Bone Marrow Stromal Cell-derived Growth Inhibitor Inhibits Growth and Migration of Breast Cancer Cells via Induction of Cell Cycle Arrest and Apoptosis*

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Genes encoding growth-inhibitory proteins are postulated to be candidate tumor suppressors. The identification of such proteins may benefit the early diagnosis and therapy of tumors. Here we report the cloning and functional characterization of a novel human bone marrow stromal cell (BMSC)-derived growth inhibitor (BDGI) by large scale random sequencing of a human BMSC cDNA library. Human BDGI cDNA encodes a 477-amino acid residue protein that shares high homology with rat and mouse pregnancy-induced growth inhibitors. The C-terminal of BDGI is identical to a novel human pregnancy-induced growth inhibitor, OKL38. BDGI is also closely related to many other eukaryotic proteins, which together form a novel and highly conserved family of BDGI-like proteins. BDGI overexpression inhibits the proliferation, decreases anchorage-dependent growth, and reduces migration of MCF-7 human breast cancer cells, whereas down-regulation of BDGI expression promotes the proliferation of MCF-7 and HeLa cervix epitheliod carcinoma cells. Interestingly, the inhibitory effect of BDGI on MCF-7 cells is more potent than that of OKL38. We demonstrate that BDGI induces cell cycle arrest in S phase and subsequent apoptosis of MCF-7 cells, which is likely to account for the antiproliferative effects of BDGI. This process may involve up-regulation of p27Kip1 and down-regulation of cyclin A, Bcl-2, and Bcl-xL. The inhibitory effect of BDGI on cell proliferation and the induction of apoptosis were also observed in A549 lung cancer cells but not HeLa cells. These results indicate that BDGI might be a growth inhibitor for human tumor cells, especially breast cancer cells, possibly contributing to the development of new therapeutic strategies for breast cancer.

Current therapeutic approaches are not particularly effective in the treatment of breast cancer, especially for patients with metastasis (1). A deeper knowledge of the genetic alterations and molecular mechanisms involved in breast cancer may allow the development of novel therapeutic strategies. Since the identification of BRCA1 and BRCA2 (2, 3), studies of tumor suppressor genes have been viewed as one of the most promising approaches to determine the mechanism of transformation and provide strategies for early diagnosis and therapy of breast cancer. A potentially valuable group of anti-tumor agents are endogenous polypeptide growth inhibitors, which function during normal tissue modeling (4). In mammary glands, several endogenous factors with growth-inhibiting or differentiation-inducing effects on mammary epithelial cells have been characterized, including transforming growth factor-β (5), mammastatin (6), neuregulin (7), mammary-derived growth inhibitor (8), and mammary-derived growth inhibitor-related gene (9). Mammastatin, a polypeptide purified from conditioned media of normal mammary epithelial cells, blocks DNA synthesis in cultured normal and transformed mammary epithelial cells (6). The more recently identified mammary-derived growth inhibitor and mammary-derived growth inhibitor-related gene factors exhibit inhibitory effects on the proliferation of breast cancer cells (8–10) and suppress breast tumor growth in the nude mouse model (11). These findings indicate that growth inhibitors have potential for use in strategies to inhibit growth of breast cancers.

In the present study, we report a novel growth inhibitor for breast cancer and other tumor cells, first identified by large scale random sequencing of a cDNA library of human bone marrow stromal cells (BMSCs),1 designated as the BMSC-derived growth inhibitor (BDGI). The C-terminal 317 residues of BDGI are identical to a previously identified pregnancy-induced growth inhibitor, OKL38 (12, 13), which may function to regulate growth and differentiation of breast epithelial cells during pregnancy and in tumorigenesis. We demonstrate that overexpression of exogenous BDGI can suppress the growth and migration of MCF-7 human breast cancer cells, inducing cell cycle arrest and cellular apoptosis, possibly by up-regulating expression of p27Kip1 and down-regulating cyclin A, Bcl-2, and Bcl-xL, whereas down-regulation of BDGI expression promotes the proliferation of MCF-7 and HeLa cervix epitheloid carcinoma cells. BDGI also inhibits proliferation and induces cellular apoptosis of A549 lung cancer cells, but not HeLa cells. Interestingly, the inhibitory effect of BDGI on MCF-7 cells is more potent than that of OKL38. These results suggest that

