Succinate Dehydrogenase 5 (SDH5) Regulates Glycogen Synthase Kinase 3β-β-Catenin-mediated Lung Cancer Metastasis

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2 The abbreviations used are: EMT, epithelial-mesenchymal transition; BLI, bioluminescent imaging; CM, conditioned medium; IHC, immunohistochemistry; MET, mesenchymal-epithelial transition; OA, okadaic acid; SDH5, succinate dehydrogenase 5; TCF, T cell factor; PP2A, protein phosphatase A; TOP, TCF-responsive promoter reporter; FOP, nonresponsive control reporter.

Background: SDH5 is in tumors. However, the functional role of SDH5 in lung cancer remains unknown.

Results: SDH5 expression regulates EMT.

Conclusion: SDH5 functions as a critical protein in the regulation of EMT by modulating the GSK-3β-β-catenin signaling pathway.

Significance: SDH5 may be a prognostic biomarker and potential therapeutic target for lung cancer metastasis.

Lung cancer is one of the most common cancers in the world (1, 2), and it is a leading cause of cancer death in men and women in China. Lung cancer is generally a treatable disease in the absence of metastasis (3). Thus, early diagnosis of patients who develop lung cancer metastasis could reduce the mortality and morbidity associated with this disease (3, 4). The development of metastasis depends on the invasion and migration of cancer cells from the primary tumor into the surrounding tissues. To acquire such invasive abilities, carcinoma cells may inherit unique phenotypic changes, such as epithelial-mesenchymal transition (EMT) (5). EMT is a process characterized by a loss of cell adhesion, repression of E-cadherin expression, and increased cell motility. EMT is a highly conserved cellular process that allows polarized, generally immotile epithelial cells to convert to motile, mesenchymal-like cells. This process was first recognized as a feature of embryogenesis, which is vital for morphogenesis during embryonic development, and more recently, it has been implicated in promoting carcinoma invasion and metastasis. During EMT, three major changes occur: (i) morphological changes from a cobblestone-like monolayer of epithelial cells to dispersed, spindle-shaped mesenchymal cells with migratory protrusions; (ii) changes in differentiation markers from cell-cell junction proteins and cytoskeleton intermediate filaments to vimentin filaments and fibronectin; and (iii) acquisition of invasiveness through the extracellular matrix (6, 7).

Decreased E-cadherin expression or gain of vimentin expression is closely correlated with various indices of lung cancer progression, including the grade, local invasiveness, dissemination into blood, and tumor relapse after radiotherapy (8, 9).

Succinate dehydrogenase 5 (SDH5), originally named EMI5/YOL07, is required for the flaviylation of succinate dehydrogenase. SDH5 is associated with increased risk for the development of several kinds of cancer, and it is mutated in paranglioma and gastrointestinal stromal tumors (10). SDH5 is down-regulated in various types of human cancers, and it plays an important role in tumor development (11–13).

Thus, SDH5 likely functions as a tumor suppressor in cancer development; however, its role and mechanism in lung cancer metastasis are largely unknown. Here, we demonstrate that loss of SDH5 facilitates EMT, leading to lung cancer metastasis.

EXPERIMENTAL PROCEDURES

Cell Culture and Clinical Specimens—Lung cancer cell lines, including NCI-H23, NCI-H1299, CRL-5908, NCI-H1975, CaLu-3, A549, SLU-02, PG49, and HTB-55 cells, were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) containing 10% fetal bovine serum (FBS; Invitrogen).

The Institutional Review Board of China approved the retrieval of cancer specimens and the connection with clinical data from
our institute (approval ID 8435672). Cell lysates were subjected to Western blot analysis or immunohistochemical staining.

**In Vitro Migration Assay**—For migration assays, 5 × 10^4 cells were plated in the top chamber of a Transwell insert (24-well insert, pore size, 8 μm; Corning), and serum-containing medium was placed in the lower chamber. After incubation for 48 h, cells that did not migrate or invade through the pores were removed with a cotton swab. The cells on the lower surface of the membrane were stained with Cell Stain (Chemicon, Tokyo, Japan) and quantified by measuring absorbance at 560.

**Analysis of the Wnt Signaling Pathway**—Cells were treated with WNT- or control-conditioned medium (Wnt-CM (ATCC number CRL-2647) and L-CM, respectively) for 24 h, and Wnt signaling was monitored by various assays, including Western blotting, GSK-3β kinase assays (Boshida, Wuhan, China), luciferase reporter gene assays (Chemicon), and fluorescence confocal microscopy (Sigma).

