**Tumor and Stem Cell Biology**

**Coexpression of Oct4 and Nanog Enhances Malignancy in Lung Adenocarcinoma by Inducing Cancer Stem Cell–Like Properties and Epithelial–Mesenchymal Transdifferentiation**

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**Abstract**

Epithelial–mesenchymal transition (EMT), a critical process of cancer invasion and metastasis, is associated with stemness property of cancer cells. Though Oct4 and Nanog are homebox transcription factors essential to the self-renewal of stem cells and are expressed in several cancers, the role of Oct4/Nanog signaling in tumorigenesis is still elusive. Here microarray and quantitative real-time PCR analysis showed a parallel, elevated expression of Oct4 and Nanog in lung adenocarcinoma (LAC). Ectopic expressions of Oct4 and Nanog in LACs increased the percentage of CD133-expressing subpopulation and sphere formation, enhanced drug resistance, and promoted EMT. Ectopic expressions of Oct4 and Nanog activated Slug and enhanced the tumor-initiating capability of LAC. Furthermore, double knockdown of Oct4 and Nanog suppressed the expression of Slug, reversed the EMT process, blocked the tumorigenic and metastatic ability, and greatly improved the mean survival time of transplanted immunocompromised mice. The immunohistochemical analysis demonstrated that expressions of Oct4, Nanog, and Slug were present in high-grade LAC, and triple positivity of Oct4/Nanog/Slug indicated a worse prognostic value of LAC patients. Our results support the notion that the Oct4/Nanog signaling controls epithelial–mesenchymal transdifferentiation, regulates tumor-initiating ability, and promotes metastasis of LAC.

**Introduction**

Lung cancer is one of the leading causes of cancer-related deaths worldwide (1). In particular, lung adenocarcinoma (LAC) is the most common histologic type. Its highly invasive and metastatic phenotypes are the major reasons for treatment failure and poor prognosis. Furthermore, a high failure rate and a low median survival rate are observed in patients undergoing chemoradiotherapy with recurrent, intractable LAC (2). To improve the patient survival, it is important to elucidate the regulatory mechanisms that control tumor-initiating and metastatic properties of LAC.

Self-renewal and pluripotency are the central features in the definition of embryonic stem cells (ESC), in which Oct4 and Nanog play a key role in the maintenance of these processes (3, 4). Oct4, a member of the Pit-Oct-Unc (POU) transcription factor family, is essential to maintain self-renewal and is normally found in totipotent or pluripotent stem cells of pregastrulation embryos (3, 5). Nanog, a downstream target of Oct4 (7), contributes to cell fate determination of the pluripotent inner cell mass during embryonic development (6) and its function requires the continued presence of Oct4 (7). Oct4 and Nanog have been suggested as 1 of 4 defined factors that render the reprogramming capability of adult cells into germ-line-competent–induced pluripotent stem cells (8–10). Previous studies also showed that mouse pulmonary stem cells endogenously express Oct4 (11). Oct4 was demonstrated to participate in tumorigenicity and malignancy of lung cancers (12). The expression of Oct4 has further been shown in human breast cancer stem-like cells, implicating its involvement in self-renewal and tumorigenesis via activating its downstream target genes (13). Similar to Oct4, immunohistochemical analysis of colorectal tumor samples showed that overexpression of Nanog was strongly correlated with poor prognosis, lymph node metastasis, and Dukes classification of colorectal cancer (14). Recently, both Oct4 and Nanog transcripts were consistently detected in human embryonic carcinomas, testicular germ cell tumors, seminomas, and bladder carcinomas (15–18). Furthermore, coexpression of Oct4 and Nanog is associated with pancreatic carcinogenesis (19) and is

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negatively correlated with the survival prognosis of oral squamous cell carcinoma patients (20). There is growing evidence of cross-talk and correlation between stemness pathways, tumor progression, and metastasis; the functional and mechanistic significance of the overexpressed stem cell markers in cancer, however, is still blurred and needs to be further clarified.