1 The abbreviations used are: BMSC, bone marrow stromal cell; BDGI, bone marrow stromal cell-derived growth inhibitor; Cdk, cyclin-dependent kinase; PI, propidium iodide; ORF, open reading frame; FBS, fetal bovine serum; RT, reverse transcription; siRNA, small interfering RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; UTR, untranslated region.
BDGI may be a functional growth inhibitor for tumor cells involved in regulating cell cycle and apoptosis. The mechanism by which BDGI suppresses growth and migration and induces apoptosis of tumor cells requires further investigation.

MATERIALS AND METHODS

Cell Culture—MCF-7 human breast cancer cells, A549 lung cancer cells and HeLa cervix epithelial carcinoma cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) in a 37 °C, 5% CO₂ atmosphere.

Isolation of BDGI cDNA—The full-length BDGI cDNA was isolated directly from a human BMSC cDNA library by large scale random sequencing, as described previously (14). Briefly, a BMSC cDNA library was constructed in the pCMV-SPORT6.0 vector (Invitrogen) using the Superscript plasmid system for cDNA synthesis and plasmid cloning (Invitrogen). The full-length cDNA clone, HNC209, was found to contain an open reading frame (ORF) potentially encoding a protein that was identical to, but 160 residues longer than, the recently identified protein OKL38, thought to play an important role in the growth regulation and differentiation of breast epithelial cells. Since the protein was derived from BMSC and later demonstrated to inhibit the growth of breast cancer cells, and lung cancer cells, it was designated as BDGI.

Blot Analysis—Human adult multiple tissue Northern blots were purchased from Clontech and hybridized according to the manufacturer's instructions. The full-length BDGI ORF was used as a template for probe synthesis. The filters were hybridized with the 32P-labeled BDGI cDNA probe and then probed with a human β-actin cDNA probe.

RT-PCR Analysis—Total cellular RNA was extracted using Trizol reagent (Invitrogen) and first-strand cDNA prepared with avian myeloblastosis virus reverse transcriptase (Promega) using an oligo(dT)12-18 primer. Synthesis of cDNA was checked by RT-PCR using β-actin primers (14). RT-PCRs with the BDGI-specific primers 5’-GCGAATTCATCGAAGCTT GCAAGGGACTGTCTTTGCGCCCTG-3’ (upstream) and 5’-GGAAGTTC AGTTTTGCTGTTCTCCTG-3’ (downstream) were subjected to denaturation (94 °C for 30 s), annealing (58 °C for 30 s), and extension (72 °C for 1 min). The PCR conditions were observed for the entire ORF of BDGI and was confirmed by DNA sequencing.

Generation of Anti-BDGI Polyclonal Antibody—Sequence encoding the N-terminal 200 residues of BDGI was inserted into the pET-24a vector (Novagen). His-tagged BDGI protein was expressed in Escherichia coli strain BL21 and purified by Ni²⁺-nitrilotriacetic acid resin affinity chromatography (Qiagen). Polyclonal antibody to recombinant BDGI protein (anti-BDGI) was generated in rabbit against purified His-tagged BDGI protein and purified using protein A affinity chromatography (Pierce).

Construction of Eukaryotic Expression Vector and Stable Transfection—Full-length coding region of BDGI cDNA was inserted into pcDNA3.1/myc-His (−) B expression vector (Invitrogen) to generate pcDNA3.1/BDGI, which was transfected by the cytomegalovirus promoter. For stable transfection, MCF-7 human breast cancer cells, A549 lung cancer cells and HeLa cervix epithelial carcinoma cells were transfected with pcDNA3.1/BDGI or mock control pcDNA3.1/myc-His (−) B using Lipofectamine reagent (Invitrogen) and screened under 800 µg/ml G418 (Merck) for ~3 weeks. Cellular clones of stably transfected cells, MCF-7/BDGI, MCF-7/mock, A549/BDGI, A549/mock, HeLa/BDGI, and HeLa/mock were obtained by limiting dilution and confirmed by Western blot analysis using anti-BDGI polyclonal antibody. Polyclone reagent (Qiagen) was used for transient transfection of MCF-7 cells.

