Tobacco Smoke Induces Polycomb-Mediated Repression of Dickkopf-1 in Lung Cancer Cells

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Abstract

Limited information is available about epigenetic mechanisms by which cigarette smoke enhances the initiation and progression of lung cancer. To examine this issue, A549 and Calu-6 lung cancer cells were cultured in normal media with or without tobacco smoke condensate (TSC) under clinically relevant exposure conditions. Ten-day TSC exposure dramatically increased the tumorigenicity of lung cancer cells in nude mice. Microarray and quantitative reverse transcription-PCR (RT-PCR) experiments revealed that this phenomenon coincided with diminished expression of Dickkopf-1 (Dkk-1). Western blot, chromatin immunoprecipitation, methylation-specific PCR, and pyrosequencing experiments showed that repression of Dkk-1 coincided with decreased H4K16Ac, increased H3K27me3, and recruitment of SirT1, EZH2, SUZ12, and Bmi1 without DNA hypermethylation within the Dkk-1 promoter despite prolonged TSC exposures. Removal of TSC from culture media resulted in loss of promoter-associated polycomb repressor complexes and reexpression of Dkk-1. siRNA-mediated knockdown of EZH2 and SirT1 partially abrogated TSC-mediated inhibition of Dkk-1 expression. Western blot and quantitative RT-PCR array experiments showed that TSC exposure as well as knockdown of Dkk-1 activated Wnt signaling and significantly up-regulated Wnt5a in lung cancer cells. Knockdown of Dkk-1 recapitulated the dramatic protumorigenic effects of TSC exposure in Calu-6 cells. Despite the transient nature of Dkk-1 repression following TSC exposure in vitro, Dkk-1 remained silenced in tumor xenografts derived from TSC-treated Calu-6 cells. Collectively, these data provide evidence that cigarette smoke directly engages polycomb machinery to activate a signaling network implicated in maintenance of cancer stem cells.

Introduction

Lung cancer ranks among the most lethal malignancies worldwide, with an incidence exceeding 1,000,000 cases per year; the vast majority of these neoplasms are directly attributable to cigarette smoking. Mounting evidence indicates that aberrant expression/activity of epigenetic regulators of gene expression contributes significantly to pulmonary carcinogenesis (1). For instance, increased DNA methyltransferase expression coincides with hypermethylation of tumor suppressor genes and diminished survival of lung cancer patients (2, 3). Aberrant expression of various histone deacetylases correlates with advanced stage of disease and adverse outcome in these individuals (4, 5).

Recently, polycomb group (PcG) proteins, which regulate stem cell pluripotency, have emerged as critical mediators of aberrant gene silencing in cancer cells (6). Stem cell PcG targets are significantly more likely to exhibit cancer-specific promoter hypermethylation than non-polycomb targets (7). Furthermore, the polycomb-mediated H3K27me3 mark is associated with promoters of all tumor suppressor genes that are hypermethylated in cancer cells (8, 9). The majority of lung cancers exhibit disruption of Rb-mediated regulation of E2F, and a number of PcG genes including EZH2, EED, and SUZ12, which mediate H3K27 trimethylation, are potential E2F targets (10). Overexpression of Bmi1 correlates with methylation-mediated silencing of p16 in lung cancer cells (11). Interestingly, loss of p16 activity in primary epithelial cells results in up-regulation of EZH2 and SUZ12 and targeting of the respective PcG proteins as well as DNA methyltransferases to HOX49, a gene frequently silenced by promoter hypermethylation in cancer cells (12). The fact that pulmonary squamous cell carcinomas and their precursor lesions exhibit increased expression of Bmi1 and EZH2 (13) suggests that dysregulation of PcG complexes occurs early during lung cancer development.

