Cancer Science
The official journal of the Japanese Cancer Association

RNAi-mediated CD73 suppression induces apoptosis and cell-cycle arrest in human breast cancer cells

Xiuling Zhi,[1] Yingjian Wang,[2] Xuerui Zhou,[3] Jerry Yu,[1,4] Rongrong Jian,[1] Shaoxian Tang[1], Lianhua Yin[1] and Ping Zhou[1,5]

[1]Department of Physiology and Pathophysiology, Shanghai Medical College, Shanghai; [2]Department of Gynaecology and Obsterics, China-Japan Union Hospital of Jilin University, Changchun; [3]Department of Biology, Huaiyin Teachers College, Huaian, Jiangsu, China; [4]Department of Pulmonary Medicine, University of Louisville, Louisville, KY, USA

(Received March 9, 2010/Revised August 25, 2010/Accepted August 25, 2010/Accepted manuscript online August 31, 2010/Article first published online September 28, 2010)

Ecto-5′-nucleotidase (CD73), a cell surface protein that hydrolyzes extracellular AMP into adenosine and phosphate, is overexpressed in many solid tumors. In this study, we tested the hypothesis that increased CD73 may promote tumor progression by examining the effect of CD73 suppression via RNA interference and CD73 overexpression on tumor growth *in vivo* and *in vitro*. Using digitized whole-body images, plate clone forming assay and TUNEL assay in frozen tissue sections, we found that the cell growth rate was significantly lower *in vivo* and *in vitro* after CD73 suppression and late apoptosis was much higher in xenograft tumors developed from the CD73-siRNA transfected MB-MDA-231 clone (P1). By flow cytometry, the P1 cell cycle was arrested in the G0/G1 phase. Moreover, Bcl-2 was downregulated, while Bax and caspase-3 were upregulated with CD73 suppression. CD73 inhibitor α,β-methylene adenosine-5′-disphosphate (APCP) functioned similarly with RNAi-mediated CD73 suppression. In addition, in transfected MCF-7 cells, we found that CD73 overexpression increased cell viability and promoted cell cycle progression, depending on its enzyme activity. More intriguingly, CD73 overexpression in MCF-7 breast cancer cells produces a tumorigenic phenotype. We conclude that CD73 plays an important role in breast cancer growth by affecting cell cycle progression and apoptosis. (*Cancer Sci* 2010; 101: 2561–2569)

Breast cancer develops in 14% of women and is a leading cause of cancer death in women around the world.[1] Understanding the molecular mechanisms of breast carcinoma progression is important for effective treatments. Ecto-5′-nucleotidase (CD73) is a 70 kDa glycosylated protein that is bound to the outer surface of the plasma membrane by a glycosyl phosphatidyl inositol anchor and co-localized with detergent-resistant and glycolipid-rich membrane sub-domains called lipid rafts.[2] CD73 hydrolyzes extracellular AMP into adenosine and phosphate. Adenosine, a proliferative factor, acting through G-protein coupled receptors, produces a spectrum of physiological functions.[3] In addition, it causes tumor growth, angiogenesis and immune suppression.[4] CD73 upregulation is associated with a highly invasive cancer phenotype, drug resistance and tumor-promoting functions.[5] In addition to acting as a hydrolytic enzyme to generate adenosine, CD73 may serve as an adhesive molecule and interact with extracellular matrix glycoprotein, such as fibronectin and laminin, to produce cancer-invasive properties.[6] Bavaresco *et al.*[7] reported that CD73 mediated glioma cell proliferation depends upon adenosine. Furthermore, CD73 is overexpressed in the progression of many human solid tumors, such as breast cancer,[8,9] papillary thyroid carcinomas,[10] melanoma [11] and prostate cancer.[12] All these factors implicate the crucial role of CD73 in tumorigenesis. To date, our knowledge on the mechanisms of CD73 on tumor growth is still limited. Previously, we showed that CD73 may promote metastasis by facilitating the migration, adhesion and invasion of human breast cancer cells.[13] The present studies examine the effect of RNAi-mediated CD73 suppression and CD73 overexpression on tumorigenicity *in vivo* and *in vitro* on cell growth, and further explore its underlying regulatory mechanisms.

Materials and Methods

Cell culture. Breast adenocarcinoma cell lines MB-MDA-231 and MCF-7 were obtained from the American Type Culture Collection and maintained in an exponential growth phase in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco, Carlsbad, CA, USA) and glutamine. The cells were incubated normoxically in 5% CO_2 at 37°C and 95% humidity.

Construction and transfection of siRNA plasmids. siRNA plasmids constructions were carried out according to a previous report.[8] Briefly, three CD73 (Gene ID: 4907) DNA sequences (TCTCAATCATGCCGCTTTA, GCCACTAGCATCTCAAATA and GATCGAGTTTGATGAAAGA) were selected for designing the siRNA target. And then three CD73 siRNA plasmids were constructed based on the U6 siRNA expression vector, pRNAT-U6.1/Neo vector (GenScript Corp., Piscataway, NJ, USA). The control RNA interference (RNAi) sequence was a randomly scrambled sequence not found in mouse, human or rat genome databases. All constructs (control plasmid, CD73 siRNA1, CD73 siRNA2, CD73 siRNA3) were confirmed by sequencing. In a pilot study, the CD73 siRNA2 plasmid was found to be the best (data not shown). Therefore, the CD73 siRNA2 and control plasmids were transfected into MB-MDA-231 cells, respectively, using Lipofectamine 2000 (Invitrogen, Carlsbad CA, USA). Clones were screened and sub-cultured for CD73 expression analysis. Constructions of CD73 mutant vector (active site His 92 of CD73 enzyme was substituted to Ala) were carried out according to Gutensohn *et al.*[14]

Analysis of CD73 enzyme activity. Surface CD73 enzyme activity was assessed by measuring the conversion of etheno-AMP (E-AMP) (Sigma-Aldrich, St Louis, MO, USA) to etheno-adenosine (E-Ado) (Sigma-Aldrich) with high performance liquid chromatography (HPLC).[15] Hank's balanced salt solution with or without CD73 specific inhibitor α,β-methylene ADP (α,β-methylene adenosine-5′-disphosphate [APCP], 10 μM) was added to an epithelial monolayer on six-well plates. After 10 min, E-AMP/E-ATP (final concentration 100 μM) was added for an additional 10 min, and then acidified to pH 3.5 with HCl, spun (10 000*g* for 20 s at 4°C), filtered (0.45 μm) and frozen (−80°C). E-AMP and E-Ado were separated with a 4–40% methanol/H_2O gradient mobile phase (1 mL/min over 20 min). CD73 enzyme activity was expressed as E-Ado production per mg protein in 10 min.

[5]To whom correspondence should be addressed.
E-mail: zping@shmu.edu.cn

doi: 10.1111/j.1349-7006.2010.01733.x

Colony formation assay. For the colony formation assay, approximately 3×10^3 cells were plated in 60 mm culture dishes, and replenished every 2–3 days with complete medium. After 14 days incubation, the cells were fixed with methanol and stained with 0.5% crystal violet. Visible colonies were manually counted. All experiments were performed in triplicate. The clone formation rate (CFR) was computed according the following formula: CFR = clone counts/seeded cell counts × 100%.

Cell counting kit-8 (CCK-8) assay. Cell proliferation was assessed by CCK-8 assay according to the manufacturer's instruction (Dojindo Laboratories, Gaithersburg, MD, USA). Cells in a 96-well plate were incubated in 10 μL CCK-8 solution for 1 h at 37°C. Absorbance of each well was quantified at 450 nm with an automated ELISA reader (Bio-Tech Instruments, Winooski, VT, USA).

Flow cytometry for analysis of cell cycle and apoptotic cells. Cell cycle analysis was performed using ethanol-fixed cells stained with propidium iodide in buffer containing RNase A. The DNA content was assessed using a FACS Calibur Flow Cytometer (Becton-Dickinson, San Jose, CA, USA).