Epithelial–mesenchymal transition (EMT), a transdifferentiation program that converts adherent epithelial cells into individual migratory cells, is critical for embryonic development and the oncogenic progression of tumor cells (21, 22). The EMT process disrupts E-cadherin mediated cell–cell adhesion during embryonic development and changes the cell phenotype into a more loosely mesenchymal-like cell, leading to the invasion of extracellular matrix. Intensive studies revealed that transcriptional factors, such as Snail, Slug, and Twist, regulate EMT process (21, 22).

The recent study suggested that EMT could promote the property of stemness in normal breast tissues as well as breast cancer cells (23). However, the detailed molecular mechanisms involved in the regulatory links between EMT and stem cell–related genes are still poorly understood. In this study, we discovered that both Oct4 and Nanog are highly expressed in CD133⁺ but not in CD133⁻ LAC cells, suggesting a positive involvement of Oct4/Nanog signaling in tumorigenesis. We further investigate the roles of Oct4 and Nanog in EMT process, cancer progression, and metastasis of lung cancer. We found a significant coexpression of Oct4 and Nanog in high-grade and metastatic lesions of patients with LAC. Ectopic expression of Oct4 and Nanog in A549 LAC cells increases tumor-initiating properties, induces EMT and drug resistance, and promotes metastasis. This report provides the evidence bridging the missing link between EMT and stemness pathways and suggests a mechanism by which the Oct4/ Nanog stem cell signaling encourages tumor malignancy and metastasis of LAC cells.

Materials and Methods

Cell culture

A549 lung cancer cell line was obtained from the American Type Culture Collection before 2007 and tested positive for human origin and for the presence of EGFR expression in the cell line. Human A549 cells were infected with 10⁵ g of the pLKO.1-based lentiviral vector, 9×10⁶ HEK293T cells were seeded onto a 100-mm culture dish 1 day before transfection. Cells were cotransfected with 10 μg of the plKO.1-based lentiviral vector, 9 μg of Δ8.9 plasmid, and 2.5 μg of vesicular stomatitis virus G protein plasmid. The medium was replaced with normal culture medium 24 hours later, and the virus-containing medium was collected 48 hours after transfection. Lentiviral infection was performed by adding virus solution to cells at the desired multiplicity of infection in the presence of 8 ng/mL polybrene. Puromycin selection was performed 24 hours after infection until all of the mock-transfected cells died. Surviving cells were pooled and cultured for further analysis.

Microarray analysis and bioinformatics

Total RNA extraction was performed as described (24). Extracted RNA was reverse transcribed with Superscript II RNase H-reverse transcriptase (Gibco BRL) to generate Cy3- and Cy5-labeled (Amersham Biosciences Co.) cDNA probes for the control and treated samples, respectively. The labeled probes were hybridized to a cDNA microarray containing 10,000 gene clone immobilized cDNA fragments. Fluorescence intensities of Cy3 and Cy5 targets were measured and scanned separately using a GenePix 4000B Array Scanner (Axon Instruments). Data analysis was performed using GenePix Pro 3.0.5.56 (Axon Instruments) and GeneSpring GX 7.3.1 software (Agilent). The average linkage distance was used to assess the similarity between 2 groups of gene expression profiles as described below. The difference in distance between 2 groups of sample expression profiles to a third was assessed by comparing the corresponding average linkage distances [the mean of all pairwise distances (linkages) between members of the 2 groups concerned]. The error of such a comparison was estimated by combining the standard errors (the standard deviation of pairwise linkages divided by the square root of the number of linkages) of the average linkage distances involved. Classical multidimensional scaling was performed using the standard function of the R program to provide a visual impression of how the various sample groups are related (25).