Whereas the link between cigarette smoking and lung cancer risk is well established, the epigenetic mechanisms by which tobacco smoke initiates and maintains the malignant phenotype of lung cancer cells remain poorly understood. Interestingly, despite the fact that tobacco carcinogens mediate inactivation of numerous tumor suppressor genes and induce expression of synuclein-γ via epigenetic mechanisms lung cancer cells (14, 15), the relevance of cigarette smoking as to overall prognosis of lung cancer patients remains controversial (16, 17). The present study was undertaken to ascertain if cigarette smoke induces epigenetic alterations, which directly enhance the malignant phenotype of lung cancer cells.

Materials and Methods

Cell lines and treatment conditions. Calu-6, A549, H226, SK-LU-1, H841, SW-480, and DLD-1 cancer lines were obtained from American Type Culture Collection. Primary normal human small airway epithelial cells (SAEC) were obtained from Lonza, Inc., and cultured per vendor instructions. Tobacco smoke condensates (TSC) derived from Kentucky Reference 1R4F research blend cigarettes (University of Kentucky) were prepared as described (18) and generously provided by Wanda L Fields (RJ Reynolds, Inc., Winston-Salem, NC) at a concentration of 9 puffs, 10 mg tar/mL in DMSO. All cancer lines were maintained in RPMI medium supplemented with 10% FCS, 10 mmol/L of glutamic acid, and penicillin/streptomycin.
For smoke exposure experiments, cells were cultured in 10-cm dishes in appropriate normal media (NM) with or without TSC (0.0005, 0.001, or 0.003 puff/ml). Medium was changed daily with the addition of fresh TSC. Cells were subcultured as necessary. Recombinant human Dickkopf-1 (rhDkk-1) was obtained from R&D Systems and used at a concentration of 300 ng/ml for 90 min according to vendor instructions.

**Proliferation assays.** Cells (1 × 10⁴) were plated per well in six-well plates and cultured in NM with or without TSC. Triplicate wells were harvested and counted by trypan blue exclusion techniques.

**RNA isolation and microarray analysis.** Total RNA was isolated from A549 or Calu-6 cells cultured for 5 d in NM with or without TSC (0.0005, 0.001, or 0.003 puff/ml) using the RNeasy minikit (Qiagen). Gene expression profiles were analyzed using Gene-Chip Human Genome U133 2.0 plus arrays (Affymetrix) according to the vendor’s instructions.

**Real-time quantitative reverse transcription-PCR.** Real-time quantitative reverse transcription-PCR (RT-PCR) was done as described (19) using Dkk-1, EZH2, SirT1, and β-actin primers obtained from Applied Biosystems.

**Methylation-specific PCR and pyrosequencing.** The previously predicted CpG island within the Dkk-1 promoter region (20) was confirmed using the online CpG island search engine. Genomic DNA was isolated from TSC-treated or control cells using the Qiagen DNeasy kit. Bisulfite modification of DNA was done using the Qiagen Epitect kit. Bisulfite-modified DNA was amplified using methylation-specific primers and PCR conditions as described (20). PCR products were resolved by 2% agarose gel electrophoresis and visualized using ethidium bromide techniques.

For pyrosequencing, PCR primer sequences (F) 5′-GGGGAGGAGGT-3′ and (R) biotin-5′TTTTGATTTTTTGAGATGGTTT3′ and sequencing primers 5′-TTTTTATTATATATAAA-3′ and 5′-TTTTGATTTTGGATTTA-3′ were designed to amplify methylated as well as unmethylated bisulfite-modified DNA using the Biotage Assay Design software. Dispensation order pyrosequencing reactions and data analysis were done using the Pyromark MD pyrosequencer with software provided by Biotage. For Wnt RT-PCR arrays, 96-well plates with RNA from control Calu-6 cells, Calu-6 cells cultured in 0.003 puff/ml TSC, or Calu-6 cells transfected with shRNA targeting Dkk-1 was used to generate cDNA using the iScript RT kit (Bio-Rad). The entire cDNA reaction was diluted and distributed among the 96 wells. Reactions were done with RT² SYBR Green/ROX PCR Master Mix in an ABI 7900HT Fast Real-Time PCR machine. Data were analyzed using the web-based Superarray program.²