Apoptotic cells were assessed following the manufacturer's protocol (Becton-Dickinson). In short, the transfected MB-MDA-231 cells were harvested, washed twice with ice-cold PBS, resuspended in 1 × binding buffer at a concentration of 1×10^6 cells/mL, incubated with Annexin V-PE (Phycoerythrin) and 7-ADD (7-Amino-actinomycin) for 15 min at room temperature in darkness, and then analyzed by flow cytometry within 1 h. The APCP pretreated cells were incubated with Annexin V-FITC and PI.

Murine xenograft model for tumorigenicity assay. The effect of CD73 on tumorigenicity was assessed by subcutaneous injection of 2×10^6 transfected MB-MDA-231 cells into athymic nude mice. Fluorescent tumors growing in live mice were imaged in real time by a whole body imaging system (Night OWL ‖ LB 983; Berthold Technologies, Bad Wildbad, Germany). The number and intensity of pixels in each image were calculated and converted to millimeter square for each tumor with a software program. For quantification of light signals, the fluorescence intensity, an average value of all detectable GFP signals, was measured in relative units defined by the gray scale in the green channel of the charge-coupled divice (CCD) camera (Berthold Technologies, Bad Wildbad, Germany) and expressed as photons/second (ph/s). An intensity visualized just above the background was used as the threshold.[16] In addition, the mean tumor volume was measured every 3 days by a vernier caliper and calculated according to the formula: $a \times (b)^2 \times 0.5$ (a, largest diameter; b, perpendicular diameter).

TUNEL (TdT-mediated dUTP nick end labeling) assay. The tumor mass was fixed with formalin and frozen tissue sections were prepared for apoptosis analysis by TUNEL assay. Apoptotic cells were identified with an *in situ* cell death detection kit (Roche, Mannheim, Germany). The sections were incubated with 50 μL TUNEL reaction mixture containing TdT for 60 min at 37°C, and then incubated with horseradish-peroxidase-avidin (0.5 mg/mL in PBS) for 30 min at 37°C, stained with 3,3-diaminobenzidine (DAB), and examined in 10 randomly selected fields. Nuclei with a brown stain indicated TUNEL-positive cells. Cell death was expressed as a percentage of the total cells counted.

Measurement of gene mRNA and protein expressions by real-time RT-PCR and western blot assays. Total RNA and proteins were isolated. Then, mRNA and protein expressions were determined by real-time RT-PCR and western blot assays, respectively, as previously described.[8] The primer sequences of the housekeeping gene β-actin and aim genes are listed in Table 1. Primary antibodies against CD73, Caspase-3, Bcl-2, Bax, respectively were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cleaved Caspase-3 (Asp175) (5A1) rabbit mAb detecting endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 was purchased from Cell Signaling Technology (Trask Lane Danvers, MA, USA).

Table 1. Primer sequences used in the study

Gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)
β-actin	GGTGGCTTTTAGGATGGCAAG	ACTGGA ACGGTG AAG GTGACAG
Caspase-3	ATGGAAGCGAATCAATGGACTC	CTGTACCAGACCGAGA TGTCA
Bcl-2	GAACTGGGGGAGGATTGTGG	CCGGTTCAGGTAC TCA GTCA
Bax	GGGTGGTTGGGTGAGACTC	AGACACGTAAGGAAA ACGCATTA

Tissue microarray assay and immunohistochemical staining. Tissue microarrays containing 80 specimens from different stages of breast cancer and 10 specimens from adjacent non-cancer tissues were obtained from Shanghai Outdo Biotech Co. (Shanghai, China), and CD73 expression was assayed by immunohistochemistry. The slides were incubated with anti-NT5E rabbit polyclonal antibody (Cat. #AP2014b, 1:50 dilution; Abgent, San Diego, CA, USA) at 37°C for 2 h, where normal rabbit serum was used as a negative control, followed by incubation with a horseradish peroxidase–conjugated anti-rabbit secondary antibody (Santa Cruz, CA, USA) at 37°C for 1 h. The signals were detected using diaminobenzidine substrate kit (Vector Laboratories, Burlingarne, CA, USA). Counterstaining was performed with hematoxylin.

An immunohistochemical score (IHS) was used to value CD73 expression in breast tissues. This method is based on the German ImmunoReactive score for image analysis-based scoring systems.[17] The IHS is calculated by multiplying the quantity and intensity scores. Theoretically, the scores could range from 0 to 12. An IHS score of 0 denotes negative, 1–4 is weak, 5–8 is moderate and 9–12 is strong immunoreactivity. The expression grade of Bcl-2 was provided by Shanghai Outdo Biotech Co.

Statistical analysis. Data from three or more separate experiments were presented as mean ± SEM. ANOVA and the Student–Neuman–Keuls post test were performed. Statistical significance was defined as $P < 0.05$.

Results

Identification of transfected cells. In our previous study, CD73 mRNA expression was significantly suppressed in six CD73 siRNA transfected clones (P1, P2, P3, P4, P5, P6).[8] Among them, P1 had the lowest expression of CD73 and therefore was used in the current study. Expression of enhanced green fluorescent protein (EGFP) was checked under fluorescent microscope and flow cytometry. The transfected MB-MDA-231 cells had a high-level of EGFP expression (Fig. 1A). As shown in Figure 1(B), the green fluorescent population of P1 cells culturing with G418 was 85.6%. Figure 1(C) was a representative HPLC chromatograph of E-AMP and E-Ado. The HPLC data show CD73 siRNA suppressed CD73 activity (Fig. 1D).

Reduction of colony formation by CD73 RNAi. Effect of RNAi-mediated CD73 suppression on colony formation was examined. In the current study, 3×10^3 cells were used for the colony formation assay. This number of cells was chosen based on a pilot study, in which different numbers of untreated MB-MDA-231 cells (100, 500, 1×10^3 and 3×10^3) were used (Fig. 2A). Transfected and non-transfected MB-MDA-231 cells were plated and colony formation assays were performed 14 days after plating. Colony numbers were much lower in