Sphere formation assay

Cells were plated in 24-well plates (Falcon; BD) at a density of 5,000 viable cells/mL and grown in a serum-free Dulbecco’s modified Eagle medium (DMEM; Sigma), supplemented with N2 plus media supplement (Invitrogen), 20 ng/mL of EGF and 20 ng/mL of bFGF (Invitrogen), and 4 μg/mL of heparin (Sigma). Cells were further allowed to grow for 12 days, and the numbers of spheres were counted by microscope.

Patients and tissue samples

LAC and adjacent nontumorous lung tissues were obtained at the time of surgery from 20 patients in Taipei Medical University Hospital and granted by the Institutional Review Board protocol number 010804. All patients gave their informed consent, and the ethics and scientific committees

Figure 1. Microarray analysis reveals key stemness-regulated transcriptomes in LAC. A, gene expression microarray analysis (gene tree) of the 987 genes that were differentially expressed in CD133⁺ and CD133⁻ LAC cells, metastatic and primary LAC, and normal lung tissues, as demonstrated by a hierarchical heat map. The changes of the expression of the 987 genes are presented as a log scale of the expression values provided by GeneSpring GX software. B, multidimensional scaling analysis illustrates the average lineage transcriptome distances between primary, metastatic LAC tissues, CD133⁻, and CD133⁺ cells. C, total RNA from 20 pairs of primary LAC and matching nontumorous lung tissues was analyzed for Oct4 and Nanog mRNA expression by quantitative real-time PCR. D, the relative mRNA levels of Oct4 and Nanog from the same patient were compared together and each bar represents the relative levels of Oct4 and Nanog in LAC versus adjacent nontumorous lung tissue. The results are means of 3 independent experiments ± SD.
of the participating institutions approved the study. Tumor types were determined according to WHO classification. At the time of surgery, all tissue samples were immediately flash-frozen in liquid nitrogen and stored at −80°C until use.

**Xenograft tumorigenicity assay**

Virus-infected A549 cells were harvested, washed with PBS, and resuspended in normal culture medium. A549 cells (1 × 10⁶) infected with Oct4/Nanog or control vector were injected subcutaneously into the right and left side, respectively, of the flank region of 8-week-old male BALB/c nude mice (Rodent Model Resource Center). Tumors were measured with calipers the days after injection as indicated. All mice were anesthetized and killed by overdose with anesthetic on day 42 after injection. Subcutaneous tumors were surgically excised, weighed, and photographed.

**Statistical analysis**

The results are reported as mean ± SD. Statistical analysis was performed using Student’s t test or a 1-way or 2-way analysis of variance (ANOVA) test followed by Tukey’s test, as appropriate. *P < 0.05* was considered to be statistically significant.

**Results**

**Microarray analysis of stemness-related gene expression profiling and linkages in primary and metastatic tissues of LAC**

