博士論文

肺癌における新規治療標的分子TMEM209の同定及びその機能解析

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Contents

1. Abstract

Through the analysis of gene expression profiles of 120 clinical lung cancers using cDNA microarray containing 27,648 genes or expressed sequence tags (ESTs), we identified *TMEM209* (Transmembrane protein 209) that was highly expressed in the majority of lung cancers. Northern-blot analysis identified that *TMEM209* was expressed only in testis among normal tissues. Immunocytochemical analysis revealed that TMEM209 protein was localized in the nuclear envelope and Golgi of lung cancer cells. Transient TMEM209 expression significantly promoted cell growth, whereas treatment with siRNAs against TMEM209 significantly suppressed the growth of lung cancer cells. Immunoprecipitation assay using lung cancer cells which was transfected with TMEM209 expression vector and subsequent mass spectrometric analysis identified NUP205 (Nucleoporin 205 kDa) as a TMEM209-interacting protein. TMEM209 was likely to interact with and stabilize NUP205 protein, and could elevate the level of c-Myc in nucleus. Expression of a portion of TMEM209 protein including NUP205-interacting region in lung cancer cells could inhibit endogenous TMEM209-NUP205 interaction and suppressed the cancer cell growth. Our findings suggest that TMEM209 overexpression as well as TMEM209-NUP205 interaction might be involved in lung cancer proliferation, and should be a promising target for lung cancer therapy.

2. Introduction

Lung cancer is one of the leading causes of death in the worldwide (1). Many genetic alterations associated with development and progression of lung cancers have been reported and contributed to the better understanding of the molecular mechanisms of pulmonary carcinogenesis (2). However, despite some advances in the early detection and recent improvements in its treatment, the prognosis of the lung cancer patients is not much improved. In addition, the quality of life (QOL) for some of those who do survive remains poor. Over the last few decades, several newly developed cytotoxic agents such as gemcitabine, paclitaxel, vinorelbine and docetaxel have offered multiple choices for treatment of patients with advanced lung cancer, but each of those regimens confers only a modest survival benefit compared with cisplatin-based therapies (3-5). In addition to these cytotoxic drugs, several molecular targeted agents, such as monoclonal antibodies (mAb) against vascular endothelial growth factor (VEGF; i.e., bevacizumab/anti-VEGF) or epidermal growth factor receptor (EGFR; i.e., cetuximab/anti-EGFR) as well as inhibitors for EGFR tyrosine kinase (i.e., gefitinib and erlotinib) and anaplastic lymphoma kinase (ALK; i.e., crizotinib) were developed and are applied in clinical practice (6, 7). However, each of the new regimens can provide survival benefits to a small subset of the patients and cause serious adverse effects such as interstitial pneumonia and acute lung injury (8). Hence, the development of molecular targeted agents providing better clinical benefits without such adverse reactions is eagerly required.

Genome-wide gene expression profiling analysis using cDNA microarray is an effective and promising approach for identification of target molecules in various types of tumors (9, 10). To isolate potential molecular targets for diagnosis, treatment, and/or prevention of lung cancer, our laboratory had performed genome-wide expression profile analysis of tumor tissues from 120 lung cancer cases by means of laser-microbeam microdissection technology and cDNA

microarray consisting of 27,648 genes or expressed sequence tags (ESTs) (11-16). Using this screening system, we identified a number of novel candidate genes that are transactivated in non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) cells but hardly detectable in various normal organs, and are indispensable for cancer cell growth (17-47). These candidate molecules are considered to be potential molecular targets for the development of anti-cancer therapeutics and prognostic and diagnostic markers. Among genes that were commonly up-regulated in clinical lung cancer cells, I here focused on TMEM209 (Transmembrane protein 209) as a potential therapeutic target for lung tumor. TMEM209 was identified as an integral protein of nuclear membrane in mouse liver by high-throughput shotgun proteomics using multidimensional protein identification technology for the first time (48). Nuclear envelope is a lipid bilayer membrane that separates the cell into two compartments, nucleus and cytoplasm, and makes a barrier which allows genomic DNA to reside in the space that are dedicated to its protection, faithful replication and regulated transcription (49, 50). It contains a large number of different proteins that have been implicated in preservation of nuclear structure, chromatin organization and gene regulation. The gates through the nuclear membrane are nuclear pore complexes (NPCs), which regulate the restricted and selective exchange of macromolecules like proteins and RNAs between the nucleus and cytoplasm. The transport of molecules through the pores are passive and active, and how the cargoes are selectively transferred through these channels is being investigated. Furthermore, several abnormality of the in- or export of transcriptional factors such as p53 and β-catenin, or in the NPC machinery itself have been observed in a variety of human cancers, hence the involvement of NPC in tumor is increasingly suggested (51). However, the pathophysiological roles and biological functions of TMEM209 in nuclear envelope or NPC of human cancers have not been reported. Here I report that TMEM209 is up-regulated in lung cancer cases and cell lines, hardly detectable in normal

tissues examined and indispensable for cancer cell growth and/or survival. TMEM209 stabilizes a component of NPC protein, NUP205 and regulates nuclear levels of c-Myc. c-Myc is one of the oncogenic transcriptional factor which play a key role in cell proliferation and tumorigenesis (52). Myc family transcription factors contain BR/HLH/LZ (basic-region/helix-loop-helix/leucine-zipper) domain. c-Myc proteins make homo- or hetero-dimers with itself or another BR/HLH/LZ interacting partners (e.g. Max) through the domain, directly binds to E-box sequence (CANNTG) and stimulate the downstream targets. The many c-Myc target genes were identified which are involved in cell proriferation, vasculartization and metastasis. Hence, nuclear localization of c-Myc and its transcriptional activity could be essential for tumorigenesis. These findings suggest that targeting TMEM209 and/or TMEM209-NUP205 interaction could be a promising therapeutic strategy for lung cancer therapy.

3. Materials and Methods

3-1. Lung cancer cell lines and tissue samples. The human lung cancer cell lines used in this study were as follows: lung adenocarcinoma (ADC) cell lines A549, LC319, PC14, NCI-H1373 and NCI-H1781; lung squamous cell carcinoma (SCC) cell lines SKMES-1, LU61, NCI-H520, NCI-H1703 and NCI-H2170; small cell lung carcinoma (SCLC) cell lines DMS114, DMS273, SBC-3, and SBC-5 and a large cell carcinoma (LCC) cell line LX1 (**Table 1**). All cells were grown in monolayers in appropriate medium supplemented with 10% FCS and were maintained at 37°C in atmospheres of humidified air with 5% CO_2. Human small airway epithelial cells (SAEC) were grown in optimized medium purchased from Cambrex Bio Science, Inc. Primary lung cancer tissue samples had been obtained with informed consent as described previously (12, 16). This study and the use of all clinical materials were approved by individual institutional ethical committees.

3-2. Semiquantitative reverse transcription-PCR. Total RNA was extracted from cultured cells using the TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's protocol. Extracted RNAs were treated with DNase I (Nippon Gene) and reversely transcribed using oligo (dT) primer and SuperScript II. Semiquantitative reverse transcription-PCR (RT-PCR) experiments were carried out with the following synthesized specific primers for *TMEM209*, *NUP205*, *CDC25A*, *CDK1* or β-actin (*ACTB*) as follows: *TMEM209*, 5'-GCAGACTCACTAAAGTATCCCCA-3' and 5'-CTCCATGGTGCTTTTAATGAAG-3'; *NUP205*, 5'-GAAACTTCTGGACATTGAAGGA-3' and 5'-TGAGGATGGAACTAGGGGAAG-3'; *CDC25A*, 5'-TGAGGTGTAGGTGGGTTTTT-3' and 5'-GCCATCCCACCTTTCTCTTT-3'; *CDK1*, 5'-ACCACTTTTCCATGGGGAT-3' and 5'-TGGATGATTCAGTGCCATTT-3'; *ACTB*, 5'-GAGGTGATAGCATTGCTTTCG-3' and 5'-CAAGTCAGTGTACAGGTAAGC-3'. PCR

reactions were optimized for the number of cycles to ensure product intensity within the logarithmic phase of amplification.

3-3. Northern blot analysis. Human multiple-tissue blots (BD Biosciences Clontech) were hybridized with ^{32}P-labeled PCR products of *TMEM209* and *NUP205*. The cDNA probes of *TMEM209* and *NUP205* were prepared by RT-PCR using following primers: *TMEM209*, 5'-AACACTTAGATTAAATTTAG-3' and 5'-GCTCCTTTCCTTTGGACATC-3'; *NUP205*, 5'-GCGCCCAGAAACGGACCCGC-3' and 5'-ACTGTTTCTGAAAGGCTAGG-3'. Prehybridization, hybridization, and washing were done according to the supplier's recommendations. The blots were autoradiographed at -80°C for 14 days with intensifying BAS screens (Bio-Rad).

3-4. Western blotting. Whole cells were lysed with NP-40 buffer [150 mM NaCl, 0.5 % NP-40, 50 mM Tris-HCl (pH 8.0)] containing Protease Inhibitor Cocktail Set III and Phosphatase Inhibitor Cocktail Set II (CALBIOCHEM). Protein fractionantion was performed with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo). Protein samples were separated by SDS-polyacrylamide gels and electroblotted onto Hybond-ECL nitrocellulose membranes (GE Healthcare Bio-Sciences). Blots were incubated with either of antibodies to TMEM209 (Catalog No. HPA031678; ATLAS Antibodies), NUP205 (Catalog No. HPA024574; ATLAS Antibodies), c-Myc (Catalog No. sc-40; Santa Cruz), Flag (Catalog No. F3165; SIGMA) or ACTB (Catalog No. A5316; SIGMA). Antigen-antibody complexes were detected using secondary antibodies conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences). Protein bands were visualized by enhanced chemiluminescence western blotting detection reagents (GE Healthcare Bio-Sciences).

