Restoration of Wnt-7a Expression Reverses Non-small Cell Lung Cancer Cellular Transformation through Frizzled-9-mediated Growth Inhibition and Promotion of Cell Differentiation

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The Wnt signaling pathway is critical in normal development, and mutation of specific components is frequently observed in carcinomas of diverse origins. However, the potential involvement of this pathway in lung tumorigenesis has not been established. In this study, analysis of multiple Wnt mRNAs in non-small cell lung cancer (NSCLC) cell lines and primary lung tumors revealed markedly decreased Wnt-7a expression compared with normal short-term bronchial epithelial cell lines and normal uninvolved lung tissue. Wnt-7a transfection in NSCLC cell lines reversed cellular transformation, decreased anchorage-independent growth, and induced epithelial differentiation as demonstrated by soft agar and three-dimensional cell culture assays in a subset of the NSCLC cell lines. The action of Wnt-7a correlated with expression of the specific Wnt receptor Frizzled-9 (Fzd-9), and transfection of Fzd-9 into a Wnt-7a-insensitive NSCLC cell line established Wnt-7a sensitivity. Moreover, Wnt-7a was present in Fzd-9 immunoprecipitates, indicating a direct interaction of Wnt-7a and Fzd-9. In NSCLC cells, Wnt-7a and Fzd-9 induced both cadherin and Sprouty-4 expression and stimulated the JNK pathway, but not β-catenin/T cell factor activity. In addition, transfection of gain-of-function JNK strongly inhibited anchorage-independent growth. Thus, this study demonstrates that Wnt-7a and Fzd-9 signaling through activation of the JNK pathway induces cadherin proteins and the receptor tyrosine kinase inhibitor Sprouty-4 and represents a novel tumor suppressor pathway in lung cancer that is required for maintenance of epithelial differentiation and inhibition of transformed cell growth in a subset of human NSCLCs.

Deregulation of developmental signaling pathways is a common theme in human cancers. In this regard, the Wnt family encodes 19 distinct proteins that serve as extracellular signaling molecules controlling diverse morphogenetic and developmental programs (1). Signaling proceeds in an autocrine and paracrine fashion and is mediated by a family of 10 distinct seven-membrane rece...
16), yet the possible dysregulation of specific Wnt proteins in lung cancer cells leading to oncogenic signaling has not been examined. In this study, we demonstrate that re-expression of Wnt-7a and signaling through Fzd-9 are associated with increased differentiation and additionally represent a novel tumor suppressor pathway in NSCLC cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Retinovirus-mediated Gene Transfer—** NSCLC cells of the adenocarcinoma (A549, H2122), large cell (H1354, H460, H661), and squamous (H226, H157) phenotypes as well as a mesothelioma (H28) cell line were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 °C in a humidified 5% CO2 incubator. Normal human short-term bronchial epithelial (STBE) cultures were obtained from Clonetics Corp. (San Diego, CA) and cultured according to the manufacturer’s protocol.

Wnt-3 and Wnt-7a cDNAs inserted into pcDNA3 encoding a C-terminal hemagglutinin (HA) epitope were kindly provided by Dr. Jan Kitajewski (Columbia University). The Wnt cDNAs were excised from pcDNA3 with HindIII and NotI and ligated between the HindIII and NotI sites of the retroviral expression vector pLNCX2 (Clontech). A murine fzd-9 cDNA was excised from pcDNA3 with HindIII and NotI and ligated between HindIII and NotI sites of the retroviral expression vector pLPCX (Clontech). The resulting vectors, LNCX2-HA-Wnt and LPCX-mFzd-9, were packaged into replication-defective retrovirus using 293T cells and the retrovirus component expression plasmids SV-MLV and SV-MPCX-AMLV and SV-Ψ -MLV and SV-Ψ’ -env -MLV as described (17–19). The secreted retroviruses were collected and incubated with NSCLC cell lines H157, A549, and H2122, and the transduced cells were selected in growth medium containing G418 (250 μg/ml) and puromycin (1 μg/ml) for LPCX vectors. NSCLC cells expressing both HA-Wnt and Fzd-9 were first transduced with LNCX2-HA-Wnt, and subclones expressing the HA-tagged Wnt proteins were subsequently transduced with LPCX-Fzd-9. The cells were maintained in medium containing G418 and puromycin.

For the measurement of transduced cell growth on plastic dishes, 50,000 cells seeded with different transfectants were seeded per well of a 24-well culture plate in complete growth medium. On subsequent days as indicated, the cells were trypsinized from the wells with 100 μl of trypsin, diluted with 400 μl of growth medium, and counted using a hemocytometer. For measurement of anchorage-independent cell growth, 2500 cells were plated in triplicate in 35-mm wells of a 6-well plate in a volume of 1.5 ml of growth medium containing 0.3% Noble agar on a base of 0.7 ml of growth medium containing 0.5% agar. The plates were incubated in a 37 °C CO2 incubator for 21 days, and the colonies were counted using a microscope. The data are presented as cloning efficiency calculated by dividing the mean number of colonies per well by the number of cells (2500) plated.