Recent reports have demonstrated that tumors contain a small subpopulation of cells, termed cancer stem-like or cancer-initiating cells, which exhibit a self-renewal capacity and are responsible for tumor maintenance and metastasis (26). Eramo and colleagues demonstrated that the small population of lung cancer–initiating cells could be identified by the CD133 surface marker (27). On the other hand, Oct4 was shown to play a crucial role in maintenance of the CD133+ lung cancer–initiating cells (12). We analyzed the genomic traits of lung cancer LC-CD133+ and LC-CD133− using gene expression microarray (Fig. 1A; Supplementary Tables S2, S3). To gain more insights into the functional consequences from differential gene expression patterns and to provide quantitative evidence, signature genes were subjected into the Gene Ontology database search to find statistically represented functional groups. The gene ontology categories of biological processes statistically represented (P < 0.01) among sphere-enriched genes are shown in Fig. 1A. The predominant processes upregulated in LC-CD133+ include those pertaining to mitosis, nuclear division, and cell-cycle regulation (Supplementary Table S2a). In contrast, the downregulated genes in LC-CD133+ include those related to immune responses, cell-to-cell adhesion, and cell biological adhesion (Supplementary Table S2b). Multidimensional scaling analysis showed that the gene expression pattern of LC-CD133+ was closer to that of metastatic lesion of LAC, but far from that of LC-CD133− cells or primary lesion of LAC (Fig. 1B). In contrast, the gene signature of LC-CD133− was closer to that of primary lesion of LAC (Fig. 1B). Interestingly, the microarray analysis showed that the expression levels of ESC-specific genes, Oct4 and Nanog, were significantly upregulated in LC-CD133+ and metastatic lesion of LAC compared to LC-CD133− or primary lesion of LAC (*P < 0.001*; Supplementary Table S3a). Twenty pairs of LAC and corresponding adjacent nontumorous lung tissues were subjected to quantitative real-time PCR analysis. The levels of Oct4 and Nanog RNA in the 20 LAC tissues were measured and represented as related levels compared to their adjacent nontumorous lung tissue (Fig. 1C). General analysis showed that both Oct4 and Nanog expressions were higher in LAC samples than in adjacent nontumorous lung tissues by an average of 2.01-fold (*P = 0.039*) and 4.76-fold (*P = 0.029*), respectively (Fig. 1D). Approximately, 55% of the LAC samples contained an Oct4 RNA level above that of patient-matched nontumorous lung tissues; similarly, 60% of the LAC samples contained a Nanog RNA level above that of patient-matched adjacent nontumorous lung tissues (Supplementary Fig. S1). Most interestingly, we observed 40% LAC cells co-overexpressed Oct4 and Nanog simultaneously (Fig. 1C).

**Oct4/Nanog overexpression enhances cancer stem-like property in LAC cells**

The co-overexpression pattern of Oct4 and Nanog in LAC tissues suggests a signal pathway induced by Oct4/Nanog circuit, which encourages tumorigenesis of LAC. We generated stable cell lines (A549-ON) from A549 human LAC cells using lentiviral infection system with plasmid vectors encoding Oct4 and Nanog cDNA. An empty vector-transfected control (A549-Ctrl) was produced simultaneously. Interestingly, Oct4 and Nanog co-overexpression induced spindle phenotype and foci formation of A549 cells, which normally exhibit a flat brick-like morphology and hardly aggregate to each other (Fig. 2A, top left). Colonies from A549-ON cells were selected and subjected to stable clone selection. The exogenously expressed Oct4 and Nanog in 3 A549-ON stable clones (Clones 1, 2, and 3) were confirmed by quantitative real-time PCR (Supplementary Figs. S2A and B) and Western Blot (Fig. 2A, bottom left). Nuclear localization of Oct4 and Nanog in A549-ON cells were confirmed by immunofluorescence staining (Fig. 2A, right).

Quantitative real-time PCR analysis showed that the cancer stem-like cell marker, CD133, was significantly elevated in all 3 A549-ON clones, ranging from 40- to 55-fold compared to their parental A549-Ctrl cells (Fig. 2B, bottom). Flow cytometry analysis showed an increased population of CD133+ cells in A549-ON clones (Fig. 2B, top). About 15% to 44% of the cells were determined as CD133+ in A549-ON clones, whereas CD133+ cells were nearly undetectable in A549-Ctrl cells. The mRNA level of another 2 stem cell–specific markers, Musashi-1 and Nestin, were also increased in A549-ON clones (Supplementary Fig. S2C and D). The elevated stem cell–specific markers suggested that A549-ON might have undergone certain process shifting cellular properties toward a state closer to stem cell or cancer stem cell.

