Identification of Smyd4 as a Potential Tumor Suppressor Gene Involved in Breast Cancer Development

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Abstract
To identify genes involved in breast tumorigenesis, we applied the retroviral LoxP-Cre system to a nontumorigenic mouse mammary epithelial cell line NOG8 to create random chromosome deletion/translocation. We found that the disruption of one allelic set of Smyd4 (SET and MYND domain containing 4) gene through chromosome translocation led to tumorigenesis. The expression of Smyd4 was markedly decreased in tumor cells. Re-expression of Smyd4 resulted in growth suppression of tumor cells and inhibition of tumor formation in nude mice. Furthermore, the RNA interference–mediated suppression of Smyd4 expression in human MCF10A mammary epithelial cells caused their growth in soft agar. Microarray studies revealed that platelet-derived growth factor receptor α polypeptide (Pdgfr-α) was highly expressed in tumor cells compared with NOG8 cells. Re-expression of Smyd4 significantly reduced the expression of Pdgfr-α in tumor cells. In human breast cancers, reverse transcription-PCR results revealed that Smyd4 expression was totally silenced in 2 of 10 specimens. These findings indicate that Smyd4, as a potential tumor suppressor, plays a critical role in breast carcinogenesis at least partly through inhibiting the expression of Pdgfr-α, and could be a novel target for improving treatment of breast cancer. [Cancer Res 2009;69(9):4067–72]

Introduction
Breast cancer is one of the leading causes of cancer deaths in women in the United States. Over the past decades, the intensive efforts on breast cancer to identify cancer genes and understand how they contribute to tumorigenesis have lead to a better understanding of molecular characterization. Identification of cancer genes has been greatly facilitated by analyzing recurring chromosome rearrangements (1) that are found in the malignant cells of most patients with leukemia, lymphoma, and solid tumors (2). Chromosomal engineering in the mouse genome has been achieved using Cre-loxP technique (3). The Cre recombinase belongs to the λ integrase family and catalyzes recombination between 2 identical 34-bp sites called loxP. Recently, to identify additional breast cancer genes, we developed Cre-loxP–based method to generate random chromosome rearrangement such as translocation and deletion by integrating two loxP sites into the mouse genome (4). If two loxP sites are integrated into DNA on two different chromosomes, the recombination catalyzed by Cre leads to the translocation. If loxP sites are integrated into DNA in the same direction on same chromosome, the recombination leads to the deletion of the interval DNA between two loxP sites. The proviral integration sites of loxP carrying vectors can be determined through inverse PCR and sequencing (4, 5).

The loss of heterozygosity or DNA methylation changes at human chromosome 17p13.3, in absence of any p53 genetic alterations at 17p13.1, seems to be frequently involved in various types of human malignant tumors including sporadic breast cancers, ovarian cancers, medulloblastomas, and small cell lung carcinomas (6–10). These results suggest that one or more tumor suppressor genes located at 17p13.3 could be involved in tumorigenesis. Among more than hundreds of genes residing in this region, previous studies have resulted in the identifications of several tumor suppressors, including MAX binding protein, hypermethylated in cancer 1, and ovarian cancer gene 1 (11–14). Loss of MAX binding protein severely disrupts mammary gland involution and leads to hyperplastic ducts (15), but no mutations in MAX binding protein have been described in human breast tumors (16–18). Hypermethylated in cancer 1 is hypermethylated and transcriptionally silent in many types of human cancers including breast cancer (13, 19). Heterozygous hypermethylated in cancer 1 mutant mice do not develop mammary gland tumors, although they are predisposed to other malignant tumors including pulmonary carcinomas, lymphomas, and sarcomas (20). There could be additional breast tumor suppressor genes located at 17p13.3.

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suppress the breast tumorigenesis at least partly through inhibiting expression of platelet-derived growth factor receptor α (Pdgfr-α) in breast tumor cells.

Materials and Methods

Constructs and cell culture. Plib-Smyd4-Bla was constructed by inserting EcoRI/Xhol fragment of Smyd4 cDNA into EcoRI/Sall sites of Plib-Bla. Plib-Bla was generated by inserting SW40-directed blasticidin resistance gene into Ccl4/Sall sites of PIB (Clontech).

NOG8 and the tumor cell line were grown in DMEM, supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% of CO2. MCF10A were maintained in DMEM/F12 containing 5% horse serum, 10 μg/mL human insulin, 0.5 μg/mL hydrocortisone, and 20 ng/mL epidermal growth factor (Sigma-Aldrich).