3-5. Immunofluorescence analysis. Cells were plated onto glass coverslips (Becton Dickinson Labware), fixed with 4% paraformaldehyde, and permeablilized with 0.1% Triton X-100 in PBS for 5 minutes at room temperature. Non-specific binding was blocked by 5 % Skim milk for 30 minutes at room temperature. Cells were then incubated for 60 minutes at room temperature with primary antibodies for anti-TMEM209 antibody (Catalog No. HPA031678; ATLAS Antibodies), anti-c-Myc antibody (Catalog No. sc-40; Santa Cruz) or anti-Golgi 58K Protein/Formiminotransferase Cyclodeaminase (FTCD) antibody (Catalog No. G2404; SIGMA) diluted in PBS containing 1% BSA. After being washed with PBS, the cells were stained by Alexa Fluor 488-conjugated or 594-conjugated secondary antibody (Molecular Probes) for 60 minutes at room temperature. After another wash with PBS, each specimen was mounted with Vectashield (Vector Laboratories, Inc.) containing 4', 6'-diamidine-2'-phenylindolendihydrochrolide (DAPI) and visualized with Spectral Confocal Scanning Systems (TSC SP2 AOBS: Leica Microsystems).

3-6. RNA interference assay. To evaluate the biological functions of TMEM209 and NUP205 in lung cancer cells, I used small interfering RNA (siRNA) duplexes against the target genes (SIGMA). The target sequences of the synthetic oligonucleotides for RNA interference were as follows: control-1: (*EGFP*, enhanced green fluorescence protein [GFP] gene, a mutant of *Aequorea gictoria* GFP), 5'-GAAGCAGCACGACUUCUUC-3'; control-2 (*LUC*, luciferase gene from *Photinus pyralis*), 5'-CGUACGCGGAAUACUUCGA-3'; si-TMEM209-#1, 5'-CUACGAACUUUGGAUACUU-3'; si-TMEM209-#2, 5'-GUGUGAAUAUAUUGUGGAU-3' si-NUP205, 5'-CUCUCUACCUGUUGGGCUU-3'. Lung cancer cells, LC319, SBC-3 and SBC-5, were plated onto 10-cm dishes, and transfected at subconfluent condition with either of the

siRNA oligonucleotides (50 μM) using 30 μL of Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. After seven days of incubation, these cells were stained by Giemsa solution to assess colony formation, and cell numbers were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide (MTT) assay; briefly, cell-counting kit-8 solution (DOJINDO) was added to each dish at a concentration of 1/10 volume, and the plates were incubated at 37°C for additional 30 minutes. Absorbance was then measured at 490 nm, and at 630 nm as a reference, with a Microplate Reader 550 (BIO-RAD).

3-7. Flow cytometry. Cells were collected in PBS and fixed in 70% cold ethanol for 30 min. After treatment with 100 μg/mL of RNase (Sigma), the cells were stained with 50 μg/mL propidium iodide (Sigma) in PBS. Flow cytometry was analyzed by using FACScan (Becknman Coulter). The cells selected from at least 20,000 ungated cells were analyzed for DNA content.

3-8. Cell-growth assay. I cloned the entire coding sequence of *TMEM209* into the appropriate site of COOH-terminal Flag-tagged pCAGGS plasmid vector. COS-7 and SBC-3 cells transfected either with plasmid expressing Flag-tagged TMEM209 or mock plasmid were grown for seven days in DMEM (COS-7) or RPMI (SBC-3) containing 10% FCS in the presence of appropriate concentrations of geneticin (G418). Cell viability was evaluated by MTT assay.

3-9. Coimmunnoprecipitation and matrix-assisted laser desorption/ionizing-time of flight mass spectrometry mapping of TMEM209-associated proteins. Cell extracts from lung cancer SBC-5 cell which was transfected with TMEM209 expression vector or mock vector were precleared by incubation at 4°C for 1 hour with 80 μL of protein G–agarose beads in a final volume of 200 μL of immunoprecipitation buffer (0.5% NP-40, 50 mM Tris-HCl, 150 mM NaCl) in

the presence of Protease Inhibitor Cocktail Set III (CALBIOCHEM). After centrifugation at 1,000 rpm for 5 minutes at 4°C, the supernatants were incubated at 4°C with anti-Flag M2 agarose (Catalog No. A2220; SIGMA) for 1 hour. The beads were then collected by centrifugation at 5,000 rpm for 1 minute and washed six times with 1 mL of immunoprecipitation buffer. The washed beads were resuspended in 30 μL of Laemmli sample buffer and boiled for 5 minutes, and the proteins were separated using 5-20% gradient SDS PAGE gel (Bio-Rad). After electrophoresis, the gel was stained with SilverQuest (Invitrogen). Protein bands specifically found in extracts from the cells which were transfected with TMEM209 vector were excised and served for matrix-assisted laser desorption/ionizationtime of flight mass spectrometry (MALDI-TOF-MS) analysis (AXIMA-CFR plus, Shimadzu Biotech).

3-10. Immunoprecipitation assay. Cell extracts from lung cancer SBC-5 cells were precleared by incubation at 4°C for 1 hour with 80 μL of protein G–agarose beads in a final volume of 200 μL of immunoprecipitation buffer (0.5% NP40, 50 mM Tris-HCl, 150 mM NaCl) in the presence of Protease Inhibitor Cocktail Set III (CALBIOCHEM). After centrifugation at 1,000 rpm for 5 minutes at 4°C, the supernatants were incubated at 4°C with normal rabbit IgG (Catalog No. sc-2027; Santa Cruz) or anti-TMEM209 antibody (Catalog No. 06-1020; MILLIPORE) for overnight. The beads were then collected by centrifugation at 5,000 rpm for 1 minute and washed six times with 1 mL of immunoprecipitation buffer. The washed beads were resuspended in 30 μL of Laemmli sample buffer and boiled for 5 minutes. Then the proteins were separated using SDS PAGE gel. The following procedure was performed as described above.

3-11. Protein synthesis and proteasome Inhibitors. Protein synthesis inhibitor,

cycloheximide (CALBIOCHEM) was dissolved in ethanol and added in culture medium at 100 μg/mL. Proteasome inhibitor, MG132 (Synonym:Z-Leu-Leu-Leu-al;SIGMA) was dissolved in Dimethyl sulfoxide (DMSO) and added in culture medium at 20 μM.

3-12. Quantitative real-time PCR. Quantitative real-time PCR was conducted with the SYBR Green I Master kit on a LightCycler 480 (Roche) according to the manufacturer's recommendations. Each experiment was done in triplicate. *GAPDH* was used for normalization of expression levels. cDNAs as templates were synthesized as described above. For quantitative RT-PCR reactions, specific primers for all human *TMEM209*, *CDC25A*, *CDK1*, and *GAPDH* were designed as follows: *TMEM209*, 5'-TCGCCCGTCAGTGGTTAT-3' and 5'-CCAACAGTGGTAGGGTACGG-3'; *CDC25A*, 5'-ATCTCTTCACACAGAGGCAGAA-3' and 5'-CCCTGGTTCACTGCTATCTCTT-3'; *CDK1*, 5'-TGGATCTGAAGAAATACTTGGATTCTA-3' and 5'-CAATCCCCTGTAGGATTTGG-3'; *GAPDH*, 5'-GCAAATTCCATGGCACCGTC-3' and 5'-TCGCCCCACTTGATTTTGG-3'.

4. Result

4-1. TMEM209 expression in lung cancers and normal tissues. To identify novel target molecules for the development of therapeutic agents and/or diagnostic biomarkers of lung cancer, Daigo, et al., Kikuchi, et al., Kakiuchi, et al. and Taniwaki, et al. had previously performed gene expression profile analysis of 120 lung carcinomas using cDNA microarray containing 27,648 genes or expressed sequence tags (11-16). In the cDNA microarray data I extracted genes that showed 3-fold or higher level of expression in the majority of 120 lung cancer samples examined, and showed no or low expression in normal tissues. Through this expression analysis, nearly 600 genes were screened. For selected genes by this criteria, I performed semi-quantitative RT-PCR and northern blot to validate the gene expression in lung cancer samples, cell lines and normal tissues, and 5 genes were validated (**Fig. 1**). And then I performed RNA interference assay using lung cancer cell lines to determine whether the extracted genes are important for cancer cell growth. Finally, two genes were screened. Through this screening, I identified *TMEM209* (Transmembrane protein 209) as an candidate molecular target. I confirmed its transactivation by semiquantitative RT-PCR experiments in 10 of 15 additional lung-cancer tissues and in 12 of 15 lung-cancer cell lines (**Figs. 2*A* and 2*B***). I also confirmed by western blot analysis high expression of TMEM209 protein in lung tumor cell lines using anti-TMEM209 antibody (**Fig. 3**). To examine the subcellular localization of endogenous TMEM209 in cancer cells, I performed immunocytochemical analysis of lung cancer SBC-5 cells that over-expressed the endogenous TMEM209 protein using anti-TMEM209 antibody. TMEM209 protein is detected in the nuclear envelope and the Golgi, and weakly in the cytoplasm of the lung cancer SBC-5 cells (**Fig. 4**). Northern-blot analysis with an amplified *TMEM209* cDNA as a probe detected a 3.5-kb transcript specifically in the testis among 16 normal human tissues examined (**Fig. 5**).

4-2. Inhibition of growth of lung cancer cells by siRNA against TMEM209. To estimate whether TMEM209 is crucial for growth or survival of lung cancer cells, I transfected synthetic oligonucleotide siRNAs against TMEM209 into lung adenocarcinoma LC319 and small cell lung cancer SBC-5 cells in which TMEM209 was highly expressed. The TMEM209 protein level in the cells transfected with si-TMEM209-#1 or -#2 were significantly decreased in comparison with cells transfected with either of control siRNAs(si-EGFP or si-LUC) (**Figs. 6*A* and 6*B***). I also observed significant decrease in the number of colonies and the number of viable cells measured by MTT assay (**Figs. 6*C* - 6*F***). On the other hand, I examined the effects of these siRNAs on the lung cancer SBC-3 cells whose endogenous TMEM209 expression is hardly detectable. MTT assay revealed that the cell viability treated with TMEM209 siRNAs (TMEM209-#1, TMEM209-#2) was equivalent to that treated with either of control siRNAs (si-EGFP or si-LUC), suggesting that suppression of cancer cell growth by treatment of TMEM209-specific siRNAs was unlikely to be off-target effects (**Fig. 7**). To further assessment of the knockdown effect of *TMEM209*, I performed cell cycle analysis using flow cytometer and found the G1 arrest of SBC-5 cells transfected with siRNA against *TMEM209* (**Fig. 8**).

