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Functional Elucidation and Methylation-Mediated Downregulation of ITGA5 Gene in Breast Cancer Cell Line MDA-MB-468

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ABSTRACT

Expression level of integrin α5 in tumor cells has been indicated to be involved in cell proliferation and organ-specific metastasis. We previously demonstrated that ITGA5 expression was downregulated in the high invasive MDA-MB-468 cells compared with other breast cancer cell lines. In this study, we found that the methylation status in the region around transcriptional start site of ITGA5 gene was increased in MDA-MB-468 cells. Overexpression of integrin α5 on MDA-MB-468 cells resulted in cell growth inhibition, which could be reversed by adhesion to fibronectin. Cell adhesion and spreading to fibronectin was enhanced after ITGA5 was overexpressed in MDA-MB-468 cells, while cell migration was attenuated. Knockdown of ITGA5 in MCF-7 cells led to cell growth inhibition but had little influence on cell migration. These findings indicated the diverse roles of ITGA5 expression in breast cancer cells. J. Cell. Biochem. 110: 1130–1141, 2010.

KEY WORDS: ITGA5; BREAST CANCER; CELL–ECM INTERACTION; CELL PROLIFERATION; MIGRATION

Integrins are transmembrane glycoproteins that consist of α and β subunits and mediate cell–matrix and cell–cell adhesions. Varieties of α and β subunits produce ligand selectivity to ECM and underlie vital cellular processes such as differentiation, migration, proliferation, and survival [Clark and Brugge, 1995; Howe et al., 1998; Giancotti and Ruoslahti, 1999; Schwartz, 2001; Askari et al., 2009].

Changes of integrin expression or its localization in transformed cells appear to contribute either positively or negatively to the transformed cell phenotype. For instance, alterations of α2β1, α3β1, and α6β1 integrins appear to be associated with malignancy in melanoma [Kramer et al., 1991; Natali et al., 1991, 1993; Moretti et al., 1993]. In breast cancer, α2β1, α5β1, or α6β4 integrins seem to be crucial for malignant transformation [Pignatelli et al., 1991; Natali et al., 1992; Zutter et al., 1993; Weaver et al., 1997]. In pancreatic carcinoma cell lines, expression of α2, α3, α6, β1, β4, and β5 integrins was detected in adenocarcinomas and ampullary tumors, in the normal pancreas, reduced levels of expression occur, or in the case of some integrins there is no expression [Hall et al., 1991].

In our previous studies, we reported the involvement of ITGA5 in integrin β1 overexpression-mediated growth arrest using different tumor cell lines [Fang et al., 2009]. We found in preliminary experiments that the expression of integrin α5 in highly invasive MDA-MB-468 cells was almost lost in comparison to other breast cancer cell lines. A recent study also reported the strong down-regulation of ITGA5 in breast cancer effusion compared with primary tumors [Konstantinovsky et al., 2010], which implied the negative correlation between ITGA5 expression and breast cancer cell metastasis. Since the expression level of ITGA5 has also been suggested to be involved in determining the organ specificity of tumor metastasis [Tani et al., 2003], the altered expression of ITGA5 in different breast cancer cells might be closely correlated with cell behavior and metastasis.

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The present work is part of our effort to understand the linkage between ITGA5 expression and breast cancer cell behavior. Here we reported that the ITGA5 gene was silenced in MDA-MB-468 cells due to the DNA hypermethylation around transcription start site of this gene. Overexpression of integrin α5 induced growth arrest in MDA-MB-468 cells, which could be reversed by attachment to fibronectin. Cell adhesion and spreading on fibronectin was increased, while cell migration was impaired after ITGA5 was transfected to MDA-MB-468 cells. These findings help us understand more about the diverse roles of integrins in breast cancer cells.

MATERIALS AND METHODS

REAGENTS

We used the following reagents: anti-integrinα5 and anti-integrinβ1 antibodies from BD Pharmingen. Crystal violet, Total RNA Isolation Kit, anti-GAPDH and anti-β-actin antibodies from Kangchen. Anti-p21 and anti-p27 antibodies from Calbiochem. SiRNA against integrin α5 as well as nonsense RNA (Santa Cruz Biotechnology), WST-1 reagent (Roche), fibronectin (Takara), lipofectamine2000 reagent (Invitrogen, CA), M-MLV reverse transcriptase (Invitrogen), formalin (Sigma), haematoxylin (Sigma), Geneclean II kit (Intermountain Scientific Corporation).

CELLS AND CULTURE CONDITIONS

Human basal-like breast cancer cell line MDA-MB-468, human breast adenocarcinoma cell line MCF-7 and human hepatocellular cancer cell line SMMC-7721 were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (PAA) and 1% penicillin/streptomycin (Life Technologies, Inc.).

PLASMIDS AND TRANSFECTION

The integrin β1 subunit expression vector, pcDNA3-β1, has been described previously. The integrin α5 subunit expression vector was a kind gift from Dr. Zhou (Biochemistry and Molecular Biology, University of South Alabama). The α5 defective-mutant expression vector contains the mutant α5 cDNA containing an alanine substitution of phenylalanine 187 [Irie et al., 1995] was a kind gift from Prof. Matsuura (Department of Pathology, School of Allied Health Science).

Cells were transfected with Lipofectamine2000 according to the manufacturer's instructions.

WESTERN BLOTTING

Cells were washed with PBS and lysed in a buffer containing 50 mM Tris-HCl (pH 6.8), 1% SDS, 10% glycerol, phosphatase inhibitors (100 mM Na_3VO_4, 10 mM NaF) and protease inhibitor (1 mM PMSF). Equal amounts of protein were loaded on a SDS–PAGE and transferred to PVDF membrane. After blocking with 5% non-fat milk in TBS-T (containing 0.1% Tween-20), the membranes were incubated with specific primary antibodies, followed by HRP-conjugated secondary antibodies. Proteins were visualized by fluorography using an enhanced chemiluminescence system.

RT-PCR AND REAL-TIME QUANTITATIVE PCR

Total RNA was isolated using the Trizol system according to the manufacturer's guidelines. Oligo(dT) 18 primer and M-MLV reverse transcriptase was used for first strand synthesis. The cDNA is then used as template for real-time PCR and RT-PCR with gene specific primers. The RT-PCR primers for ITGA5, ITGB1, and β-actin have been described previously [Fang et al., 2009]. Real-time PCR was performed with Real-time PCR Master Mix containing SYBR GREEN I and hotstart Taq DNApolymerase. GAPDH was amplified as control. The primers for ITGA5 and GAPDH are: ITGA5 (sense): 5′-TGC AAA GAT CTG TCC TCA-3′, ITGA5 (antisense): 5′-TGT GTG GCA TCT GTC CT-3′,PCR product length was 172 bp; GAPDH (sense): 5′-GAA GGT GAA GGT CGG AGT C-3′, GAPDH (antisense): 5′-GAA GAT GGT GAT GGG ATT TC-3′, PCR product length was 226 bp. Real-time detection of the emission intensity of SYBR GREEN bound to double-stranded DNAs was performed using the Icycler instrument (Bio-rad). At the end point of PCR cycles, melt curves were made to check product purity. The level of ITGA5 mRNA was expressed as a ratio relative to the GAPDH mRNA in each sample. The results were obtained from 15 reactions in each cell line and analyzed by Boxplot software.

