmannin not only inhibits ATM but also inhibits phosphoinositide 3-kinase and DNA-PK kinases as LY294002 does (44, 45). Glycogen synthase kinase-3β inactivation has been correlated with Cdc25A overproduction in human cancer cells through its regulation of Cdc25A stability by Ser76 and Tyr80 phosphorylation (46). Conversely, it is possible that inhibition of phosphoinositide 3-kinase by LY294002 or wortmannin leads to increased downstream activity of glycogen synthase kinase-3β and, thus, reduced levels of Cdc25A in the cancer cells. Based on the above findings and hypothesis, an alternative ATM inhibitor, KU55933 (not targeting phosphoinositide 3-kinase; ref. 47), was applied to interrupt the response of the ATM-Chk2-Cdc25A pathway to I3C treatment in our studies, and the results showed that the inhibitor KU55933 specially suppressed ATM activity and blocked I3C-induced Cdc25A degradation (Fig. 5).

Following the *in vitro* studies, we tested the efficacy of I3C *in vivo* on breast cancer cells with different mutated forms of Cdc25A by injecting them into nude mice. It was shown that I3C-induced degradation of Cdc25A, regulated by Cdk2 at Ser124, significantly inhibited tumorigenesis (reduction in tumor size of about 65%; G and I versus J in Fig. 6B). In the present study, a key functional test of this pathway was the demonstration that a point mutation, $Cdc25A^{S124A}$, mimicked the cancer cell response to I3C treatment *in vitro* (Figs. 3–5), and Cdc25A degradation mediated by the Ser124 site is sufficient to arrest the growth of human breast cancer cells *in vivo* (Fig. 6).

Cdc25A overexpression has been observed in a variety of human cancers, including breast, prostate, liver, esophageal, endometrial, and colorectal cancers and non–Hodgkin lymphomas (17–21), as well as Alzheimer's disease (48). Promoting cell cycle progression could be one of the major mechanisms for the oncogenic action of Cdc25A, although Cdc25A overexpression also can inhibit apoptosis in some cellular contexts (49, 50). Thus, Cdc25A would be an attractive pharmacologic target. Recent advances in the Cdc25A field have led to the identification of anti-Cdc25A reagents for cancer treatment, but it has been difficult to predict therapeutic success due to uncertainties about safety and efficacy (29). Our finding that I3C induces Cdc25A degradation may represent an effective and safe strategy for the prevention and treatment of a variety of human cancers and other human diseases associated with the overexpression of Cdc25A and awaits further investigation.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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