**Immunoblot.** Total cell proteins were extracted using the Cell Signaling lysis buffer (Cell Signaling technology) supplemented with 1× protease inhibitor (Boche, Inc.) and 1 mmol/L phenylmethylsulfonyl fluoride. Cell lysates were resolved on Bis-Tris polyacrylamide gels (Invitrogen), transferred to nitrocellulose membranes, and incubated for 1 h at room temperature with the following antibodies: Dkk-1 and β-actin (Santa Cruz Biotechnology), EZH2 (BD Biosciences), and LRP-6, phospho-LRP-6, Dvl-2, SirT1, phospho-c-jun NH₂-terminal kinase (JNK), and JNK (Cell Signaling Technology). Immunoblot signals were detected using appropriate horseradish peroxidase–conjugated secondary antibodies and SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology). Membranes were stripped and reprobed as necessary using Restore stripping buffer (Pierce).

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation was done as previously described (21) with minor modifications. Briefly, DNA-protein complexes were cross-linked with formaldehyde at a final concentration of 1% for 15 min. Immune complexes were formed with either non-specific IgG or chromatin immunoprecipitation grade antibodies recognizing H3Kme3 (Abcam), H3K9me3, H3K27me3, H4K16ac, Suz12, Bmi1 (Millipore), EZH2 (BD Biosciences), RNA polymerase II (Santa Cruz Biotechnology), or SirT1 (Cell Signaling Technology and Sigma-Aldrich). DNA was eluted and purified from complexes, followed by PCR amplification of the Dkk-1 promoter using primers and conditions as described (20).

**Luciferase promoter-reporter transient transfection experiments.** The T-cell factor responsive vector TOPFlash and the mutant negative control vector FOPFlash were purchased from Millipore. Cells (1 × 10⁴) were plated per well in 24-well plates and cultured in NM with or without TSC for 5 d, followed by transient transfection of TOPFlash or FOPFlash vectors using Lipofectin. Forty-eight hours later, cells were lysed and assayed for luciferase activity using the Promega dual luciferase reporter assay according to the manufacturer’s instruction. Renilla luciferase activity was used to normalize intersample variability.

**siRNA and shRNA knockdown.** Calu-6 cells were transiently transfected with siRNAs targeting EZH2 and SirT1 or sham siRNA sequences (Ambion) using Oligofectamine (Invitrogen). Target gene knockdown was confirmed by RT-PCR and Western blot techniques. In addition, Calu-6 cells were transfected with shRNA vectors targeting Dkk-1 and sham shRNA constructs (Origene) using Lipofectin (Invitrogen). Stable transfectants were isolated under puromycin (1 μg/ml) selection. Clonal populations exhibiting knockdown of Dkk-1 comparable to TSC-treated Calu-6 cells were expanded, with Dkk-1 knockdown confirmed by RT-PCR and Western blot techniques.

**Murine xenograft experiments.** Calu-6 and A549 cells cultured in NM with or without TSC (0.003 puff/ml) for 10 d or Calu-6 cells stably transfected with shRNA targeting Dkk-1 or control vectors were suspended in PBS at a concentration of 1 × 10⁶ cells/100 μl. Athymic nude mice were inoculated s.c. in the right flank with TSC-treated or Dkk-1-knockdown cells and in the right flank with control cells. Four weeks later, mice were euthanized and evaluated for percent tumor take and tumor mass. Xenograft tissues were harvested immediately after CO₂ euthanasia. Twenty-five animals in the TSC-treated and 20 animals in the Dkk-1 knockdown cohorts were included in at least two independent experiments. All animal procedures were approved by the National Cancer Institute Animal Care and Use Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

**Results.**

TSC enhances the tumorigenicity of lung cancer cells. A series of experiments were initiated to examine the effects of tobacco smoke on the epigenome and malignant phenotype of Calu-6 and A549 lung cancer cells. These cells were chosen for study due to their marked responses to chromatin remodelling agents suggestive of epigenomic plasticity potentially inducible by environmental carcinogens (22–24) and strikingly different tumorigenic potentials; Calu-6 cells exhibit low tumorigenicity, whereas A549 cells are highly tumorigenic in nude mice when inoculated s.c. in PBS.