BDGI siRNA Assay—19-nt sequences of BDGI siRNA were synthesized by GeneChem: 5’-CACCCUCAAGGACAGCATG (sense) and 5’-UCUGCUUCUGUGAGGUGTT (antisense). Negative control siRNA (cloned into pSPORT6) was used as the transfection control. For stable transfection, MCF-7 human breast cancer cells, A549 lung cancer cells and HeLa cervix epithelial carcinoma cells were transfected with pcDNA3.1/BDGI or mock control pcDNA3.1/myc-His (−) B using Lipofectamine reagent (Invitrogen) and screened under 800 µg/ml G418 (Merck) for ~3 weeks. Cellular clones of stably transfected cells, MCF-7/BDGI, MCF-7/mock, A549/BDGI, A549/mock, HeLa/BDGI, and HeLa/mock were obtained by limiting dilution and confirmed by Western blot analysis using anti-BDGI polyclonal antibody. Polyclone reagent (Qiagen) was used for transient transfection of MCF-7 cells.

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Proliferative Assay—3-[3H]thymidine (1 µCi, 90 Ci/mmol; Amer sham Biosciences) was used to determine the proliferative activity of stable MCF-7 transfectants. Tumor cells in exponential growth phase were trypsinized and resuspended in growth media. Cells were plated in triplicate in 96-well microtiter plates at 2 × 10⁵/well and incubated for 56 h. [3H]thymidine (1 µCi/well) was added, and cultures were incubated for a further 16 h. Incorporation of [3H]thymidine into DNA was measured using an LS 6500 scintillation counter (Beckman Coulter). Data were gathered as counts/min, representing the fraction of tumor cells that contained newly synthesized DNA. Inhibition of proliferation was determined by comparing the level of [3H]thymidine uptake with controls.

MTT Assay—Cell proliferation of stably transfected A549 and HeLa cells was measured by MTT dye reduction assay. Briefly, cells were seeded into 96-well plates, and on the day of harvest, 100 µl of spent medium was replaced with an equal volume of fresh medium containing 10% MTT 5 mg/ml stock. Plates were incubated at 37 °C for 4 h and then 100 µl of MeSO (Sigma) was added to each well, and plates were shaken at room temperature for 10 min. Cellular viability was determined by measuring the absorbance of the converted dye at a wavelength of 570 nm.

Anchorage-independent Growth Assay—Soft agar growth analyses were performed as described previously (15). In brief, stable BDGI transfected or control cells (500/well) were seeded into 0.3% Bacto-agar (Difco) over a 0.6% agar bottom layer in triplicate in 6-well plates. Both layers contained 1 × Dulbecco's modified Eagle's medium supplemented with 10% FBS. Plates were incubated at 37 °C in 5% CO₂ for 3 weeks. Colonies greater than 100 µm in diameter were counted.

Cell Wounding and Migration Assay—5 × 10⁵ stably transfected MCF-7 cells were plated in 60-mm culture dishes in Dulbecco's modified Eagle's medium plus 1% FBS. After 24 h, a 10-μl pipette tip was used to create a small wound in the cell monolayer. Cell migration was observed under the microscope to verify that no cells remained. At 36 and 48 h after wounding, cell migration was analyzed by measuring (a) the number of cells in the wound area per microscopic field and (b) the maximum migration distance by the cell front into the wound area (17). Measurements were taken from 10–15 individual microscopic fields in each experiment, and the data from three experiments are summarized.