4-3. Growth-promoting effect of TMEM209. To further estimate a potential role of TMEM209 in carcinogenesis, we constructed plasmid vector (pCAGGS vector) expressing TMEM209 with a C-terminus Flag-tag (TMEM209-Flag). I then transfected this TMEM209-Flag vector or mock plasmid into COS-7 and SBC-3 cells, in which endogenous TMEM209 was quite low, and performed cell-viability assay. I detected exogenous TMEM209 expression conferred the growth-promoting effect, compared with those transfected with mock plasmid (**Figs. 9*A* – 9*D***).

4-4. Interaction of TMEM209 with NUP205. For the clarification of the molecular function of TMEM209, I screened a TMEM209-interacting protein(s) in lung cancer cells. SBC-5 cell lysates, which were transfected with Flag-tagged TMEM209 expression vector or mock, were extracted and immunoprecipitated with anti-Flag M2 agarose. The immuno-complex was separated in the SDS-PAGE gel and stained with SilverQuest (**Fig. 10*A***). A 205-kDa band, which was observed in the lysates transfected with TMEM209 vector, but not in those with mock vector, was successfully identified by peptide sequencing to be a human NUP205 (Nucleoporin 205 kD) protein. I subsequently confirmed the interaction between endogenous TMEM209 and endogenous NUP205 in SBC-5 cells by immunoprecipitation with anti-TMEM209 antibody and subsequent immunoblotting with anti-NUP205 antibody (**Fig. 10*B***). NUP205 is a part of subcomplex of the nuclear pore complex (NPC) embedded in nuclear pore and is considered as a scaffold nucleoporin that is important for the overall integrity of the NPC. In eukaryotic cells, the spatial segregation of replication and transcription in the nucleus and translation in the cytoplasm requires transport of thousands of macromolecules including DNAs, RNAs and proteins between these two compartments. NPCs are the gateways that facilitate this transport across the nuclear envelope in co-operation with soluble transport receptors and play a crucial and essential role in cellular event (53). I found *NUP205* expression in lung cancers, but not in normal tissues such as lung, liver, kidney, heart, and brain (**Fig.11*A***). Northern-blot analysis with *NUP205* as a probe identified a 6.3-kb transcript in testis among 16 tissues examined, indicating that both TMEM209 and NUP205 are likely to be cancer-testis antigens (**Figs. 11*B***). To assess the functional relationship between TMEM209 and NUP205, I examined the NUP205 protein level after inhibition of TMEM209 expression by siRNA treatment in SBC-5 cells. I trasnfected siRNA oligonucleotides against TMEM209 (si-TMEM209) or control siRNAs (si-EGFP) into SBC-5 cells and at 24 hours after treatment with siRNAs, incubated SBC-5 cells

in growth medium supplemented with protein synthesis inhibitor cycloheimide (CHX), and monitored endogenous NUP205 protein levels in cells transfected with si-TMEM209 or si-EGFP. I observed the knockdown of *TMEM209* transcription in SBC-5 cells transfected with si-TMEM209 although no effect on *NUP205* transcription was observed. However, NUP205 protein levels were significantly reduced in a time-dependent manner, suggesting that the NUP205 protein was likely to be stabilized by its interaction with TMEM209 (**Figs. 12*A* and 12*B***).

4-5. Nuclear c-Myc levels might be regulated by TMEM209-NUP205 complex. Previous reports for large-scale mapping of human protein-protein interactions by mass spectrometry suggested NUP205 to interact with c-Myc, an oncogenic transcription factor (52, 54). Therefore, I investigated the interaction between NUP205 and c-Myc in lung cancer cells using lysates of SBC-5 cells which were transfected with Flag-tagged c-Myc expression vector or mock vector. Immunoprecipitation of the cell lysates with anti-Flag M2 agarose and subsequent immunoblotting with anti-NUP205 antibody confirmed their interaction (**Fig. 13**). Since NUP family members are known to regulate nucleocytoplasmic transport of macromolecules, I assessed the effect of TMEM209-NUP205 complex on c-Myc protein localization by fractionating cell lysates to cytoplasm and nucleus. Suppression of TMEM209 or NUP205 expression by siRNAs against TMEM209 or NUP205 appeared to reduce the levels of nuclear c-Myc protein (**Figs. 14*A* and 14*B***). In contrast, the ectopic expression of TMEM209 increased the amount of nuclear c-Myc protein (**Fig.15**). Considering that c-Myc protein stability is strictly regulated by ubiquitin-proteasome system (54), I then treated SBC-5 cells, which had been transfected with siRNAs against TMEM209 or NUP205, with proteasome inhibitor MG132. The amount of the whole c-Myc protein was not changed, while the level of cytoplasmic c-Myc protein was elevated and that of nuclear c-Myc was reduced in the cells treated with si-TMEM209 or si-NUP205 (**Fig.**

16). These data suggest that TMEM209 and NUP205 are involved in regulation of the nuclear transport of c-Myc. Furthermore, I examined the effects of other nuclear proteins, STAT3 and p65, which were reported to translocate from the cytoplasm to the nucleus in human cancer cells. The total amounts of STAT3 and p65 proteins as well as those in the nucleus were reduced in cells transfected with si-TMEM209 or si-NUP205 (**Fig. 17**). The data imply that TMEM209-NUP205 complex is associated with the nuclear import of not only c-Myc but also some nuclear proteins. To further examine whether c-Myc transcription activity could be inhibited after the knockdown of *TMEM209*, I measured the expression levels of representative c-Myc-target genes, *CDC25A* and *CDK1,* which are highly expressed in lung cancers and are reported to their involvement in carcinogenesis (**Fig. 18**). Suppression of *TMEM209* by siRNAs reduced the expression levels of *CDC25A* and *CDK1* in LC319 and SBC-5 cells as detected by quantitative real-time PCR (**Fig. 19**). These data indicate that overexpression of TMEM209-NUP205 complex proteins might prompt the nuclear transport of c-Myc and resulted in overexpression of oncogenic c-Myc target genes, such as *CDC25A* and *CDK1*.

5. Discussion

Recent advances in the study of the biological mechanisms underlying cancer development have caused the paradigm shift in designing and developing a new type of therapeutic drug, termed ‘molecular targeted drug’, that selectively interferes with molecules or pathways involved in tumor growth and/or progression. Inactivation of growth factors and their receptors in tumor cells as well as the inhibition of oncogenic pathways or specific functions in cancer cells constitutes the main rationale of novel cancer treatments (55). Molecular targeted cancer therapies are expected to treat cancer cells more selectively than normal cells, thus to be less harmful to normal cells, to reduce side effects, and to improve QOL of cancer patients. Intensive studies to screen molecular targets for development of novel drugs identified a number of possible candidates that can applicable for novel lung cancer therapies. However, suppression of some of such molecules also caused serious adverse reactions *in vivo* because of expression of molecules in certain types of normal tissue and/or off-target effects of compounds on non-target molecules. Hence, the specificity of molecules in cancer cells as well as the selectivity of compounds to a certain target should be critical to develop drugs with high efficacy and minimum toxicity.

To screen more appropriate molecular targets for the drug development, I had analyzed the whole-genome expression profiles of 120 clinical lung cancer samples using cDNA microarray data containing 27,648 genes or ESTs (12-16), and investigated loss-of-function phenotypes by RNA interference systems (17-47). On the basis of this approach, I found TMEM209 to be over-expressed in the majority of clinical lung cancer cases as well as lung cancer cell lines, while its expression was hardly detectable in normal tissues except the testis. Furthermore, I demonstrated that the knockdown of TMEM209 expression resulted in inhibition of cancer cell growth, whereas transient expression of TMEM209 resulted in the significant

promotion of cell growth. The data suggest that TMEM209 plays indispensable roles in the growth of lung cancer cells, indicating that TMEM209 could serve as a target for the development of anti-cancer agents for lung cancer. Although further analysis of TMEM209 including mutational screening and/or epigenetic alteration of this gene should be required to fully address the significant role of this gene in pulmonary carcinogenesis.

TMEM209 is a 63-kDa transmembrane protein that contains a nuclear pore complex component (NPCC) domain in its N terminus. Some proteins containing this domain are known to be components of the nuclear pore complex (NPC). One member of this family is Nucleoporin POM34 (Budding yeast) which is thought to have a role in anchoring peripheral NUP family proteins into the pore and mediating pore formation (56). My study also demonstrated that TMEM209 interacted with NUP205, a component of NPC. NUP205 was identified as a component of NPC and to interact with NUP93 and NUP53 which are involved in the integrity of the NPC in *Xenopus* (57). To date, there is no report describing the involvement of TMEM209-NUP205 complex in human carcinogenesis. I also found overexpression of NUP205 in lung cancer cells and similarly to TMEM209, its expression was scarcely detectable in normal tissues except testis, suggesting that the TMEM209-NUP205 complex could be expressed specifically in lung cancer cells and testis. I also showed that TMEM209 regulated the NUP205 protein stability by its interaction. It was reported that NUP93 and NUP53 could interact with and stabilize NUP205 in Hela cells (58). Further analysis is necessary to verify the detailed relationship between the TMEM209-NUP205 complex and other NUP proteins, but it is likely that TMEM209 protein is indispensable for the function of NPC in cancer cells.