The NOG8 cells with recombination carried an intact puromycin-resistant gene were selected and cultured in the medium containing 2 μg/mL of puromycin (Invitrogen). The NOG8 cells carrying recombination are injected s.c. into the nude mice for tumor formation. The tumor cells were harvested from the primary tumor dissected out of mouse and cultured in the medium containing puromycin.

Chromosome engineering and tumor development. The chromosome engineering on NOG8 cells was performed as described (4). Phoenix-Eco packaging cells (5 × 107) were transfected with 15 μg of LacZ/Neo-5 Pur-LoxP-TK or Hyg-5 Pur-LoxP vector through the calcium phosphate precipitation method. Forty-eight hours after the transfection, LacZ/Neo-5 Pur-LoxP-TK viral supernatant was added to NOG8 cells (5 × 106) with 8 μg/mL of polybrene. Hyg-3 Pur-LoxP retrovirus was used for infection on the second day. The cells were subjected to G418 and hygromycin selection 48 h after retroviral infection. One week after selection, cells from one 10-cm plate were infected with 106 adenovirus expressing Cre recombinase. Puromycin (1 μg/mL) was added to the medium 24 h later, and the cells were selected with puromycin for 5 d. The cells were then injected s.c. into the nude mice for tumor formation. Two months later, the tumor was harvested for further analysis.

Inverse PCR and localization of retroviral integration site. Ten micrograms of tumor DNA was digested with BamH1/Apol for localization of the 3' terminal repeat (LTR) or Sphi/Alu III for localization of the 5'LTR. The digested DNA was blunt with T4 polymerase and then circularized by ligation using T4 ligase (New England Bio Labs) in a total volume of 100 μL at room temperature overnight. Circular DNA was purified with phenol/chloroform extraction, precipitated with ethanol, and dissolved in 50 μL of water. The 100 μL of PCR reaction mixture contains 0.25 mmol/L each of deoxynucleotide triphosphates, 10 pmol of each forward or reverse primer, 30 μL of 3.3 × buffer, 4.8 μL of Mg(OAc)2 (25 mmol/L), and 2 μL of Tth polymerase (Roche). One microliter of primer PCR product was used as template in second PCR reaction. The second PCR products were separated on 1% of agarose gel, and purified with the QIA quick gel extraction kit (Qiagen). The purified second PCR products were sequenced at Northwestern University Biotech Facility. The retroviral integration site was determined through blasting the sequence against National Center for Biotechnology Information Mouse genome database. The primary and nested primers used were as follows.

For locating the 5’LTR: 5’-CATGTGCAAGTCATGGATGA-3’ and 5’-AGGAAACGGAGACACAGT-3’; its nested primers: 5’-ACGATGGTGCAAGATCTTC-3’ and 5’-GTGCAACACAGAAGGGCT-3’.

For locating the 5’LTR: 5’-CCGCTAAAGCCTGCTCCA-3’ and 5’-CGAGAAGCTCCTTCCTCAG-3’; its nested primers: 5’-CTGCTTGGGAAAGGGCT-3’ and 5’-CTGACATGCAGCAGGTG-3’.

Reverse transcription-PCR and real-time RT-PCR. RNAs from cultured cells were prepared using TRIzol (Invitrogen). Reverse transcription-PCR (RT-PCR) was performed using SuperScript III One-Step RT-PCR System (Invitrogen) with 1 μg of total RNA. PCR products were analyzed on 1% agarose gels and visualized by ethidium bromide staining. For real-time RT-PCR, cDNAs were synthesized with 2 μg total RNA using a Superscript first-strand cDNA synthesis kit (Invitrogen). The real-time PCR products were detected with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The expression of Smyd4 in mammary tumor cells was revealed by quantitative real-time RT-PCR.
was performed using Applied Biosystems 7300 Thermal cycler and a SYBR Green PCR kit (Applied Biosystems). The amplification was performed using 40 cycles. SYBR Green fluorescence was monitored in each cycle for the reference and marker gene. Samples were analyzed in triplicate, and expression was compared with β-actin.

**Cell proliferation assay and anchorage-independent growth in soft agar.** The cell proliferation was monitored by cell counting using trypan blue exclusion. For soft agar growth, 5,000 cells from each stable cell line or mock (empty vector) cells were mixed with 1.5 mL of 0.3% agar (Sigma) in 10% FBS/DMEM, then overlaid onto 1.5 mL of 0.5% agar/10% FBS/DMEM in 1 well of a 6-well plate. The medium was changed every 3 d, and colonies were grown for 4 wk. Colony formation was counted and repeated in triplicate.