Cell Cycle Analysis—MCF-7 cells transiently transfected with pcDNA3.1/BDGI or control vector were synchronized as described (18, 19). Briefly, at 24 h after transfection, cells were serum-starved for 36 h and then stimulated with 10% FBS and 2 µg/ml aprotinin (ICN Biomedical) for 24 h. Cells were washed twice with PBS to remove any remaining aprotinin, and then 10% FBS Dulbecco's modified Eagle's medium was added after 9 h. Cultures were harvested, fixed with 70% ethanol at 4 °C overnight, and stained with propidium iodide (PI) for DNA content analysis using CellQuest software 6.0.

Cellular Apoptosis Assay—MCF-7, A549 or HeLa cells transiently transfected with either pcDNA3.1/BDGI or control vector were trypsinized to detach any adherent cells. Cells were harvested and washed twice, suspended in PBS, and stained with annexin V/fluorescein isothiocyanate (Bender) for 10 min at room temperature. Cells were then washed and resuspended in binding buffer. PI was added, and cells were analyzed immediately by flow cytometry using ModFit 3.0 LTTM Software (Verity Software House). Cellular apoptosis was also confirmed by transmission electron microscopy. Briefly, cells were harvested as above, washed in PBS, and cell pellets were fixed by incubating with 2.5% glutaraldehyde for 2 h at 4 °C and then 1% osmium tetroxide for 1 h at 4 °C. After dehydration in a graded series of ethanol and infiltration in propylene oxide, cells were embedded in Epon 812. Ultrathin 60-nm sections were stained with uranyl acetate and lead citrate, and cell morphology observed by transmission electron microscopy (Philips Tecnai 10) at 80 kV.

Western Blot Analysis—Cells harvested were lysed with cold cell lysis buffer (Cell Signaling), and protein concentration was determined using the BCA protein assay ( Pierce) according to the manufacturer's instructions. Cell lysates (50 µg) were loaded onto 12% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and subjected to Western blot analysis (14). Relevant proteins were visualized using primary antibodies specific for cyclin A, p27Kip1, cyclin D, cyclin E, and cyclin C (Santa Cruz Biotechnology, CA), Bcl-2 (Oncozine), and phosphorylated Bcl-2, Bcl-xL, and Bax (Cell Signaling). Blots were then probed with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), and reactive protein bands were visualized with LumiGlo reagents (Cell Signaling).

Statistical Analysis—Pairwise comparisons were conducted using Student's t test. Group differences resulting in p values of less than 0.05 were considered to be statistically significant.
RESULTS

Identification and Sequence Analysis of BDGI, a Longer Variant of OKL38—A 1766-bp full-length novel cDNA (GenBank™ accession number AY037158) was isolated from a human BMSC cDNA library. It is composed of a single 1434-bp ORF encoding a 477-residue protein with a calculated molecular mass of 51.99 kDa, an isoelectric point of 6.63, a 5’-untranslated region (UTR) of 146 bp, and a 3’-UTR of 186 bp. The presence of an upstream in-frame stop codon in the 5’-UTR indicates that it is a full-length cDNA clone. The putative protein lacks both an N-terminal signal peptide, as predicted by the SOSUI (signal) program, and transmembrane regions, determined using the Tmpred program, indicating that it might be a nonsecretory, soluble protein. At the amino acid level, the C-terminal residues of the protein were identical to human OKL38 (pregnancy-induced growth inhibitor), a recently identified 317-residue protein thought to play an important role in the regulation of breast epithelial cell growth and differentiation. However, BDGI is 160 residues longer than OKL38 in the N terminus. Chromosome mapping of both sequences revealed that they shared an identical location on chromosome 16q23.3. Consistent with the findings of Ong et al. (13), the protein identified by us appears to be a longer variant of the previously cloned OKL38. A Blast search of the protein data base revealed significant homology with rat and mouse pregnancy-induced growth inhibitors (for overall protein sequence; 82 and 81% identity and 90 and 89% similarity, respectively; Fig. 1A). Based on its sequence identity and similarity with pregnancy-induced growth inhibitors and its capacity for inhibiting tumor growth and proliferation (shown below), the protein was designated as BDGI.

