

Effect of NELL1 on lung cancer stem-like cell differentiation

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Abstract. The cancer stem cell theory recently has received enormous attention in cancer biology. Lung cancer stem-like cells are a subpopulation of undifferentiated lung tumor cells critical for lung cancer tumorigenesis, metastasis and resistance to therapy and disease relapse. The neural EGFL like 1 (NELL1) is a potent growth factor believed to preferentially target cells committed to the osteochondral lineage; yet, its expression and function in lung cancer are largely unknown. In the present study, we used specific medium to accumulate lung cancer stem-like cells of 95-D cells in spheres and obtained these highly expressed CD133 cells through flow cytometric cell sorting of CD133-stained cells which were termed 95-D lung cancer stem-like cells (95-D LCSCs). These 95-D LCSCs highly expressed stemness genes CD133, Oct4 and Sox2 determined by western blot analysis and quantitative real-time polymerase chain reaction (qPCR) analysis. Notably, we found that overexpression of NELL1 significantly reduced colony formation and invasion of 95-D LCSCs tested by soft

agar colony formation and cell invasion assay. In addition, as determined by cell proliferation assay, overexpression of NELL1 increased the chemotherapeutic sensitivity of 95-D LCSCs to carboplatin and cisplatin. NELL1 also reduced the expression of phospho-MET (p-MET), Notch3 and HES1, which suggests that NELL1 may induce 95-D LCSC differentiation by inhibiting the expression of c-MET-Notch signaling. Our results suggest that NELL1 induces lung cancer stem-like cell differentiation, which provides a new potential therapeutic target for cancer stem cells.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide (1). It includes two major types: Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Although some lung cancer tumors are resectable or initially responsive to traditional therapies, drug resistance and poor prognosis remain high, leading to a poor 5-year survival rate of less than 15% (2-5). Approximately, non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases (6). Traditional and progressive treatments have been applied clinically, including chemotherapy, radiotherapy and biotherapy, but the resistance to radiotherapy and chemotherapy remains a critical issue for lung cancer therapy (7,8). Recently, it has been suggested that a subpopulation termed cancer stem cells (CSCs) cause initiation, drug resistance, metastasis and recurrence of cancer cells. CSCs are divided asymmetrically to stem cells that have the capacity of self-renewal, and the other cells that will differentiate and produce phenotypically diverse tumor-constitutive cancer cells (9). Researchers first reported CSCs in leukemia (10), and subsequently in solid tumors, such as colon, brain, breast and lung cancer (11-14). A small subpopulation of lung cancer cells called cancer stem-like cells (CSCs) have

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been certified in many studies using different isolation assays, including accumulation in specific medium and cell sorting by certain markers such as CD133⁺ (15-18). Human 95-D cells are highly invasive and metastatic lung cancer cells (19). In previous research, cultured in specific medium, 95-D cells can be used to accumulated lung cancer stem cells in spheres, which were called LCSCs (20). Conventional and traditional therapies that fail to eradicate CSCs may reduce tumor cells temporarily; however, resistance, metastasis and relapse are more likely to occur when treatment is suspended. After the discovery of cancer stem cells, efficient approaches targeting CSCs is considered to be indispensable for eradicating cancer cells (21).

Neural EGFL like 1 (NELL1) was originally cloned from a human fetal-brain cDNA library (22). Previous research has found that NELL1 plays an important role in osteogenic differentiation (23). NELL1 is highly expressed in patients with craniosynostosis, and NELL1 can induce bone regeneration in calvarial defects (24). Overexpression of NELL1 was found to induce apoptosis in osteoblasts during craniofacial development (25). On a cellular level, NELL1 is suggested to promote osteoblast differentiation. Thus, we aimed to ascertain whether NELL1 could induce CSC differentiation. In the present study, we investigated the effects of NELL1 on 95-D LCSCs.

Moreover, expression of the NELL1 gene has been studied in several types of cancer. In several cancers, NELL1 has been found to be related with poor prognosis. In human renal cell carcinoma, it was shown that NELL1 was significantly downregulated in renal cell carcinoma, NELL1 gene was hypermethylation in renal cell carcinoma cell lines (26). NELL1 was also found to be downregulated in esophageal squamous cell carcinoma (27). In glioblastoma cell lines, NELL1 is lowly expressed (28). However, little is known concerning the function of NELL1 in NSCLC. In the present study, we investigated the effects of NELL1 on lung cancer stem-like cells.

Materials and methods

Cell culture and reagents. The human NSCLC 95-D cell line, which is a commercial cell line, was obtained from the Chinese Academy of Science (Shanghai, China). 95-D cells were cultured with Hyclone™ RPMI-1640 medium (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with Gibco™ 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were incubated at 37°C with 5% CO₂. The 95-D LCSCs, after sorting using a BD fluorescence-activated cell sorting FACS Aria flow cytometer (BD Biosciences, San Jose, CA, USA) were culture in neuroblast medium with 20 ng/ml basic fibroblast growth factor (bFGF) (Thermo Fisher Scientific, Inc.), 20 ng/ml epidermal growth factor (EGF) (Thermo Fisher Scientific, Inc.) and B27 supplement (Thermo Fisher Scientific, Inc.).

Oncomine database analysis. Oncomine (<https://www.oncomine.org/resource/login.html>) is a bioinformatics online cancer microarray database aimed at facilitating and promoting the discovery of the functions from genome-wide expression. The differential expression of NELL1 between lung

adenocarcinoma and normal counterparts was analyzed using the Oncomine database by searching 'Gene:NELL1'; 'Analysis Type: Cancer vs. normal analysis'; 'Cancer Type: Lung cancer'.

Sphere formation. The 95-D LCSCs were accumulated by sphere formation. Basically, the 95-D cells were digested into single cells with 0.25% trypsin and washed with phosphate-buffered saline (PBS). The cells were seeded in ultra-low attachment dishes and cultured in neuroblast medium with 20 ng/ml bFGF, 20 ng/ml EGF and B27 for 7 days to form spheres. The spheres were centrifuged and digested into single cells for further studies.

Flow cytometry. To analyze the CD133 relative expression in the accumulated LCSCs in spheres, cells were harvested and washed with FACS buffer [PBS containing FBS (1%; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany)] and sodium azide (0.05%; Sigma-Aldrich; Merck KGaA). The CD133-PE/Cy7 (anti-human; dilution 1:100; cat. no. 372810; BioLegend, Shanghai, China) antibody diluted in FACS buffer was added directly to this mixture and incubated for 30 min at 4°C in the dark. Meanwhile the IgG1-PE/Cy7 (anti-human; dilution 1:100; cat. no. 401908; BioLegend) antibody was used as a negative control. Cells were washed and resuspended in FACS buffer. The cells were maintained on ice until the analysis using the FACSCalibur flow cytometer (BD Biosciences) and sorting using FACS Aria flow cytometer (BD Biosciences). Results were obtained by analyzing data with FlowJo version 7.6.1 software (FlowJo LLC, Ashland, OR, USA). The results represent the mean value of three independent experiments. The experiments were performed independently three times and a representative is shown.

