DNA Promoter Methylation-dependent Transcription of the Double C2-like Domain β (DOC2B) Gene Regulates Tumor Growth in Human Cervical Cancer*

Received for publication, June 5, 2013, and in revised form, February 18, 2014 Published, JBC Papers in Press, February 25, 2014, DOI 10.1074/jbc.M113.491506

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Background: DOC2B promoter hypermethylation is an early and frequent event in cervical cancer.

Results: DOC2B hypermethylation induces transcriptional repression, reactivated by demethylation; ectopic expression increases Ca2+ flux and inhibits key characteristics of tumorigenesis including proliferation, motility, and invasion.

Conclusion: DOC2B gene is epigenetically regulated and inhibits cervical cancer growth.

Significance: DNA methylation regulates DOC2B gene expression in cervical cancer.

Double C2-like domain β (DOC2B) gene encodes for a calcium-binding protein, which is involved in neurotransmitter release, sorting, and exocytosis. We have identified the promoter region of the DOC2B gene as hypermethylated in pre-malignant, malignant cervical tissues, and cervical cancer cell lines by methylation-sensitive dimethyl sulfoxide-polymerase chain reaction and bisulfite genome sequencing; whereas, it was unmethylated in normal cervical tissues (p < 0.05). The promoter hypermethylation was inversely associated with mRNA expression in SiHa, CaSki, and HeLa cells and treatment with demethylating agent 5-aza-2-deoxycytidine restored DOC2B expression. The region −630 to +25 bp of the DOC2B gene showed robust promoter activity by a luciferase reporter assay and was inhibited by in vitro artificial methylation with Sss1 methylase prior to transient transfections. Overexpression of the DOC2B gene in SiHa cells when compared with controls showed significantly reduced colony formation, cell proliferation, induced cell cycle arrest, and repressed cell migration and invasion (p < 0.05). Ectopic expression of DOC2B resulted in anoikis-mediated cell death and repressed tumor growth in a nude mice xenograft model (p < 0.05). DOC2B expressing cells showed a significant increase in intracellular calcium level (p < 0.05), impaired AKT1 and ERK1/2 signaling, and induced actin cytoskeleton remodeling. Our results show that promoter hypermethylation and silencing of the DOC2B gene is an early and frequent event during cervical carcinogenesis and whose reduced expression due to DNA promoter methylation may lead to selective cervical tumor growth.

Associations of genetic changes and aneuploidy with tumor growth are traditionally attributed to alterations in DNA sequence manifested as mutations, deletions, and amplifications. Inactive tumor suppressor genes cannot only serve as drivers of tumor progression due to altered or lack of protein function but may also contribute to phenotypic changes that may provide a distinct growth advantage in a hostile environment (1). Human tumors also show epigenetic alterations as heritable changes leading to abnormal gene expression; the functional consequence of which may lead to genetic changes (1). A number of key regulatory genes associated with epigenetic silencing in cervical cancer have been reported (2, 3). Elucidation of differentially methylated genes may identify new targets that could further strengthen our understanding of the molecular mechanism governing pathogenesis of cervical cancer (2).

The double C2-like domain (DOC2)3 protein family consists of two members, DOC2A and DOC2B, which are located in chromosomes 16p11.2 and 17p13.3, respectively, and share significant homology in their amino acid and nucleic acid composition. Although, DOC2A with molecular mass of 43.95 kDa is considered to be more tissue specific due to its predominant expression in brain, the 45.94-kDa DOC2B is expressed in several tissue and cell types. The domains shared by these two proteins are MUNC interacting domain and two tandem calcium binding C2 domains; however, they do not share common functions (4). DOC2B is suggested to be involved in Ca2+-dependent intracellular vesicle trafficking, ion and phospholipids binding, neurotransmitter release, and transporter activity (5, 6). It interacts with syntaxin binding protein 4 and Munc18c (7) leading to facilitation of exocytosis. Binding of calcium to DOC2B significantly increases its affinity toward phospholipids, leading to translocation of proteins from the cytosol to plasma membrane (8). Recently, DOC2B was shown as a posi-
**TABLE 1**

List of primers used

The bold and underlined sequence represents the restriction sites incorporated for cloning.

| Primer name                      | Sequence (5′-3′)                      | Product size | Annealing temperature |
|----------------------------------|--------------------------------------|--------------|-----------------------|
| **MS-DMSO-PCR**                  |                                      |              |                       |
| DOC2B-MS-DMSO-F                   | CCGGAGTATTAGTGTCGTTG                  | 1000 bp      | 64 °C                 |
| DOC2B-MS-DMSO-R                   | CTCTTGAGTCTGAGTGTC                  |              |                       |
| **BGS**                          |                                      |              |                       |
| DOC2B-BGS-F                       | GTATGTTGATATTTGATATTGCTGTG           | 412 bp       | 61.8 °C               |
| DOC2B-BGS-R                       | CCCCCAACCAC CCTAATACCCCTTACC         |              |                       |
| **RT-PCR**                       |                                      |              |                       |
| DOC2B-RT-PCR-F                    | TGGTTGATGTCTGGATCCACG               | 103 bp       | 60 °C                 |
| DOC2B-RT-PCR-R                    | TGGGACTTGGCTGATTGACCG                | 97 bp        | 60 °C                 |
| GAPDH-RT-PCR-F                    | GGCCTCCCTTGATGGATATGTT             | 132 bp       | 60 °C                 |
| GAPDH-RT-PCR-R                    | TTTATTTTGGAGGGATCTTCG               |              |                       |
| ACTB-RT-PCR-F                     | GACGACATGGAGAAATCTG                 |              |                       |
| ACTB-RT-PCR-R                     | ATGACTGGGTCTATCTTCT                 |              |                       |
| **Promoter construct: luciferase essay** |                               |              |                       |
| DOC2B-Promoter-F                  | TGTGGTACACCGAGGTCCTCGGGTATCACC      | 655 bp       | 65 °C                 |
| DOC2B-Promoter-R                  | CCGGAAGACATGGCGGCTGCGGTGCCGCT      |              |                       |