My data also indicated that TMEM209-NUP205 complex could play important roles in nuclear levels of c-Myc and then influence to the c-Myc transcriptional activity. In *Drosophila*, Nup93 is able to preferentially interact with the phosphorylated and activated form of MAD

(Human SMAD1 homolog), and could be directly involved in the nuclear import of MAD (59). One can speculate that in the process of shuttling molecules from cytoplasm to nucleus, the nuclear import of some oncogenic factors including c-Myc protein might be supported by the TMEM209-NUP205 complex. In fact, the amounts of STAT3 and p65 (a subunit of NF-kB) proteins were significantly reduced by the loss of TMEM209, indicating that the TMEM209-NUP205 complex is likely to be involved in nuclear transport of various nuclear proteins in addition to c-Myc.

In summary, human TMEM209 has an essential role in the growth of lung cancer cells through its interaction with NUP205 and regulation of the nuclear transport of c-Myc (**Fig. 20**). My data indicate that TMEM209 may be a good molecular target for the development of novel treatment for lung cancer.

6. References

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7. References

Cell line	Histology	Resource Distributer
A549	ADC	ATCC (American Type Culture Collection)
LC319	ADC	Aichi Cancer Center
PC14	ADC	RIKEN BRC (BioResource Center)
NCI-H1373	ADC	ATCC (American Type Culture Collection)
NCI-H1781	ADC	ATCC (American Type Culture Collection)
SKMES-1	SCC	ATCC (American Type Culture Collection)
LU61	SCC	Central Institute for Experimental Animals
NCI-H520	SCC	ATCC (American Type Culture Collection)
NCI-H1703	SCC	ATCC (American Type Culture Collection)
NCI-H2170	SCC	ATCC (American Type Culture Collection)
LX1	LCC	Central Institute for Experimental Animals
DMS114	SCLC	ATCC (American Type Culture Collection)
DMS273	SCLC	ECACC (European Collection of Animal Cell Cultures)
SBC-3	SCLC	JCRB (Japanese Collection of Research Bioresources)
SBC-5	SCLC	JCRB (Japanese Collection of Research Bioresources)

ADC:adenocarcinoma
SCC:squamous cell carcinoma
LCC:large cell carcinoma
SCLC:small cell carcinoma

Table. 1 Resource and histological type of lung cancer cell lines

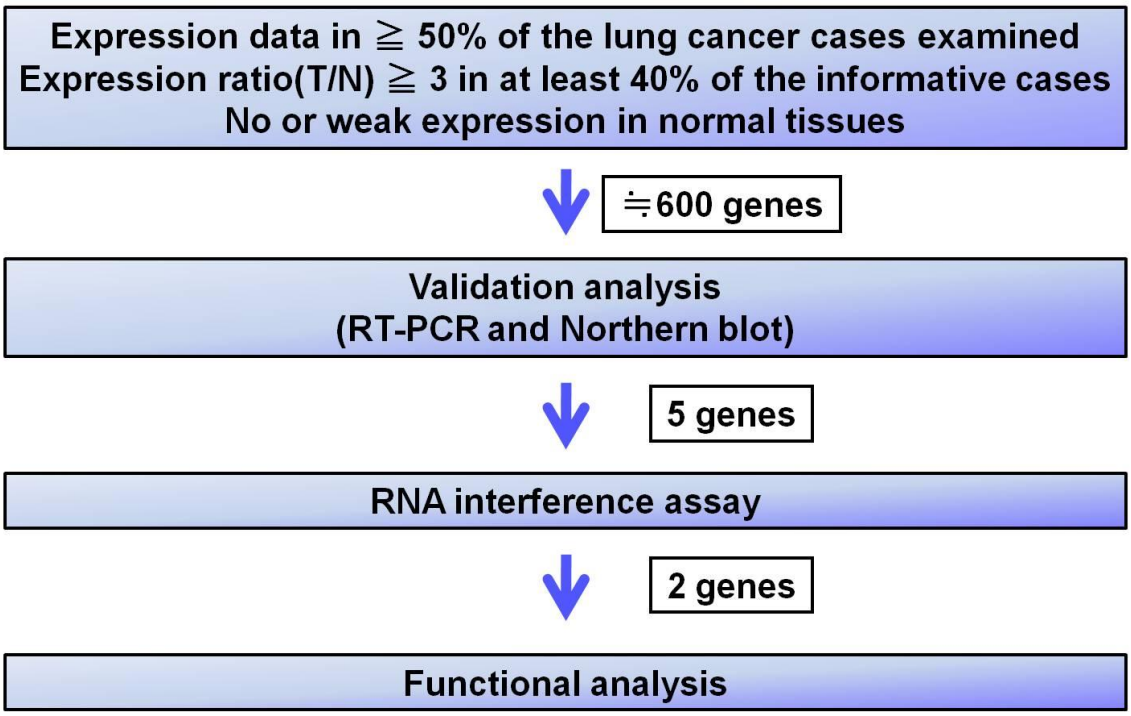

Figure. 1 Strategy for molecular target discovery

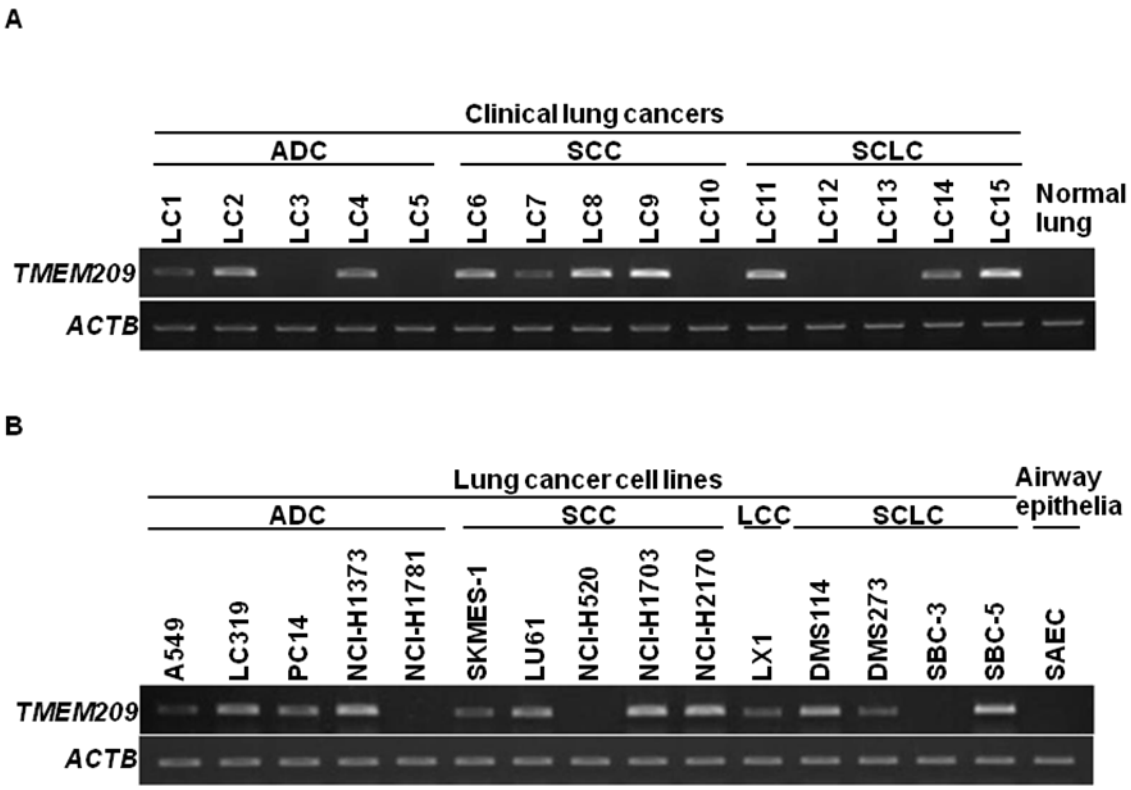

Figure. 2 TMEM209 expression in lung cancers and normal tissues.

Figure. 2 TMEM209 expression in lung cancers and normal tissues.

A, Expression of *TMEM209* in clinical samples of NSCLC and SCLC, and normal lung tissues, analyzed by semiquantitative RT-PCR. I prepared appropriate dilutions of each single-stranded cDNA generated from mRNAs of lung cancer samples, using β-actin (*ACTB*) expression as a quantitative control. ***B***, Expression of *TMEM209* in lung cancer cell lines examined by semiquantitative RT-PCR.

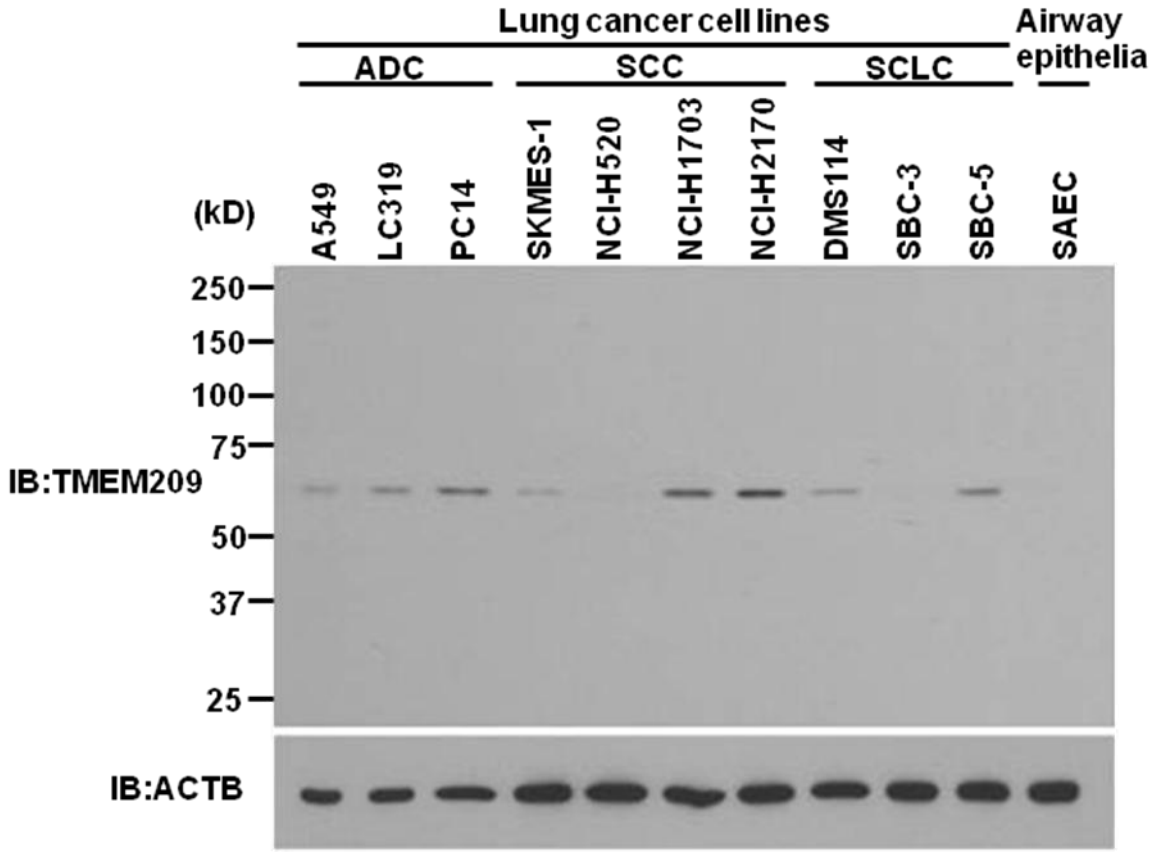

Figure. 3 Expression of TMEM209 in lung cancer cell lines.