Interestingly, homology analysis also revealed close similarity (about 40–65%) to a variety of unknown/undefined proteins originating from species such as Homo sapiens, Mus musculus, Rattus norvegicus, Danio rerio, Anopheles gambiae, Caenorhabditis elegans, C. briggsae, and Aspergillus nidulans. Phylogenetic analysis of these proteins was performed using ClustalW and TreeView software (Fig. 1B). The results revealed a close association between BDGI and rat and mouse pregnancy-induced growth inhibitors, as expected from the high sequence identity mentioned above. Structural analysis showed that most of these proteins were predicted to contain a domain belonging to a flavoprotein involved in K⁺ transport. TrkA. Of note, these proteins could be categorized into three classes, based on homology, structural features, and phylogenetic analysis (Fig. 1B). One group included human BDGI (477 residues), mouse and rat pregnancy-induced growth inhibitors (AAH22135 and NP_612513, both 478 residues in length), D. rerio AAH45855 (514 residues), and A. gambiae EAA13013 (453 residues), which all contain a C-terminal pyridine nucleotide-disulfide oxidoreductase domain except the TrkA domain; the second included human NP_004328 (505 residues), mouse BAC33436 (550 residues), and rat XP_232798 (505 residues), all proteins of greater than 500 residues, containing a C-terminal thioredoxin reductase domain; the remaining proteins, C. briggsae CAE61458 (503 residues), C. elegans AAA83493 (455 residues), and A. nidulans EAA65176 (519 residues), with no other predicted domains besides TrkA, form a third group. These disparate proteins do appear to form a family of BDGI-like proteins; however, it
remains to be determined whether these proteins share similar functions and what the nature of these functions might be.

**Expression Pattern of Human BDGI mRNA and Protein**—The mRNA expression pattern of human BDGI was analyzed by Northern blot and RT-PCR analysis. Northern blot analysis detected a major band of ~2.0 kb. Relatively high levels of mRNA expression were observed in testis, liver, and skeletal muscle, with lower levels in spleen, heart, kidney, and pancreas. No BDGI expression was detected in thymus, prostate, ovary, small intestine, colon, peripheral blood leukocyte, brain, placenta, or lung (Fig. 2A).

RT-PCR analysis revealed differences in BDGI expression levels between different cell types and cell lines. Relatively high levels of BDGI expression were seen in freshly isolated BMSC, whereas expression was not detectable in T cells, B cells, or monocytes (Fig. 2B). In hematopoietic tumor cell lines, high levels of BDGI expression were found in Daudi (B lymphoma), K562 (chronic myelogenous leukemia), Reh (acute lymphocytic leukemia), and HL-60 (promyelocytic leukemia) cells, with lower expression in Raji (B lymphoma) and Molt-4 (T lymphoblastic leukemia) cells. No BDGI mRNA expression was detectable in Jurkat or Hut78 cells. In nonhematopoietic tumor cell lines, RT-PCR detected higher levels of BDGI expression in HeLa (cervix epithelioid carcinoma), PC-3 (prostate adenocarcinoma), and CaoV-3 (ovary adenocarcinoma) cells and relatively low expression in SMMC7721 (hepatocellular carcinoma) and MCF-7 (breast adenocarcinoma) cells. BDGI expression was undetectable in HT29, LoVo, U251, or A549 cells (Fig. 2C). Since BDGI mRNA was expressed in several human tumor cell lines, protein expression of endogenous BDGI in these cells
was examined. To this end, an anti-BDGI polyclonal antibody to the N-terminal 200 residues of BDGI was produced. As shown in Fig. 2D, by Western blot analysis using anti-BDGI polyclonal antibody, a specific band of about 52 kDa was observed, strong in PC-3, HeLa, and HL-60 cells and much fainter in MCF-7 cells, but not in A549 or LoVo cells. The apparent molecular weight of endogenous BDGI protein was consistent with that predicted from its deduced amino acid sequence. Besides, no secretion of BDGI protein into culture media by HeLa cells that express endogenous BDGI was detected by Dot blot analysis (Fig. 2E), indicating that BDGI was not a secreted protein, which was consistent with the structural prediction of BDGI.