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Patient DNA Samples**—MDAMB453, THP1, Jurkat, HT29, IMR32, HCT15, HepG2, PC3, CAL24, SCC4, SaoS2, WM451, MG63, WM115, SiHa, CaSki, and HeLa cells were maintained according to American Type Culture Collection guidelines; whereas, normal skin fibroblasts were grown according to American Type Culture Collection guidelines; whereas, normal skin fibroblasts were grown in DMEM (HiMedia, Mumbai, India) containing 10% fetal bovine serum (FBS) (HiMedia, Mumbai, India). Cervical biopsy samples from patients who were diagnosed at the Kasturba Medical College, Manipal, India, for cervical cancer were included in the study. All participants provided informed consent in compliance with the Kasturba Hospital ethical committee approval. The clinical status of the samples was confirmed by histopathological examination. DNA was isolated from tissue biopsy, Pap smear, and cell lines by standard phenol–chloroform extraction and ethanol precipitation method.

**Methylation-sensitive Arbitrarily Primed PCR (MS-AP-PCR)**—For MS-AP-PCR, 2 μg of normal and tumor genomic DNA was digested with 20 units of Rsal enzyme, 20 units of Rsal and HpaII, or 20 units of Rsal and MspI (New England Biolabs) at 37 °C for 16 h. Digested DNA (100 ng) was subjected for PCR amplification using the arbitrary primers (MGC0 + MGF2) in a PTC-200 Peltier thermal cycler (MJ Research) (13). The amplicons were resolved in a 8% non-denaturing polyacrylamide gel (PAGE) and visualized by silver staining. The differentially methylated bands were isolated from PAGE, reamplified, cloned into a TA vector (Promega) and sequenced in 3130 genetic analyzer (Applied Biosystems) (12, 13). The sequences were searched for similarity using the BLAT program of the University of California Southern California against HG19 release.

**Methylation-sensitive Dimethyl Sulfoxide-Polymerase Chain Reaction (MS-DMSO-PCR)**—The MS-DMSO-PCR was performed for the −700 to +300 bp with respect to the transcription start site of the **DOC2B** gene as described previously containing 0–5% of DMSO (14). The primers used for MS-DMSO-PCR are listed in Table 1.

**Bisulfite Genomic Sequencing (BGS)**—Genomic DNA (2 μg) was used for bisulfite treatment using the EZ DNA methylation kit (Zymo Research) according to the manufacturer’s instructions. Primers were designed using Methyl primer express version 1 (Applied Biosystems) for −376 to +36 bp with respect to transcription start site of the **DOC2B** gene and is listed in Table 1. PCR products were purified and direct sequencing was performed in a 3130 Genetic analyzer according to the manufacturer’s instructions using a big dye terminator kit (Applied Biosystems). The percentage of methylation for each CpG site was calculated by comparing the peak height of cytosine (C) signals with the sum of the peak heights of the cytosine and thymine (T) signals (15).

**Demethylation and Reverse Transcription-PCR (RT-PCR)**—The SiHa, CaSki, and HeLa cells were seeded at a density of 1 × 10^5 cells in a 10-cm² culture plates and treated with 5-aza-2′-deoxycytidine (Sigma) at concentrations of 5, 10, and 20 μM daily for 3 days, total RNA was extracted using TRIzol reagent (Invitrogen), and cDNA was synthesized using a High Capacity cDNA archive kit (Applied Biosystems) according to the manufacturer’s instructions and used for RT-PCR. The nucleotide sequences used for RT-PCR are listed in Table 1.

**Human Papilloma Virus (HPV) Genotyping**—HPV genotyping was performed by nested PCR using PGMY09/11 and GP5+/GP6+ consensus L1 primers (16, 17). The PCR product was gel purified and subjected to direct sequencing in a 3130 Genetic analyzer using a big dye terminator kit according to the
manufacturer’s instructions. The HPV strains were identified by comparing using the NCBI database BLAST search.

Promoter Constructs, Artificial Methylation, Transfection, and Luciferase Assays—Luciferase reporter constructs were prepared by cloning the 655 bp (−630 to +25 bp) PCR product of the DOC2B gene into KpnI and HindIII sites of pGL3-Basic and pGL3-Enhancer vectors, respectively. The constructs were verified by DNA sequencing. All plasmid constructs were artificially methylated in vitro using SssI DNA methylase and confirmed by restriction digestion with HpaII and MspI, respectively, according to the manufacturer’s instruction (New England Biolabs). The primers used are shown in Table 1.

The methylated and unmethylated constructs (1.6 μg each) were co-transfected with pRL-SV40 vector (50 ng) into 60–70% confluent cultures of SiHa cells in a 6-well tissue culture plate. Total protein was extracted and the concentration was estimated using a Bradford assay kit (Sigma). The protein concentration was estimated by fluorometric detection with the BCA protein assay kit (Thermo Scientific) and confirmed by densitometry using ImageQuant LAS 4000 (GE Healthcare). Total proteins were separated on 8% SDS-PAGE, transferred onto Nitran membrane (Thermo Scientific), blocked with 5% nonfat dry milk, and incubated separately with anti-DDK tag primary antibody for DOC2B (Origene) and anti-DOC2B (Proteintech) (1:3000) antibodies, respectively, followed by secondary antibody total ERK1/2 (Ser473), total AKT, phosphor-ERK1/2 (Thr202/Tyr204), and phospho-AKT (Ser473). Immunoreactive bands were visualized by ECL reagent (GE Healthcare) and quantitated using ImageQuant LAS 4000 (GE Healthcare).