Extrapalation of data pertaining to cotinine levels in saliva and systemic circulation of active smokers (25), as well as nicotine content in the condensates, suggested that 0.001 to 0.003 puff/ml approximated exposure conditions in active smokers (~1 pack per day). Preliminary experiments indicated that TSC mediated modest growth inhibitory effects in A549 and Calu-6 cells (Fig. 1A). Interestingly, TSC exposure dramatically increased the tumorigenicity of Calu-6 cells (Fig. 1B). For example, only 24% of mice inoculated with control Calu-6 cells developed tumors; in contrast, 92% of mice inoculated with Calu-6 cells exposed to 0.003 puff/ml of TSC for 10 d before injection developed tumors (P = 0.000001, exact test). Furthermore, tumors arising from TSC-treated Calu-6 cells were significantly larger than those developing from control cells (439.8 ± 329.1 versus 75.2 ± 44.7 mg; P = 0.0001, two-tailed t test). Essentially 100% of mice inoculated with A549 cells developed tumors irrespective of preinjection treatment. Tumors arising from TSC-treated A549 cells tended to be somewhat larger than those developing from untreated cells (364 ± 333 versus 200 ± 329 mg; P = 0.25, two-tailed t test; data not shown).

1 http://www.urogene.org/methprimer/index1.html

2 http://www.superarray.com/pcr/arrayanalysis.php
TSC down-regulates Dkk-1 in lung cancer cells. Affymetrix microarrays were used to examine if gene expression profiles in cultured lung cancer cells coincided with enhanced tumorigenicity following TSC exposure. Using criteria of ≥2-fold up-regulated/repressed relative to controls and \( P < 0.05 \), six genes were repressed, whereas 28 genes were up-regulated in a dose-dependent manner in Calu-6 cells exposed to TSC (0.0005, 0.001, and 0.003 puff/mL). Using similar criteria, 219 genes were significantly repressed, whereas 111 genes were up-regulated in a dose-dependent manner in A549 cells following TSC exposure. \(^3\) Interestingly, Dkk-1, encoding a Wnt signaling antagonist frequently silenced by DNA methylation in cancer cells (20, 26), was down-regulated in a dose-dependent manner in both cell lines. Quantitative RT-PCR experiments (Fig. 1C) confirmed that TSC mediated dose-dependent decreases in Dkk-1 mRNA copy numbers in A549 and Calu-6 cells, which exhibited relatively high Dkk-1 expression. This phenomenon was also observed in several additional lung cancer lines such as SK-LU-1 and H226, which exhibit somewhat lower basal levels of Dkk-1. No appreciable effects were observed in H1299, H460, or H841 cells expressing very low levels of Dkk-1. Diminished Dkk-1 expression coincided with reduced Dkk-1 protein levels in A549 and Calu-6 cells following TSC exposure (Fig. 1D).


TSC-mediated repression of Dkk-1 is not associated with promoter methylation. Methylation-specific PCR and pyrosequencing experiments were done to ascertain if TSC exposure induced methylation of the Dkk-1 promoter in cultured lung cancer cells. Briefly, A549 and Calu-6 cells were treated continuously with NM+F TSC (0.003 puff/mL) for 5, 10, 15, 30, 45, and 60 days. Figure 2A depicts primer annealing sites for methylation-specific PCR and pyrosequencing relative to the transcription site in the Dkk-1 promoter region. Methylation-specific PCR analysis (Fig. 2B) confirmed that the Dkk-1 promoter is methylated in control DLD-1 colon cancer cells deficient for Dkk-1 expression, but not in SW480 colon cancer cells exhibiting high level of Dkk-1 expression (20). No appreciable increase in Dkk-1 promoter methylation was evident following methylation-specific PCR analysis of A549 or Calu-6 cells despite relatively prolonged TSC exposures. Pyrosequencing experiments (Fig. 2C) confirmed the results of methylation-specific PCR, suggesting that DNA methylation was not the primary mechanism by which TSC mediated repression of Dkk-1 in cultured lung cancer cells.