**BDGI Overexpression Inhibits Cell Proliferation and Suppresses Tumor Cell Colony Formation**—To further investigate the biological functions of human BDGI, MCF-7 human breast cancer cells were transfected with pcDNA3.1/BDGI and mock control vectors, and stable transfectants were obtained by G418 selection. A, the expression of BDGI in MCF-7 transfectants was confirmed by Western blot analysis using anti-BDGI polyclonal antibody. Values shown are mean ± S.D. for quadruplicate cultures from one experiment, representative of four independent experiments conducted. B, cell growth was determined by anchorage-independent growth assay in soft agar. 500 MCF-7 cells/well were seeded in 6-well plates. After 3 weeks, colonies greater than 100 μm in diameter were counted. Values are mean ± S.D. for triplicate cultures. *, p < 0.01 versus parental MCF-7 or MCF-7/mock cells; **, p < 0.05 versus MCF-7/OKL38 cells. C, proliferation of stably transfected MCF-7 cells was examined by [3H]thymidine incorporation assay. The result shows that overexpression of BDGI inhibited cell proliferation of MCF-7 cells. As shown in Fig. 3B, proliferation of stably transfected A549 cells was determined by MTT, p < 0.05 versus parental A549 or A549/mock cells.

We examined the effects of BDGI on MCF-7 cell proliferation, since BDGI is a longer splicing variant of the pregnancy-induced growth inhibitor, OKL38, known to inhibit cell proliferation (12). [3H]Thymidine incorporation was used to evaluate DNA synthesis consistent with proliferation of stably transfected cells. The result shows that overexpression of BDGI inhibited cell proliferation of MCF-7 cells. As shown in Fig. 3B, [3H]thymidine incorporation was 4-fold lower in MCF-7/BDGI transfectants than in MCF-7/mock (p < 0.01), which showed a similar proliferative rate to parental MCF-7 cells. Importantly, the inhibitory effect of BDGI overexpression on the proliferation of MCF-7 cells was more potent than that of OKL38 (p < 0.05). Loss of anchorage dependence for growth is a characteristic of malignant transformed cells. A soft agar colony formation assay was performed to investigate whether BDGI overexpression abolished or modulated this feature of MCF-7 cells. As shown in Fig. 3C, colonies derived from MCF-7/BDGI were significantly smaller than those of controls, in that fewer colonies were of countable size (>100 μm in diameter). This reduction in the rate of countable colony formation in MCF-7/BDGI cells was statistically significant when compared with colony formation by MCF-7/mock and parental MCF-7 cells (p < 0.01). Together, these results suggest that overexpression of BDGI inhibits in vitro growth of human breast cancer cells.
The inhibition of cell proliferation by BDGI was also observed on A549 lung cancer cells. As shown in Fig. 3D, the proliferation of pcDNA3.1/BDGI-transfected A549 cells was inhibited compared with that of mock vector-transfected or parental cells ($p < 0.05$). The result indicated that the inhibition of cell proliferation by BDGI was not limited to breast cancer cells. But for HeLa cervix epitheloid carcinoma cells, the inhibitory effect of BDGI was hardly detected by MTT assay (data not shown).

Silencing of BDGI expression promotes proliferation of MCF-7 Breast Cancer Cells—Overexpression of BDGI in MCF-7 and A549 cells showed that this protein exhibits potent inhibitory effect on cancer cell proliferation. Given its moderate expression in MCF-7 cells, the possibility that silencing BDGI expression might accelerate the cell proliferation was investigated. siRNA (target sequence within the N-terminal 160 residues of BDGI) was used to silence the expression of BDGI protein in MCF-7 breast cancer cells and HeLa cervix epitheloid carcinoma cells, with a negative control siRNA duplex and no siRNA as controls. RT-PCR confirmed the effective silencing of BDGI expression in MCF-7 and HeLa cells (Fig. 4A). $[^3]H$/Thymidine incorporation was used to detect cell growth 48 h after siRNA transfection. As shown in Fig. 4, B and C, the proliferation of BDGI-silenced MCF-7 and HeLa cells increased as compared with controls ($p < 0.05$). The result further demonstrates that BDGI inhibits proliferation of tumor cells.