Lesion Assay—Lesions were cultured in 6-well plates were coated with poly-2-hydroxyethyl methacrylate (poly-HEMA) as published previously. Control and DOC2B expressing cells were trypsinized, washed, and cultured on poly-HEMA-coated plates at a cell density of 1 × 10^5 cells/well. The number of viable cells was analyzed by FACS (FACS Calibur, BD Biosciences) after staining with propidium iodide (10 μg/ml in PBS) as published previously.

### TABLE 2
Summary of hypomethylated sequences identified by MS-AP-PCR

| Clone | Fragment size | Gene | Observed CpG/expected CpG | Chromosome location | CpG island | CNV |
|-------|---------------|------|---------------------------|---------------------|------------|-----|
| Frg1  | 243 bp        | Myomesin2 | %                         |                     |            |     |
| Frg2  | 251 bp        | hypothetical protein LOC41390 | 52.5                  |                     |            |     |
| Frg3  | 312 bp        | KLRG2  | 52.4                      |                     |            |     |
| Frg4  | 667 bp        | IKBK   | 62.7                      |                     |            |     |
| Frg5  | 359 bp        | ZBED4  | 55.6                      |                     |            |     |
| Frg6  | 406 bp        | 187-243 bp at 5’ end: hypothetical protein | 55                   |                     |            |     |
| Frg7  | 455 bp        | 440 bp at 5’ end: hypothetical protein | 57.5                 |                     |            |     |
| Frg8  | 362 bp        | 838-601 bp at 5’ end: deleted in bladder cancer 1 | 50                   |                     |            |     |
| Frg9  | 399 bp        | NXXN   | 51.6                      |                     |            |     |
| Frg10 | 613 bp        | 23,224 bp at 5’ end: MMP16 | 51.1                 |                     |            |     |
| Frg11 | 501 bp        | LOC84262 | 53.4                     |                     |            |     |
| Frg12 | 287 bp        | 3,315 bp at 3’ end: PBX/knotted 1 homeobox 2 | 51.5                 |                     |            |     |
| Frg13 | 225 bp        | DOC2B promoter | 58.5                   |                     |            |     |
| Frg14 | 415 bp        | 2,482 bp at 3’ end: proteasome assembling chaperone 3 | 61.8                 |                     |            |     |
| Frg15 | 689 bp        | 95,927 bp at 3’ end: titin immunoglobulin domain protein | 37.5                 |                     |            |     |

DOC2B Regulation by Promoter Methylation in Cervical Cancer

The presence of CpG Island was predicted using the following criteria: CG content greater than 50%, observed CpG/expected CpG greater than 0.60 and length greater than 200 bp. The copy number variation (CNV) analysis was performed by searching in genomic variant database.
Anchorage-dependent and -Independent Colony Formation Assay—Colony forming assay was performed as published previously with minor modifications (20). In brief, 100 cells were plated in duplicates in 6-cm Petri plate. After 14 days, the medium was removed and the cells were washed with PBS and stained with 0.5% crystal violet in methanol for 5 min. Excessive stains were removed by washing with distilled water, photographed, and colonies were counted. The experiments were performed in duplicates and repeated three times.

In 6-well plates, 1 × 10^3 cells/well containing 2 ml of 0.3% Nobel agar (DMEM + 10% FBS) was overlaid on top of 3 ml of 0.6% bottom agar base (DMEM + 10% FBS) and 0.5 ml of com-
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Cell Doubling and Growth Curve Analysis—About 3000 cells were plated in a 6-cm Petri plate for a 5-day growth curve analysis. The cells were trypsinized at each time point and counted using a hemocytometer. The experiments were performed in duplicates and repeated three times. The cell doubling time was calculated using the cell doubling time calculator.

Cell Migration Assay—Transfected cells were grown to confluence in 6-well plates, starved for 24 h, and a scratch test was performed using a sterile microtip. Following the scratching, fresh medium with 10% FBS was added and migration of cells into the wound area was monitored until 72 h. The images were captured at the indicated time points using a Rolera emc2 camera and Olympus Microscope CK-41 (Olympus, Japan). The migration rate/index was calculated as published previously (22).

Actin-Phalloidin Staining—The 70–80% confluency cells grown on coverslips were fixed in 4% paraformaldehyde at 37 °C for 40 min followed by permeabilization with 0.1% Triton X-100 for 20 min at room temperature, washed with PBS, and stained overnight with 15 μg of Alexa Fluor phalloidin (Sigma). The next day, the excessive stains were removed; cells were washed with PBS, stained with Hoechst stain (1:1000), and sequentially washed with 70 and 100% alcohol using a Millipore manifold. The membranes were dried and radioactivity was measured using liquid scintillation counter (PerkinElmer Life Sciences).

Intracellular Calcium Measurement—Intracellular Ca$^{2+}$ levels were measured as published previously (23). In brief, 10⁶ cells were collected by trypsinization and incubated in Hanks’ balanced salt solution, pH 7.4, containing 4 μg/ml of Fluo-3/AM ester and 0.1% pluronic F-127 (Molecular Probes) at 37 °C for 30 min. The experiment was performed in the presence and absence of external calcium. Cells treated with ionomycin (1 mg/ml) were used as positive control. The calcium flux was monitored in 10,000 cells using FACS Calibur (BD Biosciences) and data were analyzed using CellQuest software. The median fluorescence intensity was used for subsequent analysis. The experiments were performed in duplicates and repeated three times.

Cell Cycle and Apoptosis Analysis—Cell cycle distribution was measured using BrdU flow kit (BD Biosciences). Briefly, the transfected cells were cultured in the serum-free DMEM for 48 h followed by addition of BrdU (10 μM) for 30 min at 37 °C and cultured in complete medium for the indicated times, and cell cycle distribution was assessed. The cell cycle data were analyzed by a FACS Calibur flow cytometer using CellQuest software (BD Biosciences). The experiments were performed in duplicates and repeated three times.