**Semiquantitative and Quantitative PCRs—** RNA was extracted from cultured cells and RNAse (micro-dissection) extracts were prepared following microcentrifugation. RNA had been extracted from primary lung tumors as well as uninvolved lung was provided by the Specialized Program of Research Excellence Lung Cancer Tissue Procurement Core of the University of Colorado Health Sciences Center. Aliquots of the RNA (10 μg) were converted to cDNA with Superscript II (Invitrogen) and random hexamers according to the manufacturer’s specifications. Primer sets for semiquantitative and quantitative PCRs are shown in Table I. Semiquantitative PCR was carried out using a PerkinElmer Life Sciences GeneAmp System 9600 with an initial denaturation step of 94 °C for 10 min, followed by 20 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min and a final extension cycle at 72 °C for 10 min. Each reaction contained 1 μl of cDNA template, 5 pmol each of forward and reverse primers, 0.75 units of AmpliTaq Gold, 200 μM each dNTP, and 1.5 mM MgCl2. PCR products were visualized on a 1.5% agarose gel with ethidium bromide. Each sample was amplified twice using the same set of cDNAs and once using cDNAs derived from independently isolated RNAs.

Aliquots (1 μl) of reverse transcription reactions were subjected to PCR conditions of 95 °C for 10 min, followed by 95 °C for 15 s and 60 °C for 1 min for 40 cycles in a 50-ml reaction. SYBR® green Jumpstart Taq ReadyMix (Sigma) using the primer sets shown in Table I. Initial real-time PCR amplification products were resolved by electrophoresis on 5% polyacrylamide gels to verify that the primer pairs amplified a single product of the predicted size. β-Actin mRNA levels were measured by quantitative PCR in the samples as a control gene. The real-time PCR data were analyzed with the Smart Cycler® software (Version 1.2d) to calculate the threshold cycle values for the different samples and are presented as mRNA levels in arbitrary units.

**Transient Transfections and Luciferase Assays—** Aliquots of lung cancer cells (2 million cells in 100 μl) were electroporated at 220 V and 250 microfarads with a GeneZAPPER (IBI, New Haven, CT) in 0.4-cm electrode gap cuvettes (Bio-Rad). Cells were transfected with 2 μg of TOPFlash and 1 μg of pCMV-β-gal for determination of transfection efficiency and with other expression plasmids as indicated in the figure legends. For analysis of c-Jun activity in cells, cells were similarly transfected with 100 ng of plasmid c-Jun-GaIl, 2 μg of 5xUS- TK-Luc, and 1 μg of pCMV-β-gal. Following electroporation, cells were plated in 10-cm dishes in complete medium. After 3 days of incubation, the cells were collected, washed once with ice-cold phosphate-buffered saline, and resuspended in 250 μl of luciferase reporter lysis buffer (Promega, Madison, WI). The cell lysates were centrifuged in a microcentrifuge, and aliquots (80 μl) of the supernatants were assayed for luciferase activity using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Argonne, IL) and luminescence substrate (Promega). Aliquots (15 μl) of the extracts were also assayed for β-galactosidase to correct for transfection efficiency. The data are presented as relative light units/milliliter of β-galactosidase.

**Immunoblotting and Immunoprecipitation—** For immunoblotting of HA-Wnt, cell extracts were prepared in MAPK lysis buffer (0.5% Triton X-100, 50 mM β-glycerophosphate (pH 7.2), 0.1 mM sodium orthovanadate, 1 mM MgCl2, 1 mM EDTA, 1 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 4 μg/ml aprotinin, and 1 mM dithiothreitol) and force three times through a 26-gauge syringe needle to lysis the cells, and the homogenate was subjected to centrifugation at 100 × g for 10 min to pellet the cells. The supernatant was subsequently centrifuged at 10,000 × g for 10 min to collect the membrane fragments, which were then resuspended in 100 μl of MAPK lysis buffer. Aliquots of the different extracts were resolved by 10% SDS-PAGE and transferred to nitrocellulose. The filters were blocked in Tris-buffered saline (10 mM Tris-Cl (pH 7.4) and 140 mM NaCl) containing 0.1% Tween 20 and 5% nonfat dry milk and then incubated with the same blocking solution containing the indicated antibodies at 1 μg/ml for 12–16 h. The HA epitope was detected with monoclonal antibody OC15 (Roche Applied Science), and Fzd-9 was detected with a previously described rabbit polyclonal antibody (60). The filters were extensively washed with Tris-buffered saline containing 0.1% Tween 20, and bound antibodies were visualized with alkaline phosphatase-coupled secondary antibodies and LumPhos reagent (Pierce) according to the manufacturer’s directions.