Plasmid and transfection. To prepare the NELL1 expression construct, the NELL1 cDNA fragment (2932 bp, gene accession #BC069674.1) was amplified by PCR *in vitro*. The PCR products were digested with *Nhe*I and *Sgs*I restriction enzymes and inserted into corresponding sites of the PDS023_PL_IRES vector. The PDS023_PL_IRES lentivirus empty vector or vectors for NELL1 were co-transfected with lentivirus packing vector pMDLg/pRRE, RSV-rev and pMD2.G into 293T cells to obtain lentiviral supernatant. The viral supernatant was collected after 48 and 72 h. Wild-type early passage 95-D LCSCs and 95-D cells were incubated with virus-containing medium in the presence of 4 mg/ml polybrene (Sigma-Aldrich; Merck KGaA). Stable cell lines were established after 4 days of blasticidine 5 µg/ml selection.

Cell invasion assay. To investigate cell invasion, 20 µl Matrigel (BD Biosciences) and 80 µl RPMI-1640 medium were added to the upper layer of a Transwell chamber (Corning Inc., Corning, NY, USA). A total of 1×10⁴ 95-D vector (95-D EV) cells, 95-D LCSC vector (95-D LCSCs EV) and 95-D LCSC NELL1-overexpressing cells (95-D LCSCs NELL1) were gently added to the Matrigel medium, while 600 µl PRMI-1640 medium supplemented with 20% FBS was added to the lower layer of the chamber. The chambers were placed in an incubator at 37°C. After 48 h, the chambers were fixed with liquid methanol and stained with 0.5% crystal violet at 37°C for 30 min, and the chambers were washed with PBS

several times. Cotton swabs were gently used to remove the cells on the upper layers of the chamber. The cells on the lower layers were counted within 5 randomly observed images using a Nikon microscope (Nikon, Tokyo, Japan) and statistically analyzed.

Soft agar colony formation assay. The colony formation assay was performed as previously described (29). Briefly, a 5% (w/v) base agar (Sigma-Aldrich; Merck KGaA) solution was prepared and autoclaved. For the bottom agar layer, 2 ml of the 0.4% agar/RPMI-1640 bottom agar layer was added to each well of the 6-well plates and was cooled to semisolid status. Single cells (2,000) were seeded in the 0.3% top agar layer into each well. Cells were cultured at 37°C for two or three weeks. Colonies were stained with 0.05% crystal violet (Sigma-Aldrich; Merck KGaA). All assays were performed in triplicate.

Western blot analysis. For western blot analysis, samples of the 95-D EV, 95-D LCSCs EV and 95-D LCSCs NELL1 cells were harvested. An amount of 30 µg of the total protein samples was prepared, denatured at 100°C for 5 min, and inserted into the 10% gel wells for SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Darmstadt, Germany). For immunolabeling, the membranes were blocked with 5% milk in Tris-buffered saline with Tween 20 (TBS-T). The membranes were incubated with primary antibodies overnight at 4°C. These antibodies included anti-CD133 (rabbit monoclonal antibody, anti-human; dilution 1:1,000; cat. no. 64326; Cell Signaling Technology, Danvers, MA, USA), anti-Oct4 (rabbit monoclonal antibody, anti-human; dilution 1:1,000; cat. no. 2890; Cell Signaling Technology), anti-Sox2 (rabbit monoclonal antibody, anti-human; dilution 1:1,000; cat. no. 3579; Cell Signaling Technology), anti-NELL1 (rabbit polyclonal antibody, anti-human; dilution 1:500; cat. no. ab197315; Abcam, Cambridge, UK), anti-ABCG2 (rabbit monoclonal antibody, anti-human; dilution 1:1,000; cat. no. 42078; Cell Signaling Technology), anti-ABCB1 (rabbit monoclonal antibody, anti-human; dilution 1:1,000; cat. no. 13342; Cell Signaling Technology), anti-ABCC1 (rabbit monoclonal antibody, anti-human; dilution 1:1,000; cat. no. 14685; Cell Signaling Technology), anti-β-catenin (rabbit polyclonal antibody, anti-human; dilution 1:5,000; cat. no. ab32572; Abcam), anti-shh (mouse monoclonal antibody, anti-human; dilution 1:500; cat. no. sc-365112; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-p-MET (Tyr1349), (rabbit monoclonal antibody, anti-human; dilution 1:1,000; cat. no. 3133; Cell Signaling Technology), c-MET (rabbit monoclonal antibody, anti-human; dilution 1:1,000; cat. no. 8041; Cell Signaling Technology), anti-Notch3 (rabbit monoclonal antibody, anti-human; dilution 1:1,000; cat. no. 5276; Cell Signaling Technology), anti-HES1 (rabbit monoclonal antibody, anti-human; dilution 1:1,000; cat. no. 11988; Cell Signaling Technology) and anti-α-tubulin (mouse monoclonal antibody, anti-human; dilution 1:2,000; cat. no. 3873; Cell Signaling Technology). Then anti-mouse (rabbit polyclonal antibody; dilution 1:5,000; cat. no. ab6728; Abcam) or anti-rabbit (goat polyclonal; dilution 1:5,000; cat. no. ab6721; Abcam) horseradish peroxidase (HRP) conjugated antibodies were used as secondary antibodies and incubated with membranes for 1 h

Table I. Primers used for qPCR.

Primer name	Primer sequence (5'-3')	Length (bp)
CD133-F	ACACTACCAAGGACAAGGCGTTCA	154
CD133-R	CTCAGTTCAGGGTTGCTATTCA	
Oct4-F	AGCAGCGACTATGCACAACGAG	196
Oct4-R	TGACGGAGACAGGGGGAAAGGCTTC	
Sox2-F	AGACGCTCATGAAGAAGGAT	183
Sox2-R	TGGTCCTGCATCATGCTGTAGC	
NELL1-F	TATGGTGTTCACGGCTTAGTG	234
NELL1-R	ATCACGTTGGATGGTCATTTCG	
ABCG2-F	ATAAAGTGGCAGACTCCAAGGT	264
ABCG2-R	ATAAGGTGAGGCTATCAAACAA	
ABCB1-F	ACTCGTAGGAGTGTCCGTGGAT	286
ABCB1-R	CAAGGGCTAGAAACAATAGTGAAA	
ABCC1-F	CAGGCGAGTGTCTCCCTCAAAC	124
ABCC1-R	CATTCTCACGGTGATGCTGTT	
β-actin-F	TTTCCAGCCTTCCTT	150
β-actin-R	TTGGCATAACAGGTCTTT	

β-actin was used as a housekeeping gene. F, forward; R, reverse.

and blots were developed using Electrochemiluminescence (ECL)-Plus Western detection system (Thermo Fisher Scientific, Inc.) for visualization.