Invasion Assay—In vitro cell invasion analysis was performed using the agarose spot assay as published previously (24). In brief, 0.5% agarose solution containing fibronectin (20 μg/ml) or type 1 collagen (100 μg/ml) was spotted onto 6-well plates (Cell Star, Germany). Stably transfected cells (1 × 10⁶) were plated onto wells containing agarose spot in serum-free DMEM medium and incubated at 37 °C for 24–48 h. The plates were observed over a period of time to examine the movement of cells onto the agarose spot. Images of cells were captured using a Rolera emc2 camera and microscope (Olympus, Japan). The experiments were performed in duplicates and repeated three times.

In Vivo Tumorigenicity Assay—For in vivo tumorigenicity assays 5–6-week-old female BALB/c-nude mice (5 per group) were injected subcutaneously with 1 × 10⁶ cells mixed with Matrigel (1:1) into the lower flank of the animals. The tumor growth was monitored for over 2 months and tumor volume was measured 20 days post-injection. The tumor volume (V) was determined by the length (a) and width (b) as V = ab²/2. The experimental protocols were evaluated and approved by the animal ethics committee of Manipal University. Mice were sacrificed 2 months after injection and tumor tissue and organs were removed. Tumor tissue cryosection of 5-μm thickness were taken and stained with Hematoxylin & Eosin (H&E) and Masson’s trichrome and evaluated by a pathologist.

Bioinformatic and Statistical Analysis—The genomic coordinates spanning the promoter region of DOC2B was analyzed using CpG island searcher, transcriptional regulatory element database, transcription element search system (cbil.upenn.edu/
cgc-bin/tess), and AliBaba2.1. All data analysis was performed using Microsoft Excel 2007 (Microsoft) and GraphPad Instat (Trial Version). Student’s t test, one-way analysis of variance, and Kruskal-Wallis test were used for statistical analysis and significance. The p value less than 0.05 were considered statistically significant.

RESULTS

Identification of Differentially Methylated Regions—MS-AP-PCR was performed to identify differentially methylated regions in cervical cancer and non-malignant tissue samples (12, 13). We have isolated, cloned, and sequenced 15 hypermethylated fragments. A total of 10 fragments showed the characteristics of CpG islands and 6 fragments were found within the specific genes; whereas, the remaining fragments were found outside the gene coordinates. Nine hypermethylated fragments were associated with copy number variations regions (Table 2). The fragment 13 (Frg-13) was found to lie within the CpG island of the DOC2B gene promoter (−57 6bp to −342bp) and this was further analyzed for DNA methylation studies.

Mapping of Hypermethylated CpG Loci—To examine the methylation status of fragment Frg-13 and determine its frequency of methylation in cervical cancer, MS-DMSO-PCR and BGS were performed. A total of 15 non-malignant, 30 tumor samples, and 3 cervical cancer cell lines were analyzed by MS-DMSO-PCR, which showed that DNA methylation indeed changed the sensitivity of amplification to the DMSO concentration in the PCR mixture (Fig. 1B). For each sample, four different DMSO concentrations were used. i.e. 1, 2, 5, and 10% and the reaction without DMSO acting as control. In the case of the hypermethylated samples, amplification was detected only at a DMSO concentration of 2% or higher, whereas unmethylated samples showed amplification in the absence of DMSO indicating that tumor samples were hypermethylated when compared with the non-malignant samples (Fig. 1B).

To map the hypermethylated CpGs dinucleotides, BGS was performed for the (412 bp) region −376 to +36 bp of the DOC2B gene containing 58 CpG sites in 6 normal, 6 pre-malignant (4 low grade squamous intraepithelial lesion (LSIL), 2 high grade squamous intra epithelial lesion (HSIL)), 12 tumor samples, and 3 cervical cancer cell lines (SiHa, CaSki, and HeLa), respectively (Fig. 1, C and D). The CpG sites were unmethylated in normal samples, whereas hypermethylation was observed in 6 (of 6; 100%), 10 (of 12; 83.33%) and 3 (of 3; 100%) of pre-malignant, malignant, and cervical cancer cell lines, respectively (Table 3). Dense promoter methylation was observed in all the three cell lines tested, whereas partial to complete methylation of selected CpG sites were observed in LSIL, HSIL, and tumor samples (Fig. 1D). The promoter hypermethylation was found to be statistically significant between normal and tumor samples as well as between normal and LSIL/HSIL; and normal and malignant samples by one-way analysis of variance and Kruskal-Wallis test (p < 0.05), respectively. A significant association was found between HPV infection and DOC2B methylation (p < 0.005), however, more samples need to be screened before drawing any conclusion (Table 3). An independent association was observed between age and DOC2B promoter hypermethylation.

