

## 3,3'-Diindolylmethane inhibits breast cancer cell growth via miR-21-mediated Cdc25A degradation

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**Abstract** 3,3'-Diindolylmethane (DIM) is a potential cancer preventive phytochemical derived from *Brassica* vegetables. The effects of DIM on cell-cycle regulation in both estrogen-dependent MCF-7 and estrogen receptor negative *p53* mutant MDA-MB-468 human breast cancer cells were assessed in this study. DIM inhibited the breast cancer cell growth in vitro and in vivo, and caused cell-cycle arrest by down-regulating protein levels of cell-cycle related kinases CDK1, CDK2, CDK4, and CDK6, as well as Cyclin B1 and Cdc25A. Meanwhile, it was revealed that Ser<sup>124</sup> phosphorylation of Cdc25A is primarily responsible for the DIM-induced Cdc25A degradation. Furthermore, treatment of MCF-7 cells with DIM increased miR-21 expression and down-regulated Cdc25A, resulting in an inhibition of breast cancer cell proliferation. These observations collectively suggest that by differentially modulating cellular signaling pathways DIM is able to arrest the cell-cycle progression of human breast cancer cells.

**Keywords** 3,3'-Diindolylmethane (DIM) · Cdc25A · miR-21 · Breast cancer

### Introduction

Breast cancer accounts for the highest incidence of cancer and cancer-related deaths in women in both developed and developing countries [1]; hence, early diagnosis and prevention of this disease is urgently needed. Since early stage breast cancers are estrogen dependent for their growth and survival, surgery, chemotherapy, hormone therapy, or a selective combination of modalities have been used as the main therapeutic approach against the disease. Clinical practice reveals that the initial benefits of anti-estrogen treatment in breast cancer tend to decrease with time as hormone-independent cancer cells predominate, and estrogen-dependent tumors eventually become estrogen-independent that ultimately leads to patient mortality. At present there is no generally effective treatment for estrogen-independent breast cancer, which underscores the need for the development of novel alternative therapeutic strategies.

Many studies show a connection between diet and breast cancer, with cancer incidence inversely correlated with consumption of fruit and vegetables [2, 3]. Dietary glucosinolates present in *Brassica* species have been previously shown to protect against several types of cancer [4]. One of these phytochemicals, indole-3-carbinol (I3C) may prove to be a promising agent against cervical and in vitro breast cancers [5]. DIM is a stable condensation product of I3C, and previous studies have uncovered an anti-proliferative pathway that implicates Sp1/Sp3-induced expression of p21 as a target for cell-cycle control in human breast cancer cells [6]. In addition, results generated with in vitro cell culture studies have shown that DIM inhibits the proliferation of a variety of cancer cell types, including prostate [7] and breast [6] cancer cells, via the induction of cell-cycle arrest and apoptosis. Despite studies that have

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suggested the use of DIM as a cancer chemopreventive agent, relatively little is known about the molecular mechanisms by which DIM functions within cells.

In mammalian cells, progression of the cell cycle is mainly regulated by activities of the Cyclin–Cyclin-dependent kinase (CDK) complex. p21<sup>Waf1/Cip1</sup> and p27<sup>Kip1</sup> are members of the CIP/KIP family, which can inhibit the Cyclin–CDK complex. In addition, activation of CDKs requires Cdc25, an important phosphatase that dephosphorylates CDKs and promotes cell-cycle progression.

Recently, emerging evidence indicates critical roles of microRNAs (miRNAs) in the regulation of various biological and pathologic processes including the control of cell cycle [8, 9]. These small, noncoding molecules elicit their regulatory effects by imperfectly binding to the 3'-untranslated region of target mRNAs, causing either degradation of mRNAs or inhibition of their translation to functional proteins [10]. The expression of miRNAs has been recognized as integral components of many normal biological processes, such as those involving cell proliferation, differentiation, apoptosis, and stress resistance [11]. Natural agents including curcumin [12], isoflavone [13], indole-3-carbinol [14], and 3,3'-diindolylmethane [14] could alter miRNA expression profiles, leading to the inhibition of cancer cell growth, induction of apoptosis, reversal of epithelial mesenchymal transition, or enhancement of efficacy of conventional cancer therapeutics.

Experimental data presented in this study demonstrate that DIM strongly inhibits proliferation in human breast cancer cells regardless of estrogen responsiveness and p53 status, which is accompanied by a cell-cycle arrest, a reduction in cell cycle-related kinases and an increase in p21 levels. Meanwhile, Cdc25A is rapidly degraded upon DIM treatment. Interestingly, DIM-induced reduction of Cdc25A is mediated by an enhanced expression of miR-21 in MCF-7 cells. These observations collectively support a model in which DIM treatment activates miR-21 and leads to cell-cycle arrest via Cdc25A degradation in breast cancer cells.

## Materials and methods

### Cell culture, reagents, and apparatus

Human breast cancer cell lines MCF-7 and MDA-MB-468 were purchased from the American Type Culture Collection and cultured in DMEM medium (Gibco, Rockville, MD, USA), supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1 mM glutamine in a 5% CO<sub>2</sub> atmosphere at 37°C. Antibodies against CDK2, CDK4, CDK6, p21, and  $\beta$ -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against

CyclinB1 and CDK1 were from Cell Signaling Technology (Danvers, MA). Antibodies against Cdc25A were from NeoMarkers (Fremont, CA). Lipofectamine 2000 and superscript first-strand cDNA synthesis kit were purchased from Invitrogen (Eugene, OR). TaqMan MicroRNA Assay Kit, specific miRNA primer and ABI Prism 7700 Sequence Detection System were from Applied Biosystems (Bedford, MA).