DNA EXTRACTION AND SODIUM BISULFITE MODIFICATION

Total DNA was extracted by protease K-phenol extraction technique. Total DNA content and purity (A260/A280>1.8) was tested with an ultra-violet spectrophotometer. Denature 2 μg DNA by adding freshly prepared NaOH (3 M) to a final concentration of 0.3 M. Incubate at 42°C for 30 min. One thousand twenty microliters 40.5% sodium bisulfite, 60 μl 10 mM hydroquinone, 110 μl DNA (+NaOH) and 10 μl water was added to siliconize microcentrifuge tube. After the mixture was gently mixed and overlayed with mineral oil, the tube was covered with aluminum foil to shield from the light and was incubated at 55°C for 16–18 h. Then the DNA was purified using the Geneclean II kit (Intermountain Scientific Corporation). After purification, DNA was resuspended and TE was added to a final volume of 100 μl. Then the sample was denatured with freshly prepared NaOH (as above) and was incubate at 37°C for 15 min and neutralize by adding ammonium acetate (pH 7.0) to 3 M. The DNA was precipitate with three volumes of ethanol, centrifuge and wash twice with 70% ethanol and was dried under a vacuum. Then the DNA was resuspended in 50 μl TE and stored at −20°C wrapped in foil.

METHYLATION-SPECIFIC PCR DETECTION

The sequence around ITGA5 promoter was amplified by MSP method. Primers in the ITGA5 promoter sequence (Chromosome 12: 54,789,047-54,813,050) were designed. CpG island was predicted by the CpG island searcher tool on web and the MSP primers was designed using the Urogene Methprimer Design Software. ITGA5 methylation primer sets were shown in Table I. Templates of methylation contrast and un-methylation contrast were designed and synthesized by ShenGong Biotechnology Corporation. The reaction system of 25 μl PCR was: 10× Buffer 2.5 μl, dNTP2.0 μl, TaqE0.5 μl, ddH_2O 17.5 μl, Primer mix 1 μl, $MgSO_4$ 0.5 μl, DNA 1 μl. Started 35 cycles after predegeneration at 95°C 3 min: 95°C 30 s, 58°C 1 min, 72°C 45 s before finally completely extended into 72°C,

TABLE I. Primer Sets Used in MSP for ITGA5 Promoter

	Primer sequence	Position	T_m	Product length (bp)
Primer set 1				73
Methylated sense	GtTGGGGttttCGGCGtC	115	58.11	
Methylated antisense	CCCGACCCTaaaTaaCGaCG	188	57.21	
Un-methylated sense	tTGtGtTGGGGtttttGGtGttG		59.01	
Un-methylated antisense	CTAAaTTaAAaCCCCCaACCCTaaaTaaCaaCa		57.72	
Primer set 2				105
Methylated sense	GttAGAGTttttTtTttACGtCGTGtAGtTGC	83	57.59	
Methylated antisense	CCCGACCCTaaaTaaCGaCG	188	57.21	
Un-methylated sense	AtGttAGAGTttttTtTttAtGttGTGtAGtTGtG		56.52	
Un-methylated antisense	CTAAaTTaAAaCCCCCaACCCTaaaTaaCaaCa		57.72	

7 min (kept at 4°C). The strip of PCR product was observed by 2% hemoglobin agarose electrophoresis.

CELL PROLIFERATION AND COLOGENIC ASSAY

Inhibition of cell proliferation by ITGA5 overexpression was measured by WST-1 assay. Cells transfected with pcDNA3-α5, pcDNA3-α5MT and mock plasmids were plated in 96-well culture plates (1×10^4 per well), WST-1 (Roche) assay measuring the activity of mitochondrial dehydrogenases was performed following the manufacturer's instruction at 0-, 1-, 2-, 3-, 4-, 5-day time points.

To determine long-term effects, cologenic assay was used to elucidate the possible differences in long-term effects of ITGA5 expression on human breast cancer cells. MDA-MB-468 cells were transfected with pcDNA3-α5, pcDNA3-α5MT and mock plasmids for 24 h and then trypsinized and counted using a hemocytometer. Cells (2×10^4) were plated in the 6-well dishes and supplemented with geneticin (G418) 24 h later. Two weeks after the onset of drug selection, the cells were fixed and stained with crystal violet (0.1% crystal violet in 20% methanol). A cluster of a minimum of 50 cells is considered a colony.

FLOW CYTOMETRIC ANALYSIS OF CELL CYCLE

For cell-cycle assay, cells were trypsinized with 2 mM EDTA in PBS and rinsed twice with ice-cold PBS solution, then fixed by adding them drop-wise into 75% ice-cold ethanol while vortexing, followed by incubation in ice for 60 min. The fixed cells were washed with ice-cold PBS and incubated at 37°C for 30 min in 0.5 ml PBS solution containing 20 μg/ml RNaseA, 0.2% Triton X-100, 0.2 mM EDTA, and 20 μg/ml of propidium iodide. DNA content was determined by FACS analysis (Becton Dickinson). The percentage of cells in G0/G1, S, and G2/M phases was determined using the EPICS-XL flow cytometer (Beckman-Coulter, USA) and the Multicycler program.

ADHESION ASSAY

A total of 96-well plastic plates were coated with 10 μg/ml of fibronectin in phosphate-buffered saline (PBS) for 2 h at 37°C and then treated with 3% bovine serum albumin (BSA) for 1 h at 37°C, or were coated with only BSA for negative control. The cells (5×10^4 cells/well) in serum-free DMEM containing 0.1% BSA were added and incubated for 1 h at 37°C. After removal of the medium, a 0.04% crystal violet solution was added and incubation was conducted for 10 min at room temperature. The wells were washed three times with PBS and 20 μl of Triton X-100 was added for permeabilisation. After addition of distilled water to 100 μl, the optical density was measured at 550 nm. The value indicates the mean ± SE for triplicate data representing three independent experiments.

SPREADING ASSAY

Cells (5×10^3 cells/well) in serum-free DMEM containing 0.1% BSA were added to plates coated with 10 μg/ml of fibronectin (96 wells), and incubated for 3 h at 37°C. The cells were photographed on slide film under microscopy and the images on the film were analyzed by NIH Image (National Institutes of Health, Bethesda, MD, USA). The spreading area of each cell type was calculated. The value indicates the mean ± SE for 20 cells randomly sampled.

CELL MIGRATION AND BOYDEN CHAMBER ASSAY

Migration assays using fibronectin-coated insert membrane and invasion assays using Matrigel-coated chambers were carried out described previously with minor modifications [Guo et al., 2002]. Migration assays were performed using 24-well Transwell units with 8 mm pore size polycarbonate inserts (BD Biosciences). Transwells were coated overnight with 10 mg/ml of Fn in PBS at 48°C, followed by incubation with 1% BSA for 1 h at 37°C. The MDA-MB-468 cells transfected with pcDNA3-α5, pcDNA3-α5MT and mock plasmids were detached with trypsin/EDTA, washed once with DMEM containing 10% FBS, and resuspended in DMEM containing 1% FBS at 2×10^5 cells/ml. Aliquots (100 μl) of cell suspensions were directly added to the upper side of each chamber, Following incubation for 12 h, the cells on the upper side of the membrane were removed, whereas the cells that migrated to the underside were fixed with 3% formaldehyde and stained with 0.3% crystal violet for 10 min. The number of cells on the underside of the membrane was counted in five different fields with a light microscope at 100×, and the mean and SD was calculated from three independent experiments.