Quantitative real-time polymerase chain reaction (qPCR). Total RNA of 95-D EV, 95-D LCSCs EV and 95-D LCSCs EV cells were extracted using Invitrogen™ TRIzol® reagent (Thermo Fisher Scientific, Inc.). Samples were treated with TRIzol followed by chloroform and then centrifuged for 10 min at 12,000 x g at 4°C. The supernatant was discarded and the pellet was washed in cold 75% ethanol. Finally, the RNA samples were diluted with 40 µl RNase-free water. A total of 1 µg RNA was reverse transcribed using a Bio-Rad script cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR analysis of CD133, Oct4, Sox2, NELL1, ABCG2, ABCB1 and ABCC1 were performed for quantification using a Mx3000P qPCR system (Stratagene, San Diego, CA, USA) with qPCR cycling conditions (an initial denaturation at 95°C for 1 min and then 45 cycles of 15 sec at 95°C, 31 sec at 60°C). The sequences of primers used for the qRT-PCR are listed in Table I. SYBR® Premix Ex Taq™ II (Takara Bio, Inc., Tokyo, Japan) was used and 20 µl per gene was analyzed. The relative fold change was quantified by $2^{-\Delta\Delta C_q}$ (30), and β-actin was used as a housekeeping control.

Cell proliferation assay. Cell Counting Kit-8 (CCK-8) was used to conduct the proliferation assays. 95-D EV, 95-D LCSCs EV and 95-D LCSCs NELL1 cells were seeded at 1×10^4 cells per well in 96-well microtiter plate and were maintained at 37°C for 24 h. A mixture of 200 µl RPMI-1640 medium with different concentrations of carboplatin and cisplatin was added into each well. After 48 h of incubation, a mixture of 190 µl RPMI-1640 with 10% FBS and 10 µl of CCK-8 was added

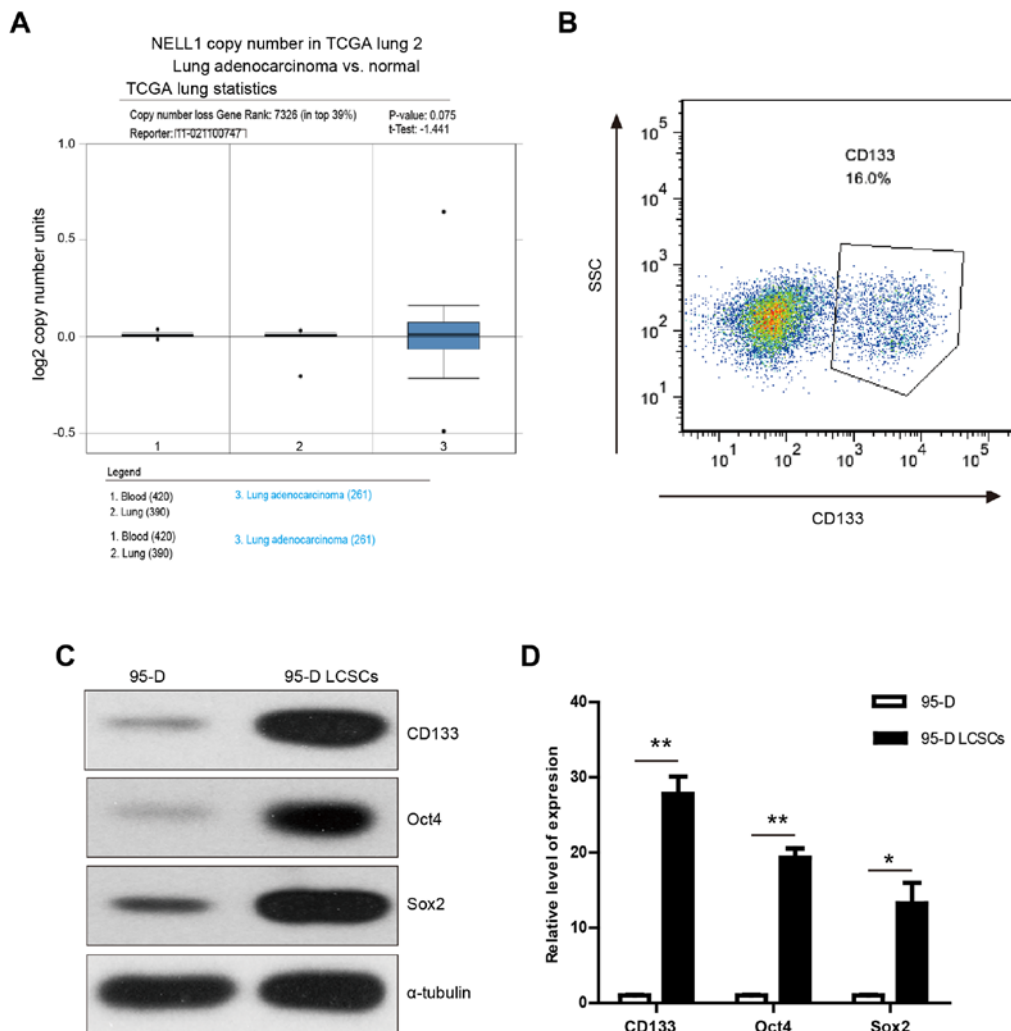


Figure 1. Putative 95-D stem-like cells express stemness genes. (A) NELL1 gene analysis in non-small cell lung cancer (Oncomine database). Box plots derived from gene expression in Oncomine comparing expression of NELL1 gene in normal (left two plots) and lung adenocarcinoma (right plot). (B) Expression of CD133 in the putative stimulated 95-D LCSCs was assessed using flow cytometry. Highly expressing CD133 cells were obtained through flow cytometric cell sorting. (C) CD133, Oct4 and Sox2 protein expression in 95-D LCSCs and 95-D cells examined with western blot analysis. (D) Quantitative RT-PCR analysis of CD133, Oct4 and Sox2 in 95-D and 95-D LCSCs. * $P < 0.05$, ** $P < 0.01$. NELL1, neural EGFL like 1; LCSCs, lung cancer stem-like cells.

into each well and measured at 450 nm. Each experiment was performed in replicates of six and background reading of the media was subtracted.

Statistical analysis. All of the experiments were performed three times independently. Statistical analysis (simple or plural) was performed using Statistical Package for Social Science (SPSS) software, version 19 (IBM Corp., Armonk, NY, USA). Groups were compared with Student's t-test or one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) post hoc test where groups were more than two. Results were considered to be statistically significant at $P < 0.05$.

Results

Putative 95-D stem cells express stemness genes. To evaluate how transcription of NELL1 correlates with NSCLC, we analyzed clinical cohorts of NSCLC patients using the Oncomine database. It was found that NELL1 expression was

lower in lung adenocarcinoma (Fig. 1A). Previous research demonstrated that NELL1 plays an important role in osteogenic differentiation (31). Thus, we aimed to ascertain whether overexpression of NELL1 could induce the differentiation of LCSCs. To measure the effect of NELL1 on LCSCs, specific medium was utilized to accumulate the LCSCs in spheres. The 95-D cancer stem-like cells were accumulated by sphere formation. To test whether the formed spheres were indeed cancer stem cells, flow cytometry was used to examine the expression of stemness gene CD133. It was found that CD133 was highly expressed in the sphere cells (Fig. 1B). These highly expressed CD133 cells were next obtained through flow cytometric cell sorting of the CD133-stained cells and these cells were termed 95-D lung cancer stem-like cells (95-D LCSCs). Then, the expression levels of stemness genes (CD133, Oct4 and Sox2) was assessed using western blot analysis. It was found that these genes were highly expressed in LCSCs (Fig. 1C). The results were confirmed using qPCR (Fig. 1D). These results demonstrated that 95-D LCSCs express tumor stem-like cell-related genes and may display tumor stem cell characteristics.