### Plasmid, site-directed mutagenesis, and transfection

Full-length *CDC25A* ORF was amplified and cloned into pcDNA4/TO doxycycline-inducible promoter (Invitrogen). To generate phosphatase-site mutants of *CDC25A*, a site-directed mutagenesis kit (Stratagene) was used. Sequences for *CDC25A* and mutated *CDC25A* primer sets are listed in Supplementary Table S1. Finally, the correct mutation derivatives were verified by DNA sequencing. To establish cell lines with inducible expression of *CDC25A* and its mutation derivatives, MCF-7 Tet-on cells were transfected using 5  $\mu$ g of plasmid DNA (pcDNA4/TO-puro-Cdc25A) using 15  $\mu$ l of lipofectamin 2000 reagent according to the manufacturer's instruction. The cells were selected with 1.5  $\mu$ g/ml puromycin. To induce *CDC25A* expression, cells were treated overnight with doxycycline (10 ng/ml) and then treated with DIM for 24–72 h.

### Cell proliferation

To determine breast cancer cell proliferation,  $5 \times 10^5$  MCF-7 and MDA-MB-468 cell lines were separately seeded in 6-well plates and allowed to grow for 24 h. The cells were then treated with 0.1% dimethyl sulfoxide (DMSO), 30 or 60  $\mu$ M DIM at 24–96 h. Fresh medium containing DMSO or DIM was changed each day. DIM was dissolved in DMSO as a 1,000 $\times$  stock solution for each treatment. Cell numbers were assessed at various time points by trypsinizing and counting cells with a Coulter Z1 cell counter.

### Cell colony formation assay

$5 \times 10^3$  MCF-7 and MDA-MB-468 cells were separately seeded in 6-well plates and allowed to grow for 24 h. The cells were then treated with 0.1% DMSO and 45  $\mu$ M DIM. After 2 weeks, breast cancer cells were washed with phosphate-buffered saline (PBS) twice, fixed with methanol and then stained with Giemsa.

### miRNA target prediction

The miRNA targets were predicted using the algorithms TargetScan<sup>3</sup> and PicTar<sup>4</sup>.

## Transfections

Transfection with 30 nmol/l anti-miR-21 or miRNA negative control (Ambion) was conducted with Lipofectamine 2000 according to manufacturer's instructions. Cells plated at 50–60% confluence in 6-well plates were transfected twice in 48 h and split into two 6-well plates. The next day, the cells were either treated with DMSO or DIM as described above. Total proteins from each sample were extracted and subjected to Western blot analysis as described below.

## Growth inhibition assay

MCF-7 cells were transfected with anti-miR-21 or miRNA negative control for 4 days as described. Then, the transfected cells were seeded in 6-well plates and then treated with DMSO or 45  $\mu$ M DIM at 24–96 h. Fresh medium containing DMSO or DIM was changed each day. Cell numbers were assessed at various time points by trypsinizing and counting cells with a Coulter Z1 cell counter.

## Real-time reverse transcription-PCR (qRT-PCR)

Total RNA was extracted using TRIZOL reagent as recommended by the manufacturer (Invitrogen). 500 ng of total RNA was subjected to reverse transcription using the Superscript first-strand cDNA synthesis kit. qRT-PCR reactions were carried out in a total of 25  $\mu$ l of reaction mixture (1  $\mu$ l of cDNA, 12.5  $\mu$ l of 2 $\times$ SYBR Green PCR Master Mix, 0.75  $\mu$ l of each 10  $\mu$ M forward and reverse primers, and 10  $\mu$ l of H<sub>2</sub>O). The PCR program was initiated for 10 min at 95°C before 40 thermal cycles, each consisting of 15 s at 95°C and 1 min at 60°C. Data were analyzed according to the comparative Ct method, with *GAPDH* as a reference. Primer sequences for PCR are listed in Supplementary Table S2.

To verify the alterations in the expression of specific miRNAs, TaqMan MicroRNA Assay Kit was used. 5 ng of total RNA from each sample was subjected to reverse transcription with a specific miRNA primer. qRT-PCRs were then carried out in a total of 25  $\mu$ l reaction mixture in an ABI Prism 7700 Sequence Detection System as described [13]. The PCR program was initiated by 10 min at 95°C before 40 thermal cycles, each at 15 s at 95°C and 1 min at 60°C. Data were analyzed according to the comparative Ct method and normalized by *U6* expression in each sample.

## Western blot analysis

After the indicated treatments, the cells were washed with PBS and harvested in a RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris)

containing 10 mg/ml aprotinin, 5 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined by the Bradford-Coomassie dye binding assay. Equal amount of proteins was fractionated by electrophoresis on 4% polyacrylamide/0.1% SDS stacking gels and 15% polyacrylamide/0.1% SDS resolving gels, transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore, Bedford, MA), and then probed with a primary antibody followed by the secondary antibody. Proteins were visualized by chemiluminescence reagent and exposure to Kodak BioMax MR film. Equal protein loading was confirmed by probing blots with antibody to  $\beta$ -actin.

## Flow cytometric analyses of DNA content

The breast cell lines were plated onto six-well tissue culture dishes (BD Biosciences, San Jose, California). Cells treated with 0.1% DMSO, 30 or 60  $\mu$ M DIM for 48 h were harvested by trypsinization, washed twice with PBS, and then fixed in 70% ethanol. Cells were treated with 100 U/ml Ribonuclease A for 15 min at 37°C, resuspended and lysed in 1 ml of PI buffer (0.5 mg/ml propidium iodide, 0.1% sodium citrate, 0.05% Triton X-100). The percentage of cells within the G<sub>1</sub>, S, and G<sub>2</sub>-M phases of the cell cycle was determined by analysis with the Multicycle computer program (version 3.11) provided by Phoenix Flow Systems (San Diego, CA, USA).