**Orthotopic Animal Model and Imaging**—All experimental procedures were approved by the Institutional Animal Care and Use Committee of China. The lungs of male nude mice (6–8 weeks of age) were exposed and injected with 5 × 10^5 cells suspended in 20 μl of phosphate-buffered saline (PBS). One week after injection, surgical staples were removed, and the tumor growth and local metastasis were monitored by bioluminescent imaging (BLI; Xenogen).

**Plasmid Constructs, Conditioned Medium, and Antibodies**—Plasmids for SDH5 and PP2A were obtained from Sigma. For cDNA transfection, cells (5 × 10^5 cells/well) were seeded in a 6-well plate (Costar) at 70–80% confluence before transfection. Transfection was carried out using Lipofectamine PLUS (Invitrogen) according to the manufacturer’s instructions. Wnt-CM and L-CM were collected according to the directions from ATCC, and they were added to the cells for 24 h. The anti-SDH5 polyclonal antibody was obtained from Biocompare. Okadaic acid (OA), anti-GSK-3β, anti-phospho-GSK-3β (Ser-9), anti-actin, anti-E-cadherin, anti-β-catenin, anti-ZEB1, and anti-vimentin were obtained from Sigma. Anti-human-specific pan-cytokeratin was purchased from Abcam. Anti-Snail, anti-Twist, and anti-TGF were obtained from Invitrogen.

**siRNA Oligonucleotides and Delivery Methods**—Three pairs of siRNA oligonucleotides for human SDH5 and PP2A were obtained from Invitrogen. siRNA oligonucleotides (20 μM) were transfected into cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.
Immunoprecipitation and Western Blot Analysis—For immunoprecipitation, transfected Slu-02 cells were washed twice with cold PBS and rinsed in 1.5 ml of cold PBS and rinsed in 1.5 ml of cold lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM pyrophosphate, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA) for 20 min on ice. The immunocomplexes were subjected to Western blot analysis according to the manufacturer’s protocol.

GSK-3β Kinase Assay—A fluorescence peptide substrate-based assay was used to assess GSK-3β kinase activity (Omnia Ser/Thr Recombinant kit; Invitrogen). Briefly, the GSK-3β complex was prepared from equal amounts of cell lysates by immunoprecipitation, and it was incubated with 10 μM of Ser/Thr peptide substrate in kinase reaction buffer (containing 1 mM ATP and 1 mM DTT) for 20 min at 30 °C. Fluorescence intensity was recorded by measuring the A485 in a 96-well plate. Relative GSK-3β activity was calculated using untreated cells (equal to 1).

Luciferase Reporter Gene Assay—For the reporter gene assay, cells seeded in 24-well plates were transfected with control or SDH5-siRNA. The subcellular localization of β-catenin was visualized by confocal microscopy (magnification, ×500) and Western blotting. B, total β-catenin mRNA was measured by RT-PCR. C, SDH5 inhibits β-catenin/TCF transcriptional activity. NCI-H23 cells treated with control- or SDH5-siRNA, and Slu-02 and NCI-H226 cells treated with control and SDH5 were transfected with the TCF-responsive promoter reporter (TOP-flash) or nonresponsive control reporter (FOP-flash); then, luciferase activity was measured as the ratio of TOP and FOP. Relative luciferase activity is presented as the mean ± S.E. (error bars) from each sample after normalizing to the control (1). The asterisk indicates statistical significance (p < 0.01). D and E, β-catenin overexpression reverses SDH5-mediated MET. Increasing amounts of β-catenin were transfected in Slu-02 (D) and NCI-H23 (E) cells for 24 h. Total cell lysates were probed with antibodies against E-cadherin, vimentin, and β-catenin. β-Catenin/TCF transcription activity was measured as described previously. F and G, the morphology of Slu-02 (F) and NCI-H23 (G) cells was observed by phase-contrast microscopy (magnification, ×100).
three mice were anesthetized, injected with 1,3-dimethyl-2-imidazolidinone (D-luciferin) (150 mg/kg, intraperitoneally), and imaged 10 min after injection for 3 min.