TSC exposure decreases H4K16 acetylation and increases H3K27 trimethylation within the Dkk-1 promoter. Chromatin immunoprecipitation techniques were used to further investigate epigenetic phenomena associated with TSC-mediated repression of Dkk-1 in lung cancer cells. Briefly, A549 and Calu-6 cells were cultured in NM+TSC (0.003 puff/mL) for 10 days before chromatin immunoprecipitation analysis. SW480 and DLD-1 were used as controls for activation (H3K4me3 and H4K16Ac) and
repression (H3K9me3 and H3K27me3) histone marks, respectively. Primers for chromatin immunoprecipitation analysis encompassed the transcription start site as well as a portion of the adjacent CpG island within the Dkk-1 promoter (Fig. 2A). As shown in Fig. 2A, chromatin immunoprecipitation analysis revealed no apparent changes in RNA polymerase II, H3K4me3, or H3K9me3 levels within the Dkk-1 promoter in lung cancer cells following TSC exposure. Interestingly, TSC exposure decreased H4K16Ac and increased H3K27me3 levels within the Dkk-1 promoter region in both lung cancer lines.

**TSC exposure induces reversible recruitment of polycomb proteins to the Dkk-1 promoter in lung cancer cells.** Because H3K27me3 levels within the Dkk-1 promoter were increased in lung cancer cells following TSC exposure, additional chromatin immunoprecipitation experiments were done to ascertain if components of polycomb repressor complexes (6, 27) were also recruited to this promoter. Calu-6 cells were cultured in NM for 10 days. Chromatin immunoprecipitation analysis (Fig. 3B) revealed a dose-dependent increase in H3K27me3, which coincided with recruitment of EZH2 and SUZ12 (components of the “initiation” polycomb repressor complex 2 that catalyze H3K27me3), as well as Bmi1, a member of “maintenance” polycomb repressor complex 1 (27). Additional analysis confirmed the dose-dependent deacetylation of H4K16, which coincided with recruitment of SirT1, a class III histone deacetylase that mediates H4K16 deacetylation (28). Consistent with previous data pertaining to polycomb-mediated transcriptional repression (29), no appreciable changes in RNA polymerase II levels within the Dkk-1 promoter were observed following TSC treatment.

Additional experiments were done to examine the stability of polycomb occupancy within the Dkk-1 promoter in TSC-treated lung cancer cells. Briefly, Calu-6 cells were cultured in NM ± TSC (0.003 puff/mL) for 10 days. Subcultures from TSC treated cells were allowed to proliferate in the absence of smoke for 5 or 10 days. Subsequent chromatin immunoprecipitation experiments (Fig. 3C) showed time-dependent reduction of H3K27me3 coinciding with diminished EZH2, SUZ12, and Bmi1 levels within the Dkk-1 promoter. A concomitant, progressive increase in H4K16Ac levels coincided with dissociation of SirT1 from the Dkk-1 promoter following cessation of TSC treatment. Real-time quantitative RT-PCR experiments with RNA isolated from cell cultures used for the chromatin immunoprecipitation experiments confirmed that the aforementioned histone modifications coincided with restoration of Dkk-1 expression in Calu-6 cells following removal of TSC from culture media (Fig. 3D).