## Human breast carcinoma xenograft study

A colony of 40 BALB/C female athymic (nu/nu) mice (4–5 weeks old) were housed in microisolator cages under standard conditions (12:12 h light/dark cycle, 50% relative humidity at 21°C) and given free access to a semi-purified AIN-76A diet and water. Half of them were implanted with a 60-day release 0.72 mg estradiol pellet in the subscapular region. The mice were then inoculated subcutaneously (S.C) in the lateral flanks with 0.1 ml of PBS solution containing  $3 \times 10^6$  MCF-7 cells. Other 20 mice were inoculated subcutaneously with 0.1 ml of PBS solution containing  $3 \times 10^6$  MDA-MB-468 cells. The mice were divided into two groups: group 1 ( $n = 2 \times 10$ ) without DIM treatment (DIM<sup>-</sup>) and group 2 ( $n = 2 \times 10$ ) with DIM treatment (DIM<sup>+</sup>). Sesame seed oil was used to facilitate gavage and avoid irritation of the esophagus (safe as shown by previous studies) [15, 16]. The mice in the intervention group were given DIM (5 mg/kg) by oral gavage once a day for five consecutive days with another 2 days off weekly. The control mice received only sesame seed oil without DIM. The treatment with DIM or seed oil begins on the next day of cell injection. The palpable tumor diameters were measured twice per week. Tumor volumes were calculated as  $ab^2/2$  (where  $a$  is length and  $b$  is cross-

sectional diameter) [17]. The treatment was terminated 7 weeks after cell injection and all the mice were killed by CO<sub>2</sub> asphyxiation. All experiments were performed in compliance with the institutional guidelines (Peking University Health Science Center, Beijing, China).

### Statistical analysis

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

## Results

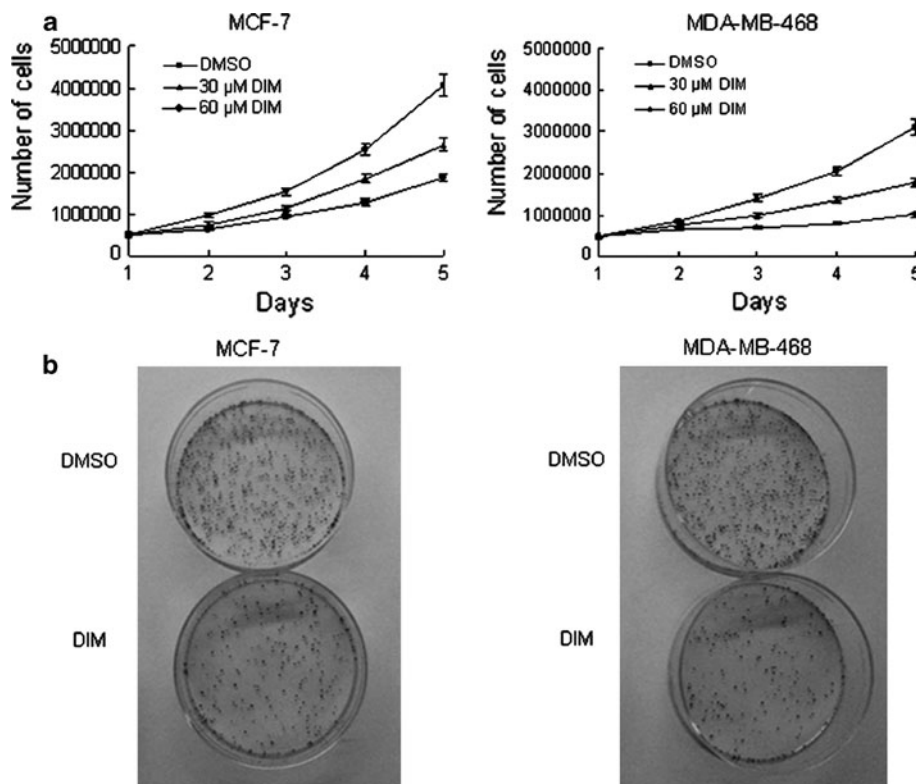
### DIM inhibits proliferation of MCF-7 and MDA-MB-468 breast cancer cells

To assess the effects of DIM on cell growth, *ER* positive, *p53* wild-type MCF-7 and *ER* negative, *p53* mutant

MDA-MB-468 cells were separately treated with 0.1% DMSO alone, or 30 or 60 μM DIM for 24–96 h. DIM inhibited the proliferation of MCF-7 and MDA-MB-468 cells in a concentration- and time-dependent manner, with up to 50% inhibition of proliferation at 72 h treatment with 60 μM DIM (Fig. 1a). A cell colony formation assay also showed that DIM treatment resulted in a dramatic reduction in the colony numbers of MCF-7 and MDA-MB-468 cells (Fig. 1b). These results suggest that DIM can directly reduce the proliferation of breast cancer cells irrespective of estrogen receptor and *p53* status.