To examine the transmigration of MCF-7 cells with different ITGA5 expression, a modified Boyden chamber assay was used. Plasma cells (1×10^5) were placed in the upper chamber of a transwell insert containing 20 μg/ml fibronectin and examined for their ability to migrate to the underside of filters after 3 h of culture. The upper side of the filter was scraped with a cotton swab. The filters were fixed with formalin (Sigma) and stained with haematoxylin (Sigma). The number of cells on the lower side was counted. The value indicates the mean ± SE for triplicate data representing three independent experiments.

RESULTS

EXPRESSION LEVEL OF ITGA5 IN MDA-MB-468 CELLS

We assessed the expression of the ITGA5 in the MDA-MB-468, MCF-7 cells (non-metastatic human breast tumor cells) and SMMC-7721 cells (hepatocellular cancer cell line as a control). As shown in Figure 1A,B, the expression of integrin β1 was detected in all the cell lines, while the mRNA and protein level of ITGA5 was hardly detected in MDA-MB-468 cells. Similar results were obtained when real-time PCR assay was carried out to estimate the mRNA amount of ITGA5 in MDA-MB-468 cells (Fig. 1C). We reported previously that solely overexpressed β1 subunit could induce the expression of diverse alpha subunit in different cell lines [Fang et al., 2009]. In this study, however, the expression level of α5 subunit in MDA-MB-468 cells could not be stimulated by β1 subunit overexpression (Fig. 1D), which also indicated the silence of ITGA5 gene in this cell line.

METHYLATION STATUS OF THE CPG DINUCLEOTIDES ON ITGA5 PROMOTER IN MDA-MB-468 CELLS

Since no ITGA5 gene abnormality was observed in MDA-MB-468 cells by sequence analysis (data not shown here), we set out to

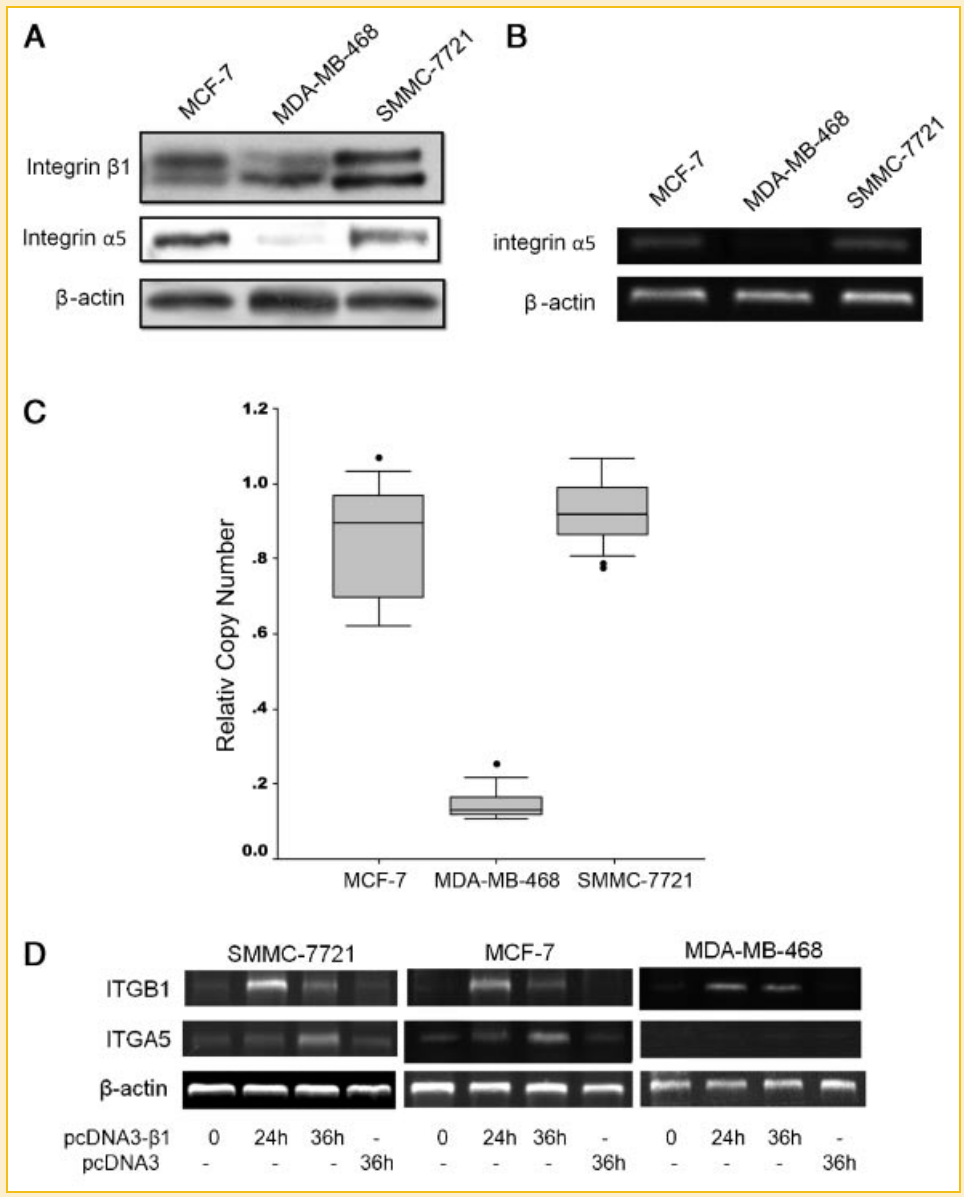

Fig. 1. Expression level of ITGA5 in MDA-MB-468 cells. A: Total cell lysates of MDA-MB-468, MCF-7, and SMMC-7721 cells were examined by Western blotting. B: mRNA levels of ITGA5 in the three cell lines were analyzed using RT-PCR assay, and normalized with the amount of β-actin. Shown is representative example of multiple experiments. C: Real-time PCR assay was carried out as described under Materials and Methods Section, and the results were obtained from 15 reactions in each cell line. Boxplots of relative copy number of ITGA5 mRNA measured with Real-Time PCR analysis showing median; box: 25th–75th percentile; bars: largest and smallest values within 1.5 box lengths; black points: outliers. D: The SMMC-7721, MCF-7, and MDA-MB-468 cells were transfected with pcDNA3-β1 or pcDNA3 plasmid for 24–36 h, mRNA level of integrin α5 and β1 were next analyzed by RT-PCR analysis as described under Materials and Methods Section, β-actin was used to normalize the amount of loaded RNA.

investigate the methylation status of the ITGA5 promoter. CpG island searcher tool on web was used to predict potential CpG island on sequence surrounding ITGA5 transcriptional start site (−2000/+1000). Two CpG Islands were found between base −47/+331(around the transcription start site) and base +670/+779 (on the first intron; Fig. 2A), and the first one contains the conserved TATA box located 25 bp upstream of the transcription site, which might be important in the regulation of ITGA5 expression. CpG Methylation-specific PCR was carried out as introduced in the Materials and Methods Section. As shown in Figure 2B, hypermethylation on the sequence around ITGA5 transcription start site was detected in MDA-MB-468 cells, but not in MCF-7 cells or SMMC-7721 cells, which might partly explained the downregulation of ITGA5 in the MDA-MB-468 cells.