For co-immunoprecipitation of Fzd-9 and Wnt-7a, H157 transfectants were lysed in radioimmune precipitation assay buffer (0.5% deoxycholic acid, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris (pH 8.0), 2 μg/ml leupeptin, 4 μg/ml aprotinin, and 1 mM dithiothreitol), and the supernatants were collected by centrifugation at 10,000 × g for 10 min. The protein complexes in 200 μg of extract were immunoprecipitated (16 h, 4 °C) using rabbit anti-Fzd-9 antibody or normal rabbit serum and protein G-Sepharose (Sigma) and washed extensively with phosphate-buffered saline. The samples were resolved by 10% SDS-PAGE and immunoblotted for HA-tagged Wnt-7a using anti-HA monoclonal antibody 12CA5.

**Three-dimensional Cell Culture, Immunofluorescence Analysis, and Image Acquisition—** Cells were grown in three-dimensional basement membrane cultures according to Debnath et al. (21) with the following modifications. Growth factor-reduced Matrigel (BD Biosciences) was combined in a 1:1 ratio with complete serum medium (RPMI 1640 medium + 10% fetal bovine serum). 90 μl was added to each well of an 8-well glass slide chamber and allowed to solidify for 2 h at a 37 °C incubator. Cells were trypsinized, counted, and diluted to 25,000 cells/ml. A 10% Matrigel solution was prepared in complete serum medium. The cell suspension was combined in a 1:1 ratio with the 10% Matrigel solution, and 200 μl of this mixture was added to each well for a final concentration of 5000 cells/well in 5% Matrigel. Cells were consequently fed complete serum medium containing 5% Matrigel every 3 days for a total period of culture. Matrigel consisted of Matrigel (Collaborative BioChem Luminex® green Jumpstart Taq ReadyMix (Sigma) using the primer sets shown in Table I. Initial real-time PCR amplification products were resolved by electrophoresis on 5% polyacrylamide gels to verify that the primer pairs amplified a single product of the predicted size. β-Actin mRNA levels were measured by quantitative PCR in the samples as a control gene. The real-time PCR data were analyzed with the Smart Cycler® software (Version 1.2d) to calculate the threshold cycle values for the different samples and are presented as mRNA levels in arbitrary units.
RESULTS

Wnt-7a mRNA Expression Is Reduced in NSCLC Cells and Primary Tumors and Re-expression Inhibits Proliferation in a Subset of Cell Lines—The Wnt proteins leading to β-catenin accumulation through stimulation of the Fzd receptors are widely invoked as an oncogenic signaling pathway in diverse tumors (1, 5). However, recent evidence indicates that specific Wnt proteins may function as tumor suppressors in certain instances where loss of Wnt expression is observed in cancer cells (22–24). In this regard, a recent study demonstrated a frequent loss of Wnt-7a mRNA expression in lung cancer cell lines and primary lung tumors (25), indicating that Wnt-7a may represent a novel tumor suppressor in lung cancer. Our quantitative reverse transcription (RT)-PCR experiments revealed that Wnt-7a mRNA levels were undetectable in six of the eight NSCLC cell lines compared with the four normal lung cell lines and primary lung tumors (25), indicating that Wnt-7a mRNA is absent in six of seven NSCLC cell lines (Fig. 1, left panel). Importantly, Wnt-7a mRNA expression was significantly lower in the 13 primary NSCLC tumors relative to the 13 matched uninvolved lung tissue samples as assessed by quantitative RT-PCR analysis (Fig. 1, right panel). By contrast, Wnt-3, Wnt-4, Wnt-5a, and Wnt-10b mRNAs were present at approximately equal levels in STBE cells relative to the panel of NSCLC cell lines (Fig. 1B) and Wnt-1, Wnt-2, Wnt-5b, Wnt-6, and Wnt-8b mRNAs were not reproducibly detected in either the STBE or NSCLC cells (data not shown). Thus, our results demonstrate decreased expression of Wnt-7a mRNA in lung cancer cell lines and primary tumor samples relative to STBE cells and uninvolved lung tissue, consistent with a previous study (25), and indicate that loss of Wnt-7a expression is a frequent molecular event that accompanies oncogenesis in the lung.

If loss of Wnt-7a expression in NSCLC cells contributes to lung cancer progression, then re-expression of Wnt-7a is predicted to negatively influence the growth of NSCLC cells lacking Wnt-7a. To this end, a retroviral vector encoding HA-tagged Wnt-7a was constructed and packaged into retrovirus (see “Experimental Procedures”). As a control, retroviruses encoding the empty LNCX vector or HA-Wnt-3, a Wnt protein ubiquitously expressed in NSCLC cell lines and STBE cells (Fig. 1, left panel), were generated. NSCLC cell lines H157, A549, and H661 were transduced with the packaged retroviruses, and stable transfectants were selected for resistance to G418. Figure 2 shows an anti-HA immunoblot of cell extracts prepared from A549 and H157 transfectants transduced with the indicated retroviruses. Whereas transfected HA-Wnt-3 was readily detected in both A549 and H157 cells, transfectant HA-Wnt-7a was detected only in transduced H157 cells, with only trace levels of HA-Wnt-7a detected in multiple independent A549 transfectants. Moreover, retroviral infection of H661 cells with the HA-Wnt-7a retrovirus failed to generate any G418-resistant H661 cells in multiple retroviral infections. This result suggests that Wnt-7a expression exerts a strong negative influence on growth of A549 and H661 cells, but not H157 cells.