| Samples | Tissue | Age | DOC2B methylation | HPV status |
|---------|--------|-----|-------------------|------------|
| N1      | Normal | 45  | 1.9               | N          |
| N2      | Normal | 50  | 7                 | N          |
| N3      | Normal | 48  | 1.9               | N          |
| N4      | Normal | 42  | 1.9               | N          |
| N5      | Normal | 48  | 1.9               | N          |
| N6      | Normal | 55  | 1.7               | N          |
| P1      | LSIL   | 40  | 25                | N          |
| P2      | LSIL   | 46  | 42.85             | HPV16      |
| P3      | LSIL   | 50  | 26.92             | HPV16      |
| P4      | LSIL   | 54  | 13.46             | HPV18      |
| P5      | HSIL   | 53  | 19.23             | HPV16      |
| P6      | HSIL   | 74  | 11.53             | HPV11      |
| Mean ± S.E. |       |     |                   |            |
| N1      | PD-SCC (II-b) | 68   | 19.23              | N          |
| N2      | UD-SCC (II-b) | 71   | 30.76              | HPV18      |
| N3      | LC-K-SCC (II-b) | 55   | 61.53              | HPV16      |
| N4      | MD-SCC (II-b) | 52   | 19.23              | HPV16      |
| N5      | LC-NK-SCC (II-b) | 43  | 19.23              | HPV18      |
| N6      | LC-NK-SCC (II-b) | 57   | 24.48              | HPV16      |
| T1      | LC-NK-SCC (I-a) | 48  | 19.23              | HPV16      |
| T2      | UD-SCC (II-b) | 55   | 17.3               | N          |
| T3      | LD-SCC (II-b) | 50   | 31.91              | HPV16      |
| T4      | LD-SCC (III-b) | 40   | 30.76              | HPV18      |
| T5      | LD-SCC (III-b) | 60   | 25                 | HPV16      |
| T6      | LD-SCC (III-b) | 49   | 86.66              | HPV16      |
| Mean ± S.E. |        |     | 23.17 ± 4.66%     |            |
| SiHa    |        |     |                   |            |
| CaSki   |        |     | 91.1               | HPV16      |
| HeLa    |        |     | 100                | HPV18      |

According to one-way analysis of variance, the percentage of methylation was found to be statistically significant between normal and malignant samples. It was also found to statistically significant between normal and pre-malignant and normal and malignant when Kruskal-Wallis test was performed. p value less than p < 0.05 was considered as statistically significant.

Aberrant Methylation and Expression of DOC2B Gene in Cell Lines—We examined DOC2B expression status in a series of cancer cell lines and normal fibroblast using semi-quantitative RT-PCR and results are summarized in Fig. 1E. Significant reduction of DOC2B expression was observed in cancer cell lines of various organs but not in normal diploid fibroblast cells. The BGS analysis revealed that methylation is not confined to any specific region but it is spread uniformly throughout the DOC2B promoter in SiHa, CaSki, and HeLa cell lines (Fig. 1D and Table 3) leading to complete loss of DOC2B gene expression and was restored after treatment with 5-aza-2DC (5 μM and above) for 3 days in all three cervical cancer cell lines suggesting its transcriptional regulation by promoter-specific DNA methylation (Fig. 1F). Taken together, our results show that DOC2B expression is inhibited or reduced in different cancer cell lines, indicating its possible role as a negative regulator in multiple cancers.

Characterization of DOC2B Promoter by Transient Transfection Assay—the DOC2B promoter construct showed nearly 9-fold higher promoter activity when compared with vector alone (Fig. 1G). The heterologous enhancer-driven (SV40) DOC2B promoter construct was 30-fold more active than the respective control (p < 0.05). Upon artificial methylation the
FIGURE 2. Effect of ectopic expression of DOC2B on cell growth and proliferation. A and B, representative figures showing expression of the DOC2B gene upon transfection by RT-PCR and Western blot, respectively. DOC2B was detected by anti-DDK tag antibody. β-Actin was used as an internal control. C, DOC2B inhibits tumor growth in vitro in SiHa and HeLa cells, respectively. D, quantitative analysis of colony forming assay represented as mean ± S.D., *, p < 0.05, shows a significant decrease in colony number after ectopic expression of DOC2B. E, representative image of soft agar colony forming assay. F, quantitative analysis of colony number represented as mean ± S.D., *, p < 0.05. G, represents the cell proliferation rate in DOC2B expressing stable clones in comparison with vector control. Ectopic expression of DOC2B significantly inhibited cell proliferation resulting in delayed cell doubling time. The cell doubling analysis was performed using the cell doubling time calculator. H, cell proliferation rate was significantly inhibited at both DNA and RNA levels in DOC2B expressing cells when compared with control cells by [3H]thymidine and [3H]uridine incorporation assays, respectively (mean ± S.D. from 3 independent experiments in duplicates), *, represents p < 0.05.

FIGURE 3. Ectopic expression of DOC2B suppresses growth and proliferation in SiHa and HeLa cells. A and B, the DOC2B ORF was cloned into retroviral vector pMX-IRES-GFP to generate pMX-DOC2B-IRES-GFP and used to transduce SiHa and HeLa cells. The expression of DOC2B upon ectopic expression was confirmed by both RT-PCR (A) and Western blot by anti-DOC2B antibody (B). β-Actin was used as internal control. C and D, representative image of the colony formation assay in SiHa and HeLa, respectively. E, representative quantitative analysis of the colony numbers in SiHa and HeLa cells, respectively. The ectopic expression of DOC2B significantly reduced the colony numbers in both SiHa and HeLa cells, respectively. F, ectopic expression of DOC2B inhibits SiHa and HeLa cell proliferation, respectively. G, representative colonies from SiHa and HeLa, respectively. Both colony number and size decreased upon DOC2B expression. H, DOC2B expression induced arrest at the G0/G1 and S phases of the cell cycle. *, p < 0.05 by independent Student's t test was considered as statistically significant. The experiments were repeated 3 times in duplicate.
**DOC2B Regulation by Promoter Methylation in Cervical Cancer**

**A**

![representative cell morphology of DOC2B expressing and control cells in SiHa and HeLa cells, respectively (×40 magnification).](image)

**B**

![representative images of actin staining showing rearrangement of actin fibers leading to increased cell adhesion and decreased lamellipodia (indicated by arrows) in DOC2B expressing cells when compared with control cells (×400 magnifications).](image)

**C**

![percent dead cells in SiHa and HeLa cells, respectively.](image)

**D**

![representative aneuploidy analysis for empty vector- and DOC2B-transfected SiHa and HeLa cells.](image)