Next, we examined if the mRNA expression of ITGA5 could be restored by demethylating the target genes. We treated MDA-MB-468 cells with 5 μM 5-aza-dc for 2 or 3 days and performed RT-PCR. As shown in Figure 2C, gene expression of ITGA5 in MDA-MB-468 cells can be restored with 5-aza-dc treatment, which further supported the hypothesis that loss of ITGA5 in this cell line may be due to promoter methylation.

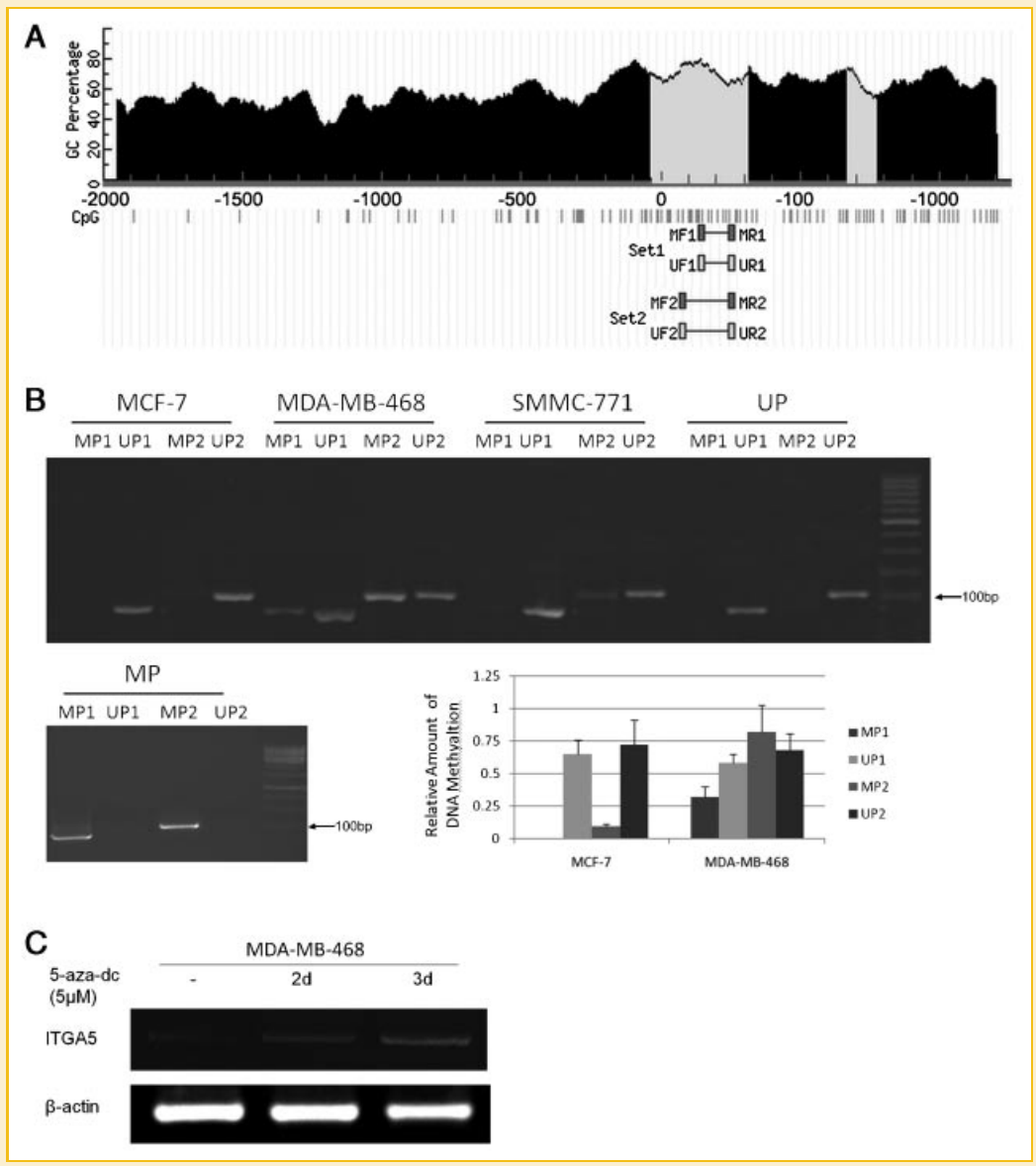

Fig. 2. Methylation-specific PCR analysis of ITGA5 promoter. A: Schematic diagram of potential CpG island around transcription start site of ITGA5 gene (GC rate > 0.6). The primer sets designed by online tool were labeled under the diagram. B: Methylation status of ITGA5 gene promoter CpG island in MDA-MB-468 and MCF-7 cells. MP1 and MP2: PCR products using methylation primer sets 1 and 2; UP1 and UP2: PCR products using un-methylation primer sets 1 and 2; methylation positive (MP): methylation contrast; un-methylation positive (UP): un-methylation contrast. Marker: 100 BP DNA ladder. Shown is the representative result of multiple independent experiments. C: The MDA-MB-468 cells were treated with 5 μM 5-aza-dc for 2 or 3 days, and RT-PCR procedure as described in the Materials and Methods Section was carried out to estimate the mRNA level of ITGA5. β-actin was used to normalize the amount of loaded RNA.

OVEREXPRESSION OF INTEGRIN α5 EXERTS AN ANTI-PROLIFERATIVE EFFECT IN MDA-MB-468 CELLS, WHICH COULD BE REVERSED BY FIBRONECTIN ATTACHMENT

The widely expressed fibronectin (FN) receptor α5β1 integrin is one of the best characterized integrins that recognize the tripeptide sequence, Arg-Gly-Asp. The association between fibronectin (FN) and α5β1 integrin is involved in regulating not only cell adhesion and migration, but also differentiation and apoptosis [Ruoslahti, 1994; Coutifaris et al., 2005; Zeng et al., 2007]. However, the MDA-MB-468 cells with high invasive potential failed to express ITGA5. To investigate the role of the methylation-mediated silence of ITGA5 in MDA-MB-468 cells, we performed transient transfection of