Additional experiments were done to ascertain if TSC could mediate similar effects in normal respiratory epithelia. Briefly, SAEC were treated with TSC under conditions identical to those used for Calu-6 and A549 cells. Quantitative RT-PCR experiments revealed that TSC markedly decreased Dkk-1 mRNA copy numbers in SAEC. Western blot and chromatin immunoprecipitation experiments showed that despite relatively low levels of EZH2 and SirT1 in SAEC compared with cultured lung cancer cells, TSC-mediated repression of Dkk-1 in SAEC coincided with recruitment of EZH2, trimethylation of H3K27, and decreased acetylation of H4K16 within the Dkk-1 promoter (Supplementary Fig. S1).

**siRNA knockdown of EZH2 and SirT1 abrogates TSC-mediated repression of Dkk-1.** Additional studies were done to further examine the role of polycomb complexes in the repression of Dkk-1 in lung cancer cells following TSC exposure. Briefly, parental Calu-6 cells or Calu-6 cells transfected with either sham siRNAs or siRNAs targeting EZH2 and/or SirT1 were cultured in NM with or without TSC (0.001 or 0.003 puff/mL) for 2 days. This abbreviated exposure duration was selected to minimize cumulative toxicities of transfections, target gene knockdown, and TSC exposures that might confound experimental results. Quantitative

**Figure 2.** Analysis of Dkk-1 promoter methylation in A549 and Calu-6 lung cancer cells following TSC exposure. **A,** schematic representation of a portion of the CpG island flanking the transcription start site within the Dkk-1 promoter. Thin black arrow, transcription start site. White and black arrows, positions of methylation-specific PCR and pyrosequencing primers, respectively; 18 CpG sites were analyzed by pyrosequencing. Striped arrows, chromatin immunoprecipitation primer sites. **B,** methylation-specific PCR analysis of Dkk-1 in A549 and Calu-6 cells cultured in NM ± TSC (0.003 puff/mL) for 5 to 60 d. DLD-1 and SW480 colon cancer cells served as positive and negative controls, respectively. **C,** pyrosequencing analysis confirming no significant increase in Dkk-1 promoter methylation in A549 and Calu-6 cells despite relatively prolonged TSC exposures.
RT-PCR (data not shown) and Western blot experiments (Fig. 4A) showed no appreciable changes in EZH2 or SirT1 expression in control or transfected Calu-6 cells following TSC exposure. Additional analysis confirmed specific target gene knockdown in transfected cells (Fig. 4B). Quantitative RT-PCR analysis showed that knockdown of EZH2 increased Dkk-1 expression in untreated Calu-6 cells and partially abrogated TSC-mediated repression of Dkk-1 (Fig. 4C). Knockdown of SirT1 also seemed to somewhat increase Dkk-1 expression in untreated Calu-6 cells and partially abrogate TSC-mediated repression of Dkk-1 (Fig. 4C). The effects of SirT1 knockdown were less pronounced than those resulting from EZH2 depletion, possibly due to less efficient target gene inhibition [53% knockdown relative to sham control for siSirT1 versus 83% knockdown for siEZH2 by quantitative RT-PCR analysis (data not shown)]. Simultaneous knockdown of EZH2 and SirT1 also seemed to increase Dkk-1 expression and partially abrogate TSC-mediated repression of Dkk-1. This phenomenon was most likely mediated primarily by EZH2 knockdown (73%); the contribution of SirT1 knockdown (44%) in the combination knockdown experiment was not readily apparent. Correlative Western blot experiments showed that EZH2, SirT1, and combined EZH2/SirT1 knockdown modestly increased Dkk-1 protein levels in untreated Calu-6 cells and diminished the depletion of Dkk-1 in these cells following TSC exposure (Fig. 4D).

**Dkk-1 repression modulates noncanonical Wnt signaling in lung cancer cells.** A series of experiments were undertaken to ascertain if down-regulation of Dkk-1 enhances Wnt signaling in lung cancer cells. As shown in Fig. 5A, TSC treatment resulted in a modest, statistically insignificant increase in T-cell factor reporter activity reflective of canonical Wnt signaling (30, 31) in A549 and Calu-6 cells. Subsequent Western blot experiments revealed a ~2-fold increase in cyclin D levels but no appreciable alterations in β-catenin localization in these cell lines following TSC exposure (data not shown).