**E**

![representative images showing an increase in intracellular Ca\(^{2+}\) flux when compared with control cells.](image)

**FIGURE 4.** DOC2B expression changes cell morphology, induces remodeling of cytoskeletons, inhibits cell division, and increases intracellular calcium flux. A, representative cell morphology of DOC2B expressing and control cells in SiHa and HeLa cells, respectively (×40 magnification). B, representative images of actin staining showing rearrangement of actin fibers leading to increased cell adhesion and decreased lamellipodia (indicated by arrows) in DOC2B expressing cells when compared with control cells (×400 magnifications). C, representative aneuploidy analysis for empty vector- and DOC2B-transfected SiHa and HeLa cells. Cells were cultured on poly-HEMA-coated tissue culture plates and cell death was analyzed by measuring the sub-G\(_1\) population of cells by propidium iodide staining and FACS analysis. Ectopic expression of DOC2B showed a significantly higher sub-G\(_1\) population of 18.66 versus 36.33% and 24.47 versus 48.96% between DOC2B and empty vector-transfected cells in both SiHa and HeLa cells, respectively. D, quantification of DNA content and cell cycle phase distribution in empty vector- and DOC2B-transfected SiHa cells at different time points as analyzed by a BrdU pulse-chase experiment. Ectopic expression of the DOC2B gene resulted in a significant cell cycle arrest at G\(_0/G_1\) and S phases of the cell cycle. E, representative figures showing an increase in intracellular Ca\(^{2+}\) flux when compared with control cells. There was a significant increase in intracellular Ca\(^{2+}\) flux upon ectopic expression of DOC2B. Values were the median fluorescence intensities ± S.D. of at least three independent experiments performed in duplicates. *p < 0.05 by independent Student’s t test: p value < 0.05 was considered statistically significant.

**DOC2B promoter and enhancer-driven constructs showed diminished expression as opposed to mock methylated constructs in SiHa cells (Fig. 1G). These results confirmed that the DOC2B promoter was indeed under tight regulation by DNA methylation.**

**DOC2B and Tumor Suppressive Function**—Frequent silencing of DOC2B in cervical cancer cell lines suggests it is likely to be associated with cell proliferation. To explore this, the DOC2B gene was overexpressed in SiHa cells, which do not inherently express DOC2B. After confirmations of ectopic DOC2B expression by RT-PCR (Fig. 2A) and Western blot analysis (Fig. 2B), SiHa and HeLa cells were subjected to colony formation, cell doubling, and growth curve assays. The colonies formed by DOC2B expressing cells were significantly less and smaller in size when compared with empty vector (p <0.005) transfected stable clones (Fig. 2, C and D). Moreover, DOC2B expression suppressed the colony size and decreased the number of colonies to 40% in anchorage-independent experiments (Fig. 2, E and F). Ectopic expression of DOC2B also inhibited cell proliferation and increased doubling time in both SiHa and HeLa, respectively (Fig. 2G). Overexpression of DOC2B showed a substantial increase in doubling time from 37.49 to 48.52 h in SiHa cells, whereas 22.79 to 27.88 h in HeLa cells. There was also a significant reduction in the rates of DNA and RNA synthesis (Fig. 2H) in DOC2B expressing cells as opposed to control cells (p <0.05). Therefore, DOC2B expression may provide growth disadvantage function to cervical cancers. We have validated some of these findings by overexpressing DOC2B using pMX-IRE-RES-GFP retroviral vector and confirmed by using RT-PCR and Western blot (Fig. 3, A and B) that DOC2B expression inhibited cell growth (Fig. 3C-3E) and proliferation (Fig. 3F) in SiHa and HeLa cells, respectively. The sizes of the colonies were smaller in DOC2B expressing cells when compared with control cells (Fig. 3G). Similar to single cell clones, polyclonal cells expressing retrovirally transduced DOC2B also inhibited cell cycle progression at the G\(_0/G_1\) and S phases of cell cycle when compared with DOC2B-deficient control cells (Fig. 3H).

**Ectopic Expression of DOC2B Changes Cell Morphology**—DOC2B expressing cells showed distinct morphological changes compared with control cells such as increased cell-cell adhesion and decreased cell scattering (Fig. 4). Increased cell-cell adhesion and decreased cell scattering may have been a
contributing factor toward the decreased ability of DOC2B expressing cells to migrate. To investigate the role of DOC2B in regulating the properties of tumor cell invasion and metastasis, actin-phalloidin staining was performed (Fig. 4B). Ectopic expression of DOC2B resulted in actin rearrangement and decreased lamellipodia formation when compared with control cells, indicating that DOC2B could induce cytoskeleton remodeling leading to inhibition of cervical cancer cell invasion (Fig. 4B).

DOC2B Expression Induces Anoikis in SiHa Cells—To determine the mechanism of DOC2B induced inhibition of colony formation, delay in cell doubling, and proliferation, we analyzed the effect on apoptosis and cell cycle progression. Normal cells may undergo cell death due to anoikis when detached from the extracellular matrix; whereas tumor cells escape anoikis due to oncogenetic transformation. Therefore we investigated apoptosis under anoikis conditions. Cells were cultured on poly-HEMA-coated tissue culture plates and cell death was analyzed by measuring the sub-G1 population of cells by propidium iodide staining and FACS analysis. Ectopic expression of DOC2B showed a significantly higher sub-G1 population 18.66 versus 36.33% and 24.47 versus 48.96% between DOC2B and...
empty vector-transfected cells in both SiHa and HeLa cells, respectively (Fig. 4C). These data suggest that DOC2B induces aneikis in SiHa and HeLa cells.