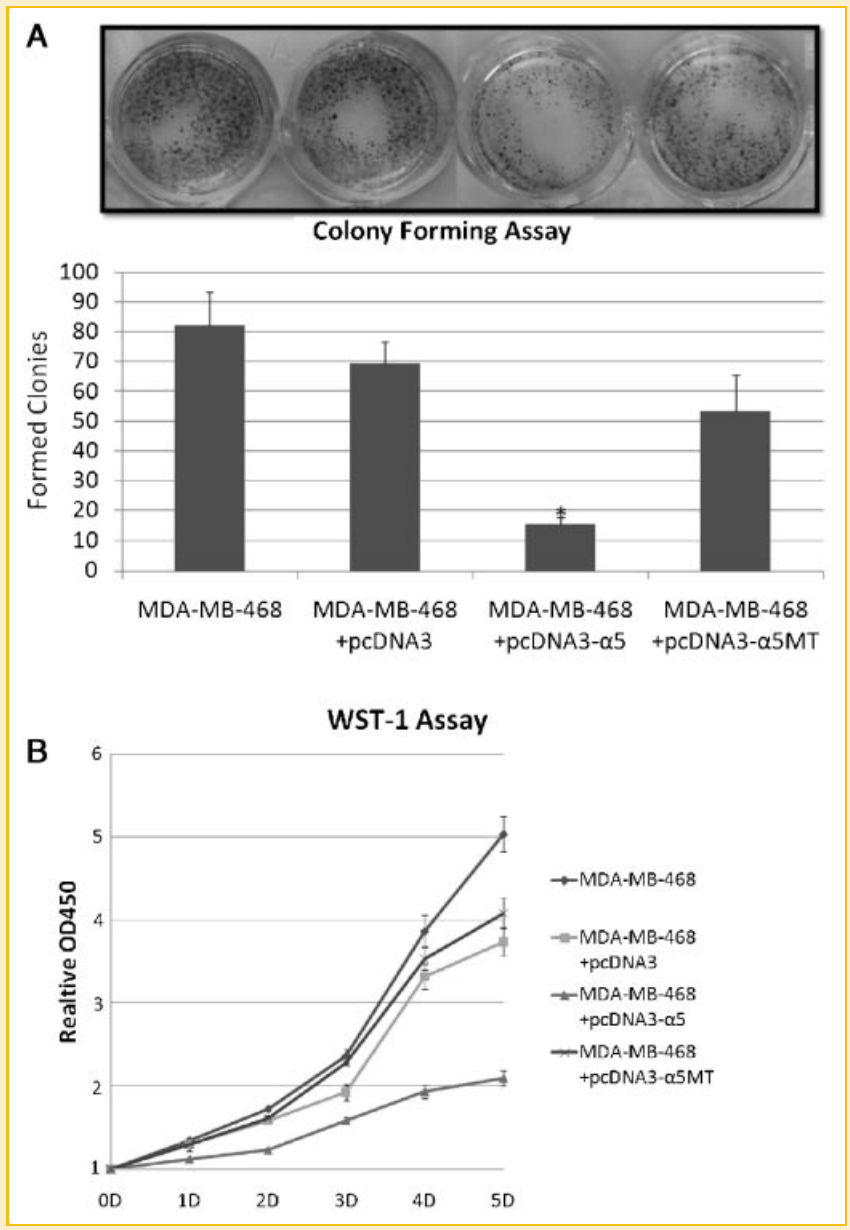

Fig. 3. Growth suppression in MDA-MB-468 cells induced by ITGA5 overexpression. A: Colony-formation assay. MDA-MB-468 cells were transfected with Mock plasmid, ITGA5 expression vector and its defective-mutant using Lipofectamine 2000 (Invitrogen), selected in G418 for 14 days, and the colonies were stained with Giemsa. The bar graph shows the absolute colony (≥50 cells) number SE in duplicate experiments. B: WST-1 (Roche) assay measuring the activity of mitochondrial dehydrogenases was performed following the manufacturer's instruction at 0-, 1-, 2-, 3-, 4-, 5-day time points. Error bars represent standard deviation of the mean. C: MDA-MB-468 cells were transfected with pcDNA3-α5, pcDNA3-α5MT and mock plasmids for 48 h, and cell-cycle distribution was determined by propidium iodide flow cytometry analysis. $^{*}P < 0.05$ versus Control, Mock. D: MDA-MB-468 cells were treated as in (C), and the total cell lysates of were examined by western blotting. E: MDA-MB-468 cells were transfected with the ITGA5 expression vector for 24 h, then plated in 96-well culture plates (1×10^4 per well) coated with FN or VN, and WST-1 assay was carried out to estimate the cell proliferation as described in (B). F: MDA-MB-468 cells were transfected with the ITGA5 expression vector and cultured on VN-coated, FN-coated or FN-free 6-well dishes. And cell colony forming ability was determined by cologenic assay as described in the Materials and Methods Section.

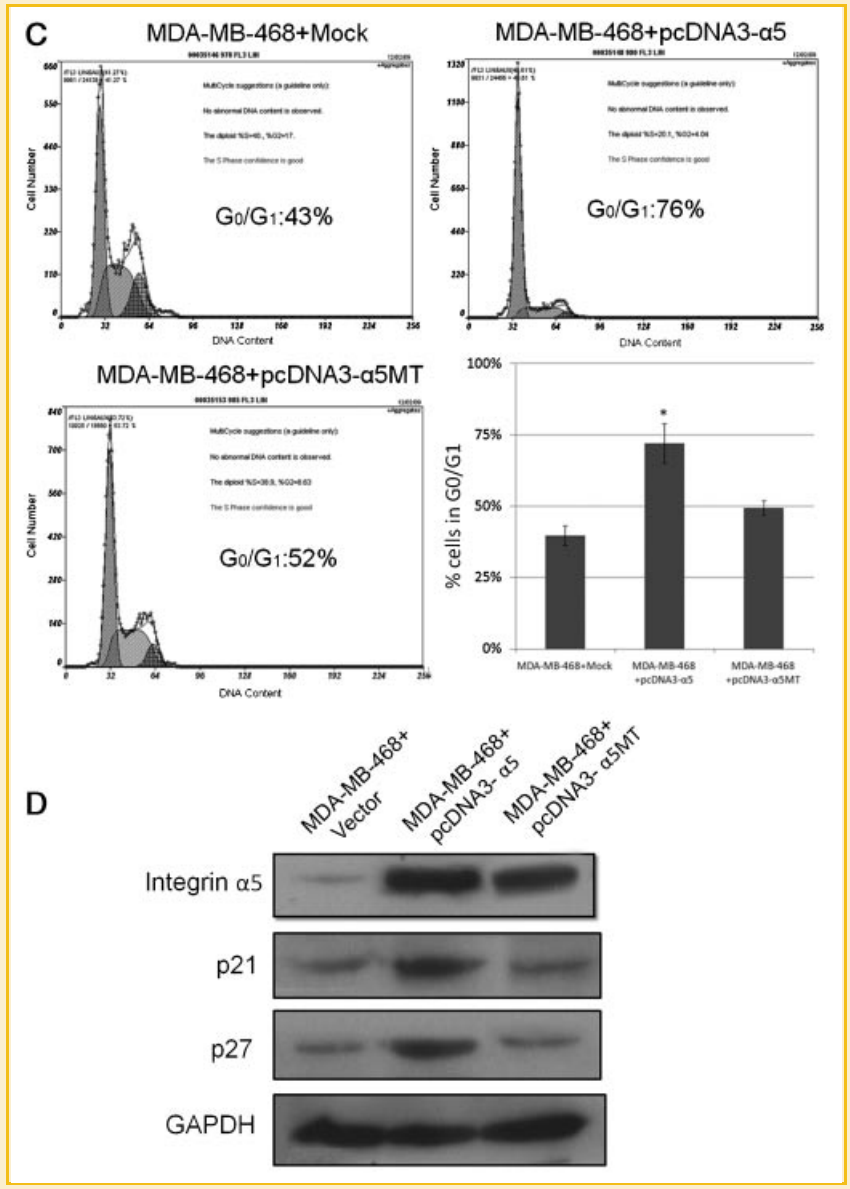

Fig. 3. (*continued*)

ITGA5 expression vector and its mutant in these cells. WST-1 assay and colony formation assay were carried out to estimate the cell proliferational ability. As shown in Figure 3A,B, overexpression of ITGA5 in MDA-MB-468 cells resulted in marked cell growth arrest and attenuated colony forming ability. And the results of flow cytometry also showed that S-phase delay was induced in MDA-MB-468 cells by ITGA5 overexpression (Fig. 3C), which was accompanied by the accumulation of cell cycle-related CDK inhibitors, p21 and p27 (Fig. 3D).