Sphere formation and drug resistance are 2 of the important measurements used to define malignant cancers and cancer stem-like cells. In functional analyses, we found that A549-ON
Figure 2. Oct4/Nanog overexpression enhances cancer stem-like property in LAC cell line. A, A549 cells were infected with lentiviral vectors encoding cDNA of Oct4 and Nanog (A549-ON) or a control empty vector (A549-Ctrl). A549-ON cells lost the epithelial phenotype and formed foci 12 days after infection. In contrast, foci did not appear in A549-Ctrl cells. The scale bar represents a length of 50 μm (top left). A549-ON cells were subjected to stable clone selection. Three A549-ON clones (#1, #2, and #3) were analyzed by Western blot for Oct4 and Nanog expression (bottom left). Immunofluorescence staining was conducted for evaluating nuclear expression of Oct4 and Nanog (right). B, 3 A549-ON clones or A549-Ctrl were subjected to flow cytometry (top) and quantitative real-time PCR (bottom) to analyze the population of CD133⁺ cell and CD133 mRNA expression, respectively. C, A549-ON clones and A549-Ctrl were subjected to sphere formation assay. The sphere formation was photographed (top) and quantified (bottom). D, A549-ON clones (#1, #3), A549-Ctrl, and none transfected A549 cells (A549) were treated with cisplatin (5, 10, and 20 μmol/L) for 48 hours. The viable cells were distinguished by Trypan blue staining and counted using hemacytometer (left). A549-ON clones (#1, #2, and #3) or A549-Ctrl was subjected to quantitative real-time PCR analysis for ABCB1 and ABCG5 multidrug resistant gene expression (right). Data shown are the mean ± SD of 3 independent experiments.
cells have acquired the ability to form sphere in suspension culture (Fig. 2C) and were more sustainable to cisplatin treatment (Fig. 2D, left). Quantitative real-time PCR also showed that \( ABCB1 \), a member of the ABC family of multidrug resistant genes, was highly enhanced in all selected A549-ON clones (Fig. 2D, right).

**Oct4/Nanog overexpression promotes in vivo tumorigenic and metastatic abilities of A549 cells**

A549-ON clones and A549-Ctrl cells were subcutaneously injected in 8-week-old male BALB/c nude mice. Tumor growth was monitored with calipers on the days after injection as indicated (Fig. 3A). Significant increase in the growth rate of the A549-ON tumors was observed. The tumors were then surgically excised and weighed 8 weeks after injection (Fig. 3B). The tumors generated by A549-ON cells were 5- to 8-fold heavier than those by A549-Ctrl (Supplementary Fig. S3A). Interestingly, significant invasion into muscle layers was observed in hematoxylin and eosin (H&E) staining of the A549-ON tumor sections (Fig. 3C, yellow arrow heads in the top indicate muscle tissues). Compared to A549-Ctrl, a 12-fold increase of mitotic cell number in the A549-ON tumor was observed (Fig. 3C, bottom). Furthermore, staining of Alcian blue and periodic acid-Schiff (PAS), which detect mucosubstances or glycoproteins in normal lung tissue, indicated a decrease of lung-specific differentiation markers in A549-ON.
tumor sections, suggesting a poorly differentiated phenotype of A549-ON cells (Fig. 3D).

A serial dilution experiment was performed to evaluate the in vivo tumorigenicity of A549-ON cells. Nude mice were injected with different number of cells as indicated. A549-ON, but not A549 or A549-Ctrl, generated tumors with the cell number as low as $3 \times 10^3$ cells (Table 1). Furthermore, tail vein injection experiments showed that all 3 of the A549-ON–injected mice contained metastatic lung tumors and 2 of 3 contained metastatic liver tumors, whereas only 1 A549-Ctrl–injected mice contained metastatic lung tumors and none of them contained metastatic liver tumors (Table 2).

Oct4/Nanog-mediated pathways regulate EMT in lung cancer cells

As EMT is associated with tumor malignancy and metastasis, we investigated the effect of Oct4/Nanog signaling on EMT process of LAC. We first observed that A549-ON cells contained a mesenchymal-like phenotype, whereas the A549-Ctrl cells stay in their original epithelial-like morphology (Fig. 4A, left). Western blotting analysis showed that the EMT-related transcription factors, Snail and Slug, and the mesenchymal markers, Vimentin and N-cadherin, were elevated in A549-ON clones, whereas the epithelial markers, E-cadherin and Cytokeratin 18 (28), were suppressed (Fig. 4A, right). Functional analyses further demonstrated that A549-ON cells exhibited higher mobility and less dependence on anchorage for their growth, respectively, compared to A549-Ctrl cells (Fig. 4B).