The influence of Wnt-7a expression on cell proliferation measured on standard tissue culture plastic in complete medium was tested. The findings revealed that expression of Wnt-7a significantly reduced the growth rate of A549 cells compared with cells transduced with the empty vector (Fig. 3A) despite the fact that the HA-tagged Wnt-7a protein was only weakly expressed (Fig. 2A). By contrast, the growth rate of H157 cells expressing substantially higher levels of HA-Wnt-7a protein was not different from that of the LNCX controls (Fig. 3B). In preliminary studies, expression of Wnt-7a also failed to influence the growth rate of H2122 cells (data not shown). Importantly, expression of Wnt-3 did not significantly affect the growth of any of the cell lines tested (Fig. 3, A and B), indicating that the growth inhibition observed in A549 cells with Wnt-7a was not simply a general effect of Wnt overexpression.

Fzd-9 Is Differentially Expressed in NSCLC Cell Lines and Mediates the Growth Inhibitory Effect of Wnt-7a—Wnt proteins are ligands for the family of seven-membrane Fzd receptors (3). The finding that the growth of neither H157 nor H2122 cells...
was affected by Wnt-7a expression, but that the growth of A549 and presumably H661 cells was reduced or strongly inhibited by Wnt-7a expression suggests that A549 and H661 cells express a specific Fzd protein that is not present in H157 or H2122 cells and that transmits signals leading to cell growth inhibition. To investigate the expression status of different Fzd proteins expressed by the indicated retroviruses is shown in Fig. 2A. Consistent with the quantitative RT-PCR data, an immunoblot of membrane fractions prepared from the panel of NSCLC cell lines demonstrated high levels of Fzd-9 protein expression in A549 and H661 cells, but weak expression in the other NSCLC cell lines (Fig. 4B). In addition, Fzd-9 mRNA levels in total RNA from 13 primary lung tumor samples and 13 matched uninvolved normal lung samples were measured by quantitative PCR and revealed that Fzd-9 mRNA expression levels were significantly higher in primary lung tumors relative to uninvolved lung tissues (Fig. 4B), consistent with a general increase in expression of Fzd-9 in the NSCLC cell lines.

Inspection of the data in Figs. 1 and 4 revealed that the ability to successfully express Wnt-7a in the NSCLC cell lines correlated with a lack of expression of Fzd-9 mRNA. The H157 cell line, in which Wnt-7a could be efficiently expressed, showed little or no Fzd-9 mRNA, whereas A549 and H661 cells, in which transfection of Wnt-7a proved extremely difficult, expressed readily detectable Fzd-9 mRNA. In fact, H661 cells, which failed to yield stable Wnt-7a transfectants, expressed detectable endogenous Wnt-7a mRNA (Fig. 1), were similarly transduced with LPCX or LPCX-Fzd-9. An anti-Fzd-9 immunoblot of membrane fractions prepared from H157 and H2122 cells in which transfection of Wnt-7a proved extremely difficult, expressed readily detectable Fzd-9 mRNA. In fact, H661 cells, which failed to yield stable Wnt-7a transfectants, expressed detectable endogenous Wnt-7a mRNA (Fig. 1), were similarly transduced with LPCX or LPCX-Fzd-9. An anti-Fzd-9 immunoblot of membrane fractions prepared from H157 and H2122 cells in which the fzd-9 cDNAs were expressed by the indicated retroviruses is shown in Fig. 2B and
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Table 1
Primer sequences for RT-PCR