Naturally, integrins and the extracellular matrix (ECM) collaborate to regulate gene expression associated with cell growth, differentiation and survival. Abundant expression of ITGA5 might lead to the relative lack of integrin ligand in vitro. So we next examined cell growth after the cells were attached to fibronectin and other matrix proteins. As shown in Figure 3E,F, cells attached to fibronectin regained its proliferational and colony forming ability while adhesion to vitronectin failed to reverse the growth inhibition. These results were consistent with Varner et al.'s [1995] findings.

ADHESION AND MIGRATION OF MDA-MB-468 CELLS WITH DIFFERENT ITGA5 EXPRESSION TO FIBRONECTIN IN VITRO

To estimate whether it is cell adhesion or migration that is supposed to be crucial for tumor cell metastasis, the behavior of MDA-MB-468 cells transfected with different ratio of ITGA5 versus fibronectin were analyzed in vitro. In the adhesion assay, MDA-MB-468 cells with high α5 expression effectively adhered to fibronectin, whereas cells transfected with mock plasmid or mutated α5 subunit did not adhere to fibronectin (Fig. 4A). Likewise, MDA-MB-468 cells

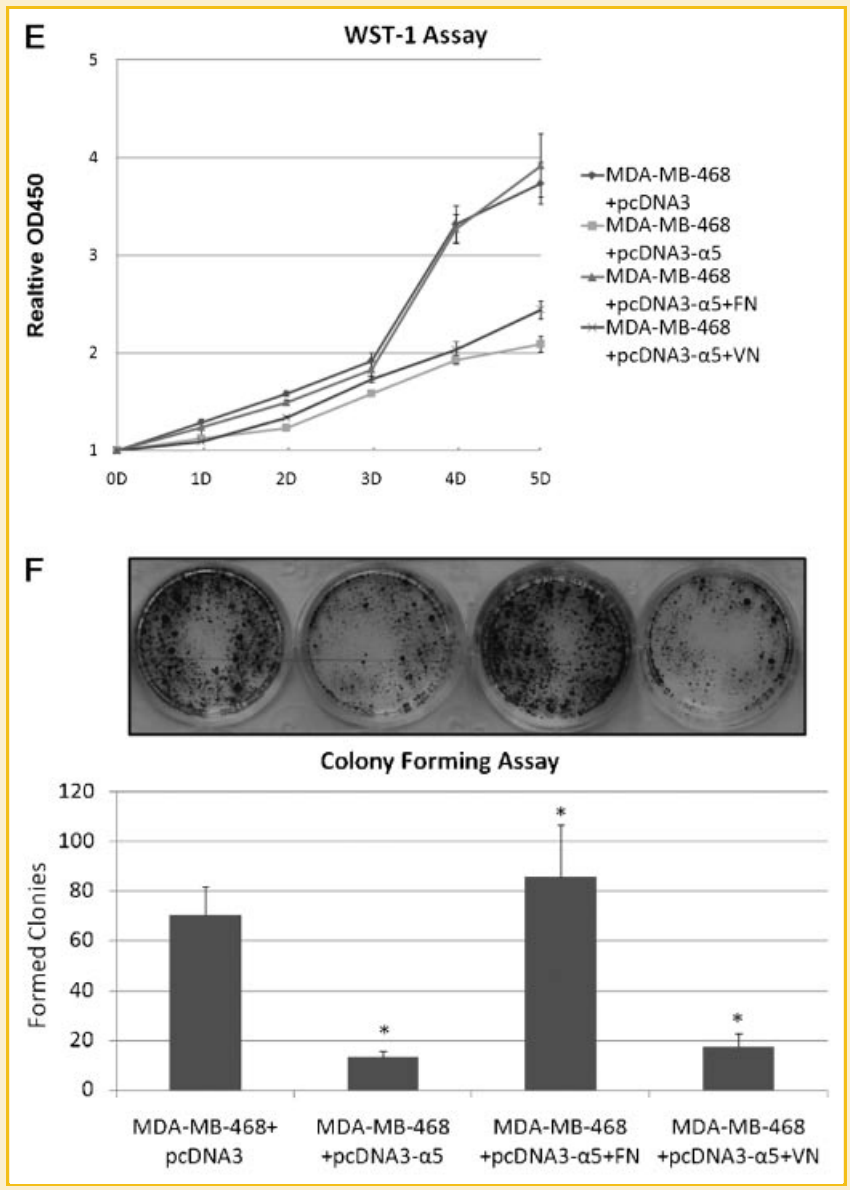

Fig. 3. (*continued*)

showed an increase in cell spreading that correlated with the overexpression of α5 (Fig. 4B).

Since tumor cells in intravasation penetrate into blood vessels containing abundant soluble fibronectin (300 μg/ml) in serum [Mosesson and Umfleet, 1970], the cell migration of MDA-MB-468 cells transfected with different ITGA5 expression vectors were estimated by transwell assay. However, it was found that the MDA-MB-468 cells with the native level of integrin α5 migrated most effectively through transwell membrane (Fig. 4C). These data are consistent with the theory that cell migration is controlled by dynamic interactions between cell receptors and substratum ligands in a manner representing events at the front and rear of the migrating cells [Regen and Horwitz, 1992; Palecek et al., 1996, 1997]. These findings suggested that there is an appropriate level of α5 expression on tumor cells that leads to metastasis. We also noticed that the migration ability of the cells overexpressing mutated integrin α5 was almost abrogated, and this might be due to the inactivation of integrin α5β1 heterodimer (Fig. 4C).

KNOCK DOWN OF ITGA5 IN MCF-7 IMPAIRS CELL PROLIFERATION AND SLIGHTLY REDUCES CELL MIGRATION

To further investigate the role of ITGA5 expression in breast cancer cell behavior, ITGA5 siRNA was used to abrogate the integrin α5 expression (Santa Cruz Biotechnology) in non-metastasis MCF-7 cells (Fig. 5A). And the experiments described previously were carried out to estimate the proliferation, adhesion, and migration of cells. As shown in Figure 5B, reduced ITGA5 expression resulted in impaired cell growth in MCF-7 cells, which was not consistent with