Figure. 3 Expression of TMEM209 in lung cancer cell lines.

Expression of TMEM209 protein in lung cancer cell lines examined by western blot analysis.

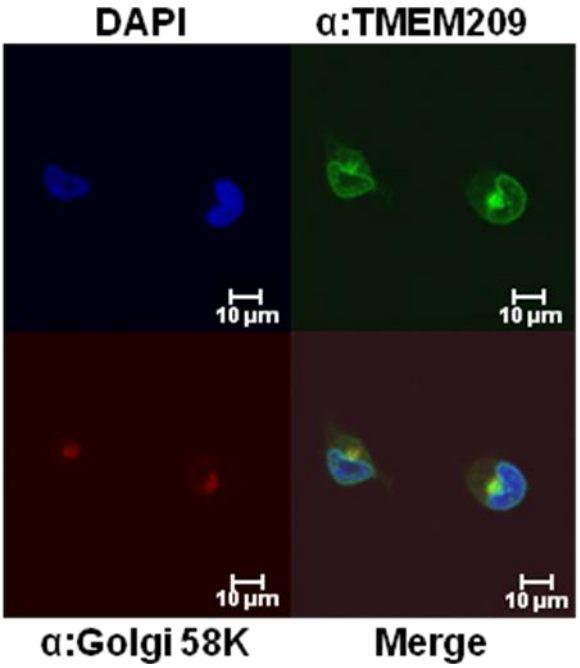

Figure. 4 Subcellular localization of TMEM209 in lung cancer cells.

Figure. 4 Subcellular localization of TMEM209 in lung cancer cells.

Subcellular localization of endogenous TMEM209 protein in lung cancer SBC-5 cells.

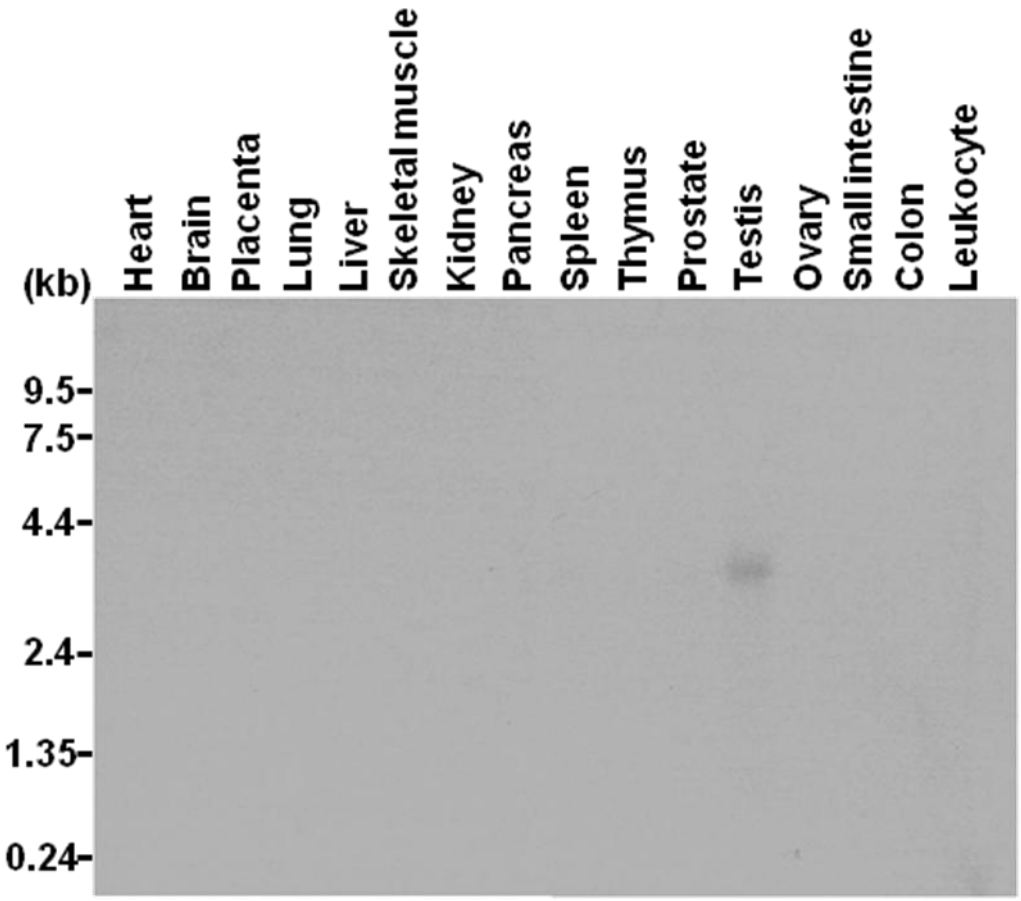

Figure. 5 Expression of *TMEM209* in normal human tissues.

Figure. 5 Expression of *TMEM209* in normal human tissues.

Expression of *TMEM209* in normal human tissues detected by northern blot analysis.

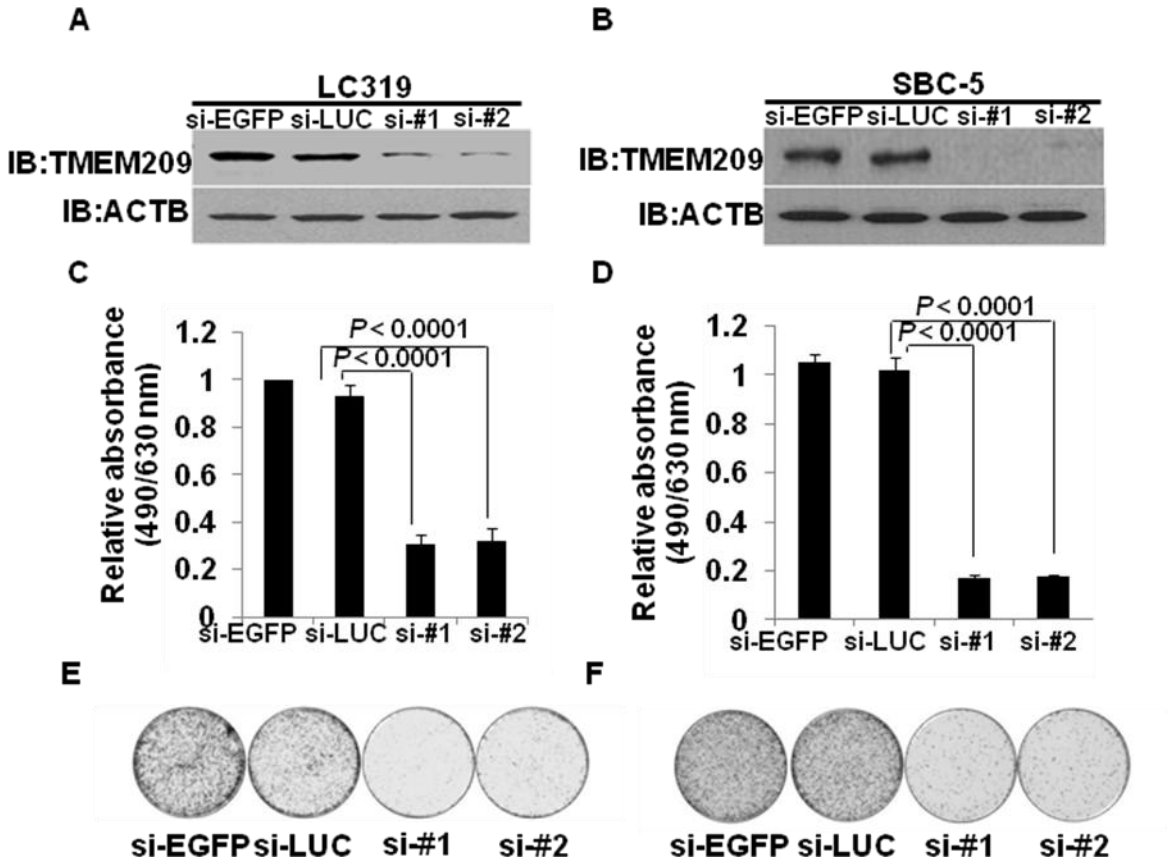

Figure. 6 Growth suppression by siRNA against TMEM209.

Figure. 6 Growth suppression by siRNA against TMEM209.

A, ***B***, Expression of TMEM209 by the treatment with si-TMEM209 (si-#1 or si-#2) or control siRNAs (si-EGFP or si-LUC) in LC319 cells and SBC-5 cells, analyzed by western blot analysis. ***C***, ***D***, Viability of LC319 cells and SBC-5 cells evaluated by MTT assay by the treatment with si-TMEM209 (si-#1 or si-#2), si-EGFP, or si-LUC. All assays were done thrice, and in triplicate wells. ***E***, ***F***, Colony formation assays of LC319 cells and SBC-5 cells transfected with si-TMEM209 (si-#1 or si-#2) or control siRNAs.