| RT-PCR primers | Forward primer | Reverse primer | Fragment size | Accession No. |
|----------------|----------------|----------------|---------------|---------------|
| **Semi-quantitative** |              |                |               |               |
| Wnt-1          | TGGGCTTCCCTCATGACC | TGACGTGGCCACGACAG | 472           | X03072        |
| Wnt-2          | TGATGCGATATATTGCC | ACATAGCGGCTCACCC | 530           | X07876        |
| Wnt-3          | AGAGAAGCCAGGTGAGTCC | AAACACACCCATCAG | 559           | AB067828      |
| Wnt-4          | CGCAACGAGCTTTCAAGCC | CTTCCGACATGCCTACC | 402           | NM_030761     |
| Wnt-5a         | GGCATGTGTCACATCCGAAG | CTGATGGCAACACATTAC | 654           | L2861         |
| Wnt-5b         | AGGCTGCTGCTGCTGTTTC | CAAAGAATCTTCGGCCTC | 550           | AB069066      |
| Wnt-7a         | TCTGCGGACATTCCTGACACC | GCAGTTGATAGAGAGGCA | 552           | U53476        |
| Wnt-8b         | GTGGCTGTGATGTGGTCTCC | CTGATGGCAACACATTAC | 454           | X91940        |
| Wnt-10b        | CCCAAGGCCATATACTGATG | CGCTCTCCAGATGCAG | 522           | X97057        |
| Wnt-11         | CCTCTCCACCGCCTTCAC | GAAACTACTGCCCAGCACC | 520           | U82169        |
| Fzd-2          | TGTGCTCTCATTAGACCACTG | ACAGAACCGGCGGATGAT | 622           | AB017364      |
| Fzd-3          | TGATGAGCCATATCCTCGCC | ATTTACACCCACATACCTG | 519           | AJ272427      |
| Fzd-5          | GGCCTACAACCTGAGCGAC | TGGAGTAGTGGACAGCAC | 616           | U33318        |
| Fzd-6          | TTTTTCCTCCTCTGCAAACT | AAAGTCTACAGCTCTCACTG | 628           | AB012811      |
| Fzd-7          | GGCTCAGACGCTCTTACC | GAGGACAGCAAGTAGCACCC | 450           | AB017365      |
| Fzd-9          | CTTCCTCAGCGCGCTTCCAC | GAAACTACTGCCCAGCACC | 520           | U82169        |
| **Quantitative** | atgecgactetcagac | GCTCTGGGTGGTGCAGCAG | 115           |               |
| Fzd-9          | GCAGTGGTTCCTCTGACCG | TCTCGGTGTTGGGCNGCC | 82            |               |

**Fig. 4.** RT-PCR analysis of Fzd mRNAs in NSCLC cell lines and primary lung tumors. **A**, total RNA was isolated from the indicated NSCLC cell lines and submitted to semi-quantitative RT-PCR with the primers listed in Table I as described under “Experimental Procedures.” The PCRs were resolved by agarose gel electrophoresis, and the DNA was stained with ethidium bromide and photographed. The results shown are representative of at least three independent RT-PCRs for each sample with two independent RNA samples. **B**, total RNAs from the cell lines in A or from primary NSCLC tumors and uninvolved lung tissues were submitted to quantitative RT-PCR with the Fzd-9 primer pair as described under “Experimental Procedures.” **C**, membrane fractions purified from the indicated NSCLC cell lines were immunoblotted with anti-Fzd-9 antibody.

demonstrates the successful expression of Fzd-9 in these cells. The H2122 cells expressing Fzd-9 exhibited a markedly reduced growth rate relative to cells transfected with the empty LPCX vector (Fig. 3C). Likewise, H157 cells transfected with both Wnt-7a and Fzd-9 exhibited a decreased growth rate relative to empty vector-transfected cells (Fig. 3D). As with expression of Wnt-7a alone (Fig. 3B), H157 cells transfected with Fzd-9 alone proliferated at a rate equal to empty vector-transfected cells. Importantly, coexpression of Wnt-3 alone or combined with Fzd-9 had no effect on the rate of H157 cell proliferation (Fig. 3, B and D), indicating the specificity of Wnt-7a for growth inhibition. In addition to the rate of proliferation on tissue culture plastic, coexpression of Wnt-7a and Fzd-9 in H157 and H2122 cells inhibited anchorage-independent growth in soft agar-containing medium. The soft agar cloning efficiency of H2122 cells expressing Fzd-9 was <1% of that of cells transfected with the empty LPCX vector (Fig. 5). The cloning efficiency of H157 cells transfected with Fzd-9 alone or with Wnt-7a alone was not different from that of cells transfected with the empty LPCX vector, but the cloning efficiency of cells expressing both Fzd-9 and Wnt-7a was inhibited by ~65% (Fig. 5). Thus, coexpression of Wnt-7a and Fzd-9 in either H2122 or H157 cells inhibited cell proliferation as well as anchorage-independent growth, the latter being an excellent in vitro measure of cellular transformation. These data combined suggest that re-establishment of a Wnt-7a and Fzd-9 signaling system exerts a tumor suppressor phenotype.

The simplest interpretation of growth inhibition dependent upon coexpression of Fzd-9 and Wnt-7a is that Wnt-7a binds to Fzd-9 and stimulates growth inhibitory signaling pathways. To test whether Wnt-7a can bind to Fzd-9, cell extracts from H157 cells expressing the empty LNCX and LPCX vectors or coexpressing HA-Wnt-7a and Fzd-9 were submitted to an anti-Fzd-9 immunoprecipitation reaction, and the immunoprecipitated proteins were immunoblotted for the presence of HA-tagged Wnt-7a. As shown in Fig. 2C, HA-tagged Wnt-7a was detected in anti-Fzd-9 immunoprecipitates, but not in complexes precipitated with normal rabbit serum, providing evidence for the direct physical interaction of Wnt-7a and Fzd-9.