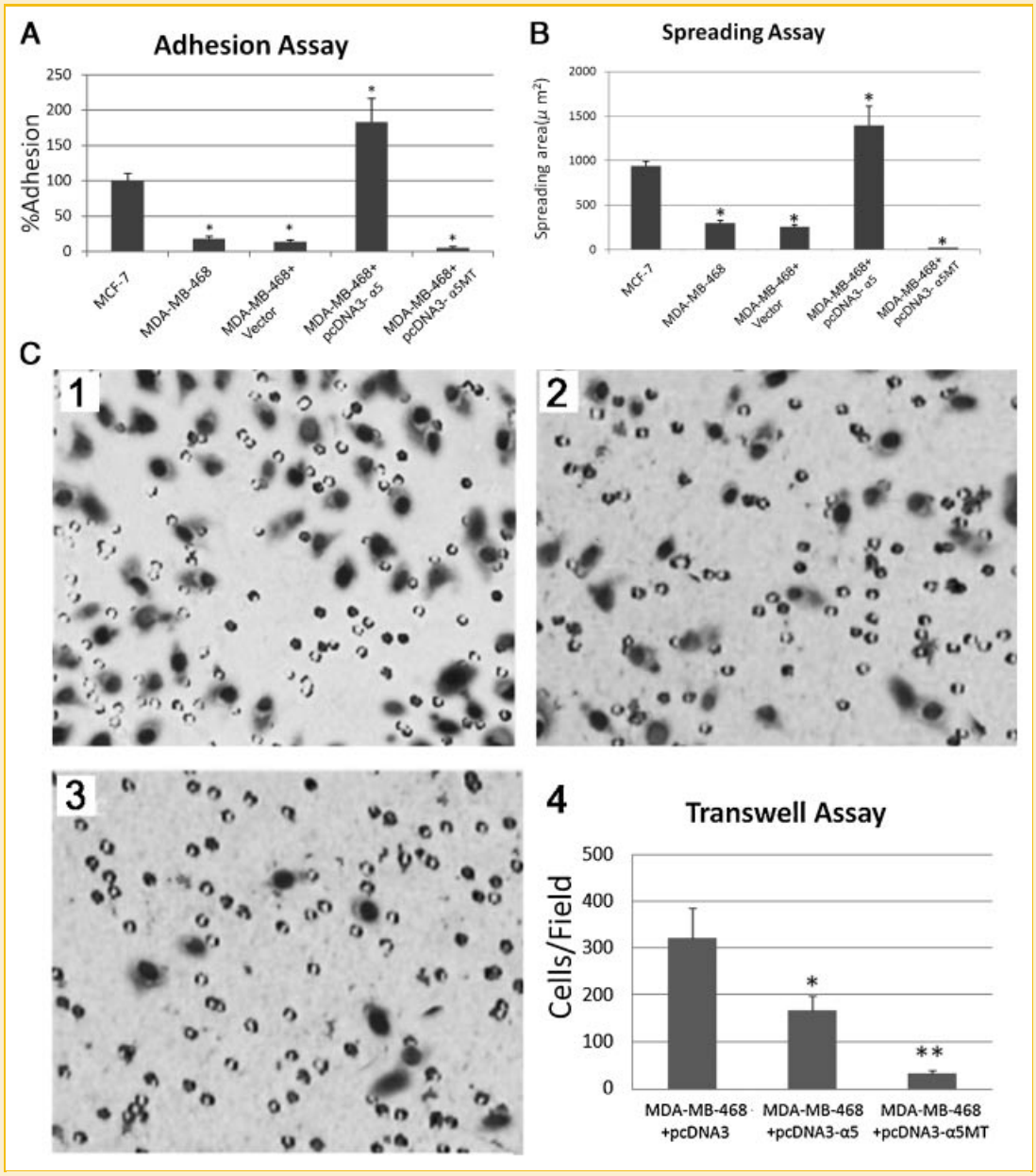

Fig. 4. Adhesion and migration of MDA-MB-468 cells to fibronectin. A: Adhesion assay demonstrating that adhesion activities to fibronectin in each MDA-MB-468 cell depend on α5 expression level. After transfected with α5, α5MT and mock plasmids for 36 h, MDA-MB-468 cells (5×10^4/well) in serum-free DMEM containing 0.1% BSA were plated on coated fibronectin (10 μg/ml) and incubated for 1 h at 37°C. Cells binding to fibronectin that were stained with crystal violet were assessed by measuring optical density at 550 nm. Results are expressed as the percentage compared with MCF-7 cells. Each value is the mean ± SE of triplicate data representative for three independent experiments. $^*P < 0.05$ versus Control, MCF-7. B: Spreading assay quantifying increased binding area in cell adhesion to fibronectin compared with cell spreading on BSA. MDA-MB-468 cells were treated as in (A), and each cell in serum-free DMEM containing 0.1% BSA was plated on coated fibronectin (10 μg/ml) and incubated for 3 h at 37°C. Each value is the mean ± SE of 20 cells randomly selected. The same results were obtained in two independent experiments. $^*P < 0.05$ versus Control, MCF-7. C: Cell migration was determined using a Transwell assay as described in the Materials and Methods Section. (1, 2, 3) Microscopic image of migrated MDA-MB-468 cells transfected with mock plasmid, ITGA5 expression vector and its mutant, respectively. Original magnification: 200×. (4) Diagrams of migrating cells from the different transfectants are shown, which from more than three independent experiments. $^*P < 0.05$ versus Control, MDA-MB-468. $^{**}P < 0.001$ versus Control, MDA-MB-468.

the findings in MDA-MB-468 cells. This might be due to the altered interaction between integrin α5β1 and fibronectin after silence of ITGA5, for the MDA-MB-468 cells with ITGA5 overexpressed could regain its proliferational ability after attachment to fibronectin.

Cell adhesion and spreading to fibronectin was markedly attenuated (Fig. 5C,D) while cell migration was only slightly influenced by α5 integrin overexpression (Fig. 5E). These data also suggested that the amount of integrin α5 expression on breast cancer cells might vary due to their tissue origin and surrounding microenvironment cells during tumorigenesis, and tumor cells with native integrin α5 expression proliferate and metastasize most effectively.

DISCUSSION

Expression level of integrin α5 on tumor cells has been previously reported to be involved in cell proliferation [Kuwada and Li, 2000; Ritzenthaler et al., 2008] and organ-specific metastasis [Tani et al., 2003; Qian et al., 2005]. However, the ITGA5 expression in MDA-MB-468 cell line was almost lost compared to other breast cancer cell line, which might be closely correlated with cell behavior and metastasis. Isolated from a pleural effusion [Cailleau et al., 1974, 1978], the MDA-MB-468 line is from basal-like cell origin and has isoenzyme markers similar to those of the HeLa line. In this study, MDA-MB-468 cells accompanied by another breast cancer cell line with positive ITGA5 expression were employed to reveal the role of ITGA5 expression in breast cancer cells. We found that altered expression of ITGA5 on MDA-MB-468 cells had an influence on both cell proliferation and metastasis. These effects seemed to be closely correlated with the interaction between integrin and extracellular matrix because both cell growth and migration could be influenced by integrin ligand treatment. Furthermore, after the expression of integrin α5 was knockdown in ITGA5-positive MCF-7 cells, the cell proliferation was also impaired. These results indicated that the appropriate amount of interaction between integrin α5β1 and fibronectin lead to cell proliferation. The adhesion and spreading of breast cancer cells to fibronectin were closely correlated with protein level of integrin α5 on cell membrane, which is consistent with other's findings [Yao et al., 1997; Tani et al., 2003]. The migration of MDA-MB-468 cells was attenuated after ITGA5 overexpression while silence of ITGA5 in MCF-7 also resulted in slightly diminished migration, which indicated that the breast cells with native level of integrin α5 migrate most effectively. These findings suggested the diverse roles of integrin α5 in breast cancer cell metastasis. It was interesting that the MDA-MB-435 cells, another breast cancer cell line, had extremely high ITGA5 expression (Fig. S1). However, the MDA-MB-435 line was currently considered from M14 melanoma cells [Rae et al., 2004, 2007] and it might be questionable to employ it as a model system for human breast cancer [Christgen and Lehmann, 2007].