**DOC2B Expression Regulates G_{0}/G_{1}-S Phase Transition and Increases Ca^{2+} Flux**—DOC2B expression resulted in an increase in percent of G_{0}/G_{1} cells and decrease in S phases cells ($p < 0.05$), respectively (Fig. 4E). Our result indicates that expression of DOC2B inhibits tumor cell growth by suppressing cell proliferation and influences delay in cell cycle progression and induces aneikis. Concurrently, expression of DOC2B showed an increase in intracellular Ca^{2+} flux (median fluorescence intensity; 153.2 versus 56.5, $p < 0.05$) when compared with control cells (Fig. 4F) as measured using Fluo-3-AM probe.

**DOC2B Inhibits Migration and Invasion in Cervical Cancer Cells**—Expression of the DOC2B gene reduced cell migration (Fig. 5), which was evident from analysis of the migration index ($p < 0.05$) (Fig. 5, E and F). Quantitative analysis at 24, 48, and 72 h showed a progressive decrease in wound closure rate (14.5 versus 29.4%, 25.4 versus 53.9%, 40.1 versus 75.9%, and 25.26 versus 51.47%, 33.5 versus 63.6% and 47 versus 85.7%) between DOC2B and empty vector-transfected cells in both SiHa and HeLa, respectively (Fig. 5, C–F). DOC2B overexpression using pMX-IRE-6-GFP retroviral vector reduced cervical cancer cell migration in both SiHa and HeLa cells, respectively (Fig. 6). Moreover, at the end of 96 h, the wound was completely closed in vector-transfected cells, whereas it remained incomplete in DOC2B expressing cells. Regardless of DOC2B expressing clonal isolates or retroviral transduced cells, their invasion toward fibronectin and type I collagen-coated plates were significantly inhibited ($p < 0.05$) (Fig. 7, A–H). These results suggest that DOC2B may play a role as an inhibitor of invasion of cervical tumor cells in our experimental systems. Because DOC2B regulates cell survival, proliferation, growth, and cell cycle progression in SiHa cells, we examined the phosphorylation status of AKT1 and ERK1/2 proteins. DOC2B expressing cells inhibited AKT1 (Ser^{473}) and ERK1/2 (Thr^{202}/Tyr^{204}) phosphorylation without showing significant changes in their total protein level (Fig. 7, I–L).

**DOC2B Inhibits Tumor Growth in Vivo**—Animal studies were conducted to evaluate the role of DOC2B in influencing tumor growth in athymic nude mice. The transfected cells were injected into the mice and progressive tumor growth was analyzed. The results demonstrated that cells in the control group formed a progressively growing tumor. In contrast, animals injected with DOC2B expressing cells produced a significantly smaller tumor ($p < 0.05$) (Fig. 8, A and B). Thus, these results indicate that, ectopic expression of DOC2B results in in vivo inhibition of tumor growth. The H&E staining of DOC2B expressing cells showed a decreased nucleus to cytoplasmic ratio, less abnormal nucleus and decreased tumor cell density, less pleomorphic (less atypical) and densely aggregated cells, and reduced the number of abnormal mitosis in contrast to DOC2B negative cells (Fig. 8C). Expression of the DOC2B gene may inhibit collagen degradation and/or synthesis, which is evident by Masson’s trichrome staining of tumor xenografts grown in nude mice (Fig. 8D).

**DISCUSSION**

To investigate the role of differential methylation in cervical cancer and identify novel regions silenced in these tumors, we performed MS-AP-PCR that has previously been shown to frequently select methylated CpG islands (12, 13, 25). Although some of the CpG islands identified were near or within the genes such as IKBKG, KLRG2, Myomesin-2, and NXN, we chose DOC2B due to its proximity to functional promoter (Table 2). In silico analysis of −700 to +300 bp of the DOC2B by CpG Island searcher, TESS and AliBaba2.1 predicted a CpG island, E Box (CACGTG, −393/−387), and identification of binding
sites for several methylation-sensitive transcription factor such as Sp-1, Ap-2, USF, and E2F; suggesting that transcription of DOC2B could be regulated by promoter methylation. Our experiment shows that methylation of the CpG island proximal to the transcription start site of the DOC2B gene leads to transcriptional repression in SiHa cells. Inactivation of the DOC2B gene by other mechanisms such as deletions, copy number variation, and mutations will also need to be evaluated to understand the role of the DOC2B gene in the pathogenesis of cancer in general and cervical cancer in particular.

DNA methylation is an epigenetic process that regulates expression of genes during a variety of physiological conditions (26, 27). Transcriptional repression of the tumor suppressor gene and activation of oncogenes via aberrant methylation is common in cancers. Interestingly, specific drugs have been shown to reactivate the down-regulated genes in cancers via inhibition of epigenetic machineries. We showed evidence for the first time that the DOC2B gene is frequently hypermethylated in cervical cancer leading to loss of its expression, which can be restored upon treatment with 5-aza-2DC, suggesting that promoter methylation directly contributes to DOC2B gene silencing. Thus we suggest that binding of chromatin remodeling complexes and associated proteins to the DOC2B promoter might lead to transcriptional repression by inhibiting the interaction with transcription factors (26, 27).

Our study shows that DOC2B may act as a pro-apoptotic tumor suppressor gene in the absence of adhesion to the matrix via inhibiting AKT/ERK signaling by inducing actin cytoskeleton remodeling and increase in intracellular Ca$^{2+}$. Reduced cell-cell adhesion, inhibition of cell growth, proliferation, invasion, and migration, which all may be regulated by decreased phosphorylation of AKT/ERK proteins by interfering with their signaling pathways. Our study is the first to demonstrate the causes and consequences of DOC2B silencing in cervical cancer and supports the notion that DOC2B might be a putative tumor suppressor gene in multiple cancers.