Coexpression of Wnt-7a and Fzd-9 Stimulates Epithelial Differentiation Accompanied by Induction of Sprouty-4 and Cadherin Proteins—Normal epithelia maintain cell-to-cell contacts that contribute to the proper development and maintenance of epithelial polarity and architecture (26–29). The loss of these inputs has been proposed as a mechanism whereby epithelial cells lose their characteristic phenotype and acquire motile and invasive properties (29). There is ample evidence to support that an epithelial-to-mesenchymal transition permits dissemination of single carcinoma cells from the sites of primary tumors and is involved in the dedifferentiation program that leads to malignant carcinoma (30). To examine the effect of Wnt-7a and Fzd-9 coexpression on cell polarity and the epithelial phenotype, a three-dimensional Matrigel culture assay was employed (see “Experimental Procedures”). Fig. 6 demonstrates that transfection of Fzd-9 had a dramatic effect on the morphologic architecture of LNCX cells cultured in a three-dimensional Matrigel matrix. Compared with H2122 cells transfected with the empty LPCX vector (H2122-LPCX cells), H2122 cells transfected with Fzd-9 (H2122-Fzd-9 cells) showed a polarized deposition of laminin V, a basement membrane...
Transfectant

**FIG. 5.** Anchorage-independent growth of H157 and H2122 transfectants. The indicated transfectants were seeded in agarose-containing medium as described under "Experimental Procedures." After 8 days, the cultures were fixed and stained with anti-ERM or anti-laminin V antibody. The filter was stripped and reprobed for the EGF receptor (EGFR) as indicated. Anti-phosphotyrosine antibody-stained polypeptides of ∼50 kDa were reduced in the extracts from H2122-Fzd-9 cells.

**FIG. 6.** Immunofluorescence analysis of H2122-Fzd-9 transfectants propagated in three-dimensional culture. H2122 cells transfected with the empty LPCX vector or Fzd-9 were cultured in a three-dimensional Matrigel matrix as described under "Experimental Procedures." After 8 days, the cultures were fixed and stained with anti-ERM or anti-laminin V antibody. Z-Stacked confocal fluorescent images of H2122-LPCX and H2122-Fzd-9 cells stained for phospho-ERM and laminin V are shown.

Cells (Fig. 7). Neither the phosphotyrosine content of the EGF receptor nor the expression level of the EGF receptor was affected by Wnt-7a and Fzd-9 transfection (Fig. 7). This finding suggests that the ability of the EGF receptor to phosphorylate cellular targets is reduced upon expression of Wnt-7a and Fzd-9. Sprouty proteins are known intracellular antagonists of receptor tyrosine kinases (32, 33), and immunoblot analysis of the expression levels of Sprouty proteins in the transfected NSCLC cell lines revealed that Sprouty-4 was induced by 2–3-fold in H157 cells expressing Wnt-7a and Fzd-9 and by 5–10-fold in H2122 cells transfected with Fzd-9 (Fig. 8). Sprouty-1 and Sprouty-2 were not induced in either cell line (data not shown). These data suggest coexpression of Wnt-7a and Fzd-9 induces Sprouty-4, an antagonist of receptor tyrosine kinase activity, and may contribute to the reduced *in vitro* growth of transfected NSCLC cells.

The cadherin family of proteins is critical for maintenance of normal epithelial function and architecture (28, 34). Immunoblot analysis of E-cadherin revealed a marked induction in H2122 cells transfected with Fzd-9 relative to empty vector-transfected control cells (Fig. 8). Interestingly, expression of E-cadherin in H157 cells cotransfected with Wnt-7a and Fzd-9 was not different from that in empty vector-transfected control cells. Rather, expression of E-cadherin was markedly induced (Fig. 8). Thus, increased epithelial differentiation by Wnt-7a and Fzd-9 coexpression is associated with increased expression of specific cadherin proteins, which are likely to contribute to the epithelial differentiation response observed in Fig. 6.