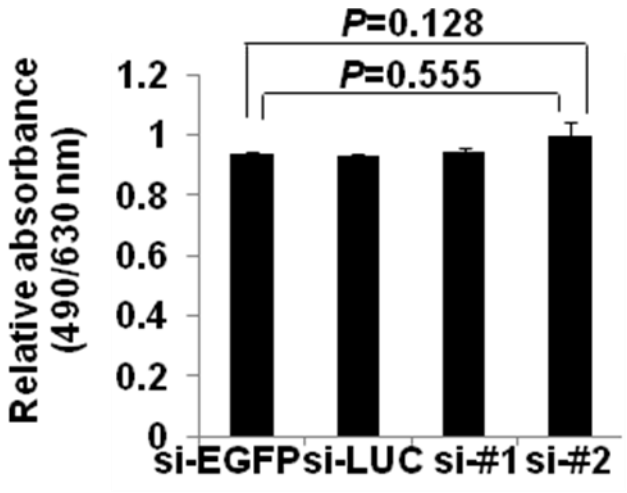

Figure. 7 Cell growth assay.

Figure. 7 Cell growth assay.

Viability of SBC-3 cells evaluated by MTT assay after treatment with si-TMEM209 (si-#1 or si-#2), si-EGFP, or si-LUC.

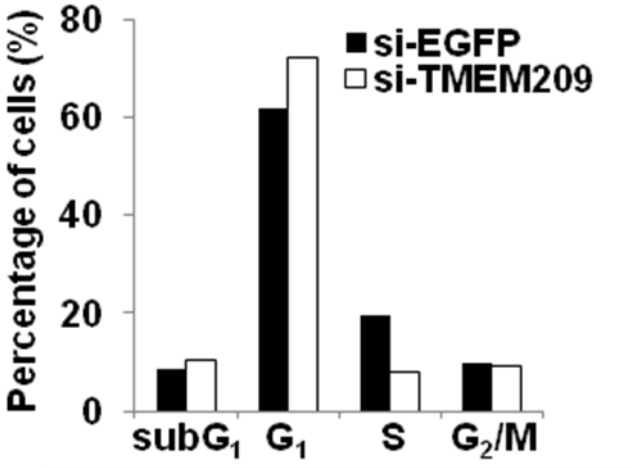

Figure. 8 Suppression effect of TMEM209 on cell cycle.

Figure. 8 Suppression effect of TMEM209 on cell cycle.

Cell cycle population change in SBC-5 cells after treatment of siRNA against TMEM209 detected by FACS analysis.

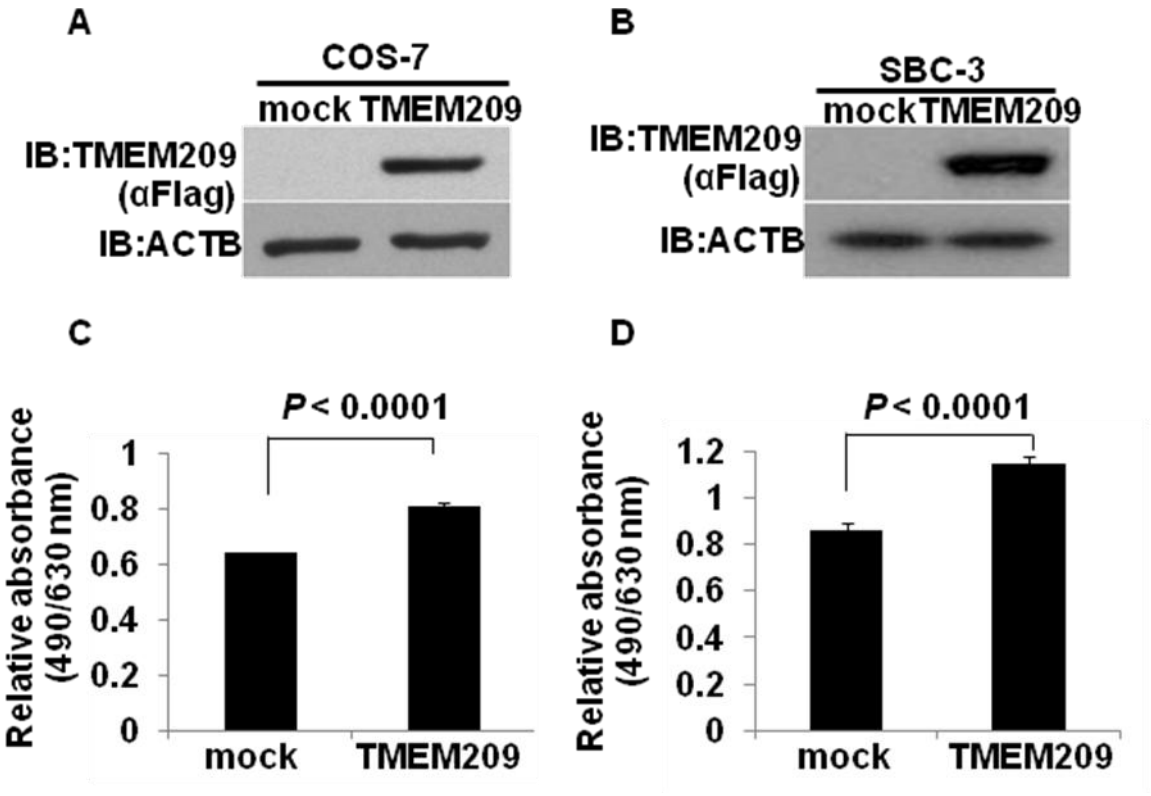

Figure. 9 Growth promoting effect of TMEM209.

Figure. 9 Growth promoting effect of TMEM209.

A, ***B***, Transient expression of TMEM209 in COS-7 and SBC-3 cells detected by western blot analysis. ***C***, ***D***, Assays demonstrating the growth promoting effect of transient introduction of TMEM209 in COS-7 and SBC-3 cells. Assays were done thrice and in triplicate wells.

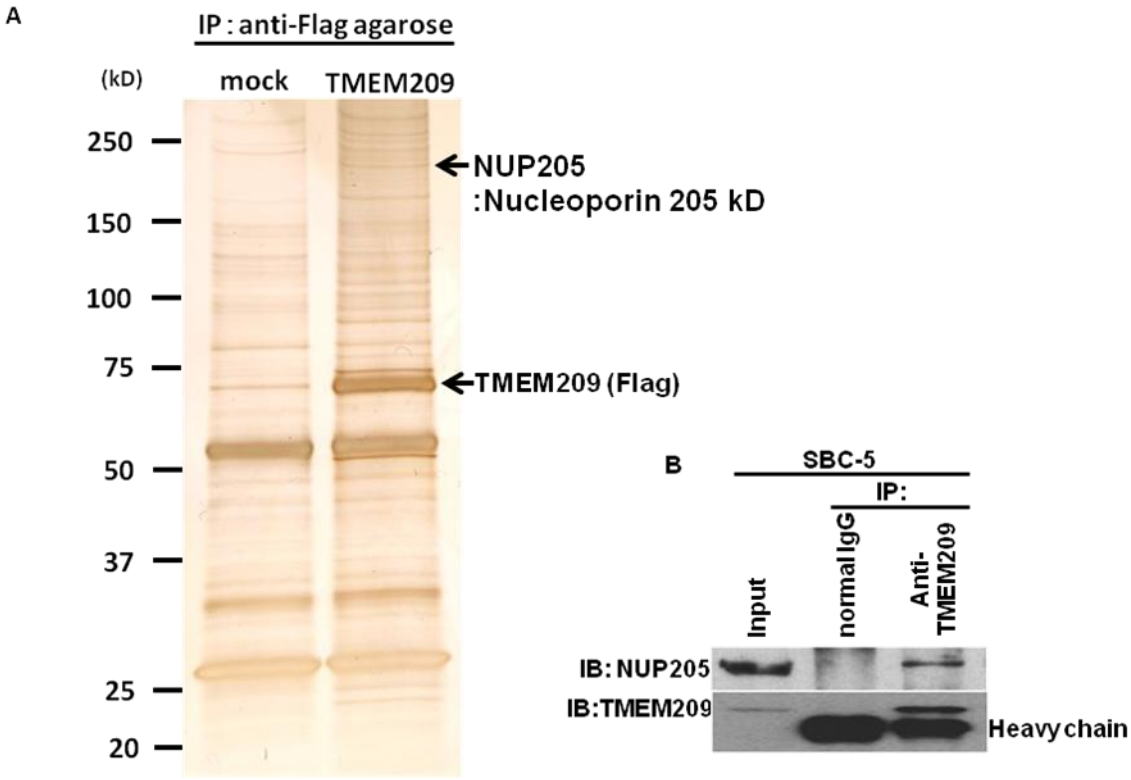

Figure. 10 Interaction of TMEM209 with NUP205.

Figure. 10 Interaction of TMEM209 with NUP205.

A, Silver staining of SDS-PAGE gels that contained immunoprecipitated lysates of lung cancer SBC-5 cells, which were transfected with Flag-tagged TMEM209 expression vector or mock vector, with anti-Flag M2 agarose. ***B***, Interaction of endogenous TMEM209 with NUP205. Lysates of SBC-5 cells were immunoprecipitated with TMEM209 antibody. Precipitated proteins were separated by SDS-PAGE and western blot analysis was performed with NUP205 antibody.