doi: 10.1111/j.1349-7006.2010.01733.x

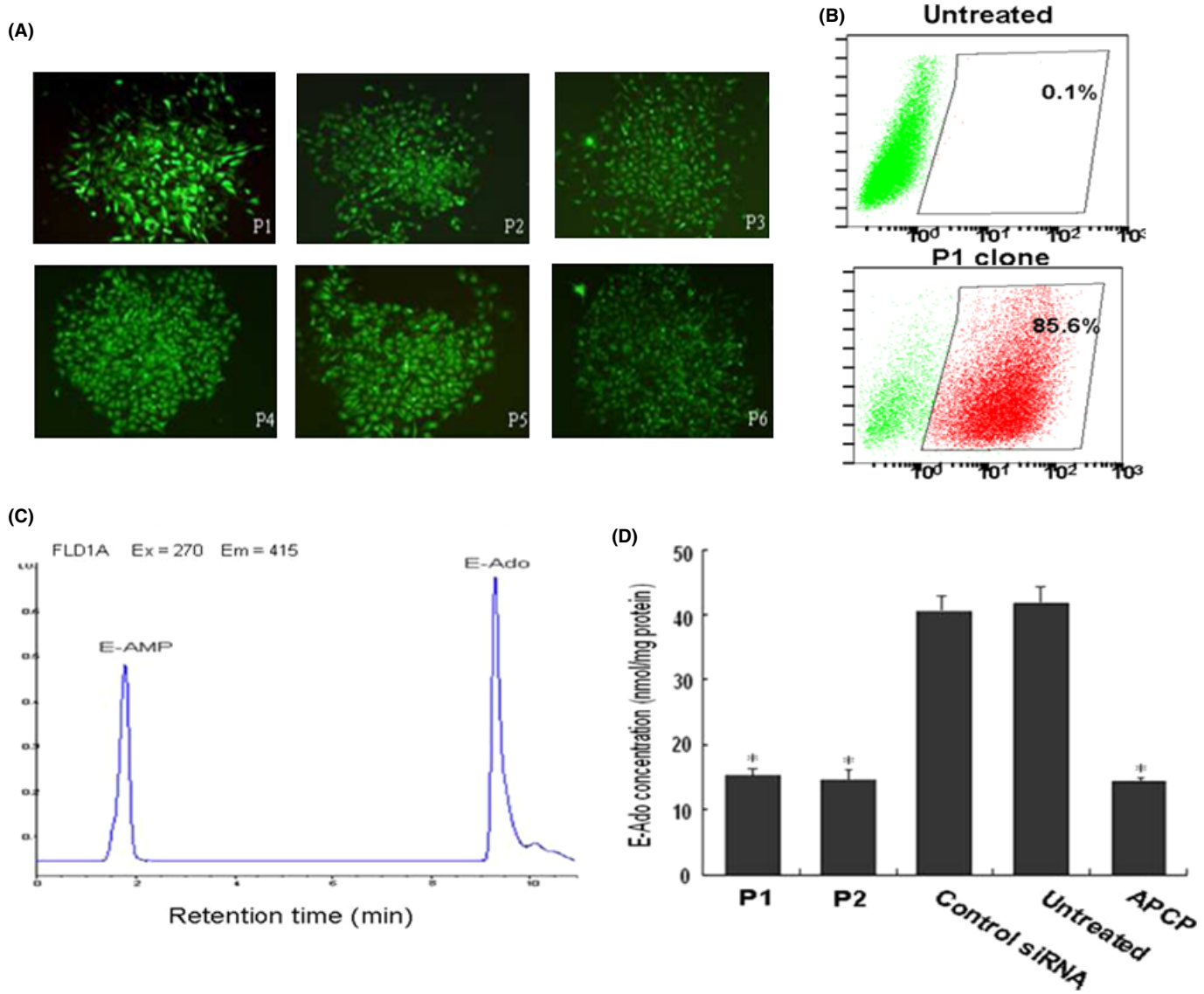

Fig. 1. Identification of stably transfected cells. (A) High-level GFP expression in transfected MB-MDA-231 cells detected using fluorescence microscope. (B) Analysis of the green fluorescent population of P1 cells cultured with G418 by flow cytometry (FCM). (C) Representative high performance liquid chromatography (HPLC) of E-AMP and E-Ado. Fluorescence was detected at a wavelength of 270/418 nm (excitation/emission). (D) Surface CD73 enzyme activity assessed by measuring E-Ado with HPLC. The enzyme activity decreased in CD73 siRNA transfected cells and in APCP (10 μM) treated cells (n = 8; *P < 0.05 *vs* control siRNA and untreated groups, respectively).

transfected versus non-transfected and control siRNA transfected groups (Fig. 2B).

CD73 downregulation retarding tumorigenicity in nude mice. A subcutaneous tumor assay was conducted to examine the effect of RNAi-mediated CD73 suppression on tumor growth *in vivo* in nude mice. The EGFP fluorescence area and intensity of xenografts from P1 cells were significantly smaller than those in the control siRNA group at 21 days (Fig. 2C1). Our data showed that CD73 siRNA inhibited growth of breast cancer cells *in vivo* significantly when compared with the control siRNA groups (Fig. 2C2,C3).

Effect of CD73 RNAi on the cell cycle. The cell cycle was analyzed to elucidate the mechanisms underlying the siRNA-mediated growth inhibition. Compared with control siRNA transfected and non-transfected cells, P1 cells increased 9.9 ± 1.2% in the G1/G0 phase, and decreased 10.7 ± 0.5% in the S phase (n = 3, P < 0.05, Table 2).

Effect of CD73 overexpression. CD73 was overexpressed in pcDNA3.0-NT5E transfected MCF-7 cells (Fig. 3A). Interestingly, as Figure 3(B) shows, none of the control cells or parental MCF-7 cells gave rise to tumors after 21 days, while 3/5 mice injected with pcDNA3.0-NT5E transfected MCF7 cells produced tumors that grew in a rapid fashion. In addition, the viability of pcDNA3.0-NT5E transfected cells increased when examined by CCK-8 assay (Fig. 3C) and cell-cycle progression was promoted when assessed by flow cytometry (Table 3). However, transfection of mutant constructions with substituted active site (His 92) to Ala does not promote MCF-7 cell viability and cell-cycle progression (Fig. 3C and Table 3).

Increased apoptosis with CD73 RNAi. We measured apoptosis using two methods (flow cytometry and TUNEL assay) to determine if the growth inhibition caused was due to apoptotic cell death. By Annexin V/7-AAD or Annexin V/PI double staining, flow cytometry data can show early (labeled with Annexin V alone) and late (labeled with Annexin V/7-AAD or Annexin V/PI) apoptotic cells. Our data demonstrated that the early and late apoptotic rate of P1 cells increased significantly compared with the control siRNA cells (Fig. 4A1,A3). CD73