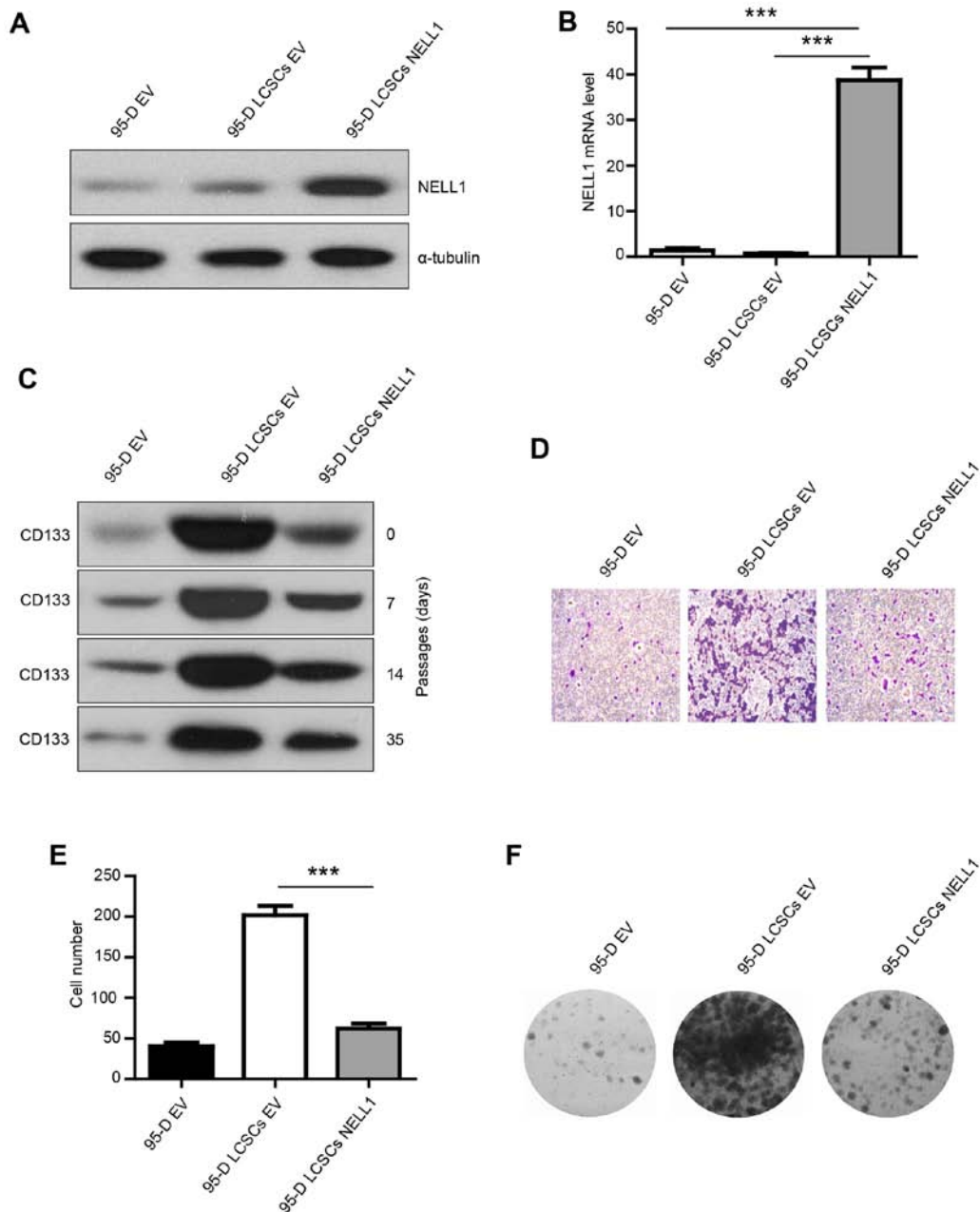


Figure 2. NELL1 affects the invasion and colony formation of 95-D LCSCs. (A and B) Expression of NELL1 in 95-D EV, 95-D LCSCs EV and 95-D LCLs cells. NELL1 was examined with western blot analysis and quantitative RT-PCR. (C) CD133 expression in 95-D EV, 95-D LCSCs EV and 95-D LCLs NELL1 cells at early and multiple passages. (D and E) Transwell invasion assay for 95-D EV, 95-D LCSCs EV and 95-D LCLs NELL1 cells. (F) Effect of NELL1 overexpression on 95-D colony formation. ***P<0.001. NELL1, neural EGFL like 1; LCSCs, lung cancer stem-like cells.

NELL1 overexpression in 95-D LCSCs results in decreased colony formation and invasion. To test whether NELL1 inhibits 95-D LCSC growth, 95-D cells were transfected with a lentivirus empty vector and 95-D LCSCs cells with a lentivirus empty vector or a lentivirus carrying NELL1. The transfection efficiency was confirmed (Fig. 2A and B). NELL1 was highly expressed in the 95-D LCSCs NELL1 group. To test whether the isolated 95-D LCSCs could maintain high expression of CD133 in different passages, the CD133 expression was evaluated in early and multiple passages. We found that CD133 was highly expressed in the different passages (Fig. 2C). Then, we examined the effect of NELL1 on 95-D LCSCs. Transwell invasion assay was used to examine the change in the invasive ability. Overexpression of NELL1

reduced the invasive capability of the 95-D LCSCs compared with the control cells, and the number of invasive cells was significantly decreased (Fig. 2D and E). Overexpression of NELL1 also significantly inhibited the colony formation and growth of 95-D LCSCs (Fig. 2F). These results indicate that NELL1 expression decreased the colony formation and invasion of 95-D LCSCs.