BDGI Overexpression Reduces the Migratory Ability of MCF-7 Breast Cancer Cells—Tumor growth and metastasis are multifaceted processes that involve cell adhesion, proteolytic degradation of the extracellular matrix and cell migration. Loss of invasive/metastatic potential is a key event in tumor regression. Cell mobility/migration of MCF-7 breast cancer cells was examined by in vitro culture wounding assay. Wounds were created in confluent cell cultures, and repopulation of the wound space was evaluated by counting the number of cells that migrated into the wound area and by measuring the maximum distance these cells traveled. MCF-7/BDGI and MCF-7/mock transfectants displayed differential migration in wounded cultures. BDGI overexpression significantly inhibited migration of MCF-7 cells, decreasing the number of MCF-7 cells migrating into the denuded area ($p < 0.05$; Fig. 5A) and also significantly decreasing the maximum distance MCF-7 cells migrated ($p < 0.05$; Fig. 5B).

BDGI Overexpression Blocks the Cell Cycle at S Phase and Induces Apoptosis of MCF-7 Cells—To investigate whether the inhibition of MCF-7 proliferation and migration by BDGI was mediated, at least in part, by regulating the cell cycle, MCF-7
cell cycle progression was analyzed by fluorescence-activated cell sorting. In agreement with the inhibitory effects of BDGI on cell proliferation, BDGI-overexpressing cells accumulated in S phase (≈37% at 9 h postsynchronization, compared with about 25% S phase cells in mock control), with a corresponding decrease in cell numbers in the G2/M phase (Fig. 6A). These results indicated that BDGI reduced MCF-7 proliferation by arresting cells in the S phase of the cell cycle, which was consistent with the [3H]thymidine incorporation finding that DNA synthesis, a hallmark of S phase progression, was also inhibited.

Following transient transfection, some MCF-7 cells displayed shrinkage and a loss of adherence, which suggested that BDGI overexpression might be inducing apoptosis. To assess whether apoptosis contributed to the BDGI-mediated decrease in cell proliferation, we performed fluorescein isothiocyanate-conjugated annexin V/PI staining followed by fluorescence-activated cell sorting analysis to detect cell apoptosis. The percentages of early apoptotic cells (annexin V-positive, PI-negative) and late apoptotic or necrotic cells (annexin V/PI-double positive) were calculated. The displayed result is representative of three independent experiments. C, detection of BDGI-induced apoptosis in MCF-7 cells by transmission electron microscopy analysis. 48 h after transfection, the cells were trypsinized, harvested, fixed, embedded in Epon 821, and stained with uranyl acetate and lead citrate. Ultrathin sections (60 nm) were analyzed by transmission electron microscopy.

**FIG. 6.** Effect of BDGI overexpression on apoptosis and cell cycle progression of MCF-7 cells. A, flow cytometric cell cycle analysis. Transiently transfected or parental MCF-7 cells were synchronized as described under “Materials and Methods.” Cells were harvested 9 h after aphidicolin removal and then fixed and stained with PI for DNA content analysis by fluorescence-activated cell sorting. B, flow cytometric analysis of cellular apoptosis. Transiently transfected or parental MCF-7 cells and A549 cells were stained with annexin V/fluorescein isothiocyanate and PI 48 h post-transfection. Percentages of early apoptotic cells (annexin V-positive, PI-negative) and late apoptotic or necrotic cells (annexin V/PI-double positive) were calculated. The displayed result is representative of three independent experiments. C, detection of BDGI-induced apoptosis in MCF-7 cells by transmission electron microscopy analysis. 48 h after transfection, the cells were trypsinized, harvested, fixed, embedded in Epon 821, and stained with uranyl acetate and lead citrate. Ultrathin sections (60 nm) were analyzed by transmission electron microscopy.
induction of apoptosis might thus be one of mechanisms by which BDGI inhibits proliferation of MCF-7 and A549 cells.