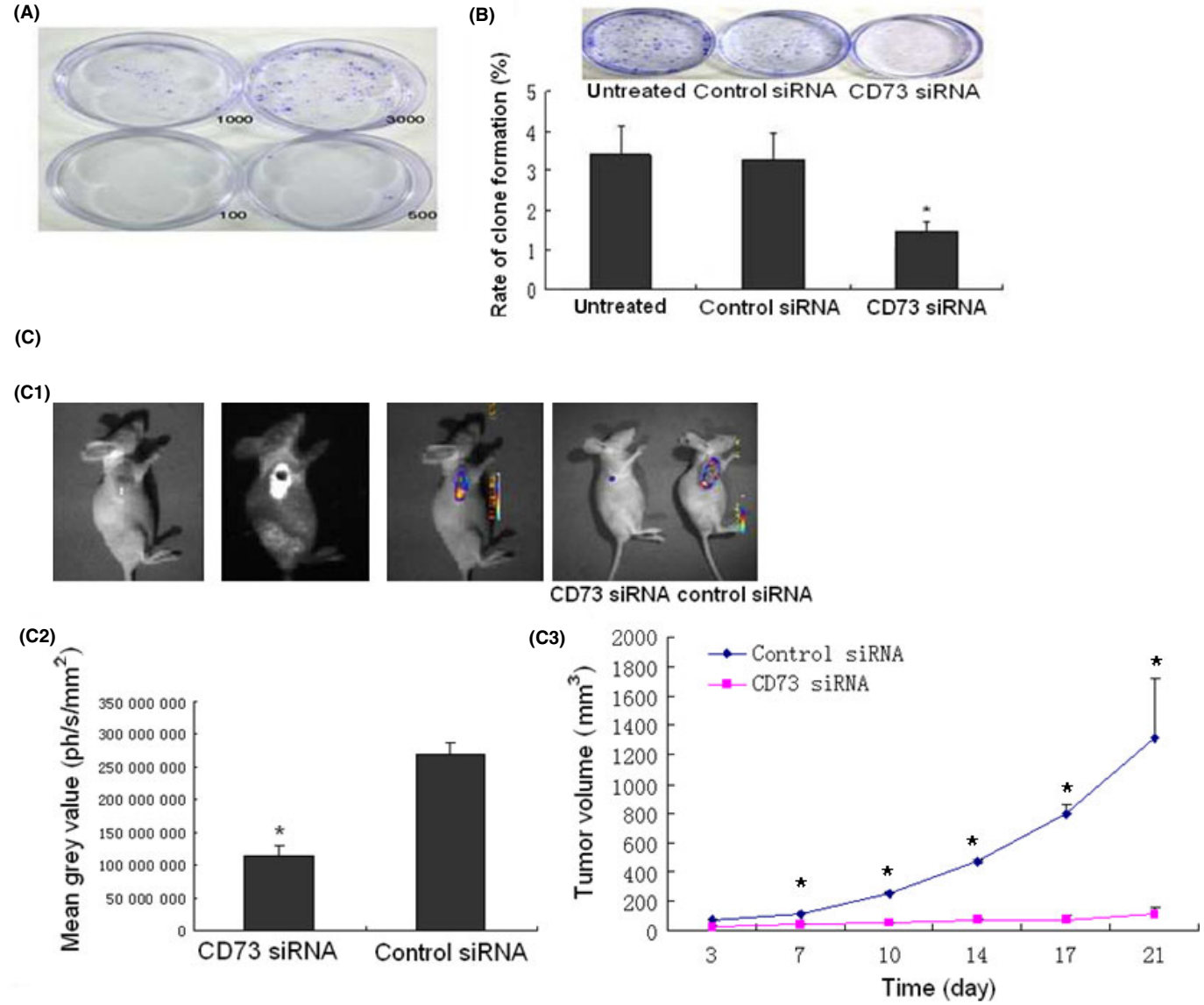

Fig. 2. Effect of CD73 on cell growth *in vivo* and *in vitro*. (A) MB-MDA-231 cells (100, 500, 1×10^3 and 3×10^3) were used for colony formation assays and 3×10^3 was the optimal number of cells. (B) Colony formation assays for three groups (untreated, control siRNA and CD73 siRNA) were performed. The colony numbers were much lower in CD73 siRNA transfected cells than in the untreated and control transfected cells ($n = 3$; $^*P < 0.05$ *vs* untreated and control siRNA groups). (C) *In vivo* tumor growth. C1: whole body imaging of nude mice. The left two pictures are photographed with optic and fluorescent imaging systems, respectively. The third picture is the integrated photo of the two. The fourth picture depicts two tumor-bearing mice showing that CD73 RNAi inhibited growth of transplanted tumor. C2: the GFP fluorescence area and intensity were substantially greater in the control xenograft than those from P1 cells at 21 days. C3: tumor growth curve by regular measurement. CD73 RNAi inhibited growth of transplanted tumor ($n = 5$; $^*P < 0.05$ *vs* control siRNA groups).

Table 2. Cell cycle analysis in different MB-MDA-231 cells

	CD73 siRNA	Control siRNA	Untreated
G0G1	65.83 ± 4.37*	55.02 ± 8.09	56.74 ± 7.65
S	8.86 ± 0.53*	19.24 ± 1.67	19.96 ± 2.40
G2M	17.93 ± 3.08	18.10 ± 3.58	18.43 ± 2.74

Data are expressed as mean ± SEM (%). $^*P < 0.05$, compared with control siRNA transfected and untreated cells. CD73 siRNA cells (P1) in the G1/G0 phase increased by 9.9 ± 1.2%, whereas P1 cells in S phase reduced by 10.7 ± 0.5% ($n = 3$).

inhibitor APCP functioned similarly with RNAi-mediated CD73 suppression (Fig. 4A2,A3). The TUNEL assay, based on the labeling of DNA strand breaks for the detection of apoptosis in frozen sections of xenograft tumor, revealed more intense brown staining in the P1 group than in the control group (Fig. 4B).

Effect of CD73 RNAi on activation of the apoptotic pathways. We examined different apoptotic pathways to look at the mechanism of CD73-shRNA-induced apoptosis by assessing protein (western blot) and mRNA (real-time PCR) expression. Caspase-3 expression increased both at mRNA and protein levels (Fig. 5A1,A3). The approximately 35 kDa pro-Caspase-3 protein was detected in all samples while active/cleaved Caspase-3 was detected only in CD73 inhibited cells (Fig. 5A2). In the presence of CD73-shRNA, the anti-apoptotic Bcl-2 expression decreased, whereas the pro-apoptotic Bax expression increased significantly (Fig. 5B,C). CD73 inhibitor (APCP) produced similar effects on caspase-3, Bcl-2 and Bax expressions (Fig. 5).

doi: 10.1111/j.1349-7006.2010.01733.x

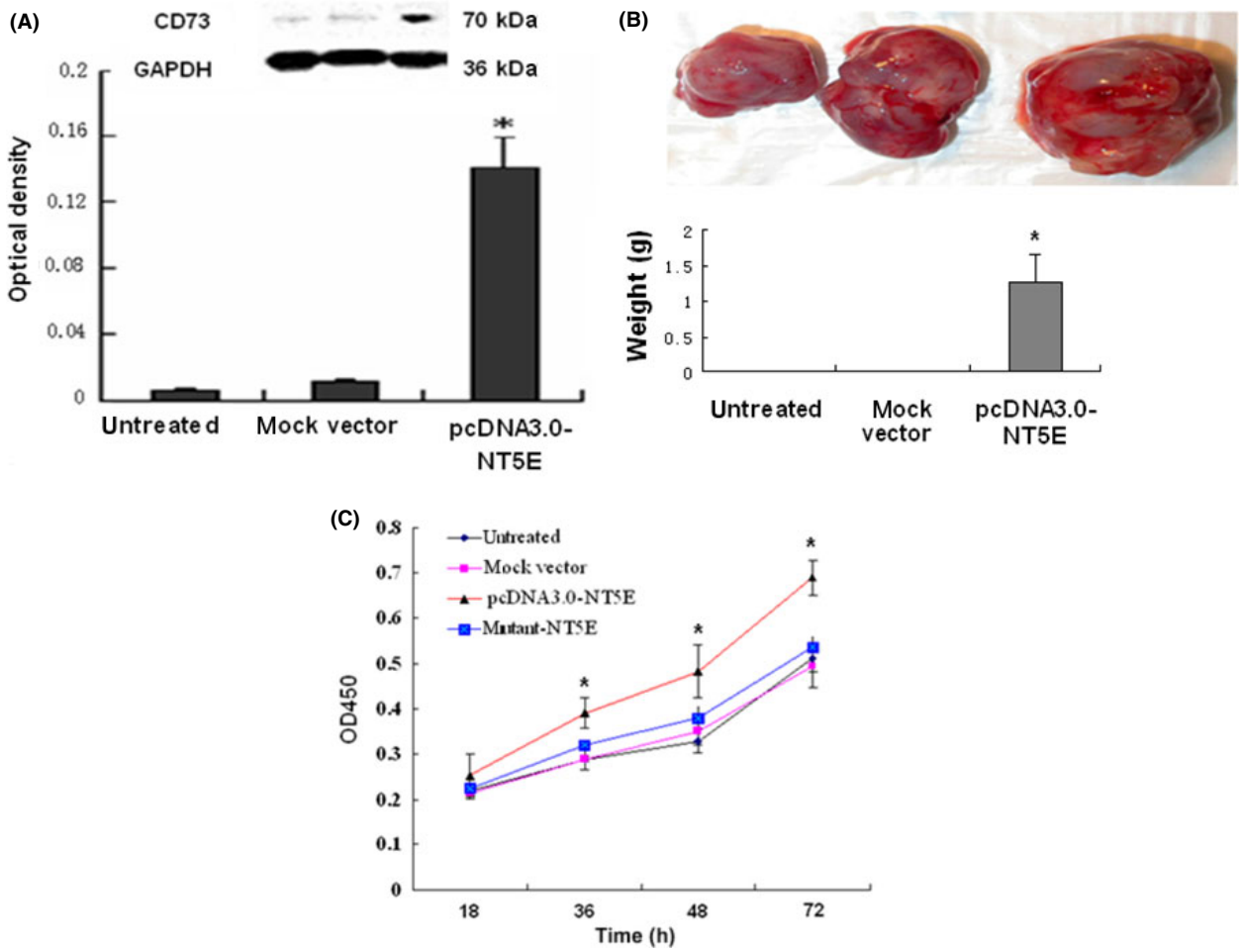

Fig. 3. The effect of CD73 overexpression on cell growth. (A) CD73 expression analysis by western blot. CD73 expression was increased in MCF-7 cells after pcDNA3.0-NT5E transfection. (B) *In vivo* tumor growth. None of the control cells or parental MCF-7 cells gave rise to tumors after 21 days, while 3/5 mice injected with pcDNA3.0-NT5E transfected MCF7 cells produced tumors that grew in a rapid fashion ($n = 5$; $^*P < 0.05$). (C) CCK-8 assay. Cell viability was increased in pcDNA3.0-NT5E transfected MCF-7 cells, while transfection of mutant constructions substituted active site (His 92) to Ala does not promote MCF-7 cell viability ($n = 3$; $^*P < 0.05$ *vs* untreated, mock vector and mutant-NT5E groups).