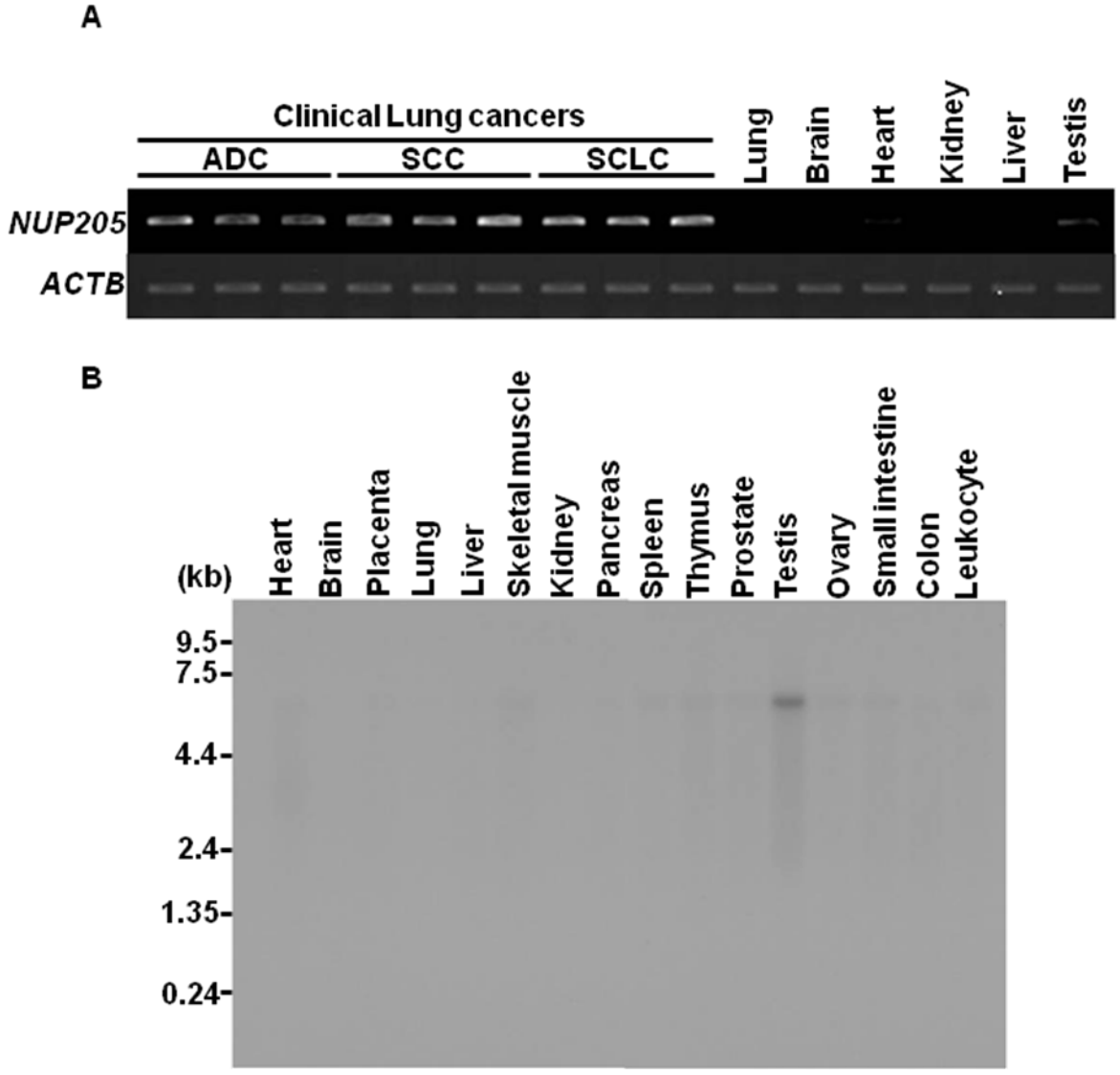

Figure. 11 Expression of NUP205 in lung cancer and normal human tissues.

Figure. 11 Expression of NUP205 in lung cancer and normal human tissues.

A*,** Expression of *NUP205* in clinical samples of NSCLC and SCLC, and in normal tissues, analyzed by semiquantitative RT-PCR. ***B, Expression of *NUP205* in normal human tissues detected by northern blot analysis.

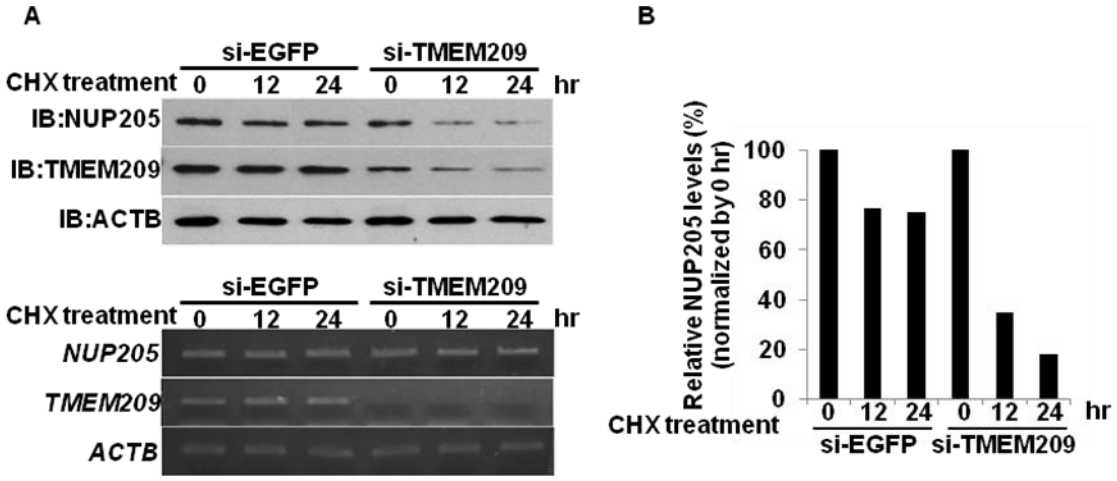

Figure. 12 TMEM209 might stabilize NUP205 protein.

Figure. 12 TMEM209 might stabilize NUP205 protein.

A, The levels of TMEM209 and NUP205 proteins as well as *TMEM209* and *NUP205* mRNAs detected by western blot analysis and semi-quantitative RT-PCR analysis in SBC-5 cells that had been transfected with si-TMEM209 after the treatment with cycloheximide (CHX). ***B***, Relative NUP205 protein levels at each time point (0, 12, and 24 hours) quantified by image J software and normalized by its levels before the treatment with protein synthesis inhibitor cycloheximide (0 hour).

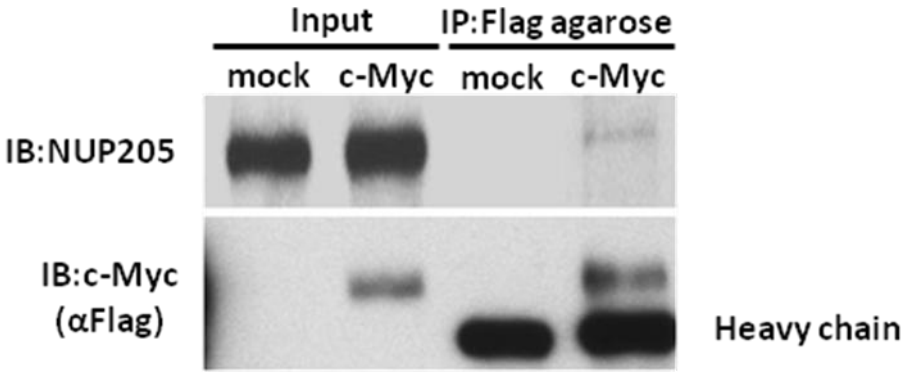

Figure. 13 Interaction of NUP205 with c-Myc.

Figure. 13 Interaction of NUP205 with c-Myc.

The lysate of SBC-5 cells that were transfected with mock or c-Myc expression vector were immunoprecipitated with ant-Flag agarose. Precipitated proteins were separated by SDS-PAGE and western blot analysis was performed using anti-NUP205 antibody.

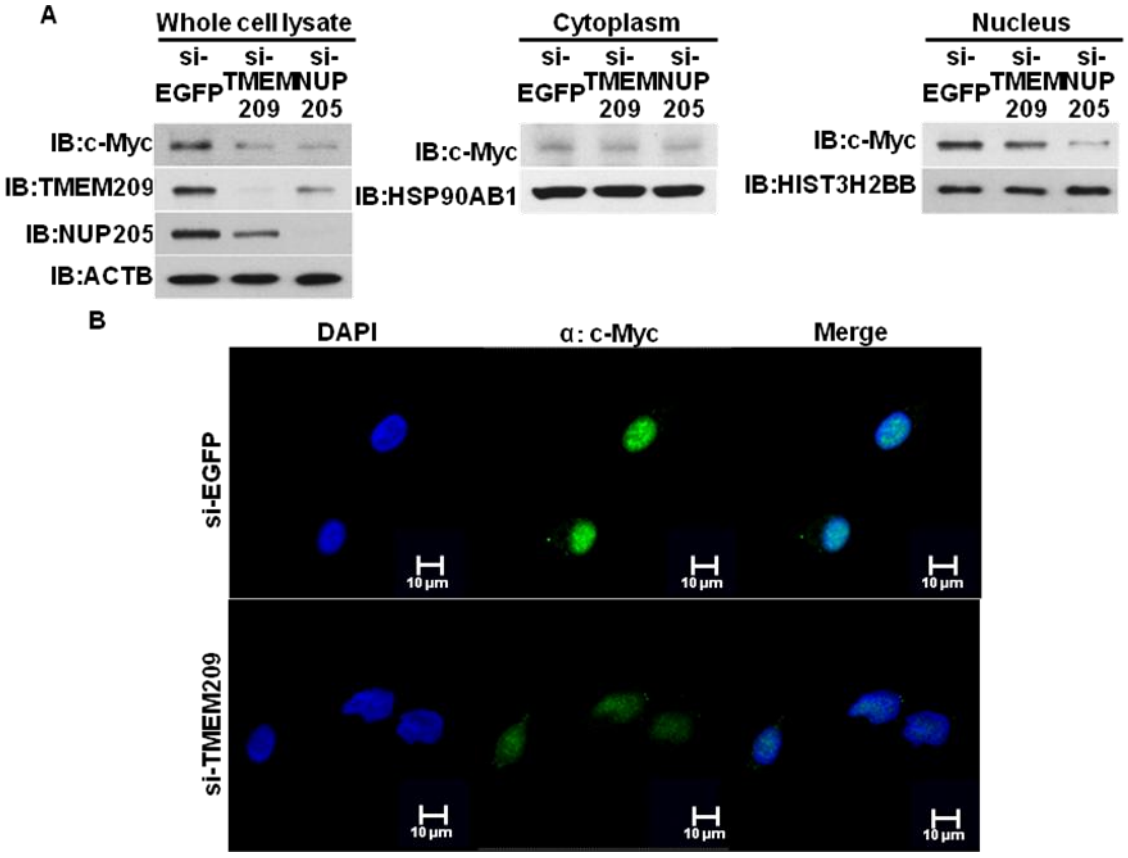

Figure. 14 Reduction of c-Myc protein after transfection of si-TMEM209 or NUP205 without MG132 treatment.