**BDGI Overexpression Induces Up-regulation of p27Kip1 and Down-regulation of Cyclin A, Bcl-2, and Bcl-xL—Apoptosis and cell cycle involve complex molecular cascades; dysfunction of any of a variety of genes may lead to cell cycle blockage and apoptotic progression. To gain insight into the mechanisms by which BDGI induces apoptosis and S phase arrest of MCF-7 cells, we examined the expression levels of cell cycle and apoptotic regulators by Western blot analysis. Since the appropriate temporal activation of cyclin E-cyclin-dependent protein kinase 2 (Cdk2), cyclin D1-Cdk2, and cyclin A-Cdk2 is required for progression through the G1 and S phases, we first examined expression of cyclins A, E, and D1, as well as cyclin C, following BDGI transient transfection. Interestingly, the level of cyclin A was decreased considerably at 24, 36, and 48 h post-transfection, compared with mock-transfected or parental MCF-7 cells, whereas the levels of cyclins D1, E, and C remained unchanged (Fig. 7A). The Cdk inhibitor p27Kip1 is the first identified to bind to and inhibit cyclin E-Cdk2 and cyclin A-Cdk2. The ability of p27 and related proteins to act as Cdk inhibitors and repress cell proliferation is well established. Overexpression of p27 leads to cell cycle arrest (22, 23), and antisense inhibition of p27 expression prevents quiescence following growth factor withdrawal (24). Of the various cyclin-Cdk complexes, p27Kip1 has its greatest inhibitory effect on Cdk-2 activity. p27 overexpression completely inhibits Cdk-2 activity and associated cell growth without significantly affecting Cdk-4 or Cdk-6 activity (25). p27 has also been reported to block cyclin A transcription in various cell types (27). Given that we have demonstrated up-regulated p27 expression accompanied by down-regulation of cyclin A in BDGI-overexpressing MCF-7 cells, the activation of cyclin A/Cdk2, a critical requirement for both the initiation and elongation of DNA replication in the late G1, and S progression (28), is probably impaired in this case. This might result from both the cyclin A degradation and p27 up-regulation together inhibiting the formation and/or inactivating the activity of the Cdk2 complex. In addition, whereas cyclin E levels de-
BDGI might be one of the local mammary-related inhibitory and breast cancer cell lines, together with the inhibition of differences in BDGI expression in normal mammary glands inancy and lactation (12) and is also expressed at lower levels in increased expression in normal mammary glands during pregnancy and lactation (36, 37). The breast cancer prevention. As hormonally related processes, it is associated with more potent inhibitory effects on cell proliferation and results in inhibition of cell growth and migration, consistent with the observations that p27 phosphorylated Bcl-2 was up-regulated, whereas Bax expression overexpression attenuates human vascular endothelial cell migration. Our data show that BDGI overexpression selectively up-regulates p27 expression in MCF-7 breast cancer cells and results in inhibition of cell growth and migration, consistent with recent reports that an elevated p27 protein level is associated with more potent inhibitory effects on cell proliferation and migration.

The impact of pregnancy and lactation on breast cancer risk has recently been of great interest in terms of approaches for breast cancer prevention. As hormonally related processes, it is widely accepted that pregnancy at an early age and subsequent breastfeeding reduce the risk of breast cancer (36, 37). The shorter variant of BDGI, OKL38, has been reported to exhibit increased expression in normal mammary glands during pregnancy and lactation (12) and is also expressed at lower levels in breast cancer cell lines, just as we have observed for BDGI. The differences in BDGI expression in normal mammary glands and breast cancer cell lines, together with the inhibition of breast cancer growth and migration by BDGI, suggest that BDGI might be one of the local mammary-related inhibitory factors that prevent benign cells from malignant progression during pregnancy and lactation or in situ lesions developing a metastatic phenotype. However, BDGI functions in other tissues or cells are not limited. The inhibitory effect on cell proliferation and the induction of cellular apoptosis by BDGI was also observed in A549 lung cancer cells. The potential application of BDGI as a cytostatic agent for the gene therapy of cancer warrants further investigation.

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