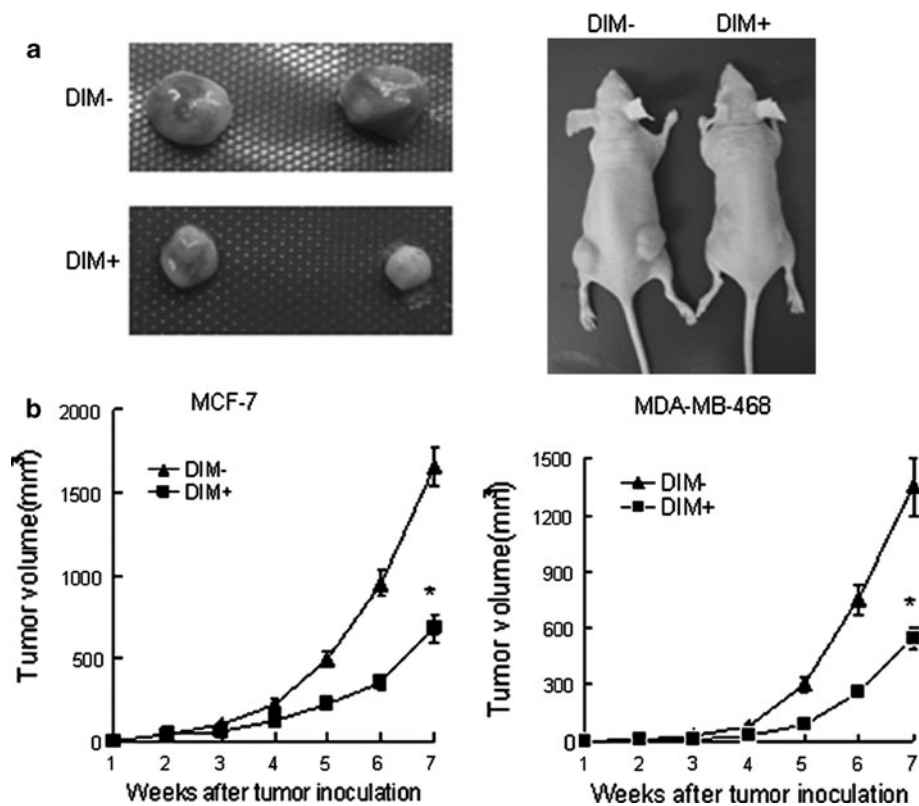
DIM inhibits the growth of transplanted human breast carcinoma cells

Since an inhibition of *in vitro* cell growth by DIM was observed, it was interesting to know whether DIM could inhibit the growth of transplanted breast carcinoma cells in female athymic (nu/nu) mice. Mice were injected subcutaneously with MDA-MB-468 or MCF-7 cells in the bilateral flanks as described in the “Materials and methods” section. Compared to untreated controls DIM



**Fig. 1** DIM inhibits the breast cancer cell growth and cell colony formation *in vitro*. **a** The effect of DIM on MCF-7 and MDA-MB-468 proliferation. Plates were seeded in 6-well plates with  $5 \times 10^5$  cells and incubated overnight to allow attachment before starting the treatments. Treatments were DMSO as a control, 30 or 60 μM DIM. Cell numbers were assessed at various time points by trypsinization and counting with a Coulter Z1 cell counter. Results are

representative of at least three independent experiments and values are expressed as mean with standard deviation. **b**  $5 \times 10^3$  MCF-7 and MDA-MB-468 cells were separately seeded in 6-well plates and allowed to grow for 24 h. The cells were then treated with 0.1% DMSO and 45 μM DIM. After 2 weeks, breast cancer cells were washed with PBS twice, fixed with methanol and then stained with Giemsa



**Fig. 2** DIM inhibits the breast cancer cell growth in vivo. **a** DIM strongly inhibits the development of human breast tumor in xenograft model. DIM treatment caused a 60% reduction in tumor volume in xenograft model. The representative figure of mice from each group, photographed at the time of killing. **b** Statistical analysis for tumor volume from control and treatment groups at different time points. The mice were inoculated subcutaneously (SC) in the lateral flanks with 0.1 ml of PBS solution containing  $3 \times 10^6$  MCF-7 or

MDA-MB-468 human breast cancer cells. The mice in the intervention group ( $n = 10$ ) were DIM (5 mg/kg) by oral gavage five times weekly, and the majority of the implants began to appear as a tumor after the inoculation of each cell line for 1–2 week. The control mice ( $n = 10$ ) received only sesame seed oil without DIM. The palpable tumor diameters were measured and volumes were calculated twice per week. Tumor volumes were calculated as  $ab^2/2$ . Asterisk indicates significant difference from control at a level of  $P < 0.05$

treatment led to a 60% decrease in tumor volume in both MCF-7 and MDA-MB-468 cells ( $P < 0.05$ ) (Fig. 2). These results are consistent with previously published data from other investigators that DIM treatment inhibited the growth of MCF-7 cells [18, 19].