Additional experiments were done to examine alternative Wnt pathways that might be modulated by inactivation of Dkk-1. Western blot analysis (Fig. 5B) revealed that TSC-mediated repression of Dkk-1 coincided with dose-dependent phosphorylation of LDL receptor related protein 6 (LRP-6) and dishevelled-2 (Dvl-2) indicative of activation of Wnt receptor complex, as well as JNK, a downstream effector of noncanonical Wnt signaling. Exogenous recombinant Dkk-1 abrogated Wnt receptor as well as JNK activation by TSC. Additional experiments revealed that shRNA-mediated knockdown of Dkk-1 enhanced phosphorylation...
of JNK in Calu-6 cells (Fig. 5C), suggesting that activation of JNK in these cells following TSC exposure was due, at least in part, to activation of Wnt signaling, rather than nonspecific responses to genotoxic stress. Subsequent quantitative RT-PCR array experiments confirmed that TSC exposure enhances Wnt signaling in lung cancer cells (Fig. 5D); interestingly, Wnt5a (which encodes a ligand implicated in noncanonical Wnt signaling; ref. 32) was markedly induced in TSC-treated Calu-6 cells as well as Calu-6 cells transfected with shRNA targeting Dkk-1. Taken together, these data suggest that repression of Dkk-1 contributes to activation of noncanonical Wnt signaling in lung cancer cells following TSC exposure.

**Dkk-1 knockdown enhances the tumorigenicity of Calu-6 cells in nude mice.** Additional experiments were done to directly assess the relevance of Dkk-1 repression with respect to the tumorigenic potential of lung cancer cells mediated by TSC. Briefly, nude mice were inoculated s.c. with Calu-6 cells stably expressing a shRNA targeting Dkk-1 or a vector control. Western blot experiments (Fig. 5C) confirmed Dkk-1 knockdown. As shown in Fig. 6A, mice inoculated with Calu-6 cells exhibiting knockdown of Dkk-1 were considerably more likely to develop tumors relative to mice injected with vector control cells (18 of 20 versus 6 of 20; \( P = 0.0002 \), exact test). In addition, tumors arising from Dkk-1 knockdown cells were markedly larger than those derived from vector control cells (574.3 ± 410.1 versus 69.3 ± 33.4 mg; \( P = 0.00011 \), two-tailed \( t \) test). Quantitative RT-PCR analysis confirmed very low levels of Dkk-1 expression in Dkk-1 knockdown xenografts but not in those derived from vector control cells (Fig. 6B). Interestingly, additional quantitative RT-PCR and pyrosequencing analyses revealed persistently low levels of Dkk-1 expression without apparent promoter methylation in TSC-treated Calu-6 tumor xenografts, suggesting a strong \textit{in vivo} selection pressure to maintain silencing of this tumor suppressor gene.

**Discussion**

Whereas the epidemiologic association between tobacco smoke and lung cancer is irrefutable, the epigenetic events contributing to the initiation and progression of this malignancy have yet to be fully elucidated. Pertinent to this study, ~30% of lung cancer patients continue to smoke following diagnosis (17, 33). Surprisingly, the effect of cigarette smoking on the lung cancer epigenome relative to cancer-specific mortality has not been firmly established.