We generated Oct4/Nanog double knockdown cells (A549-ON-shOct4+shNanog) from the previously established A549-ON cells using shRNA approach to examine the effect of Oct4/Nanog signaling on EMT. A randomly scrambled shRNA-transfected control (A549-ON-SC) was established simultaneously. The knockdown efficiency was confirmed by both quantitative real-time PCR (Fig. 4C) and Western blot (Supplementary Fig. S3B). The mRNA level of stem cell–specific marker, CD133, and EMT-related transcription factor, Slug, were reduced upon Oct4/Nanog double knockdown (Fig. 4C, left). A reduced protein level of Snail and Slug, and the elevation of E-cadherin and Cytokeratin 18 were also observed in the A549-ON-shOct4+shNanog cells (Fig. 4C, right). Moreover, Oct4/Nanog silencing was found to suppress anchorage-independent cell growth and cell migration and decrease sphere formation ability of A549-ON cells (Fig. 4D).

Knockdown of Oct4/Nanog signaling in A549-ON cell retards its tumorigenicity and mobility

Subrenal injections were performed by transplanting $3 \times 10^3$, $3 \times 10^4$, or $3 \times 10^5$ of A549-ON-shOct4+shNanog or A549-ON-SC cells in nude mice. As shown in Table 1, the tumorigenicity of A549-ON-shOct4+shNanog cells was significantly weaker than that of A549-ON-SC cells. The A549-ON-shOct4+shNanog–injected mice generated tumors only when transplanted with $3 \times 10^5$ cells or more (Table 1). A tail vein injection experiment was then conducted with A549-ON-shOct4+shNanog or A549-ON-SC cells. The metastatic tendency to lung or liver in A549-ON cells was prominently blocked by the double knockdown of Oct4 and Nanog (Table 2). Moreover, the number of tumor nodules and tumor volume in lung of the transplanted mice were measured by ex vivo I&H.

### Table 1. Different numbers of nontransfected A549 parental cells, A549-Ctrl, A549-ON, and A549-ON cells with Oct4/Nanog double knockdown (A549-ON-shOct4+shNanog) or scrambled shRNA control (A549-ON-SC) injected in the subrenal space of nude mice

<table>
<thead>
<tr>
<th>Subrenal injection (cells)</th>
<th>$3 \times 10^3$</th>
<th>$3 \times 10^4$</th>
<th>$3 \times 10^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>0/3</td>
<td>1/3</td>
<td>3/3</td>
</tr>
<tr>
<td>A549-Ctrl</td>
<td>0/3</td>
<td>0/3</td>
<td>2/3</td>
</tr>
<tr>
<td>A549-ON</td>
<td>2/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>A549-ON-SC</td>
<td>2/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>A549-ON-shOct4+shNanog</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
</tr>
</tbody>
</table>

NOTE: Experiments were performed in triplicate and the tumor formation was detected 4 weeks after injection.

### Table 2. Tail vein injection performed with $3 \times 10^5$ of each cell line

<table>
<thead>
<tr>
<th>Tail vein ($3 \times 10^5$)</th>
<th>Lung</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td>A549-Ctrl</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td>A549-ON</td>
<td>3/3</td>
<td>2/3</td>
</tr>
<tr>
<td>A549-ON-SC</td>
<td>3/3</td>
<td>2/3</td>
</tr>
<tr>
<td>A549-ON-shOct4+shNanog</td>
<td>1/3</td>
<td>0/3</td>
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NOTE: Mice were sacrificed 6 weeks after injection and the metastases to lung or liver were examined.
staining (Fig. 5A). In comparison to A549-ON cells, double knockdown of Oct4 and Nanog reduced the number of metastatic nodules and size of tumor by more than 5-fold (Fig. 5B). Furthermore, mice transplanted with the A549-ON-shOct4+shNanog cells had a significantly prolonged mean survival rate compared to those implanted with A549-ON-SC or the A549-ON cells ($P < 0.05$; Fig. 5C).