Table 3. Cell cycle analysis in different MCF-7 cells

	Untreated	Mock vector	pcDNA3.0-NT5E	Mutant-NT5E
G0G1	67.27 ± 0.41	65.94 ± 0.76	52.83 ± 0.91*	65.31 ± 1.64
S	15.91 ± 0.11	15.26 ± 0.18	29.98 ± 0.9*	17.48 ± 1.23
G2M	16.83 ± 0.52	18.58 ± 0.27	17.2 ± 1.81	17.2 ± 0.41

Data are expressed as mean ± SEM (%). Mutant-NT5E, mutant-NT5E vector (active site His 92 of CD73 enzyme was substituted to Ala) transfected MCF-7 cells; pcDNA3.0-NT5E, pcDNA3.0-NT5E transfected MCF-7 cells; untreated, control MCF-7 cells; mock vector, control transfected cells. $^*P < 0.05$ compared with untreated, mock vector and mutant-NT5E groups ($n = 3$).

CD73 and Bcl-2 co-expressed in breast cancer tissues. In normal breast tissue, CD73 was differentially expressed in lobular, ductal and myoepithelial cells (Fig. 6A). Figure 6(B) and Table 4 showed that CD73 was also expressed in tumor cells. The tumor specimens had positive staining in 95% of cases; 52.6% were scored as moderate and strong. CD73 expression was higher in invasive duct carcinoma than in normal mammary glands, although the difference was not statistically significant (Fig. 6C). Figure 6(D,E) suggests that CD73 expression was positively related to lymph node metastasis but not to grading. CD73 expression positively correlated with Bcl-2 expression (Fig. 6F,G, $n = 25$, $r = 0.397$, $P < 0.05$). The Bcl-2 data were provided by Shanghai Outdo Biotech Co.

Discussion

A major characteristic of malignant tumor is the ability to proliferate without limit, which is dependent on the synthesis of a large amount of nucleotide, while CD73 is a key enzyme in the generation of adenosine in the extracellular medium. Although CD73 is widely distributed in a variety of normal tissue,[18] its expression is low. However, CD73 expression is high in many kinds of solid tumors, correlates with tumor grade and localization,[19] and is closely associated with tumor aggression and a poor prognosis in melanoma,[11] breast cancer[20] and glioblastomas[21]. Furthermore, adenosine controls cell proliferation, angiogenesis and the immune response.[4] Therefore, we hypothesized that CD73, a key enzyme in purine and pyrimidine salvage, may play an important role in tumor progression.

In this study, we first investigated the role of CD73 in breast cancer growth *in vivo* and *in vitro* by RNAi-mediated suppression. By RNA interfering, we successfully obtained MB-MDA-231 cells with CD73 suppression and stable EGFP expression. In the nude mice tumorigenicity study, using a whole body imaging system, we observed that CD73 suppression inhibited

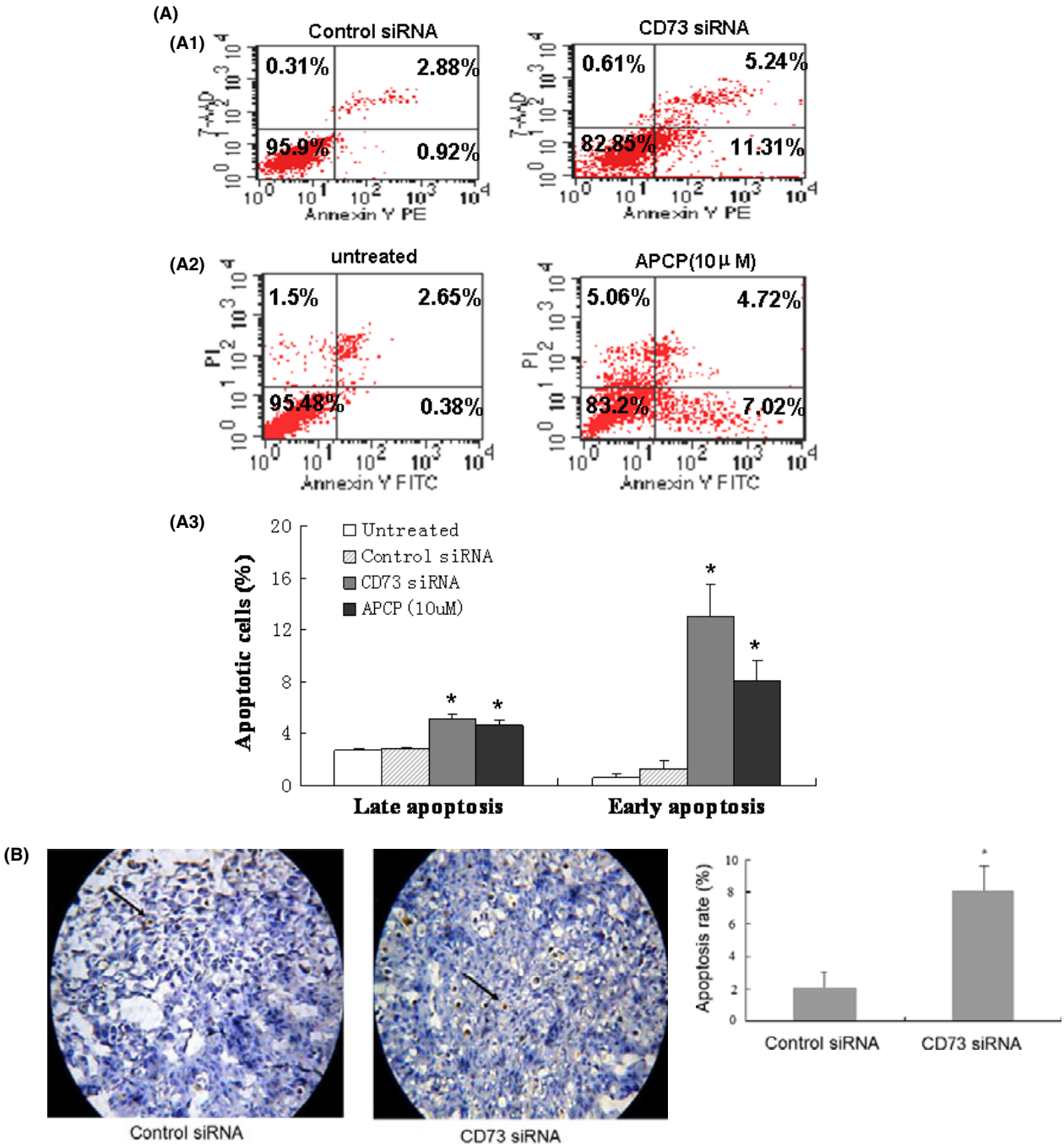

Fig. 4. Cell apoptosis detected by flow cytometry and TUNEL assay. (A) Both early and late apoptosis rates were significantly higher in the CD73 siRNA group than in the control siRNA group, while APCP functioned similarly ($n = 3$; $^*P < 0.05$ vs control siRNA and untreated). The lower right and upper right show early and late apoptosis, respectively. (B) A TUNEL assay based on the labeling of DNA strand breaks for detection of apoptosis was performed on frozen sections of xenograft tumor. P1 showed intense brown staining when compared with the control group ($n = 10$; $^*P < 0.01$ *vs* control siRNA).