Polycomb repressor complexes maintain pluripotency in germ cells by establishing a bivalent chromatin structure and silencing...
genes that regulate development and differentiation (6, 34). Several recent studies indicate that aberrant promoter DNA methylation in cancer cells frequently involves genes with relatively low transcriptional activity exhibiting bivalent histone marks; DNA demethylating agents and histone deacetylase inhibitors derepress these aberrantly silenced genes but do not revert them to fully euchromatic states (35, 36). Data presented in this article strongly suggest that tobacco smoke induces a bivalent chromatin structure within the Dkk-1 promoter, coinciding with polycomb-mediated repression of this Wnt antagonist in lung cancer cells. Whereas various components of Wnt signaling including Dkk-1 are known to be potential polycomb targets (8, 37), our data provide the first evidence that tobacco smoke induces a bivalent chromatin structure within the Dkk-1 promoter, coinciding with polycomb-mediated repression of this Wnt antagonist in lung cancer cells. Polycomb targets seem to be preferentially silenced by de novo methylation mechanisms during malignant transformation (7–9). Our data suggest that recruitment of polycomb repressor complexes precedes promoter-specific hypermethylation of Dkk-1 during pulmonary carcinogenesis (26). Our observations pertaining to repression of Dkk-1 in the absence of promoter methylation in cultured lung cancer cells despite prolonged TSC exposures are consistent with recent studies showing that polycomb-mediated gene silencing in cancer cells can occur independent of DNA methylation (40). Whereas the mechanisms underlying these phenomena have not been established, several recent reports have shown that EZH2 promotes formation of intrachromosomal and interchromosomal loops that mediate long-range transcriptional repression; intrachromosomal loops frequently involve genes with relatively low transcriptional activity that are “poised” for activation or further repression via DNA methylation (41, 42). Conceivably, chromatin looping contributes to polycomb-mediated repression of Dkk-1 in cells exposed to tobacco smoke.

Figure 5. Analysis of Wnt signaling mediated by TSC or Dkk-1 knockdown in cultured lung cancer cells. A, TOP-FLASH promoter-reporter activity in A549 and Calu-6 cells following TSC exposure. Columns, mean of triplicate experiments measured as a ratio of TOP/FOP values, normalized to Renilla luciferase; bars, SD. B, Western blot analysis of Wnt signaling components in Calu-6 cells exposed to TSC in the presence or absence of exogenous recombinant Dkk-1 (rhDkk-1). C, Western blot analysis of Dkk-1, phospho-JNK, and total JNK levels in Calu-6 lung cancer cells exposed to TSC or stably transfected with shRNA targeting Dkk-1. D, focused quantitative RT-PCR analysis of Wnt signaling in Calu-6 lung cancer cells exposed to TSC (0.003 puff/mL) or stably transfected with shRNA silencing Dkk-1. Results depicted are a summation of triplicate arrays. Lines above and below the center regression lines indicate 4-fold changes in gene expression.
The fact that knockdown of Dkk-1 seemed to be sufficient to recapitulate the effects of TSC exposure clearly suggests that epigenetic silencing of this Wnt antagonist enhances the tumorigenic potential of lung cancer cells. These data are consistent with recent studies indicating that Dkk-1 inhibits the tumorigenicity of colon and breast cancer cells (20, 43). Interestingly, TSC- and shRNA-mediated repression of Dkk-1 dramatically induced expression of Wnt5a, which encodes a noncanonical Wnt ligand critical for distal airway development (44). Although the effects of Wnt5a vary depending on tissue histology and receptor context, recent data indicate that this ligand activates a planar cell polarity pathway mediated via JNK, which confers a proinvasive phenotype in cancer cells (32). Of particular relevance in this regard, Huang and colleagues (45) observed that Wnt5a expression in non–small-cell lung cancers correlates with proliferation index, stromal expression of vascular endothelial growth factor A, and diminished patient survival.

Because Wnt signaling seems to be critical for maintenance of normal as well as cancer stem cells (46–48), it is not surprising that numerous Wnt antagonists are silenced via epigenetic mechanisms in lung cancers (26, 49). Data presented in this article clearly show that cigarette smoke can engage polycomb repressor complexes to mediate epigenetic silencing of Dkk-1, thereby enhancing the malignant phenotype of lung cancer cells. These observations provide a potential mechanism by which continued smoking could adversely affect survival in lung cancer patients and support further analysis of the epigenetic effects of tobacco smoke during pulmonary carcinogenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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