Growth Inhibition by Transfected Wnt-7a and Fzd-9 Is Associated with Activation of the JNK Pathway, but Not the β-Catenin/TCF Pathway—Stabilization of β-catenin leading to transcriptional activation of gene expression through the TCF/lymphoid enhancer factor is a major signaling pathway regulated in response to Wnt proteins (1, 3). H157 cells stably transfected with empty vector, Wnt-3, or Wnt-7a with or without Fzd-9 (Figs. 1 and 4) were transiently cotransfected with the TOPflash reporter containing tandomized TCF-binding sites linked to firefly luciferase. Wnt-7a and Wnt-3 coexpressed with or without Fzd-9 were expressed at comparable levels (data not shown). As shown in Fig. 9, coexpression of Wnt-3 and Fzd-9, which did not inhibit cell proliferation (Fig. 3A) or anchorage-independent growth, increased TOPflash activity by 3–4 fold in H2122 cells transfected with Fzd-9 relative to empty vector-transfected control cells.
distinct Fzd protein can engage different signaling pathways in NSCLC cells. Thus, this experiment demonstrates that a negative Rac/RacN19 decreased c-Jun-Gal4 activity to 0.3-fold stimulation of luciferase activity in cells transduced with the Rho-associated kinase inhibitor Y-27632 partially inhibits constitutive phosphorylation of the transfected MKK7-JNK1α fusion polypeptide (40). This construct encodes a fusion protein of JNK1α and MKK7, its dual-specificity kinase activator. The forced proximity of MKK7 and JNK1 inducer activated phosphorylation of the JNK1 moiety, leading to constitutive activation (41). Fig. 10A demonstrates the constitutive phosphorylation of the transfected MKK7-JNK1α fusion protein detected with anti-phospho-JNK antibody. Also, increased c-Jun Ser73 phosphorylation was detected in MKK7-JNK1α-transfected cells, indicating a functional activation of the JNK pathway (Fig. 10A). Two independent H2122 cell lines transfected with MKK7-JNK1α exhibited markedly reduced anchorage-independent growth in soft agar-containing medium (Fig. 10B). Thus, consistent with the increased JNK activity resulting from coexpression of Wnt-7a and Fzd-9, constitutive activation of the JNK pathway in H2122 cells reduces transformed cell growth, indicating that the JNK pathway is a likely mediator of the reduced transformed growth stimulated by Wnt-7a and Fzd-9.

DISCUSSION

Based on ample precedent in the literature for a transforming role of Wnt proteins in human cancers, we anticipated that one or more Wnt mRNAs would be induced in NSCLC cell lines. By contrast, our study instead unveiled the loss of a specific Wnt (Wnt-7a) in NSCLC cell lines and primary tumors. Importantly, our findings that re-expression of Wnt-7a in NSCLC cell lines reverses multiple indicators of cellular transformation provide evidence to support the function of Wnt-7a as a tumor suppressor in NSCLC. Although the overwhelming majority of published reports invoke a transforming role for Wnt proteins, a limited number of studies have also invoked a tumor suppressor role for specific Wnt proteins (23, 42). Wnt-7a is upregulated in endometrial tissue by progestogens, a finding that may account for the antineoplastic effect of these hormones on the endometrium. Also, analysis of uterine leiomyomas demonstrated a frequent reduction in Wnt-7a expression relative to the adjacent myometria (22). The latter two findings may be related to the established importance of Wnt-7a in development and differentiation of the female reproductive tract (43). Loss of Wnt-5a expression has been observed in several tumor cell types, including renal cell carcinoma and breast cancer (24, 42). Transfection of Wnt-5a into RCC23 renal cell carcinoma cells (42) or transformed uroepithelial cells (44) reverses the transformed phenotype, similar to our findings with transfection of Wnt-7a. In addition, an antisense strategy to decrease Wnt-5a expression induces transformation of C57MG mammary epithelial cells (42). Finally, Wnt-5a has been recently shown to inhibit proliferation of B cells and to function as a tumor suppressor in hematopoietic tissue (23). It is likely that expression of specific Wnt proteins may have dominant roles in tissue homeostasis and maintenance of epithelial cell differentiation and that loss of expression of these Wnt proteins will contribute to tumorigenesis in specific tissues.

A well recognized program of molecular events occurs upon cellular transformation and mediates an epithelial-to-mesenchymal transition (30). Normal epithelia possess cadherin-dependent cell-cell contacts that promote proper development of these tissues during embryogenesis and maintenance and homeostasis of adult epithelial structures. It has been proposed that epithelial tumor cells progressively lose their epithelial phenotype, they obtain a more mesenchymal phenotype, which is associated with increased cell motility and migration (29). The reverse of epithelial-to-mesenchymal transition is mesenchymal-to-epithelial transition, and evidence supports the involvement of the Wnt proteins in mesenchymal-to-epithelial transition during morphogenesis (30). Our findings provide support for a role of Wnt-7a in maintenance of lung epithelial...
differentiation. Expression of Wnt-7a in the context of Fzd-9 inhibited NSCLC cell proliferation, transformed growth in soft agar, and induced a more differentiated epithelial phenotype as assessed in three-dimensional culture. In addition, E-cadherin and N-cadherin, markers of differentiated epithelial cells, were induced by coexpression of Wnt-7a and Fzd-9. Interestingly, Fzd-9 expression in H2122 adenocarcinoma cells induced E-cadherin, whereas coexpression of Wnt-7a and Fzd-9 in H157 squamous carcinoma cells resulted in N-cadherin (but not E-cadherin) induction. The induction of E-cadherin or N-cadherin by the Wnt-7a/Fzd-9 interaction may in part explain the mesenchymal-to-epithelial transition observed in this study.