Histology and Immunohistochemical Staining—Tumors were removed, weighed, fixed in 5% formalin, and prepared for histological analysis. Consecutive tumor sections were stained with H&E, SDH5, E-cadherin, vimentin, and β-catenin. Immunohistochemical staining was carried out using the ABC staining kit (Santa Cruz Biotechnology) and a secondary biotinylated antibody to mouse IgG (Invitrogen). Lung cancer patient tissues were washed with PBS, inflated, and fixed with 10% buffered formalin. The sections were paraffin-embedded, cut into 5-μm sections, and stained with routine H&E.

Targeted Inactivation of the SDH5 Gene by Homologous Recombination—We recently reported the KO strategy (9). The linearized targeting construct was electroporated into 129/C57BL/6 ES cells, and the targeted clones were selected with G418 and ganciclovir. Resistant clones were screened for homologous recombination by PCR and confirmed by Southern blot analysis. Two independent AIP1/lox ES clones were injected into WT blastocysts. Chimeras were further bred with WT females for germ line transmission.

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RESULTS

SDH5 Regulates EMT in Vitro—We detected SDH5 expression in the following lung cancer cell lines: EKVX, HOP-62, and NCI-H23 cells express high levels of SDH5; Slu-01 expresses low levels of SDH5; and Slu-02, NCI-H226, NCI-H460, and NCI-H522 do not express SDH5 (Fig. 1A). We also detected E-cadherin, vimentin, and ZEB1 expression in these lung cancer cell lines: NCI-H23 and Slu-01 express high levels of vimen-
that SDH5 is a potent inhibitor of EMT. As was reported previously, cells undergoing an EMT or mesenchymal-epithelial transition (MET) display transient morphologic and biologic changes that modify cell polarity, contact with neighboring cells, and cell motility. These phenotypic changes are reminiscent of those observed in Slu-02 and NCI-H226 cells, which, when transfected with SDH5, displayed a clear morphological transition from spindle-like fibroblastic (control) to cobblestone-like cells (transfected with SDH5) with well organized cell contact and polarity (Fig. 1D). The effect of SDH5 expression on increasing E-cadherin and reducing vimentin expression was observed in Slu-02 and NCI-H226 (Fig. 1E). In contrast, when endogenous SDH5 expression was knocked down in two different human lung cancer cells (NCI-H23 and Slu-01), EMT clearly occurred, based on changes in cell morphology and biomarker expression (Fig. 1, F and G). Moreover, the expression of SDH5 significantly impacted cell motility in vitro (Fig. 1H). Taken together, these data indicate that SDH5 is a potent inhibitor of EMT.

**SDH5 Prevents β-Catenin Nuclear Translocation, and β-Catenin Overexpression Reverses SDH5-mediated MET**—To understand the possible mechanism by which SDH5 inhibits EMT, we examined the effect of SDH5 on the GSK-3β-β-catenin signaling pathway. In the canonical Wnt pathway, GSK-3β-mediated β-catenin degradation is inhibited, leading to the accumulation of β-catenin in the nucleus and the trans-activation of β-catenin/T cell factor (TCF) target genes (14). Thus, the hallmark of β-catenin signaling in both normal and neoplastic tissues is nuclear translocation. By knocking down endogenous SDH5 with siRNA, we observed accumulation of cytoplasmic β-catenin, nuclear translocation of β-catenin, and reduced membrane-associated β-catenin (Fig. 2A).

Total β-catenin mRNA did not change after siRNA knockdown of SDH5 in NCI-H23 cells (Fig. 2B), but β-catenin/TCF transcriptional activity (TOP/FOP) increased (Fig. 2C). Consistently, overexpression of SDH5 in Slu-02 and NCI-H226 cell lines decreased β-catenin/TCF transcriptional activity (TOP/FOP) (Fig. 2C).

Because SDH5 can activate GSK-3β, which decreases cytosolic β-catenin protein levels and nuclear β-catenin transcriptional activity (Fig. 2, A and C), we examined whether the inhibitory effect of SDH5 could be reversed by overexpressing β-catenin. In SDH5-transfected cells, increasing amounts of β-catenin cDNA restored EMT, as determined by marker expression, and increased β-catenin transcriptional activity and morphology (Fig. 2, D and F). Similarly, elevated expression of β-catenin protein levels and nuclear β-catenin transcriptional activity in NCI-H23-KD cells induced EMT in a dose-dependent manner (Fig. 2E). The morphology of these cells also changed (Fig. 2G).