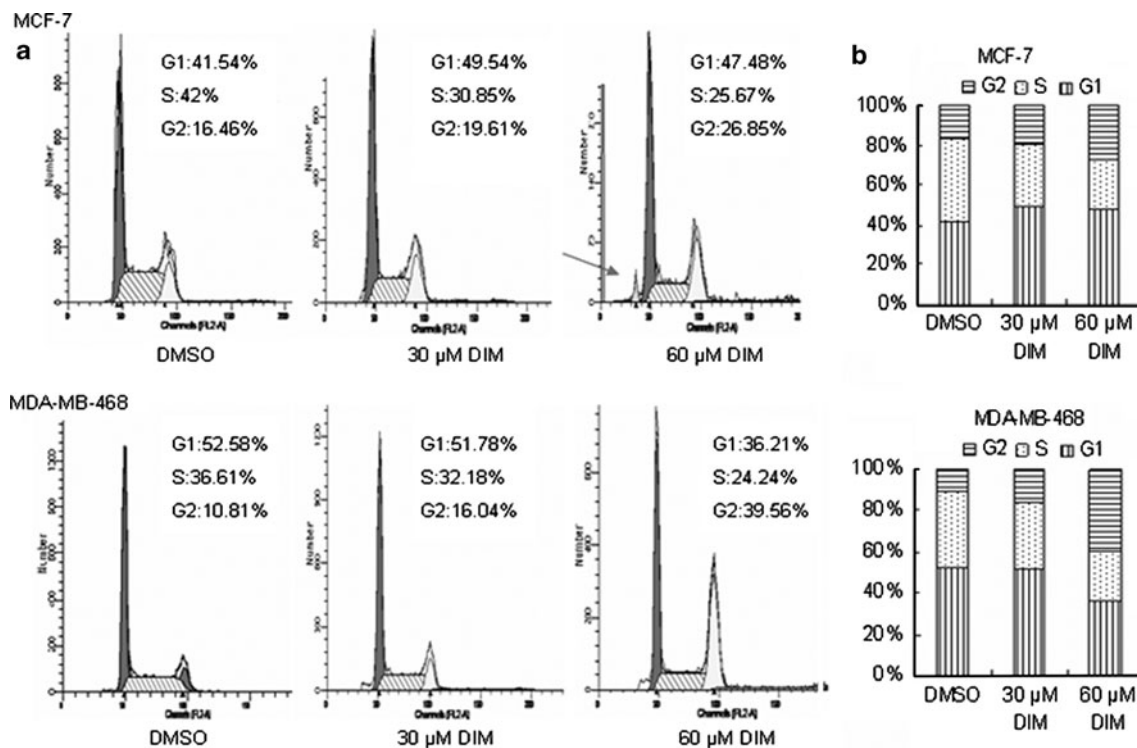
DIM induces cell-cycle arrest at  $G_1$  and  $G_2/M$  phase in breast cancer cells

Based on the growth inhibitory responses of MCF-7 and MDA-MB-468 cells to DIM, its effects on cell-cycle regulation was further pursued. Both cells treated with either 0.1% DMSO alone or 30 or 60  $\mu\text{M}$  DIM for 48 h were analyzed by flow cytometry. Figure 3 shows representative histograms of cell-cycle distribution in the control, 30 and 60  $\mu\text{M}$  DIM-treated MCF-7 and MDA-MB-468 cells. DIM treatment increased the cell population in the  $G_1$  phase and the  $G_2/M$  phase of MCF-7 cells and caused  $G_2/M$  arrest in MDA-MB-468 cells. Furthermore, after treating MCF-7 cells with 60  $\mu\text{M}$  DIM for 48 h, the apoptotic peak was

detected by FACS, which was not found in MDA-MB-468 cells.

Effects of DIM on cell-cycle regulatory molecules in MCF-7 and MDA-MB-468 cells

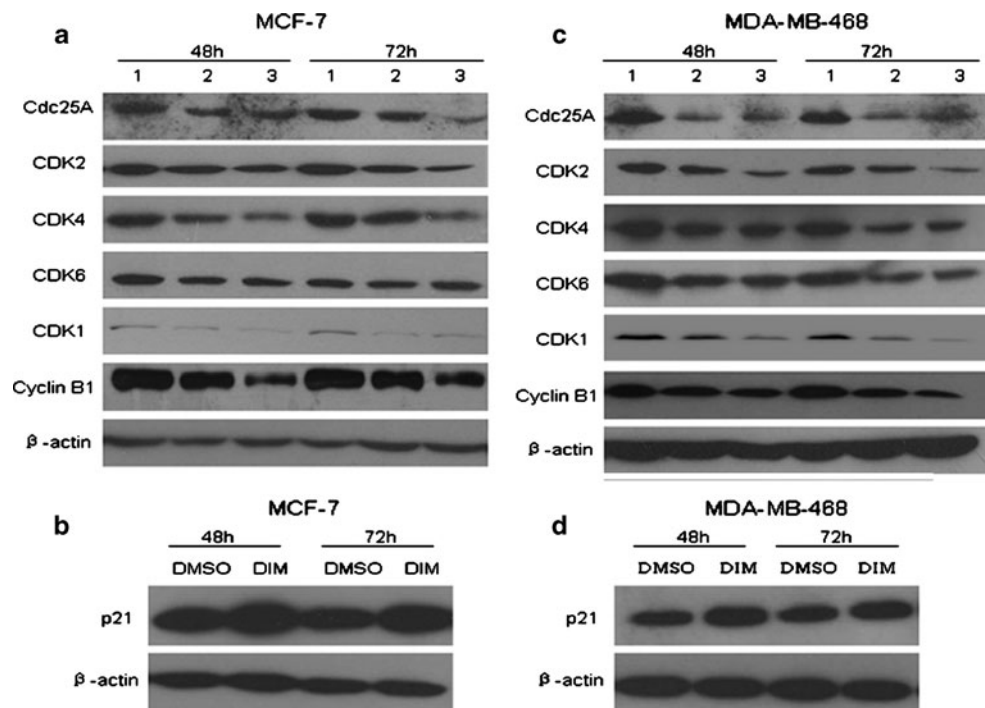
The observation that DIM induces a  $G_1$  block in cell-cycle progression of MCF-7 cells suggests that DIM selectively regulates activities of  $G_1$  cell-cycle regulating components. To examine this possibility, the expression of  $G_1$  cell-cycle components was investigated by using western blot analysis. Results shown in Fig. 4a indicate that DIM treatment reduced CDK4 and CDK2 protein levels but not their transcript levels (Supplementary Fig. S1a and b), and meanwhile enhanced the cellular  $p21^{\text{Waf1/Cip1}}$  (Fig. 4b). DIM also down-regulated CDK family members and up-regulated  $p21^{\text{Waf1/Cip1}}$  in MDA-MB-468 cells (Fig. 4c, d). In contrast, the cellular CDK6 level was not altered by DIM treatment (Fig. 4a, c). In addition, in both MCF-7 and MDA-MB-468 cell lines, DIM can noticeably down-