tumor growth. In the plate colony formation assay, the colony numbers were much lower in P1 cells than in non-transfected and control siRNA transfected cells. We then observed the effect of CD73 on breast cancer cell growth by overexpression in pcDNA3.0-NT5E transfected MCF-7 cells. Our data showed CD73 overexpression increased cell viability. More intriguingly, CD73 overexpression in MCF-7 breast cancer cells produced a tumorigenic phenotype while none of the control cells or parental MCF-7 cells gave rise to tumors. This is in accordance with the report that MCF-7 cells are non-tumorigenic in nude mice because their growth is dependent on exogenous estrogen.[22,23] The mechanisms of CD73 overexpression leading to acquiring the estrogen-independent phenotype in breast cancer cells need to be further studied. In addition, transfection of mutant constructions does not promote MCF-7 cell viability, which indicates that CD73 promotes breast cancer cell growth depending on its enzyme activity. Our data support that CD73 plays an important role in breast cancer cell growth both *in vivo* and *in vitro*, which is consistent with Braganhol *et al.*'s[24] study in the U138MG glioma cell line.

doi: 10.1111/j.1349-7006.2010.01733.x

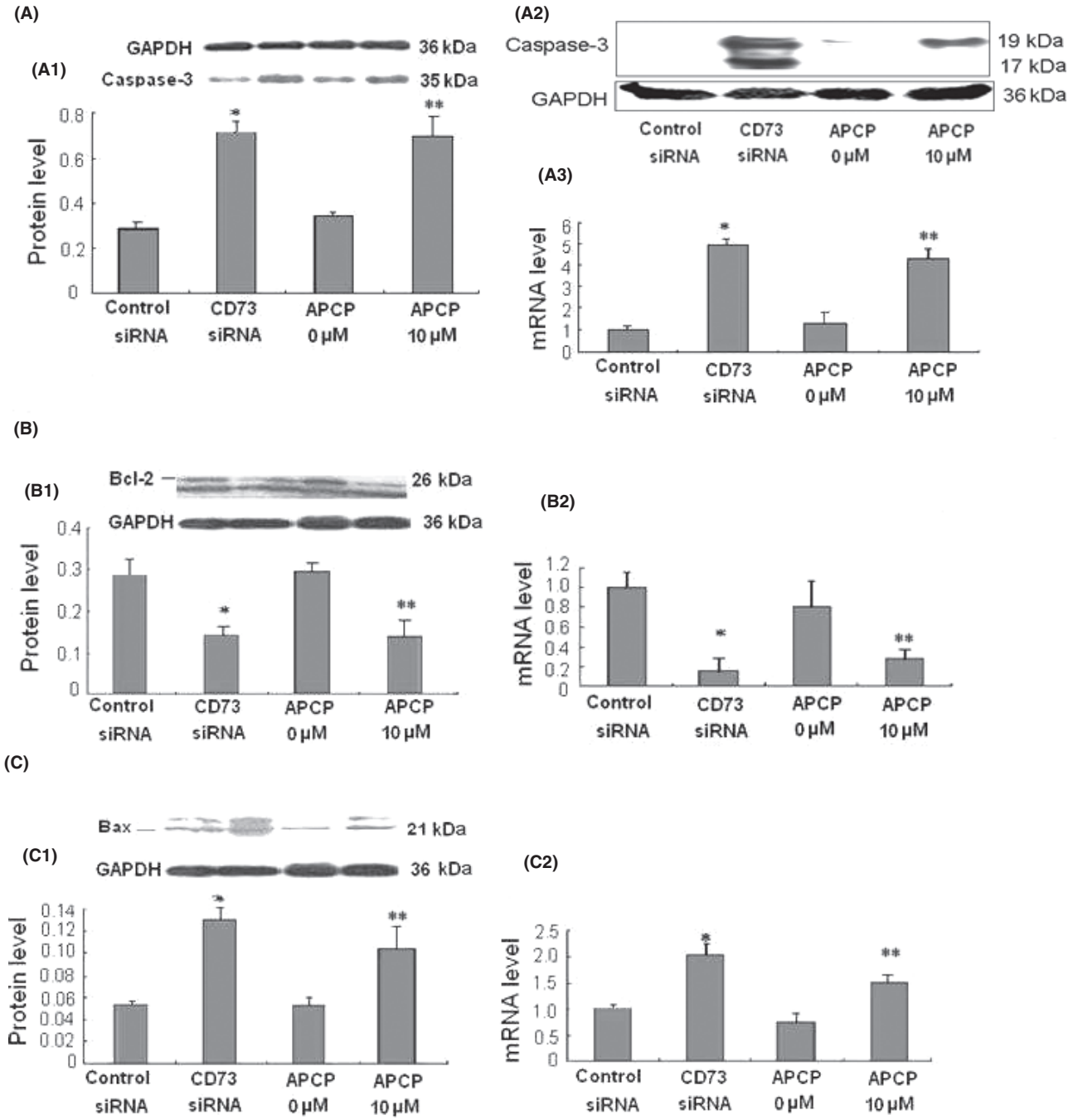

Fig. 5. Effect of CD73 suppression on apoptotic pathways. After CD73 was suppressed by either siRNA or α,β-methylene adenosine-5′-disphosphate (APCP) (10 μM), (A) Caspase-3 expression increased at protein (A1) and mRNA (A3) levels with Caspase-3 activation (A2); (B) Bcl-2 expression decreased at protein (B1) and mRNA (B2) levels; but (C) Bax expression increased at both protein (C1) and mRNA (C2) levels ($n = 3$; $^*P < 0.05$ *vs* control siRNA; $^{**}P < 0.05$ *vs* 0 μM APCP).

During development, cell-cycle arrest is critical to controlling cell number, the proliferation rate and organ size. Dysfunction of the cell cycle is a fundamental reason for uncontrolled proliferation of tumor cells. Previously, we found that APCP (a specific CD73 inhibitor) decreased MDA-MB-231 viability in a dose-dependent manner. α,β-methylene adenosine-5′-disphosphate (APCP) increased the percentage of G0/G1 cells, but decreased the S and G2/M cells. In the current study, we examined the cell cycle in breast cancer growth by suppressing or overexpressing CD73. We found that CD73 suppression induced an accumulation of MB-MDA-231 cells in the G0/G1 phase and a reduction in the S phase, while it is quite the contrary in pcDNA3.0–NT5E transfected MCF-7 cells. Our data on mutant constructions indicates that promotion of CD73 on cell-cycle progression also needs its enzyme activity. Taken together, our results suggest that CD73 may arrest cells in the S phase and promote cell proliferation.

The tumor growth rate is critically influenced by the ratio of neoplastic cell proliferation to cell apoptosis. Apoptosis is a cell death process that plays a critical role in tissue development, homeostasis and development of disease.[25] CD73 hydrolyzes extracellular AMP into adenosine and the extracellular concentration of adenosine profoundly affects cell apoptosis.[26] Some studies indicate that it reduces cell viability, arrests the cell