Overexpression of NELL1 inhibits the proliferation of 95-D LCSCs cells. One of the major points of cancer stem cells is their chemoresistance. Carboplatin and cisplatin are common chemotherapeutic drugs. To investigate the role of NELL1 expression in chemotherapeutic drug resistance, a CCK-8 assay was performed (Fig. 3A and B). Our results showed that the

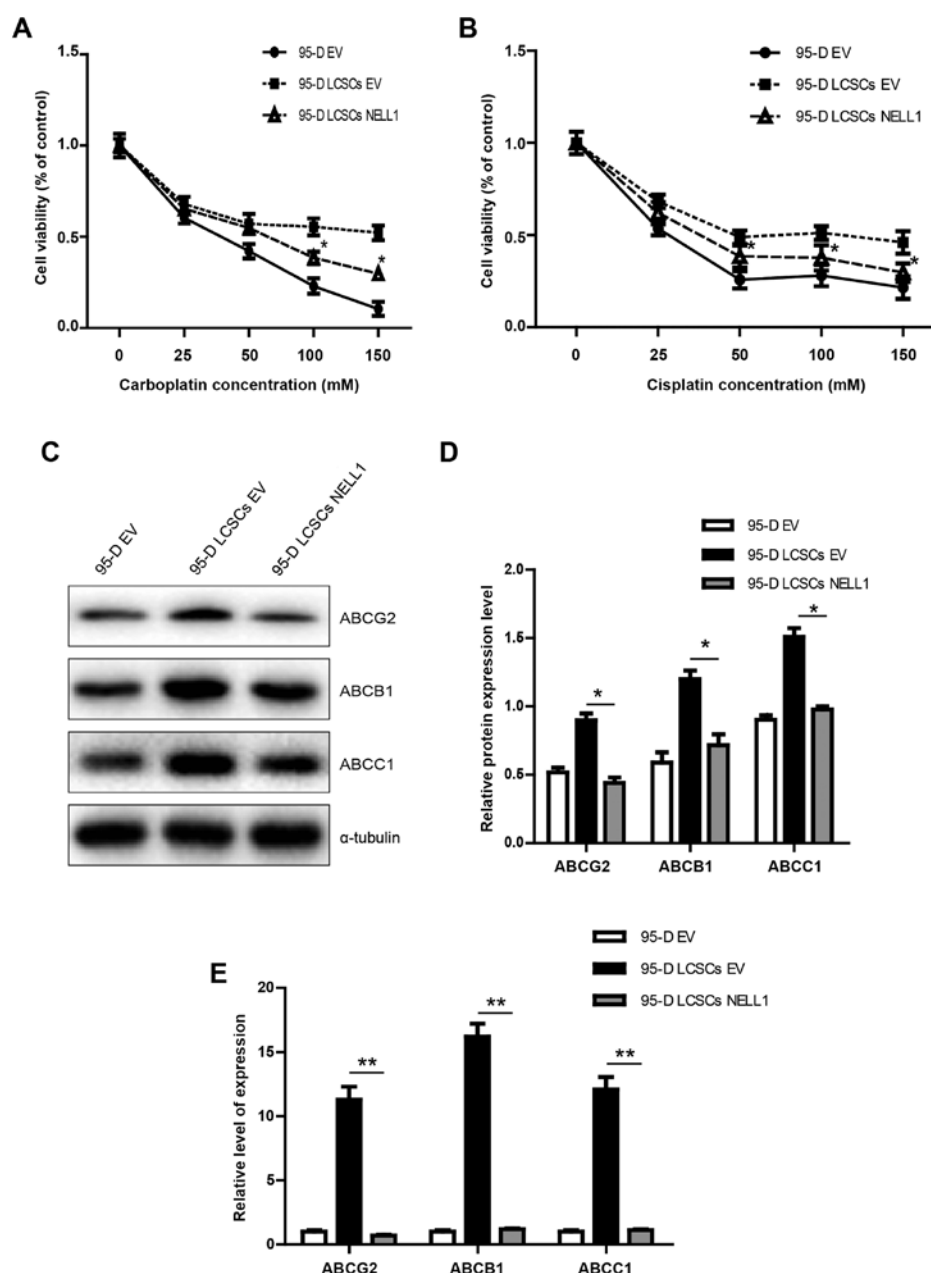


Figure 3. Cell viability of 95-D EV, 95-D LCSCs EV and 95-D LCSCs NELL1 cells. (A and B) Cell viability analysis of 95-D EV, 95-D LCSCs EV and 95-D LCSCs NELL1 cells following treatment with chemotherapeutic agent carboplatin or cisplatin for 48 h (25, 50, 100 and 150 μ M). (C and D) Western blot analysis results of ABCG2, ABCB1 and MDR1 proteins in 95-D EV, 95-D LCSCs EV and 95-D LCSCs NELL1 cells. (E) Quantitative RT-PCR results of ABCG2, ABCB1 and MDR1 in 95-D EV, 95-D LCSCs EV and 95-D LCSCs NELL1 cells. * $P < 0.5$, ** $P < 0.01$. NELL1, neural EGFL like 1; LCSCs, lung cancer stem-like cells.

chemosensitivity of 95-D LCSCs NELL1 cells was significantly increased compared with the 95-D LCSCs. Subsequently, we analyzed the protein levels of common multi-drug resistance markers ABCG2, ABCB1 and ABCC1. As shown in Fig. 3C and D, the expression of ABCG2, ABCB1 and ABCC1 was significantly decreased in the 95-D LCSCs NELL1 cells. Upon mRNA analysis, the same trends for ABCG2, ABCB1 and ABCC1 protein expression were also identified (Fig. 3E). These data show that overexpression of NELL1 in 95-D LCSCs cells increased the chemosensitivity of these cells.

NELL1 induces differentiation of 95-D LSCSs. From the aforementioned results, we confirmed that 95-D LCSCs expressed stem cell genes, CD133, Oct4 and Sox2, and

overexpression of NELL1 could inhibit 95-D cell growth. However, the mechanism remained unclear. Studies have shown that Sonic Hedgehog and Wnt pathways are important in cancer stem cells (32,33). We assessed Sonic Hedgehog (Shh) and β -catenin which are important proteins in these two pathways (34,35). Yet, we did not find any change of expression in these two proteins (Fig. 4A). c-MET was found to be activated in cancer stem cells (36,37). We examined the expression of these proteins. As shown in Fig. 4B and C, we found that NELL1 overexpression downregulated p-MET. Previous research has demonstrated that Notch signaling plays an important role in cancer stem cells (38). In the present study, it was also demonstrated that Notch3 and HES1 were downregulated in the 95-D LCSCs NELL1 cells. These results