**Figure 4.** Oct4/Nanog-mediated pathways regulate EMT in lung cancer cells. A, the morphology of A549-ON clones and A549-Ctrl was investigated under microscope (left). Total protein of 3 A549-ON clones and A549-Ctrl was extracted and analyzed by Western blot using specific antibodies (right). B, 3 A549-ON clones and A549-Ctrl cells were subjected to TransWell cell migration assay (left) and soft agar colony formation assay (right). C, knockdown of Oct4 and Nanog (shOct4+shNanog) in A549-ON clones was performed by lentiviral infection system using vectors encoding shRNA against Oct4 and Nanog. A randomly scrambled shRNA control (SC) was used as an internal control. A quantitative real-time PCR analysis was conducted to assess the mRNA level of stemness genes as indicated (left). The protein levels of EMT markers were determined by Western blot (right). D, Oct4 and Nanog double knockdown (shOct4+shNanog) and the scramble control (SC) cells were subjected to soft agar, migration, and sphere formation assays. Data shown are the mean ± SD of 3 independent experiments.

Poor overall survival rate of patients with LAC was positively associated with Oct4, Nanog, and Slug expression

We studied the levels of Oct4, Nanog, and Slug proteins by immunohistochemical staining of a panel of specimens from 118 patients with LAC (Fig. 6A). Patient characteristics are summarized in Supplementary Table S3. The elevated
expressions of Oct4, Nanog, or Slug were positively associated with high-grade LAC with moderate to poor differentiation (Fig. 6A). Kaplan–Meier survival analysis was then conducted to determine the prognostic significance of Oct4, Nanog, or Slug expression in patients with LAC. First, the results showed that the Oct4-positive cases were associated with a considerably worse overall survival rate compared with Oct4-negative ones (Fig. 6B; *P < 0.05). Second, patients with lower Nanog expression had a better survival prognosis than the Nanog highly expressing patients (Fig. 6B; *P < 0.01). Third, Slug+ patients had a worse survival prognosis (Fig. 6B; *P < 0.01). Finally, patients positive for all 3 molecules (Oct4+ Nanog+ Slug+) had the worst survival rate compared to other LAC patients (Fig. 6C; group 4 vs. other groups), whereas the triple negative for Oct4, Nanog, and Slug had the most favorable survival as compared with other groups (Fig. 6C; group 1 vs. other groups). The correlation...
between Oct4/Nanog/Slug level and LAC patient survival rate may provide a novel index for predicting the disease progression and clinical outcome.

**Discussion**

Aberrant upregulation of EMT transcriptional factors, Twist, Snail, and Slug, is associated with poor overall and metastasis-free survival in patients with non–small cell lung cancer (29). However, upstream regulatory pathways leading to EMT-related metastasis in lung cancer remain unclear. It has been shown that poorly differentiated tumors preferentially overexpress genes normally enriched in ESCs, and downstream targets of Oct4 and Nanog are more frequently overexpressed in poorly differentiated tumors than in well-differentiated ones (14, 20, 30–34).