We suggest that Wnt-7a and Fzd-9 signaling inhibits growth of NSCLC cell lines in part through activation of the JNK pathway, but not the β-catenin/TCF pathway. Our data showing that Wnt-7a and Fzd-9 failed to engage the β-catenin signaling pathway are consistent with a previous study demonstrating that Wnt-7a failed to engage the β-catenin signaling pathway when coexpressed with Fzd-9 in 293 cells (45). Importantly, genetic analyses in Drosophila revealed Wnt-dependent regulation of two epithelial programs (planar polarity and dorsal closure) that proceed through JNK activation, but independently of β-catenin (36). Recent studies have established that the mammalian counterpart of planar polarity, termed convergent extension, is also a Wnt-5a-dependent, β-catenin-independent program (35, 46). Moreover, studies in Xenopus revealed that Wnt-5a-regulated convergent extension requires the JNK pathway (35). Although the JNKs are widely invoked as components of pro-apoptotic signaling cascades (47), substantial literature has emerged to support the JNK pathway as a required element in development, morphogenesis, and cell differentiation (48). We have previously demonstrated a role for the JNK pathway in neural differentiation modeled in PC12 cells (49) and increased muscle gene expression in vascular...
smooth muscle cells (50). In both instances, a modest JNK activation similar to the activation observed in response to Wnt-7a and Fzd-9 coexpression (Fig. 9) was observed. With regard to epithelial cell differentiation and related to our own findings, a recent study demonstrated a JNK requirement for the in vitro formation of polarized acinar structures in a breast epithelial cell line (51).

Our data indicate that the growth inhibitory action of Wnt-7a is mediated by Fzd-9 and imply the existence of signaling specificity by distinct Wnt and Fzd combinations. The fact that coexpression of Wnt-3 and Fzd-9 did not have an effect on in vitro cell growth or anchorage-independent growth provides additional support for the specificity of Wnt/Fzd interactions yielding distinct cellular responses. This has in fact long been appreciated with other members of the seven-membrane receptor superfamily such as the adrenergic and cholinergic receptors. In Drosophila, DFz1 signals through the JNK pathway rather than β-catenin (36). In addition, Sheldahl et al. (52) have shown that signaling through Fzd-1, Fzd-7, and Fzd-8 results in β-catenin activation, whereas signaling through Fzd-2, Fzd-3, Fzd-4, and Fzd-6 stimulates protein kinase C activity through a G protein-dependent mechanism. Beyond signaling specificity inherent to the different Fzd proteins, our findings shown in Fig. 9A indicate that Fzd-9 engages the β-catenin/TCF pathway when stimulated by Wnt-3, but the JNK pathway when stimulated by Wnt-7a. Thus, distinct Fzd molecules appear to engage different signaling pathways depending on the specific Wnt protein they bind. Our results shown in Fig. 2C indicate that Wnt-7a directly interacts with Fzd-9 to induce growth inhibition, increased epithelial differentiation, and JNK activation.

Overexpression of EGF receptor family members is frequently observed in NSCLC (53–55). More recently, gain-of-function EGF receptor mutations have been identified in some NSCLC cells (56, 57). In this regard, our observation that Wnt-7a and Fzd-9 coexpression resulted in the induction of the receptor tyrosine kinase antagonist Sprouty-4 is novel and potentially important for the reduced anchorage-dependent and -independent growth of NSCLC cells. Severe defects in lobulation and lung hypoplasia during lung development are observed in the mammalian fetus overexpressing Sprouty-4, suggesting a role for Sprouty-4 as a growth-inhibiting protein (58).

Our findings showing little or no Wnt-7a mRNA expression in NSCLC cell lines and primary NSCLC tumors are consistent with a previous report by Calvo et al. (25). The mechanism for reduced expression of Wnt-7a was not addressed in the present study, but it is interesting to note that the wnt-7a gene maps to chromosome 3p:25.1, a recognized site of frequent genomic deletion in lung cancer (10, 25, 42). Although genomic deletion is a possible mechanism for loss of Wnt-7a expression, decreased Wnt-7a mRNA expression has been observed in pancreatic carcinoma cell lines, and treatment with inhibitors of DNA methylation or histone deacetylases restores Wnt-7a expression (59). Furthermore, direct analysis revealed that the wnt-7a gene is frequently (59%) methylated in pancreatic carcinoma cell lines (59). We are presently pursuing the mechanism for loss of Wnt-7a expression in NSCLC cell lines and primary tumors. The results of our studies predict that reinduction of Wnt-7a expression in human lung cancers would significantly decrease the transformed phenotype of the cancer cells in situ.

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