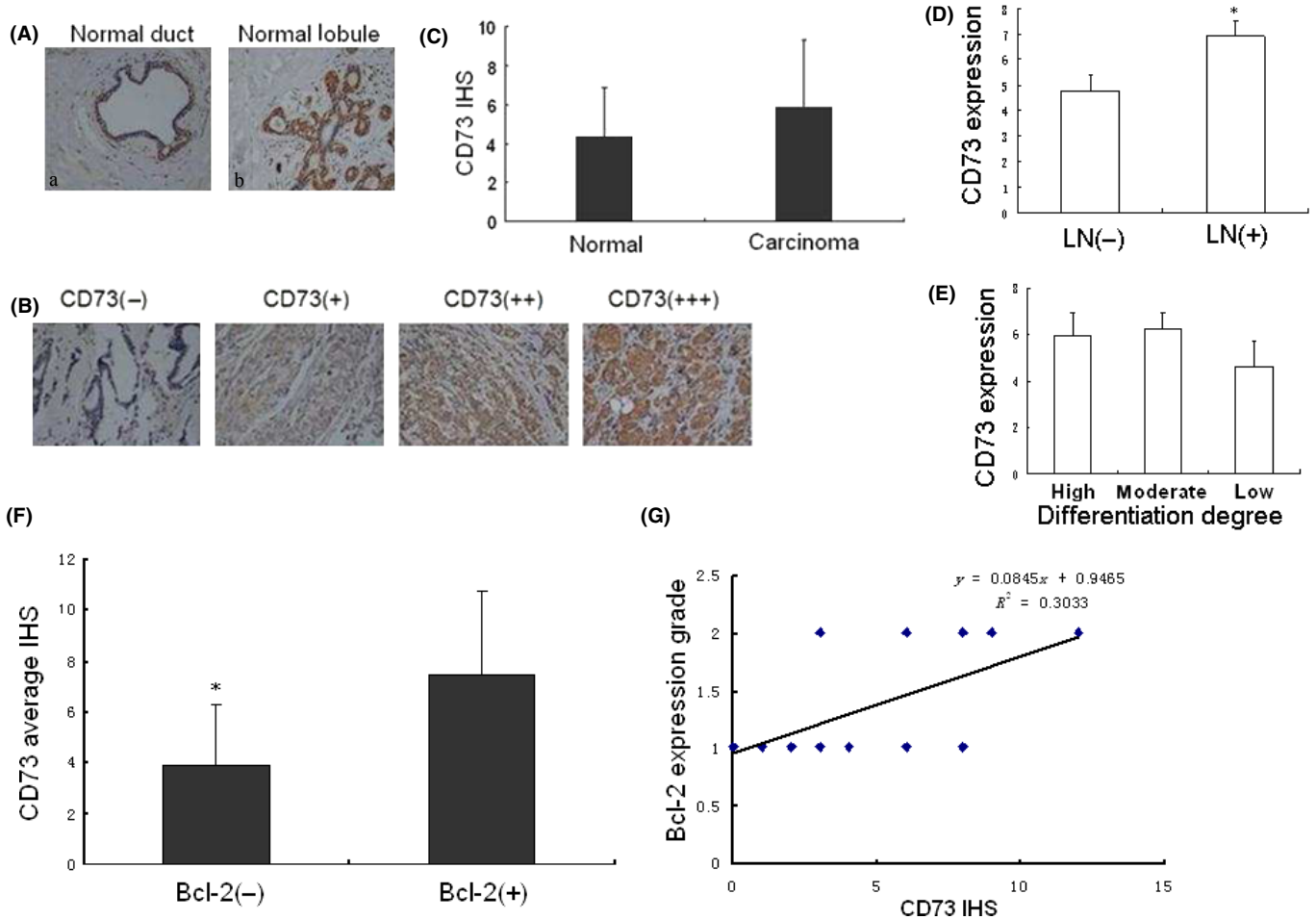

Fig. 6. Tissue microassay. (A) Expression of CD73 in normal mammary gland (×200). Left, normal mammary gland; right, lobule of normal mammary gland. (B) Expression of CD73 in breast tumor specimens with different grades. CD73 was differentially expressed in tumor cells. The glandular stroma contained fibrocytes and a subset of the fibrocytes was CD73 positive. (C) The CD73 expression level in invasive duct carcinoma (n = 49) was higher than normal mammary gland (n = 10), although the difference was not statistically significant ($P > 0.05$). (D,E) CD73 expression was positively related to lymph node (LN) metastasis but not to grading. (F,G) There was significant positive correlation between CD73 and bcl-2 expressions in the breast cancer specimens (n = 25; r = 0.397; $^*P < 0.05$). IHS, immunohistochemical score.

Table 4. CD73 expression in 80 breast cancer cases

CD73 expression	Cases (n)
Strong	12
Moderate	28
Weak	36
Negative	4

The tumor specimens were assayed by immunohistochemistry, showing staining products in 95% of cases. 52.6% of the positive cases were graded as moderate and strong.

cycle and induces apoptosis,[27–33] whereas other studies support a protective role including anti-apoptosis.[34,35] In the present study, CD73 overexpression increased cell viability, which indicates that adenosine generated by tumor CD73 increased cancer cell viability and could prevent cell death. The diverse effects of adenosine in apoptosis appear to depend on the pattern of adenosine receptor activation (endogenous or exogenous) and on the types of adenosine receptors activated (A1, A2A, A2B, or A3). CD73 also has functions independent of its enzyme activity. However, little information is available on whether CD73 has a direct effect on cell apoptosis. In our study using flow cytometry and TUNEL assay, shRNA-targeted CD73 increased apoptosis of MB-MDA-231 cells. It also decreased the percentages of early and late apoptotic cells. As in our previous report,[36] CD73 inhibitor APCP functioned similarly with RNAi-mediated CD73 suppression in apoptosis. However, the effect of RNAi-mediated CD73 suppression in apoptosis was stronger than APCP, which indicates that CD73 may have an effect on apoptosis independent of its activity. Mikhailov *et al.*[37] reported that CD73-mediated resistance against TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis was independent of the enzymatic activity of CD73, but was reliant on the interaction of CD73 with death receptor 5.

Among apoptotic regulatory proteins, the Bcl-2 family, including both anti-apoptotic (Bcl-2, Bcl-XL, Mcl-1) and pro-apoptotic (Bid, Bax, Bad) members, is particularly important.[38] The levels of anti-apoptotic protein Bcl-2 and pro-apoptotic proteins Bax were identified to determine the mechanism of CD73-shRNA-induced apoptosis; CD73 inhibition caused a significant reduction in levels of the Bcl-2 gene and increased levels of Bax gene mRNA and protein compared with the controls. Furthermore, data from the case history of Bcl-2 expression provided by Shanghai Outdo Biotech Co. and our CD73 immunohistochemical (HIC) assay showed that CD73 and Bcl-2 were co-expressed in breast cancer tissues. We also examined the

doi: 10.1111/j.1349-7006.2010.01733.x

expression of Caspase-3 by real-time PCR and western blot, given its pivotal role in apoptosis. Our results revealed that CD73 inhibition significantly increased Caspase-3 expression and activation. These findings suggest that both the Bcl-2 family of proteins and caspase-3 are involved in apoptosis induced by CD73 siRNA.

Although not statistically significant, which may be due to the small sample size, CD73 was expressed more in breast cancer than in normal mammary glands in a tissue microassay study, supporting the role of CD73. Tumor growth is usually closely associated with a hypoxic environment. Hypoxia produces hypoxic-induced factor, which can upregulate CD73.[3] This may explain why CD73 is overexpressed during cancer progression. In turn, the increased CD73 promotes cancer progression, including angiogenesis, tumor growth, migration, invasion and metastasis. In addition, our data suggested that CD73 expression was positively related to lymph node metastasis but not to grading. Besides the direct tumor-promoting effects of CD73, tumor CD73 can promote tumor metastasis by an adaptive immune system. Stagg *et al.*[9] identified tumor-derived CD73 as a mechanism for tumor immune escape and tumor metastasis.

In summary, we demonstrated that CD73 overexpression promoted MCF-7 cell viability and cycle progression depending on its enzyme activity, while CD73 suppression significantly reduced breast cancer growth *in vivo* and *in vitro*. CD73 might be promoting breast cancer growth by arresting the cell cycle at the S phase and reducing cell apoptosis. Thus, targeting CD73 expression might be an important means for breast cancer therapy.

Acknowledgment

The authors are grateful to Xu Chen for his sincere help and technical support. This article is funded by project of Science and Technology Commission of Shanghai Municipality, project code: 0852nm04400.

Disclosure Statement

The authors have no conflict of interest.