In the present study, we showed that DOC2B inhibits growth, proliferation, migration, and invasion of cervical cancer cells in vitro. However, to begin to understand the molecular mechanism of the anti-tumor effect exerted by DOC2B, we focused on its effect on AKT1 and ERK1/2 signaling as it has been previously reported that the inhibition of both AKT and ERK activation by phosphorylation results in inhibition of cell growth, proliferation, migration, and invasion (28–31). In our study, phosphorylated ERK1/2 and AKT1 decreased upon DOC2B expression suggesting the role of ERK/MAPK and AKT signaling on its effects. It has been shown that the regulation of rearrangement of the actin cytoskeleton occurs via phosphorylating proteins such as EPLIN, MLCK, FAK vinexin by ERK1/2, and phosphorylation of girdin, fascin, and $\alpha$-plastin by AKT1.10647

FIGURE 7. DOC2B inhibits invasion and phosphorylation of AKT-1 and ERK1/2. A–D, representative images showing the inhibition of invasion onto the agarose spot containing fibronectin and type I collagen upon ectopic expression of DOC2B in a single cell clone (A and B) and retrovirally transduced polyclonal cells (C and D). E–H, quantitative analysis of a number of tumor cells invading fibronectin and type I collagen in a single cell clone (E and F) and retrovirally transduced polyclonal cells (G and H), respectively. The bar graph represents mean ± S.D. of triplicate experiments performed in duplicates. *, p < 0.05 by independent Student’s t test was considered as statistically significant. I–L, ectopic expression of DOC2B regulated both AKT and ERK signaling. A Western blot was performed using antibodies against total AKT, phospho-AKT, total ERK1/2, and phospho-ERK1/2. β-Actin was used as internal control. DOC2B expression inhibited AKT phosphorylation, whereas there was a decrease in ERK1/2 phosphorylation when compared with vector-transfected control SiHa cells in both single cell clone (I and J) and polyclonal cells (K and L), respectively. Relative phosphorylation levels of AKT and ERK1/2 were determined by normalization with total AKT and total ERK1/2, respectively.
In conclusion, our study demonstrates for the first time that (i) promoter hypermethylation is a regulator of DOC2B gene transcription and is down-regulated in cervical cancer; (ii) DOC2B expression leads to an increase in intracellular Ca$^{2+}$, remodeling of cytoskeleton, and inhibition of AKT and ERK phosphorylation to interfere with their downstream signaling indicating the involvement of multiple pathways; and (iii) expression of DOC2B may result in inhibition of key biological characteristics of tumor growth, migration, and invasion in cervical cancer cells. Therefore these characteristics and features of DOC2B can be exploited for therapeutic application in cervical cancer.
REFERENCES

1. Shen, H., and Laird, P. W. (2013) Interplay between the cancer genome and epigenome. Cell 153, 38–55
2. Dueñas-González, A., Lizano, M., Candelaria, M., Cetina, L., Arce, C., and Cervera, E. (2005) Epigenetics of cervical cancer. An overview and therapeutic perspectives. Mol. Cancer 4, 38
3. Esteller, M. (2008) Epigenetics in cancer. N. Engl. J. Med. 358, 1148–1159
4. Duncan, R. R., Shipston, M. J., and Chow, R. H. (2000) Double C2 protein. A review. Biochimie 82, 421–426
5. Orita, S., Sasaki, T., Naito, A., Taguchi, Y., Asahina, K., Miyamoto, S., Uraki, S., Oka, Y., and Tanizawa, Y. (2009) DOC2B: a novel syntaxin-4 binding protein mediating insulin-regulated GLUT4 vesicle fusion in adipocytes. Diabetes 58, 377–384
6. Groomen, A. J., Martens, S., Diez Aranzola, R., Cornelisse, L. N., Rozovaya, N., de Jong, A. P., Goriounova, N. A., Habets, R. L., Takai, Y., Borst, J. G., Brose, N., McMahon, H. T., and Verhege, M. (2010) Doc2b is a high-affinity Ca2+ sensor for spontaneous neurotransmitter release. Science 327, 1614–1618
7. McMahon, H. T., Kozlov, M. M., and Martens, S. (2010) Membrane curvature in synaptic vesicle fusion and beyond. Cell 140, 601–605
8. Enomoto, A., Murakami, H., Asai, N., Morone, N., Watanabe, T., Kawai, K., Murakumo, Y., Usukura, Y., Kaibuchi, K., and Takahashi, M. (2005) Akt/PKB regulates actin organization and cell motility via Girdin/AEF. Dev. Cell 9, 389–402
9. Cohan, C. S., Welshofer, E. A., Zhao, L., Matsumura, F., and Yamashiro, S. (2001) Role of the actin bundling protein fascin in growth cone morphogenesis: localization in filopodia and lamellipodia. Cell Motil. Cytoskeleton 48, 109–120
10. Flanagan, J. M. (2007) Host epigenetic modifications by oncogenic viruses. Br. J. Cancer 96, 183–188
11. Uozaki, H., and Fukayama, M. (2008) Epstein-Barr virus and gastric carcinoma: viral carcinogenesis through epigenetic mechanisms. Int. J. Clin. Exp. Pathol. 1, 198–216
12. Guerin, S., Moualil, M., Deplus, R., Lampe, X., Krys, N., Calonne, E., Delbecque, K., Kridelka, F., Fuchs, E., Ennaji, M. M., and Delvenne, P. (2012) Aberrant promoter methylation and expression of UTF1 during cervical carcinogenesis. PLoS One 7, e42704
13. Lai, H. C., Lin, Y. W., Huang, T. H., Yan, P., Huang, R. L., Wang, H. C., Liu, J., Chan, M. W., Chu, T. Y., Sun, C. A., Chang, C. C., and Yu, M. H. (2008) Identification of novel DNA methylation markers in cervical cancer. Int. J. Cancer 123, 161–167