**SDH5 Regulates PP2A to Active GSK-3β**—After Slu-02 cells were transfected with SDH5, GSK-3β appeared to directly associate with SDH5 based on immunoprecipitation experiments (Fig. 3A). Moreover, GSK-3β activity was significantly elevated, as determined by Ser-9 (negative regulatory site) phosphorylation levels and a decrease in β-catenin/TCF transcriptional activity assays (TOP/FOP) (Fig. 3, B and C). Because SDH5 is not a phosphatase, the mechanism of GSK-3β activation by SDH5 may be mediated by a separate phosphatase associated with this complex. PP2A is a heterotrimERIC complex containing a catalytic subunit, a structural subunit, and a variable regulatory subunit. PP2A has been shown to regulate GSK-3β phosphorylation. In our study, co-immunoprecipitation data (Fig. 3D) indicated that SDH5 could form a complex with GSK-3β and PP2A.

To further assess the direct effect of PP2A on GSK-3β-β-catenin activity, we examined the role of endogenous PP2A in SDH5-mediated modulation of GSK-3β-β-catenin signaling. SDH5-expressing cells were treated with a PP2A inhibitor, OA, or PP2A-siRNA. Both OA and PP2A-siRNA treatment abolished SDH5-mediated dephosphorylation of GSK-3β on Ser-9, EMT (Fig. 3F), and GSK-3β kinase activity (Fig. 3E). These data indicate that PP2A is critical for SDH5-mediated GSK-3β activation and MET responses. Thus, SDH5 inhibits GSK-3β activity through PP2A.
SDH5 Activates GSK-3β and Antagonizes Wnt-mediated EMT—Wnt signaling is a key inducer of EMT during embryonic development and cancer progression. We tested whether manipulating SDH5 levels in various cell lines could modulate Wnt-induced EMT. Whereas Wnt only slightly elicited EMT (Fig. 4A, left two lanes) in NCI-H23, it significantly increased EMT when endogenous SDH5 was knocked down by SDH5-siRNA (Fig. 4A, right two lanes). Consistently, β-catenin/TCF transcriptional activity was increased upon knockdown of SDH5 (Fig. 4B).

In contrast, restoring SDH5 expression in Slu-02 (SDH5-negative cell) prevented Wnt-induced EMT and β-catenin/TCF transcriptional activity (Fig. 4, C and D), strongly suggesting that SDH5 is an antagonist of Wnt-mediated EMT. However, overexpression of SDH5 did not affect the expression of Frizzled-1 (Wnt receptor) in NCI-H226 cell lines (supplemental Fig. S3).

Down-regulation of SDH5 Promotes Tumor Metastasis in Vivo—Because NCI-H23 cells have low metastatic potential, decreased SDH5 expression, and can initiate EMT (Fig. 1), we examined the metastatic potential of KD (SDH5 knockdown) versus Con-expressing NCI-H23 cells using an orthotopic mouse model. Stable luciferase activity was confirmed in each subline to ensure equal levels before injection. BLI was used to monitor tumor growth and the onset of metastases. One week after injection, BLI (Fig. 5A) detected multiple metastatic lesions in various sites in animals injected with NCI-H23-KD cells. In contrast, control mice exhibited only small primary tumors 5 weeks after injection, and none of these animals showed any signs of metastases (Fig. 5A). All mice bearing KD (SDH5 knockdown) cells developed metastasis (Fig. 5A), and H&E staining showed that all of these mice developed lung cancer, with or without metastasis (Fig. 6). Immunohistochemistry (IHC) showed that the majority of tumor cells strongly expressed vimentin (Fig. 6), whereas they exhibited weak staining of E-cadherin and cytokeratin (Fig. 6). Moreover, in the localization experiment, SDH5 was expressed in lung epithelial cells, and in the co-localization experiment, we SDH5 was...
expressed in lung epithelial cells but not in bronchial cells (supplemental Fig. S2).