**Tumor formation assay in vivo.** The nu/nu athymic mice were used under Institutional Animal Care and Use Committee approval. A total of $1 \times 10^6$ of Smyd4 re-expressing cancer cells or control cancer cells in 200 μL of media were combined with 200 μL of Matrigel (BD Biosciences) and injected s.c. into the dorsal neck region of nu/nu athymic mice. Tumor growth was monitored weekly.

**Small interfering RNA.** Four of Smyd4 shRNA constructs (V2HS_88307, V2HS_88308, V2HS_88309, and V2HS_255847) and one mock shRNA (pSM2 empty vector) constructs were purchased from Open Biosystems. Retrovirus was produced by transfecting Ampho 293 cells with shRNA vectors for 48 h. MCF10A cells were infected for 24 h with viral supernatant and selected for 6 to 8 d with 0.5 μg/mL of puromycin. The cells were then combined for further analysis. The four shRNA constructs showed similar degree of suppression. The results presented here are from experiments using V2HS_88307.

**Results**

**Decreased expression of Smyd4 gene due to chromosome translocation.** We previously reported our effort on efficient generation of random chromosome rearrangement with Cre-loxP system (4). In this study, Cre-loxP recombination system was used to create chromosome deletion/translocation in nontumorigenic mouse mammary epithelial cell line NOG8. NOG8 cells were sequentially infected with LacZ/Neo-5’Pur-LoxP-TK virus, Hyg-3’Pur-loxP virus, and Cre adenovirus. Recombinant between these two vectors can generate an intact puromycin-resistant gene with correct reading frame. The cells with combination carrying an intact puromycin-resistant gene would survive against puromycin. The NOG8 cells with chromosome rearrangement were injected s.c. into nude mice for tumor formation. The tumor cells were harvested from the primary tumor and subcultured.

For one tumor, inverse-PCR and sequencing revealed that the 5’ Pur-LoxP-TK was inserted into the 10th intron of Smyd4 gene on mouse chromosome 11, whereas 3’ Hyg-Pur-LoxP was inserted into Figure 2. Smyd4 functions as a tumor suppressor. A, cell proliferation assay shows that Smyd4 suppressed the growth of tumor cells in vitro. Exponentially grown breast tumor cells infected with retroviruses expressing Smyd4 or control retroviruses were monitored by cell counts using trypan blue exclusion. The expression of Smyd4 mRNA was determined by real-time RT-PCR. B, Smyd4 suppressed anchorage-independent growth in soft agar growth. Both the size and number of colony were decreased with Smyd4 re-expression. C, Smyd4 inhibited tumor growth in vivo. Two million breast tumor cells re-expressing smyd4 (R1, R2) or control tumor cells (L1, L2) were injected into 5-wk-old athymic nude mice for tumor formation. Tumor growth was monitored weekly. Smyd4 re-expression dramatically suppressed the tumor growth in nude mice compared with control tumor cells with disrupted Smyd4 expression.
translocation, one allele of from these two genes act in opposite direction. As the result of tumorigenesis, we focused on involved in neurodegenerative diseases and unlikely plays a critical role in breast epithelial cells.

Expression of Smyd4 in tumor cells was less than one third of that from NOG8 cells examined if Smyd4 expression is altered due to translocation.

As Smyd4 expression was reduced in the tumor cells, demonstrating the colonies formed by cells with Smyd4 knockdown or control MCF10A cells, the markedly increased number of colonies formed by MCF10A cells with Smyd4 knockdown compared with control MCF10A cells. Columns, mean from two independent triplicate experiments; bars, SD.

transcriptional recombination led to translocation between chromosome 11 and 14, creating a fusion gene including the promoter, exon 1 to 10 of Smyd4 gene (Fig. 1B). After determining the integration sites, the recombination between the two vectors integrated into different sites was further confirmed by PCR using primers specific for recombination (Fig. 1B). One 6.7-kb band were amplified by PCR with one primer (5’-TGACAAGAGCTTCACGAG-3’) located in the Smyd4 on chromosome 11 and another primer (5’-TCGGACATC0GCAAGGTG-3’) specifically for Hyg-3 Pur-loxP vector. Another 2.2-kb band were amplified by PCR with 1 primer (5’-CATGGTCACCTAAGAGGG-3’) located on chromosome 11 and another primer (5’-CTCCGTCGGAACCTCGAG-3’) specifically for LacZ/Neo-5’ Pur-loxP-TK vector. The promoters from these two genes act in opposite direction. As the result of translocation, one allele of Smyd4 gene is disrupted but no fusion protein is created.