**Fig. 3** DIM induces cell-cycle arrest at G<sub>1</sub> or G<sub>2</sub>/M phases in breast cancer cells. **a** Histograms of distribution of DNA content in MCF-7 and MDA-MB-468 with either 0.1% DMSO alone or 30 or 60 μM DIM treatment for 48 h followed by flow cytometry. Cells were lysed in 1 ml of propidium iodide solution. Nuclear fluorescence was measured and analyzed. The percentage of cells in G<sub>1</sub>, S, and G<sub>2</sub>

phase of the cell cycle were determined by analysis with the Multicycle computer software. The apoptosis peak is indicated as an arrow. **b** Distribution of cell population in G<sub>1</sub>, S, and G<sub>2</sub> phases in control and 30, 60 μM DIM-treated MCF-7 and MDA-MB-468. Results are representative of at least three independent experiments

**Fig. 4** Effects of DIM on cell-cycle regulatory molecules in MCF-7 and MDA-MB-468 cells. DIM reduces CDK4, CDK2, Cdc25A, CDK1, and of Cyclin B1 protein levels (**a** and **c**) and increases the level of p21 protein (**b** and **d**) in both MCF-7 (**a** and **b**) and MDA-MB-468 (**c** and **d**) cells. Cell lysates were analyzed via Western blotting with the indicated antibodies. Photographs of the chemiluminescent detection of the blots, which were representatives of three independent experiments, are shown. The relative abundance of each band to its own β-actin was quantified. 1 represents control cells treated with 0.1% DMSO. 2 and 3 represent cells treated with 30 and 60 μM DIM, respectively

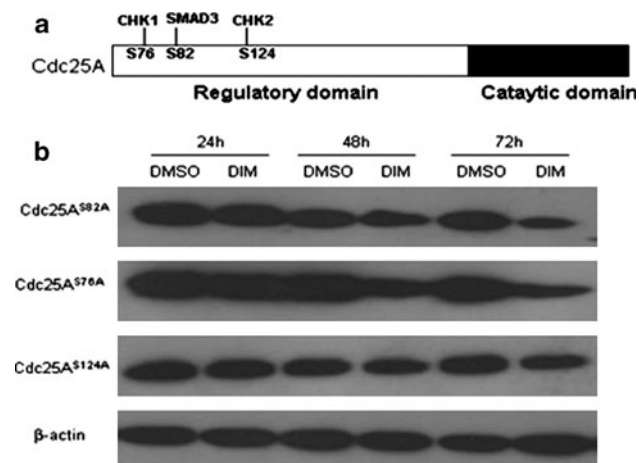


regulate Cdc25A (Fig. 4a, c), which plays an important role in the G<sub>1</sub> and G<sub>2</sub>/M checkpoints [20–22]. Some other G<sub>2</sub>/M-related molecules were also examined, and it was found that DIM reduced the protein levels of Cyclin B1 and CDK1 in MCF-7 and MDA-MB-468 cells (Fig. 4a, c). These results indicate that DIM induces a cell-cycle arrest in MCF-7 and MDA-MB-468 cells, which is accompanied by a down-regulation of the expression of G<sub>1</sub>/S-related and G<sub>2</sub>/M-related kinases.

Ser<sup>124</sup> site of Cdc25A partially respond to DIM treatment

To explore the mechanism by which DIM targets Cdc25A, the mRNA of Cdc25A was measured in MCF-7 cells, which did not reveal significant changes in the *CDC25A* mRNA level between control and DIM treatment by qRT-PCR (Supplementary Fig. S1c). Thus, it is hypothesized that the reduced Cdc25A in the cells with DIM treatment was due to degradation of the Cdc25A protein.

Regulation of Cdc25A protein levels through the cell cycle requires phosphorylation at multiple sites by different kinases and the presence of intact recognition motifs on Cdc25A, including Ser<sup>76</sup>, Ser<sup>82</sup>, and Ser<sup>124</sup>, which are related to the degradation pathways mediated by Chk1, Smad3, and Chk2, respectively [23, 24]. To assess whether Cdc25A phosphorylation is responsible for its down-regulation mediated by DIM, a Tet-on system with doxycycline-inducible expression of human Cdc25A<sup>WT</sup> or the derivatives Cdc25A<sup>S76A</sup>, Cdc25A<sup>S82A</sup>, or Cdc25A<sup>S124A</sup> in the breast cancer cells as described before was employed



**Fig. 5** The Ser<sup>124</sup> site of Cdc25A partially responds to DIM treatment. **a** Sketch for multiple pathways related to Chk1, Smad3 and Chk2 regulation of Cdc25A degradation. **b** Detecting the response of the mutated Cdc25A derivatives to DIM treatment in MCF-7 breast cancer cells. Cell lysates were analyzed via Western blotting with the indicated antibodies. The relative abundance of each band to its own  $\beta$ -actin was quantified. Representative data from one experiment are shown ( $n = 3$ )