SDH5 Knock-out Mice Exhibit Mesenchymal Characteristics in Lung Epithelial Cells—To further determine the impact of SDH5 on phenotypic changes in normal lung tissue, SDH5 knock-out (KO; SDH5/H11002/H11002) mice were employed (15). The strategy for targeted deletion of the mouse SDH5 gene is outlined in our previous study (15). This strategy effectively disrupts the coding regions of SDH5 that are required for its activity. Moreover, after homologous recombination, it can introduce a translation stop codon in-frame, preventing translation downstream of the SDH5 sequence. Following heterozygous mating, homozygotes were identified and distinguished from heterozygotes and wild-type mice by PCR (Fig. 7B). RT-PCR and Western blot analyses failed to detect SDH5 expression in SDH knock-out mice (Fig. 7, C and D). In SDH5WT (SDH5+/+) mice, SDH5 expression was associated with lung epithelial cells (Fig. 7E). Consistent with in vitro data (Figs. 3 and 4), Ser-9 phosphorylation of GSK-3β was higher in SDH5−/− mice than that in the SDH5+/+ mice (Fig. 7F), suggesting that GSK-3β activity was suppressed in SDH5−/− mice. Additionally, lung epithelial cells in SDH5−/− mice exhibit decreased E-cadherin and elevated vimentin expression (Fig. 7E).

SDH5 Expression Regulates EMT Markers in Lung Cancer Patients—Next, we examined the relationship between SDH5 expression and EMT markers in lung cancer patients. Specimens from different stages of human lung cancer patients with or without lymph nodes metastasis were chosen by positron emission tomography/computed tomography (Fig. 8A). Based on the TNM Staging System for Lung Cancer, we selected patients from stage I and stage III. Detailed information for the chosen patients is listed in Table 1. H&E staining verified lung cancer patients with or without lymph node metastasis (Fig. 8B). Loss of SDH5 and E-cadherin, as well as increased vimentin, p-GSK-3β, and β-catenin levels were clearly detected in tissues from lung cancer patients with lymph node metastasis (Fig. 8C). There was a significant correlation between the levels of SDH5 and E-cadherin and an inverse correlation between the levels of SDH5 and vimentin in all of the samples tested. Taken together, our human in vivo data are consistent with our in vitro data from various cancer cell lines.

**TABLE 1**

| Tumor   | Sex | Age | TNM        | Stage |
|---------|-----|-----|------------|-------|
| Primary | Male| 44  | T1N0M0     | I     |
| Primary | Male| 56  | T2N0M0     | I     |
| Primary | Female| 45  | T1N0M0    | I     |
| Primary | Male| 76  | T2N0M0     | I     |
| Primary | Female| 56  | T1N0M0   | I     |
| Metastasis | Female| 66  | T2N2M0   | III |
| Metastasis | Female| 42  | T1N2M0    | III |
| Metastasis | Female| 68  | T2N2M0    | III |
| Metastasis | Male| 67  | T2N2M0    | III |
| Metastasis | Male| 54  | T1N2M0   | III |

DISCUSSION

SDH5 is mitochondrial protein. In a recent study, Rutter and co-workers (10) showed that SDH5 is required for the flavina-
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The physical interaction between SDH5 and GSK-3β facilitates GSK-3β activation through Ser-9 dephosphorylation, which decreases nuclear β-catenin accumulation and transcriptional activity. These results reveal the potent inhibitory function of SDH5 on Wnt-β-catenin signaling. Within the SDH5-GSK-3β complex, SDH5 functions as a negative regulator of Wnt-β-catenin signaling. The role of Ser-9 phosphorylation of GSK-3β in Wnt/β-catenin signaling is still controversial (16–19). For example, Ser-9 phosphorylation of GSK-3β is not correlated with Wnt-mediated GSK-3β activity in certain cell types (20, 21). However, other studies demonstrated that many growth factors, such as insulin growth factor, transforming growth factor-β, and epidermal growth factor, could increase β-catenin accumulation through Ser-9 phosphorylation of GSK-3β (21, 22). Inactivation of GSK-3β through Ser-9 phosphorylation is involved in hepatitis B virus-x protein-mediated β-catenin stabilization in hepatocellular carcinoma cells (23). Our analysis of several lung cancer cell lines revealed that Ser-9 phosphorylation of GSK-3β is involved in SDH5-mediated β-catenin stability and transcriptional activity, suggesting that the effect of Ser-9 phosphorylation on β-catenin signaling is cell type-dependent.

We further examined the relationship between SDH5 expression and EMT markers in lung cancer patients. Loss of SDH5 and E-cadherin, as well as increased vimentin and expression and EMT markers in lung cancer patients. Loss of SDH5 in EMT, which explains how loss of SDH5 in lung cancer underlies the onset of aggressive metastatic lung cancer. We believe that assessing SDH5 expression in lung cancer specimens could be a valuable prognostic biomarker for the risk of lung cancer metastasis, and the delineation of SDH5 function could provide a potential intervention strategy for lung cancer metastasis.

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