Given that Msra, as a regulator of antioxidant defense, is mainly involved in neurodegenerative diseases and unlikely plays a critical role in tumorigenesis, we focused on Smyd4 gene. At first, we examined if Smyd4 expression is altered due to translocation. Real-time RT-PCR revealed that the level of Smyd4 mRNA from tumor cells was less than one third of that from NOG8 cells expression (Fig. 1B).

Growth suppression of tumor cells through Smyd4 re-expression. As Smyd4 expression was reduced in the tumor cells, we assessed if Smyd4 plays a role in inhibiting cell proliferation. The tumor cells were infected with retroviruses expressing Smyd4 or with control retroviruses. The re-expression of Smyd4 was confirmed by real-time RT-PCR (Fig. 2A). Cell proliferation assay revealed that Smyd4 re-expression dramatically decreased the growth of cancer cells (Fig. 2A).

The effect of Smyd4 re-expression on the anchorage-independent growth of cells in soft agar was tested. Compared with the tumor cells infected with control viruses, the numbers of colonies formed in soft agar were dramatically reduced in Smyd4 re-expression tumor cells (Fig. 2B), indicating that Smyd4 suppresses the anchorage independent growth of tumor cells.

To further show that Smyd4 is a tumor suppressor, we tested whether the recovery of Smyd4 expression in tumor could suppress tumor growth in vivo in athymic nude mice. Cells (2 × 10^5) were injected s.c. into the dorsal neck region of nu/nu athymic mice. Four weeks later, control tumor cells formed tumors, whereas Smyd4 re-expressing tumor cells were incapable of forming tumors (Fig. 2C). Both in vitro and in vivo data conclusively indicate that Smyd4 suppressed the tumor growth.

Transformation of human breast epithelial cell MCF10A by Smyd4 RNA interference. To determine if Smyd4 knockdown could lead to transformation, we knocked down Smyd4 expression in human mammary gland epithelial cell MCF-10A using RNA interference. MCF10A cells are of mammary origin and exhibit anchorage-dependent growth property in vitro (30). Smyd4-shRNA vectors or control vector were integrated and expressed in MCF10A cells using standard retroviral infection. Real-time RT-PCR revealed that Smyd4 expression was efficiently suppressed in MCF10A cells expressing Smyd4 shRNA (Fig. 3A). Smyd4 knockdown cells and the control cells were seeded into soft agar and allowed to grow for 4 weeks. As a result, the control MCF10A cells were incapable of forming foci in soft agar, whereas the MCF10A cells with Smyd4 knockdown readily formed foci, indicating that Smyd4 knockdown promotes the transformation of breast epithelial cells (Fig. 3B and C).

The expression of Pdgfr-α is repressed by Smyd4. To identify the down-stream genes of Smyd4, microarray analysis were performed with the tumor cells and NOG8 cells. By comparing expression profiles of tumor cells and NOG8 cells, we selected four genes (Pdgfr-α, semaphorin 5A, myogenic factor 6, and nerve growth factor receptor) that were known for their potential roles in tumorigenesis and up-regulated in tumor cells for further studies.

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Real-time RT-PCR confirmed that Pdgfr-α expression was dramatically elevated in tumor cells (Fig. 4A) but not detectable in NOG8 cells during 40 cycles of amplification. In addition, the microarray data (data not shown here but in the Supplementary Data) indicates that the NOG8-derived tumors highly express Pdgfa and Pdgfb, which are the ligands of Pdgfr-α homodimer (31). Furthermore, real-time RT-PCR revealed that Pdgfr-α expression was significantly suppressed by re-expressing exogenous Smyd4 in tumor cells (Fig. 4B). For the rest of the three genes, their expression was increased in tumor cells but the re-expression of Smyd4 could not suppress their expression (data not shown). As Pdgfr-α overexpression was reported in breast cancer and associated with tumor progression (32), the finding indicates that Smyd4 acts as a tumor suppressor at least partly through controlling Pdgfr-α expression.