Reference

1 Walt G. WHO's World Health Report 2003. *BMJ* 2004; **328**: 6.
2 Bianchi V, Spychala J. Mammalian 5′-nucleotidases. *J Biol Chem* 2003; **278**: 46195–8.
3 Colgan SP, Eltzschig HK, Eckle T, Thompson LF. Physiological roles for ecto-5′-nucleotidase (CD73). *Purinergic Signal* 2006; **2**: 351–60.
4 Spychala J. Tumor-promoting functions of adenosine. *Pharmacol Ther* 2000; **87**: 161–73.
5 Buffon A, Wink MR, Ribeiro BV *et al.* NTPDase and 5′ ecto-nucleotidase expression profiles and the pattern of extracellular ATP metabolism in the Walker 256 tumor. *Biochim Biophys Acta* 2007; **1770**: 1259–65.
6 Sadej R, Spychala J, Skladanowski AC. Expression of ecto-5′-nucleotidase (eN, CD73) in cell lines from various stages of human melanoma. *Melanoma Res* 2006; **16**: 213–22.
7 Bavaresco L, Bernardi A, Braganhol E *et al.* The role of ecto-5′-nucleotidase/CD73 in glioma cell line proliferation. *Mol Cell Biochem* 2008; **319**: 61–8.
8 Zhi X, Chen S, Zhou P *et al.* RNA interference of ecto-5′-nucleotidase (CD73) inhibits human breast cancer cell growth and invasion. *Clin Exp Metastasis* 2007; **24**: 439–48.
9 Stagg J, Divisekera U, McLaughlin N *et al.* Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. *Proc Natl Acad Sci USA* 2010; **107**: 1547–52.
10 Kondo T, Nakazawa T, Murata SI, Katoh R. Expression of CD73 and its ecto-5′-nucleotidase activity are elevated in papillary thyroid carcinomas. *Histopathology* 2006; **48**: 612–4.
11 Sadej R, Spychala J, Skladanowski AC. Ecto-5′-nucleotidase (eN, CD73) is coexpressed with metastasis promoting antigens in human melanoma cells. *Nucleosides Nucleotides Nucleic Acids* 2006; **25**: 1119–23.
12 Hastie C, Saxton M, Akpan A, Cramer R, Masters JR, Naaby-Hansen S. Combined affinity labelling and mass spectrometry analysis of differential cell surface protein expression in normal and prostate cancer cells. *Oncogene* 2005; **24**: 5905–13.
13 Zhou P, Zhi X, Zhou T *et al.* Overexpression of Ecto-5′-nucleotidase (CD73) promotes T-47D human breast cancer cells invasion and adhesion to extracellular matrix. *Cancer Biol Ther* 2007; **6**: 426–31.
14 Gutensohn W, Resta R, Misumi Y, Ikehara Y, Thompson LF. Ecto-5′-nucleotidase activity is not required for T cell activation through CD73. *Cell Immunol* 1995; **161**: 213–7.
15 Bonitati AE, Agarwal KC, Rounds S. A simple assay for ecto-5′-nucleotidase using intact pulmonary artery endothelial cells Effect of endotoxin-induced cell injury. *Biochem Pharmacol* 1993; **46**: 1467–73.
16 Yang M, Luiken G, Baranov E, Hoffman RM. Facile whole-body imaging of internal fluorescent tumors in mice with an LED flashlight. *BioTechniques* 2005; **39**: 170–72.
17 Bloomer CW, Kenyon L, Hammond E *et al.* Cyclooxygenase-2 (COX-2) and epidermal growth factor receptor (EGFR) expression in human pituitary macroadenomas. *Am J Clin Oncol* 2003; **26**: S75–80.
18 Zimmermann H. 5′-Nucleotidase: molecular structure and functional aspects. *Biochem J* 1992; **282** (Pt 2) : 345–65.
19 Eroglu A, Canbolat O, Demirci S, Kocaoglu H, Eryavuz Y, Akgul H. Activities of adenosine deaminase and 5′-nucleotidase in cancerous and noncancerous human colorectal tissues. *Med Oncol* 2000; **17**: 319–24.
20 Spychala J, Lazarowski E, Ostapkowicz A, Ayscue LH, Jin A, Mitchell BS. Role of estrogen receptor in the regulation of ecto-5′-nucleotidase and adenosine in breast cancer. *Clin Cancer Res* 2004; **10**: 708–17.
21 Ludwig HC, Rausch S, Schallock K, Markakis E. Expression of CD 73 (ecto-5′-nucleotidase) in 165 glioblastomas by immunohistochemistry and electronmicroscopic histochemistry. *Anticancer Res* 1999; **19**: 1747–52.
22 Clarke R, Dickson RB, Brunner N. The process of malignant progression in human breast cancer. *Ann Oncol* 1990; **1**: 401–7.
23 Smith LM, Wise SC, Hendricks DT *et al.* cJun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype. *Oncogene* 1999; **18**: 6063–70.
24 Braganhol E, Tamajusuku AS, Bernardi A, Wink MR, Battastini AM. Ecto-5′-nucleotidase/CD73 inhibition by quercetin in the human U138MG glioma cell line. *Biochim Biophys Acta* 2007; **1770**: 1352–9.
25 Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *J Intern Med* 2005; **258**: 479–517.
26 Ohana G, Bar-Yehuda S, Barer F, Fishman P. Differential effect of adenosine on tumor and normal cell growth: focus on the A3 adenosine receptor. *J Cell Physiol* 2001; **186**: 19–23.
27 Barry CP, Lind SE. Adenosine-mediated killing of cultured epithelial cancer cells. *Cancer Res* 2000; **60**: 1887–94.
28 Peyot ML, Gadeau AP, Dandre F, Belloc I, Dupuch F, Desgranges C. Extracellular adenosine induces apoptosis of human arterial smooth muscle cells via A(2b)-purinoceptor. *Circ Res* 2000; **86**: 76–85.
29 Schrier SM, van Tilburg EW, van der Meulen H, Ijzerman AP, Mulder GJ, Nagelkerke JF. Extracellular adenosine-induced apoptosis in mouse neuroblastoma cells: studies on involvement of adenosine receptors and adenosine uptake. *Biochem Pharmacol* 2001; **61**: 417–25.
30 Appel E, Kazimirsky G, Ashkenazi E, Kim SG, Jacobson KA, Brodie C. Roles of BCL-2 and caspase 3 in the adenosine A3 receptor-induced apoptosis. *J Mol Neurosci* 2001; **17**: 285–92.
31 Zhao Z, Kapoian T, Shepard M, Lianos EA. Adenosine-induced apoptosis in glomerular mesangial cells. *Kidney Int* 2002; **61**: 1276–85.
32 Yasuda Y, Saito M, Yamamura T, Yaguchi T, Nishizaki T. Extracellular adenosine induces apoptosis in Caco-2 human colonic cancer cells by activating caspase-9/-3 via A(2a) adenosine receptors. *J Gastroenterol* 2009; **44**: 56–65.
33 Wang MX, Ren LM. Growth inhibitory effect and apoptosis induced by extracellular ATP and adenosine on human gastric carcinoma cells: involvement of intracellular uptake of adenosine. *Acta Pharmacol Sin* 2006; **27**: 1085–92.
34 Boucher M, Wann BP, Kaloustian S, Cardinal R, Godbout R, Rousseau G. Reduction of apoptosis in the amygdala by an A2A adenosine receptor agonist following myocardial infarction. *Apoptosis* 2006; **11**: 1067–74.
35 Hasegawa T, Bouis D, Liao H, Visovatti SH, Pinsky DJ. Ecto-5′ nucleotidase (CD73)-mediated adenosine generation and signaling in murine cardiac allograft vasculopathy. *Circ Res* 2008; **103**: 1410–21.
36 Zhou X, Zhi X, Zhou P *et al.* Effects of ecto-5′-nucleotidase on human breast cancer cell growth in vitro and in vivo. *Oncol Rep* 2007; **17**: 1341–6.
37 Mikhailov A, Sokolovskaya A, Yegutkin GG *et al.* CD73 participates in cellular multiresistance program and protects against TRAIL-induced apoptosis. *J Immunol* 2008; **181**: 464–75.
38 Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2002; **2**: 647–56.