![Figure 6. Correlation of Oct4, Nanog, and Slug expressions to the clinical grading and survival rate of LAC patients. A, representative results of immunohistochemical staining for Oct4, Nanog, and Slug in 118 LAC patients at different grades (top, low-grade; bottom, high-grade). B, Kaplan–Meier analysis of overall survival in 118 LAC patients according to single Oct4 expression (left; *, P < 0.01), single Nanog expression (middle; **, P < 0.01), single Slug expression (right; **, P < 0.01), and C, the mean survival times of 118 patients with LAC in the different combination of these 3 markers of Oct4, Nanog, and Slug were measured by Kaplan–Meier analysis. The combined expression of triple positivity for Oct4”Nanog”Slug” presents the worse prediction for the patient’s survival outcome (***, P < 0.0001). Inset box, group 1 (Oct4”, Nanog”, and Slug” cells) was used as the reference for comparison with other groups (2–4).](image-url)
In the present study, we demonstrated that Oct4 and Nanog are significantly upregulated in LAC patients (Figs. 1 and 6). The bioinformatics and quantitative real-time PCR analysis identified that both Oct4 and Nanog are co-overexpressed in LAC (Fig. 1). Ectopic coexpression of Oct4 and Nanog converted A549 cells to a mesenchymal-like phenotype. Moreover, there is evidence to support that Oct4 and Nanog encourage the malignancy of lung cancer cells. First of all, A549-ON cells exhibit enhanced sphere formation ability, elevated anchorage-independent growth, and increased mobility (Figs. 2 and 4). Second, A549-ON cells are highly tumorigenic and metastatic, and this is reversed by Oct4/Nanog silencing in transplanted mice (Figs. 3 and 5; Tables 1 and 2). Third, immunohistochemical analysis showed that xenograft A549-ON tumor exhibits poorly differentiated and fast mitotic phenomenon (Fig. 3). Finally and most importantly, ectopically overexpressed Oct4/Nanog elevates mesenchymal markers and suppresses epithelial markers (Fig. 4). We propose that Oct4/Nanog might positively regulate tumor metastasis through enhancing EMT in LAC.

We have shown that Slug is a possible target for Oct4 and Nanog and exerts their effect on the regulation of EMT. The mRNA and protein levels of Slug are increased upon Oct4/Nanog overexpression and decreased by RNAi-mediated Oct4/Nanog knockdown (Fig. 4). The reporter assay further supported the regulatory role of Oct4/Nanog signaling on Slug promoter (Supplementary Fig. S4). Because Oct4 binds Nanog to activate gene expression in ESCs (35), it is possible that Oct4 and Nanog work together in their target genes to induce EMT. Further characterizations are required to illustrate how Oct4 and Nanog regulate Slug or other EMT-related factors, if any.

Lung cancer is notorious for its difficult diagnosis at early stage and poor recurrence-free prognosis. Advanced diagnostic methods and novel prognosis markers are urgently needed to improve the clinical treatments of the disease. The studies on hepatocellular carcinoma (HCC) proposed that Oct4 mRNA might be a biomarker for assessing the prognosis of HCC (32). Recently, the elegant study by Bass and colleagues (36) demonstrated that the DNA copy numbers of Sox2 were highly amplified in lung and esophageal squamous cell carcinomas. A detailed analysis done by Hassan and colleagues (31) has demonstrated that the expression profile of ESC-like genes, including overlapped targets of Oct4, Nanog, and Sox2, is preferentially detected in histologically poorly differentiated LAC, but not lung squamous cell carcinoma, suggesting that ESC genes may be involved in prognosis of LAC. In line with their findings, our data further showed that the expression levels of Oct4, Nanog, and Slug, individually or simultaneously, are oppositely correlated with the 5-year survival rate of LAC patients (Fig. 6). The clinical significance of Oct4/Nanog/Slug would be worth exploring in the future.

In conclusion, the present study has demonstrated that Oct4 and Nanog induce cancer stem cell–like properties and enhance EMT, contributing to the tumorigenesis and metastasis in LAC. The Oct4/Nanog-induced EMT could be regulated partly, if not fully, via increasing Slug transcription. Moreover, we have shown a correlation between the worse prognosis of LAC patients and the high expression of Oct4/Nanog/Slug. We propose that the expression of Nanog/Slug would be a potential marker of prognosis and a novel target of therapy for LAC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Coexpression of Oct4 and Nanog Enhances Malignancy in Lung Adenocarcinoma by Inducing Cancer Stem Cell–Like Properties and Epithelial–Mesenchymal Transdifferentiation

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