Smyd4 expression is silenced in some of human breast cancers. Human Smyd4 gene is located at chromosome 17p13.3, which is a region commonly exhibiting loss of heterozygosity in breast cancers. After having shown that Smyd4 is a potential tumor suppressor, we asked if Smyd4 is involved in human breast cancers. We analyzed the expression of Smyd4 gene in 10 of human breast carcinomas. RT-PCR results reveal that Smyd4 gene expressions were completely silenced in two breast cancer cases and markedly decreased in 3 additional cases (Fig. 5A). To find if there is correlation between the expression of Smyd4 and Pdgfr-α, we also examined the Pdgfr-α expression. For the human breast cancers in which Smyd4 expression were totally silenced or markedly decreased, the concordance was found in two of human breast cancers (HBT-5, HB-10) but not found in the others (HBT-1, HBT-9; Fig. 5B). In addition, the expression of Pdgfr-α was not suppressed in some breast tumors expressing Smyd4 (HBT-3). These findings indicated that Smyd4 is not the only factor determining the expression of Pdgfr-α in breast cancers.

Discussion

In present work, we found that disruption of Smyd4 expression is associated the tumorigenesis of a nontumorigenic mammary cell line. Re-expressing Smyd4 in tumor cells suppressed the proliferation of cancer cells, the anchorage-independent growth of tumor cells, and tumor formation in nude mice. RNA interference–mediated Smyd4 knockdown in MCF10A led to anchorage-independent growth. Smyd4 was found to suppress Pdgfrα expression. From 10 human breast cancer cases, Smyd4 gene expressions were revealed to be completely silenced in 2 breast cancer cases and markedly decreased in 3 additional cases. These findings suggest that Smyd4 is a potential novel breast tumor suppressor.

Chromosome deletion of 17p13.3 is frequently involved in various types of human malignant tumors (6–10). Although previous studies has identified three tumor suppressor genes including MAX binding protein, Hic1, and ovarian cancer gene 1 (11–14) in this region, there is evidence suggesting the presence of additional novel tumor suppressors. Our studies reveal that Smyd4 is another potential novel tumor suppressor from 17p13.3. The involvement of Smyd4 in other types of tumors needs to be further investigated.

Haploinsufficiency of Smyd4 could be an explanation as to why the loss of one allele of Smyd4 gene led to the tumorigenesis. However, the detail of pathway through which Smyd4 is involved in breast cancer development needs to be further studied. Considering the potential methyltransferase activity of Smyd family on lysine residues, Smyd4 could suppress tumor growth through modifying histone or nonhistone proteins. The study on the activity of Smyd4 in the modification of histone or nonhistone proteins is undergoing.

The tumor developed after the chromosome translocation was likely to undergo additional genetic changes, as evidenced by the finding that the expression of three of four genes that are up-regulated in the tumor could not be suppressed by Smyd4 re-expression. Nevertheless, the decreased expression of Smyd4 is critical for the tumorigenesis as the re-expression of Smyd4 abolished the tumor formation.

Pdgfr-α is involved in the proliferation and survival of a variety of tumors (33, 34). Pdgfr-α–activating mutations were detected in gastrointestinal stromal tumor and brain tumors. High expression of Pdgfr-α is detected in many types of tumors including breast cancers. Autocrine activation of Pdgfr-α promotes the progression or aggressiveness of ovarian cancer and breast cancer (32, 35). The importance of Pdgfr-α signaling was corroborated by the
observed that disrupting Pdgfr-α-mediated signaling results in significant inhibition of tumor growth (36). Recent findings revealed that inhibition of Pdgfr-α restrains the growth of human breast cancer in the bone of nude mice (37, 38), indicating its role in organ-specific metastasis. We found that Pdgfr-α was highly expressed in the mammary cancer cells with reduced expression of Smyd4, re-expressing Smyd4 in the breast cancer cells significantly suppressed the expression of Pdgfr-α. In our present study, the concordance between the loss of Smyd4 expression and up-regulation of Pdgfr-α RNA was found in some of our human breast cancer samples but not in all human breast cancers with loss of Smyd4 expression. Our present study provides a novel mechanism through which abnormal expression of Pdgfr-α occurs in breast cancers and possibly in other cancers. Smyd4 may modify the expression of Pdgfr-α directly or indirectly through affecting the expression of factors that control Pdgfr-α. Given that Smyd4 carries a conserve SET domain that often possesses methyltransferase activity, Smyd4 is likely to act as a repressor for certain transcription factor that regulates the expression of Pdgfr-α gene.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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