Alterations in the p53 locus are a common occurrence in human breast cancer. The MDA-MB-468 line harbors a G → A mutation in codon 273 of the p53 gene resulting in an Arg → His substitution [Runnebaum et al., 1991], which was correlated with cell's neoplastic phenotype [Wang et al., 1993]. And others have

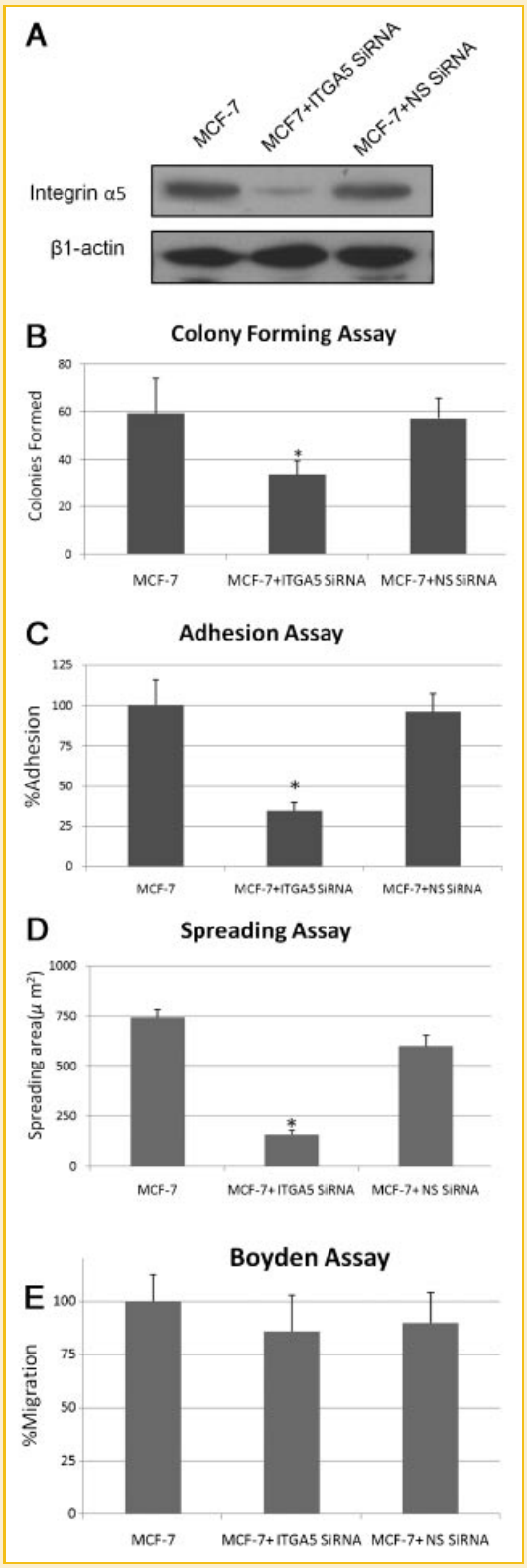

Fig. 5. ITGA5 knockdown in MCF-7 cells impairs cell proliferation and slightly influences cell migration. A: MCF-7 cells were transfected with ITGA5 SiRNA and nonsense SiRNA for 36 h, and the protein level of integrin α5 was determined by Western blotting. B: MCF-7 cells were treated as in (A), and cell proliferation ability was determined by cologenic assay as described in the Materials and Methods Section. The results were obtained in four independent experiments. $^{*}P < 0.05$ versus Control, MCF-7. C: MCF-7 cells transfected with ITGA5 siRNA and nonsense siRNA were used for adhesion assay as described in Figure 4A. The results were obtained in two independent experiments. $^{*}P < 0.01$ versus Control, MCF-7. D: MCF-7 cells transfected with ITGA5 siRNA and nonsense siRNA were used for spreading assay as described in Figure 4B, the results were obtained in three independent experiments. $^{*}P < 0.01$ versus Control, MCF-7. E: Boyden chamber assay evaluating migration activity of each MDA-MB-468 cells to soluble fibronectin. A chamber with 10^5/cells well in the upper chamber and 20 μg/ml fibronectin in the lower chamber was incubated for 3 h at 37°C in 10% CO_2. The number of cells migrating to the lower side of the filter was assessed as a percentage compared with cells transfect with mock plasmid. Each value is the mean SE of triplicate data, representative for three independent experiments. $P = 0.059$ versus Control, MCF-7.

established that loss of p53 expression promotes RhoA-mediated signaling leading to cell migration [Gadea et al., 2007]. However, it remains unclear whether p53 plays a role in the IGTA5-related cell migration process, since mutation of p53 and loss of IGTA5 both happen in MDA-MB-468 cell line. So, in the future, it would be necessary to test the IGTA5 function in other cell lines (e.g., p53+/+ and IGTA5−/−). The activation of Rho GTPases has also been shown to contributes to cell adhesion and spreading [Marignani and Carpenter, 2001; Gakidis et al., 2004] through integrin signaling. But our study did not address the possible link between Rho GTPases and ITGA5. Thus, further studies focusing on the possible synergistic effects of Rho GTPases and ITGA5 are needed. This also provides us the future directions to more extensively investigate the role of ITGA5 in tumorigenesis and metastasis.

Though the abnormally expressed integrin in tumor has been reported by different groups [Lu et al., 2008], little is known about the mechanism of altered integrin expression regulation during tumorigenesis. In this study, we found that the methylation level of the CpG island around the ITGA5 gene transcription start site was increased in MDA-MB-468 cells, which might be the reason for the impaired ITGA5 expression in this high invasive cell line. This might be a mechanism of transcriptional repressed integrins in certain breast cancer cells.

Tumor cells or mice with genetic changes of integrin expression have been used for analyzing the effects of integrin on tumorigenesis and organ-preferential metastasis. Rhabdomyosarcoma (RD) cells transfected with cDNA encoding the α_2 subunit enhanced metastasis in nude mice, demonstrating the specific effect of $\alpha_2\beta_1$ integrin on metastasis [Chen et al., 1991]. CHO cells transfected with α4 cDNA caused experimental bone metastasis in nude mice, suggesting that bone metastasis was induced by interactions between $\alpha 4\beta 1$ on tumor cells and VCAM-1 on stromal cells in the bone marrow [Matsuura et al., 1996]. In this study, we mainly focused on the relation between the change in $\alpha 5\beta 1$ levels and tumor cell metastasis. Since different α5 integrin expression might lead to different organ-preferential metastasis, next we should constructed MDA-MB-468 transfectants expressing $\alpha 5\beta 1$ at various levels and examined metastatic sites in nude mice.

In conclusion, we demonstrated that DNA hypermethylation was involved in the transcriptional repressed integrin α5 expression in MDA-MB-468 cells. An appropriate level of α5 expression on breast cancer cells plays an important role in cell proliferation and metastasis.

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