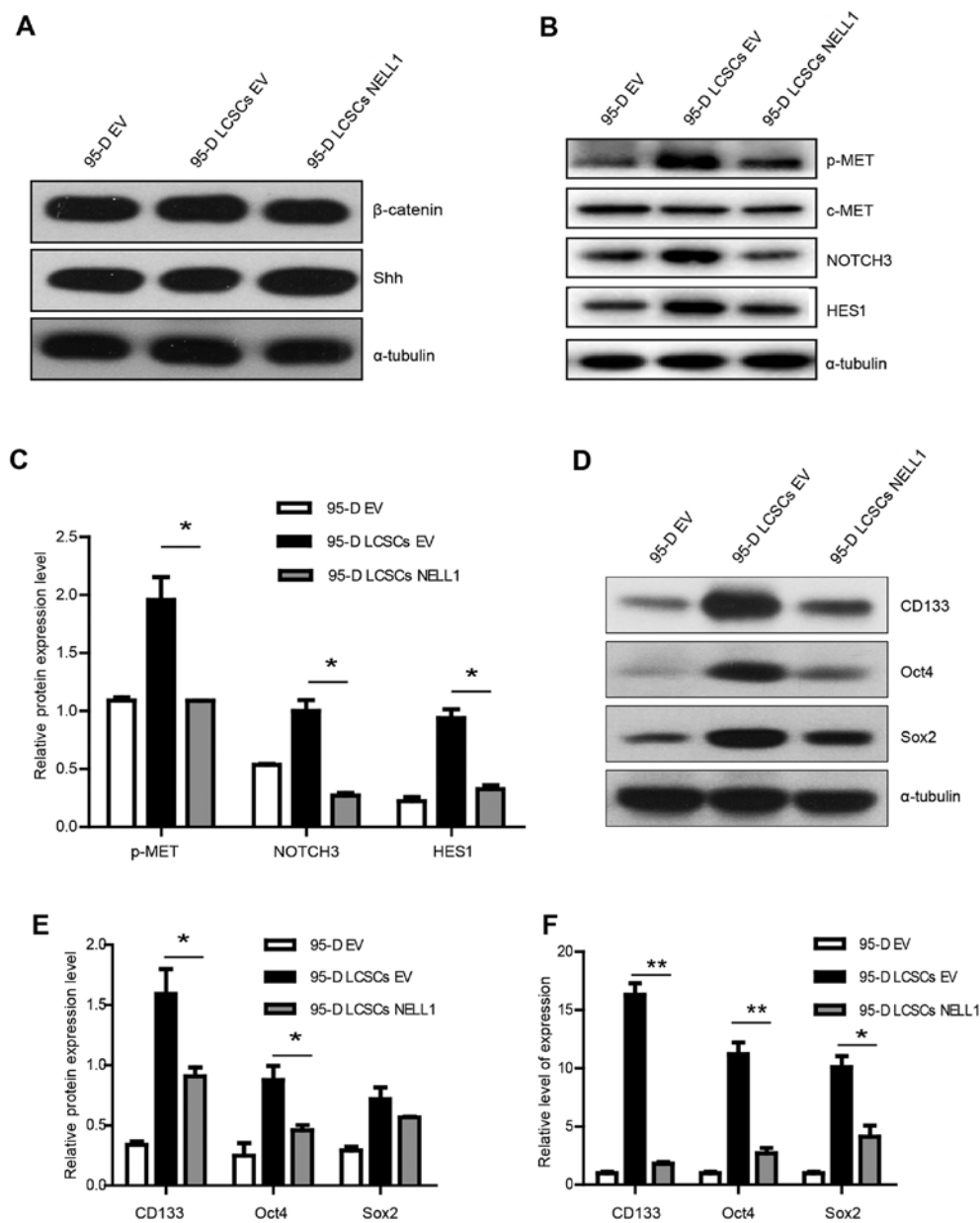


Figure 4. Relative expression of β -catenin, Shh, p-MET, Notch3 and HES proteins and stemness genes in 95-D EV, 95-D LCSCs EV and 95-D LCSCs NELL1 cells. (A) Western blot results of β -catenin and Shh in 95-D EV, 95-D LCSCs EV and 95-D LCSCs NELL1 cells. (B and C) Western blot results of p-MET, c-MET, NOTCH3 and HES1 proteins in 95-D EV, 95-D LCSCs EV and 95-D LCSCs NELL1 cells. (D and E) Western blot results of CD133, Oct4 and Sox2 proteins in 95-D EV, 95-D LCSCs EV and 95-D LCSCs NELL1 cells. (F) Quantitative RT-PCR results of CD133, Oct4 and Sox2 in 95-D EV, 95-D LCSCs EV and 95-D LCSCs NELL1 cells. * $P < 0.05$, ** $P < 0.01$. NELL1, neural EGFL like 1; LCSCs, lung cancer stem-like cells; Shh, Sonic Hedgehog; HES1, hairy and enhancer of split-1.

demonstrated that NELL1 may affect 95-D LCSC growth through c-MET/Notch signaling. In addition, overexpression of NELL1 decreased CD133, Oct4 and Sox2 at the protein and mRNA levels (Fig. 4D-F). Based on these data, we conclude that NELL1 may induce differentiation of 95-D LCSCs by inhibiting c-MET/Notch signaling (Fig. 5). Our results showed that NELL1 could be a potential target for lung cancer stem-like cells.

Discussion

Despite significant advances in the field of oncology over the previous decade, lung cancer mortality rates remain high (39).

Currently, traditional therapies for lung cancer are confronted with issues such as cancer resistance and poor prognosis. Searching for an effective and safe method is urgent for lung cancer therapy. The cancer stem cell theory hypothesizes that small populations of cancer cells may play critical roles in cancer (40). Researchers are looking for an effective way to target these cancer stem cells.

NELL1 is a novel growth factor that promotes osteoblast differentiation (41). NELL1 may exert significant activity in enhancing mesenchymal stem cells into osteoblast cells. A previous study reported that NELL1 plays an important role in human renal cell carcinoma (26), liver cancer (42) and esophageal adenocarcinoma (27). Notably, overexpression of

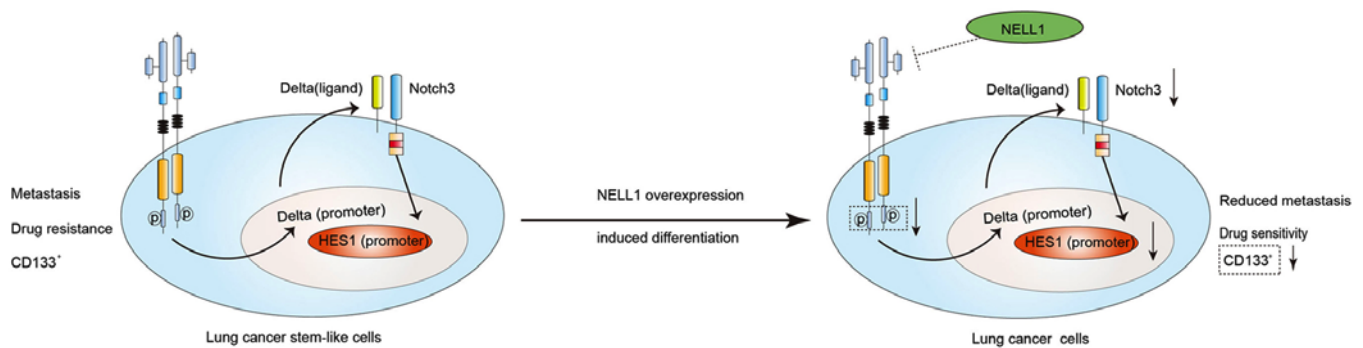


Figure 5. NELL1 induces the differentiation of lung cancer stem-like cells (LCSCs). A working model of NELL1 function in 95-D LCSCs is shown. In 95-D LCSCs, p-MET promotes the transcription of Delta, a Notch3 ligand, which upregulates HES1. NELL1 inhibits this signaling. NELL1, neural EGFL like 1; HES1, hairy and enhancer of split-1.

NELL1 was found to induce cancer stem cell differentiation and decrease their proliferation in glioblastoma (43). However, how NELL1 affects lung cancer stem cells has never been reported. The 95-D human cell line is a highly invasive and metastatic lung carcinoma cell line (19). It has been found that 95-D cells cultured in specific medium can be used to accumulate lung cancer stem-like cells in spheres, which are called lung cancer stem cells (LCSCs) (20). Thus, we investigated the role of NELL1 in 95-D cancer stem-like cell differentiation.