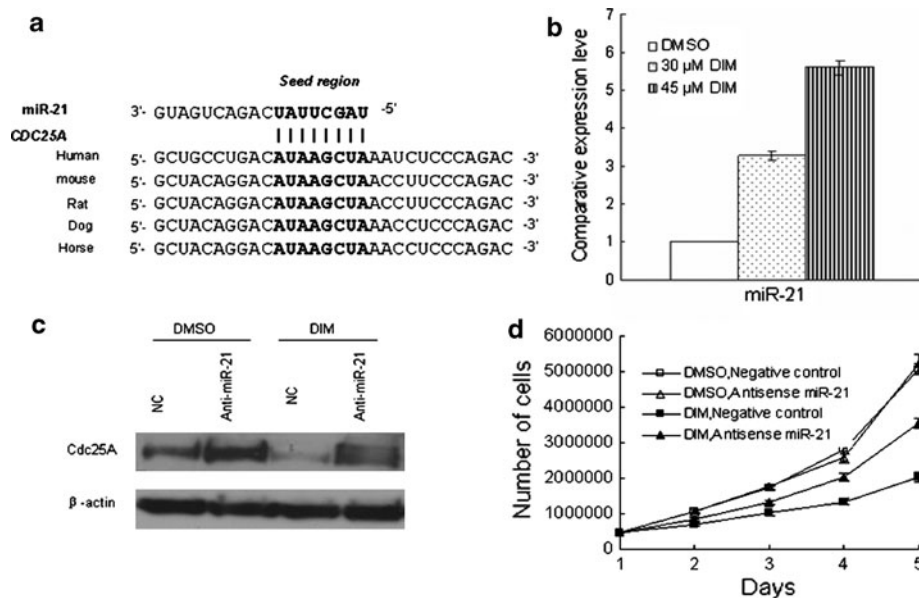
[25]. The stability of the three mutation derivatives Cdc25A<sup>S76A</sup>, Cdc25A<sup>S82A</sup>, and Cdc25A<sup>S124A</sup> was assayed in MCF-7 cells with or without DIM treatment by Western blot. Cdc25A<sup>S76A</sup> and Cdc25A<sup>S82A</sup> were rapidly degraded with DIM treatment; in sharp contrast, the degraded fraction of Cdc25A<sup>S124A</sup> was much lower compared to that of Cdc25A (data not shown), Cdc25A<sup>S76A</sup> or Cdc25A<sup>S82A</sup>, although it may still be partially degraded with DIM treatment (Fig. 5b). The differential stability of Cdc25A mutant proteins was unlikely due to their effects on cell proliferation in response to the DIM treatment, since all three mutant cells grow at a rate indistinguishable from that of wild-type Cdc25A transfectants, regardless of the presence or absence of DIM (Supplementary Fig. S2). These results suggest that mutation of Cdc25A at the Ser<sup>124</sup> site can partially block DIM-induced Cdc25A degradation.

miR-21 inhibits cell proliferation following DIM treatment through Cdc25A

It has been reported that miR-21 regulates Cdc25A through the miR-21 binding site in its 3'-UTR and Cdc25A is a direct target of miR-21 [26]. On a closer inspection, a putative miR-21 binding site located in the 3'-UTR of Cdc25A gene was predicted by two algorithms (TargetScan and PicTar, Fig. 6a). Importantly, this putative miR-21 binding site is 100% conserved in five species in the region that pairs with the seed sequence (Fig. 6a). To ask whether miR-21 is also regulated by DIM, the miR-21 qRT-PCR was conducted and was surprisingly found that miR-21 was up-regulated in DIM-treated MCF-7 cells (Fig. 6b), which was not observed in *ER* negative, *p53* mutant MDA-MB-468 cells (Supplementary Fig. S3). To further address whether miR-21 mediates the regulation of Cdc25A by DIM, MCF-7 cells were transfected with anti-miR-21 and then treated with DIM for 48 h. Transfection of anti-miR-21 elevated Cdc25A and partially restored the Cdc25A level in MCF-7 cells treated with DIM (Fig. 6c). Cdc25A is an important regulator of cell-cycle progression during G<sub>1</sub>/S and G<sub>2</sub>/M transition [20, 22]. To evaluate whether miR-21 affects cell-cycle progression, the growth rate of cells transfected with anti-miR-21 or miRNA negative control, and cultured in medium containing DMSO or 45  $\mu$ M DIM was monitored over a course of 5 days. The growth rate of cells transfected with negative control and anti-miR-21 was indistinguishable after DMSO treatment. However, anti-miR-21 transfected MCF-7 cells exhibited enhanced proliferation over control cells after DIM treatment (Fig. 6d).

## Discussion

Despite major advances in early detection and adjuvant therapy, advanced breast cancer remains a major clinical



**Fig. 6** miR-21 inhibits cell proliferation following DIM treatment through Cdc25A. **a** Conserved *miR-21* binding site in the 3'-UTR of *CDC25A*. Schematic representation of *CDC25A* transcript with its 3'-UTR. The predicted *miR-21* binding sites in the *CDC25A* gene of five species are shown with *miR-21* targeting sequences aligned (GenBank accession numbers NM\_201567.1, NM\_007658.3, NM\_133571.1, NM\_001145215.1, and NM\_001145213.1, respectively). The base pairing nucleotides are in **bold**. **b** DIM induces *miR-21* expression in MCF-7 cells. Cells were treated with DIM as described in Materials and Methods. Total RNA was isolated and qRT-PCR was performed. Levels of mature *miR-21* expression were normalized to those of *U6*.

Values were expressed as means with standard deviation. Results are representative of at least three independent experiments. **c** DIM-mediated decrease of Cdc25A protein level was reversed by anti-miR-21. MCF-7 cells transfected with or without anti-miR-21 were treated with DMSO or 45 μM DIM and subjected to Western blot analysis. NC negative control. **d** MCF-7 cells transfected with anti-miR-21 or miRNA negative control were cultured in medium containing DMSO or 45 μM DIM for 5 days. Cell numbers were determined by counting as mentioned above. Results are representative of at least three independent experiments

problem. Therefore, there is an urgent need for new therapeutic approaches. The perceived health benefits of micronutrient supplements and phytochemicals have generated considerable interest in recent years because of their potential roles in cancer prevention and treatment.