In the present study, we investigated the significance of NELL1 overexpression in the differentiation of 95-D stem-like cell line, and the molecular mechanisms underlying the effects of NELL1 overexpression.

Firstly, NELL1 overexpression was suggested to result in decreased colony formation and invasion (Fig. 2C-E). NELL1 has been previously reported in other cancers using cell lines and clinical samples. For example, NELL1 gene loss was noted in more than 40% Hodgkin's lymphoma patients (44). In gastric cancer, NELL1 expression was lower in cancer tissues relative to normal tissue (45). In colon cancer, researchers found that NELL1 is a promising tumor-suppressor gene candidate (46). NELL1 protein also participates in the growth, differentiation, and oncogenesis of cancer cell lines (47). NELL1 was found to inhibit renal cell carcinoma cell migration and adhesion (26). These data suggest that NELL1 may be involved in cancer development.

In order to investigate the effect of NELL1 on chemotherapeutic resistance, we detected the resistance of these cells to cisplatin and carboplatin. It was demonstrated that 95-D LCSCs overexpressing NELL1 had increased sensitive to cisplatin and carboplatin (Fig. 3A and B) and exhibited lower expression of ABCG2 and ABCC1 (Fig. 3C and D) compared with the 95-D LCSCs EV cells. 95-D LCSCs NELL1 cells differed from the control 95-D LCSCs EV cells following treatment with 50 μ M carboplatin and cisplatin. These results demonstrated that 95-D LCSCs overexpressing NELL1 were sensitive to chemotherapeutic drugs.

Another confounding issue was to determine the signaling pathway associated with NELL1. In osteogenesis differentiation, studies have shown that NELL1 activates MAPK signaling cascades (48,49), while in the present study we did not detect change in the expression of these proteins (data not shown). However, it was found that NELL1 inhibited the

expression of p-MET, Notch3 and HES1. We also found that after overexpression of NELL1, CD133, Oct4 and Sox2 expression was downregulated. Based on these data, we conclude that NELL1 may induce 95-D LCSC differentiation by inhibiting c-MET-Notch signaling.

In summary, our findings revealed that NELL1 may induce 95-D LCSC differentiation, resulting in decreased invasion, migration and proliferation abilities. Therefore, NELL1 is a promising potential target for lung cancer stem-like cells.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

GL, XJ and HW conceived and designed the study; YZ wrote the manuscript and performed most of the experiments; RW, SS and CL assisted with the quantitative RT-PCR and the western blot analysis. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriate investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2016. *CA Cancer J Clin* 66: 7-30, 2016.
2. Alamgeer M, Peacock CD, Matsui W, Ganju V and Watkins DN: Cancer stem cells in lung cancer: Evidence and controversies. *Respirology* 18: 757-764, 2013.
3. Kelsey CR, Marks LB, Hollis D, Hubbs JL, Ready NE, D'amico TA and Boyd JA: Local recurrence after surgery for early stage lung cancer. *Cancer* 115: 5218-5227, 2009.
4. Murray N, Coy P, Pater JL, Hodson I, Arnold A, Zee B, Payne D, Kostashuk EC, Evans WK and Dixon P: Importance of timing for thoracic irradiation in the combined modality treatment of limited-stage small-cell lung cancer. The national cancer institute of Canada clinical trials group. *J Clin Oncol* 11: 336-344, 1993.
5. Wisnivesky JP, Yankelevitz D and Henschke CI: Stage of lung cancer in relation to its size: Part 2. Evidence. *Chest* 127: 1136-1139, 2005.
6. Devesa SS, Bray F, Vizcaino AP and Parkin DM: International lung cancer trends by histologic type: Male:female differences diminishing and adenocarcinoma rates rising. *Int J Cancer* 117: 294-299, 2005.
7. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. *CA Cancer J Clin* 61: 69-90, 2011.
8. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2015. *CA Cancer J Clin* 65: 5-29, 2015.
9. Donnenberg VS and Donnenberg AD: Multiple drug resistance in cancer revisited: The cancer stem cell hypothesis. *J Clin Pharmacol* 45: 872-877, 2005.
10. Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA and Dick JE: A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 367: 645-648, 1994.
11. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ and Clarke MF: Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 100: 3983-3988, 2003.
12. Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J and Dirks PB: Identification of a cancer stem cell in human brain tumors. *Cancer Res* 63: 5821-5828, 2003.
13. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C and De Maria R: Identification and expansion of human colon-cancer-initiating cells. *Nature* 445: 111-115, 2007.
14. Eramo A, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A, Conticello C, Ruco L, Peschle C and De Maria R: Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ* 15: 504-514, 2008.
15. Bertolini G, Roz L, Perego P, Tortoreto M, Fontanella E, Gatti L, Pratesi G, Fabbri A, Andriani F, Tinelli S, *et al*: Highly tumorigenic lung cancer CD133⁺ cells display stem-like features and are spared by cisplatin treatment. *Proc Natl Acad Sci USA* 106: 16281-16286, 2009.
16. Leung EL, Fiscus RR, Tung JW, Tin VP, Cheng LC, Sihoe AD, Fink LM, Ma Y and Wong MP: Non-small cell lung cancer cells expressing CD44 are enriched for stem cell-like properties. *PLoS One* 5: e14062, 2010.
17. Qiu X, Wang Z, Li Y, Miao Y, Ren Y and Luan Y: Characterization of sphere-forming cells with stem-like properties from the small cell lung cancer cell line H446. *Cancer Lett* 323: 161-170, 2012.
18. Jiang F, Qiu Q, Khanna A, Todd NW, Deepak J, Xing L, Wang H, Liu Z, Su Y, Stass SA and Katz RL: Aldehyde dehydrogenase 1 is a tumor stem cell-associated marker in lung cancer. *Mol Cancer Res* 7: 330-338, 2009.
19. Yang X, Yang Y, Tang S, Tang H, Yang G, Xu Q and Wu J: Anti-tumor effect of polysaccharides from *Scutellaria barbata* D. Don on the 95-D xenograft model via inhibition of the C-met pathway. *J Pharmacol Sci* 125: 255-263, 2014.
20. Yue H, Huang D, Qin L, Zheng Z, Hua L, Wang G, Huang J and Huang H: Targeting lung cancer stem cells with antipsychological drug thioridazine. *Biomed Res Int* 2016: 6709828, 2016.
21. Dalerba P, Cho RW and Clarke MF: Cancer stem cells: Models and concepts. *Annu Rev Med* 58: 267-284, 2007.
22. Watanabe TK, Katagiri T, Suzuki M, Shimizu F, Fujiwara T, Kanemoto N, Nakamura Y, Hirai Y, Maekawa H and Takahashi Ei: Cloning and characterization of two novel human cDNAs (NELL1 and NELL2) encoding proteins with six EGF-like repeats. *Genomics* 38: 273-276, 1996.
23. Zhang X, Kuroda S, Carpenter D, Nishimura I, Soo C, Moats R, Iida K, Wisner E, Hu FY, Miao S, *et al*: Craniosynostosis in transgenic mice overexpressing Nell-1. *J Clin Invest* 110: 861-870, 2002.
24. Aghaloo T, Cowan CM, Chou YF, Zhang X, Lee H, Miao S, Hong N, Kuroda S, Wu B, Ting K and Soo C: Nell-1-induced bone regeneration in calvarial defects. *Am J Pathol* 169: 903-915, 2006.
25. Zhang X, Carpenter D, Bokui N, Soo C, Miao S, Truong T, WU B, Chen I, Vastardis H, Tanizawa K, *et al*: Overexpression of Nell-1, a craniosynostosis-associated gene, induces apoptosis in osteoblasts during craniofacial development. *J Bone Miner Res* 18: 2126-2134, 2003.
26. Nakamura R, Oyama T, Tajiri R, Mizokami A, Namiki M, Nakamoto M and Ooi A: Expression and regulatory effects on cancer cell behavior of NELL1 and NELL2 in human renal cell carcinoma. *Cancer Sci* 106: 656-664, 2015.
27. Jin Z, Mori Y, Yang J, Sato F, Ito T, Cheng Y, Paun B, Hamilton JP, Kan T, Oлару A, *et al*: Hypermethylation of the nel-like 1 gene is a common and early event and is associated with poor prognosis in early-stage esophageal adenocarcinoma. *Oncogene* 26: 6332-6340, 2007.
28. Maeda K, Matsuhashi S, Tabuchi K, Watanabe T, Katagiri T, Oyasu M, Saito N and Kuroda S: Brain specific human genes, NELL1 and NELL2, are predominantly expressed in neuroblastoma and other embryonal neuroepithelial tumors. *Neurol Med Chir (Tokyo)* 41: 582-589, 2001.
29. Shohet JM, Ghosh R, Coarfa C, Ludwig A, Benham AL, Chen Z, Patterson DM, Barbieri E, Mestdagh P, Sikorski DN, *et al*: A genome-wide search for promoters that respond to increased MYCN reveals both new oncogenic and tumor suppressor microRNAs associated with aggressive neuroblastoma. *Cancer Res* 71: 3841-3851, 2011.
30. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
31. Ting K, Vastardis H, Mulliken JB, Soo C, Tieu A, Do H, Kwong E, Bertolami CN, Kawamoto H, Kuroda S and Longaker MT: Human NELL-1 expressed in unilateral coronal synostosis. *J Bone Miner Res* 14: 80-89, 1999.
32. Cochrane CR, Szczepny A, Watkins DN and Cain JE: Hedgehog signaling in the maintenance of cancer stem cells. *Cancers (Basel)* 7: 1554-1585, 2015.
33. de Sousa e Melo F and Vermeulen L: Wnt signaling in cancer stem cell biology. *Cancers (Basel)* 8: E60, 2016.
34. Xu X, Lu Y, Li Y and Prinz RA: Sonic hedgehog signaling in thyroid cancer. *Front Endocrinol (Lausanne)* 8: 284, 2017.
35. Behrens J, von Kries JP, Kühl M, Bruhn L, Wedlich D, Grosschedl R and Birchmeier W: Functional interaction of β -catenin with the transcription factor LEF-1. *Nature* 382: 638-642, 1996.
36. Sun J, Zhang C, Liu G, Liu H, Zhou C, Lu Y, Zhou C, Yuan L and Li X: A novel mouse CD133 binding-peptide screened by phage display inhibits cancer cell motility in vitro. *Clin Exp Metastasis* 29: 185-196, 2012.
37. Firtina Karagonlar Z, Koç D, Şahin E, Avci ST, Yilmaz M, Atabey N and Erdal E: Effect of adipocyte-secreted factors on EpCAM+/CD133+ hepatic stem cell population. *Biochem Biophys Res Commun* 474: 482-490, 2016.
38. Suman S, Das TP and Damodaran C: Silencing NOTCH signaling causes growth arrest in both breast cancer stem cells and breast cancer cells. *Br J Cancer* 109: 2587-2596, 2013.
39. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 136: E359-E386, 2015.
40. Eyler CE and Rich JN: Survival of the fittest: Cancer stem cells in therapeutic resistance and angiogenesis. *J Clin Oncol* 26: 2839-2845, 2008.
41. Wang J, Liao J, Zhang F, Song D, Lu M, Liu J, Wei Q, Tang S, Liu H, Fan J, *et al*: NEL-like molecule-1 (Nell1) is regulated by bone morphogenetic protein 9 (BMP9) and potentiates BMP9-induced osteogenic differentiation at the expense of adipogenesis in mesenchymal stem cells. *Cell Physiol Biochem* 41: 484-500, 2017.