DIM has been demonstrated to be effective against the proliferation of MCF-7 human breast cancer cells in vitro and in vivo [18, 19]. Mechanisms for the protective effects of DIM against tumorigenesis in MCF-7 cells have also been suggested including reduced expression of ER-alpha [27], increased interferon-gamma secretion [28], enhanced generation of mitochondrial reactive oxygen species leading to cell-cycle arrest through increased expression of p21 [29] and suppression of cyclooxygenase-2 expression [30]. Our results are in full agreement with the previously published data showing that DIM treatment inhibits the growth of MCF-7 cells in vitro and in vivo. However, it is important to note that the present study describes that DIM can also target the more aggressive MDA-MB-468 cells. The potency of DIM in our rodent assays of tumorigenesis is similar to the published findings in which a DIM dose of 5 mg/kg body weight inhibited carcinogen-induced mammary carcinogenesis in rodents [31]. In this animal study,

DIM effectively inhibited tumor growth at a dose as low as 5 mg/kg without noticeable weight loss or toxicity to major organs of the animals.

In this study, it is revealed that DIM causes not only  $G_1$  arrest but also  $G_2/M$  arrest in MCF-7 and MDA-MB-468 cells. Cell cycle is driven by the periodic association of CDKs with their cyclin partners and is controlled by kinase inhibitors. p21, a CDK inhibitor known to bind to the CDK1-Cyclin B complex [32, 33] and negatively regulate the  $G_2/M$  phase progression. Experimental data presented in this study demonstrate that constitutive CDK4, CDK2, CDK1, and Cyclin B1 levels are significantly down-regulated, while the protein level of p21 is noticeably induced by DIM treatment. In mammals, the Cdc25 family includes three homologs, namely Cdc25A, B, and C. In the past, Cdc25B and C were regarded as mitotic regulators [34] while Cdc25A was regarded as a  $G_1/S$  regulator. However, recent data show that Cdc25A, in addition to its S-phase-promoting effect, physically and functionally interacts with the main mitosis-promoting Cyclin-CDK complex and generates a rate-limiting stimulus for the  $G_2/M$  transition; the lack of its activity can delay completion of the cell division cycle [20, 21]. This

study shows that DIM treatment significantly reduces Cdc25A levels, which further inactivated the CDK1-CyclinB1 complex, leading to a blockade of cells in the G<sub>2</sub>/M phase.

Cdc25A is a critical regulator of cell-cycle progression and checkpoint response; its overexpression is found in about 50% of breast cancer patients and is associated with poor prognosis [35, 36]. The major mechanism of rapid turnover of Cdc25 family proteins is regulated by ubiquitin-mediated proteolysis [24]. For example, hyperphosphorylation of Cdc25A by the ATR-Chk1 signaling leads to its degradation and contributes to a delay in the cell cycle, which allows either DNA repair or apoptosis, depending on the extent of DNA damage [23, 24]. However, extensive effort in the mapping of phosphorylation sites in Cdc25A and the use of cells deficient in Chk2 or ATM indicate that many such sites are not required for Cdc25A-mediated G<sub>2</sub>-M checkpoint following DNA damage [37, 38]. Data presented in this report also reveal that Ser<sup>76</sup> and Ser<sup>82</sup> of Cdc25A are not necessary for DIM-induced Cdc25A degradation but Ser<sup>124</sup> is partially responsible. It is well known that Cdc25A-Ser<sup>124</sup> is a target of the ATM-Chk2 kinase cascade. We have previously found that I3C induces Cdc25A degradation by the Chk2 kinase in human breast cancer cells [25], which make it conceivable that DIM may also induce Cdc25A degradation partially via Chk2.

This study reveals a novel mechanism in which the full extent of Cdc25A inactivation requires miR-21 in response to DIM treatment in MCF-7 cells, but not in MDA-MB-468 cells, suggesting that DIM-induced decrease in Cdc25A levels in MDA-MB-468 cells is probably mediated by a different mechanism. Both MCF-7 and MDA-MB-468 cells are human breast adenocarcinoma cells, but the MCF-7 cells are positive for cytoplasmic estrogen receptors, while the MDA-MB-468 is a well-characterized estrogen receptor negative cell line, which expresses the mutant p53 protein. Thus, no change in miR-21 expression with DIM treatment in MDA-MB-468 cells may result from loss of estrogen receptor expression or the specific p53 mutation. The involvement of miR-21 in cell-cycle progression following DIM treatment is supported by several recent studies, as it is induced by the chemotherapeutic drug 5-fluorouracil in colon cancer cells [39] and by UV irradiation in primary fibroblasts [40] or colon cancer cells [26]. Interestingly, the elevated Cdc25A level in anti-miR-21 transfected cells do not appear to affect proliferation but profoundly affect cell progression following DIM treatment, indicating the importance of inactivating Cdc25A under these conditions. These results demonstrate that DIM significantly reduces cell proliferation of human breast cancer cells via cell-cycle arrest, which is in part due to up-regulation of miR-21, and the subsequent down-regulation of Cdc25A.

Apoptosis is one of the most vital pathways through which chemopreventive agents inhibit the overall growth of cancer cells. When MCF-7 cells were treated with 60 μM DIM for 48 h, the apoptotic cell population become visible. It was also found that DIM can affect some apoptosis-associated gene expression, such as *Survivin* and *FOXMI* (Supplementary Fig. S4). The inhibition of *FOXMI* expression by DIM may disrupt survivin-microtubule interactions and result in the loss of survivin's anti-apoptosis function. Hence, induction of apoptosis by DIM may enhance its inhibitory efficacy in breast cancer cells.

In conclusion, findings presented in this study demonstrate pre-clinical proof of efficacy of DIM in breast cancer cell lines; thus DIM should be evaluated further as a potential agent for estrogen-dependent and estrogen-negative breast cancer.

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