42. Ding D, Lou X, Hua D, Yu W, Li L, Wang J, Gao F, Zhao N, Ren G, Li L and Lin B: Recurrent targeted genes of hepatitis B virus in the liver cancer genomes identified by a next-generation sequencing-based approach. *PLoS Genet* 8: e1003065, 2012.
43. Jiang X, Xu M, Yin D, Zhang Z, Yu J, Black K and Liu G: Expression and functional analysis of Nell-1 on cancer stem cells and glioma patients' survival. *Am Association Cancer Res* 68: 12-16, 2008.
44. Slovak ML, Bedell V, Hsu YH, Estrine DB, Nowak NJ, Delioukina ML, Weiss LM, Smith DD and Forman SJ: Molecular karyotypes of Hodgkin and Reed-Sternberg cells at disease onset reveal distinct copy number alterations in chemosensitive versus refractory Hodgkin lymphoma. *Clin Cancer Res* 17: 3443-3454, 2011.
45. Gao C, Zhang Q, Kong D, Wu D, Su C, Tong J, Chen F and Zhang Q: MALDI-TOF mass array analysis of Nell-1 promoter methylation patterns in human gastric cancer. *Biomed Res Int* 2015: 136941, 2015.
46. Mori Y, Cai K, Cheng Y, Wang S, Paun B, Hamilton JP, Jin Z, Sato F, Berki AT, Kan T, *et al*: A genome-wide search identifies epigenetic silencing of somatostatin, tachykinin-1, and 5 other genes in colon cancer. *Gastroenterology* 131: 797-808, 2006.
47. Kuroda S, Oyasu M, Kawakami M, Kanayama N, Tanizawa K, Saito N, Abe T, Matsuhashi S and Ting K: Biochemical characterization and expression analysis of neural thrombospondin-1-like proteins NELL1 and NELL2. *Biochem Biophys Res Commun* 265: 79-86, 1999.
48. Bokui N, Otani T, Igarashi K, Kaku J, Oda M, Nagaoka T, Seno M, Tatematsu K, Okajima T, Matsuzaki T, *et al*: Involvement of MAPK signaling molecules and Runx2 in the NELL1-induced osteoblastic differentiation. *FEBS Lett* 582: 365-371, 2008.
49. Cowan CM, Jiang X, Hsu T, Soo C, Zhang B, Wang JZ, Kuroda S, Wu B, Zhang Z, Zhang X and Ting K: Synergistic effects of Nell-1 and BMP-2 on the osteogenic differentiation of myoblasts. *J Bone Miner Res* 22: 918-930, 2007.