Figure. 14 Reduction of c-Myc protein after transfection of si-TMEM209 or NUP205 without MG132 treatment.

A, Suppression of TMEM209 or NUP205 reduced c-Myc protein in SBC-5 cells without proteasome inhibitor, MG132, detected by western blot analysis. B, Reduction of c-Myc levels in SBC-5 cells detected by immunocytochemistry after knockdown of TMEM209 expression with siRNAs without proteasome inhibitor, MG132.

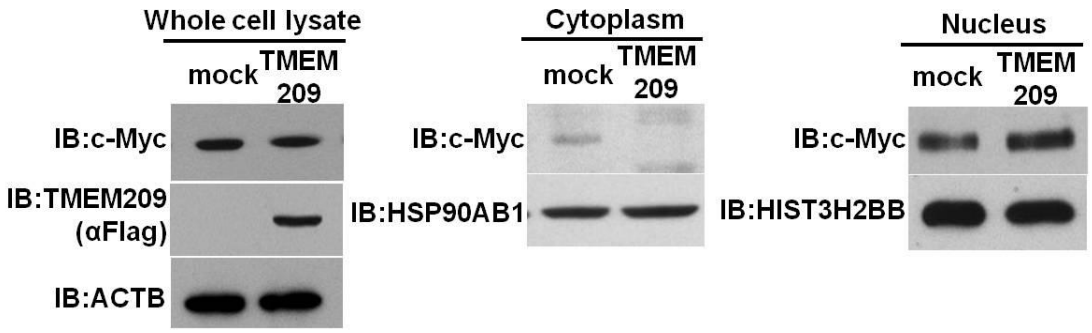

Figure. 15 Increase of c-Myc protein after introduction of TMEM209 expression vector without MG132 treatment.

Figure. 15 Increase of c-Myc protein after introduction of TMEM209 expression vector without MG132 treatment.

A, Ectopic expression of TMEM209 increased c-Myc protein in SBC-3 cells without proteasome inhibitor, MG132, detected by western blot analysis.

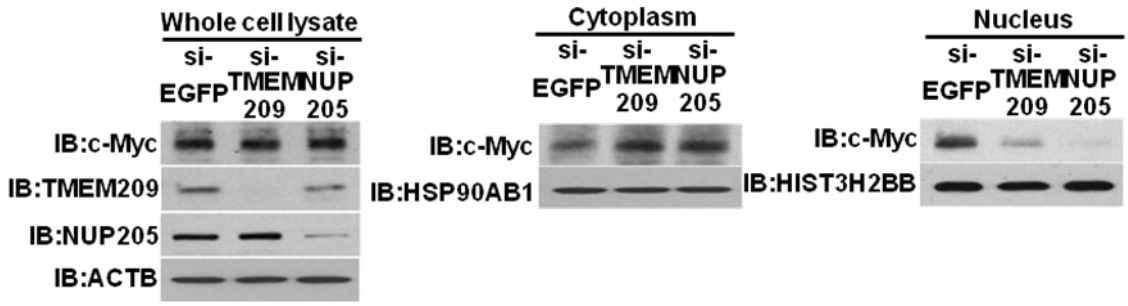

Figure. 16 Nuclear levels of c-Myc might be regulated by TMEM209-NUP205 complex.

Figure. 16 Nuclear levels of c-Myc might be regulated by TMEM209-NUP205 complex.

Attenuation of nuclear levels of c-Myc protein in SBC-5 cells transfected with si-TMEM209 or si-NUP205 in the culture condition with proteasome inhibitor, MG132.

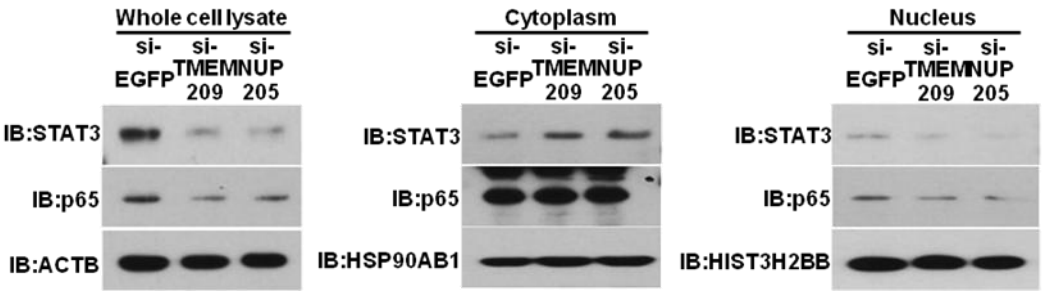

Figure. 17 Reduction of STAT3 and p65 proteins after transfection of si-TMEM209 or NUP205 with MG132 treatment.

Figure. 17 Reduction of STAT3 and p65 proteins after transfection of si-TMEM209 or NUP205 with MG132 treatment.

Suppression of TMEM209 or NUP205 reduced STAT3 and p65 proteins in SBC-5 cells with proteasome inhibitor, MG132, detected by western blot analysis.

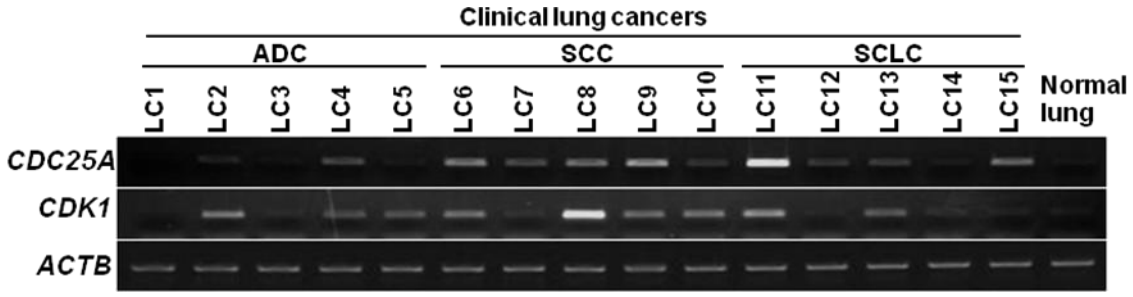

Figure. 18 Expression of CDC25A and CDK1 in clinical lung cancer samples.

Figure. 18 Expression of CDC25A and CDK1 in clinical lung cancer samples.

Expression of *CDC25A* and *CDK1* in clinical samples of NSCLC and SCLC, and normal lung tissue, analyzed by semiquantitative RT-PCR.

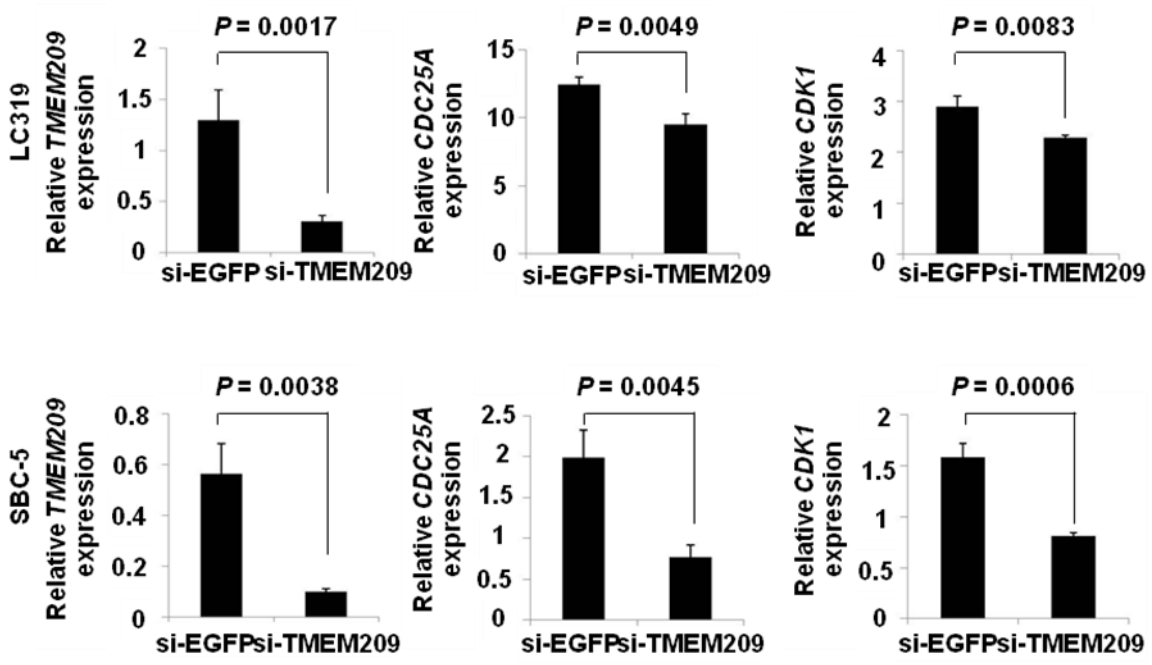

Figure. 19 The c-Myc target genes might be regulated by TMEM209.

Figure. 19 The c-Myc target genes might be regulated by TMEM209.

The down-regulation of c-Myc target genes, *CDC25A* and *CDK1* in LC319 and SBC-5 cells after the knockdown of TMEM209 expression with siRNAs.

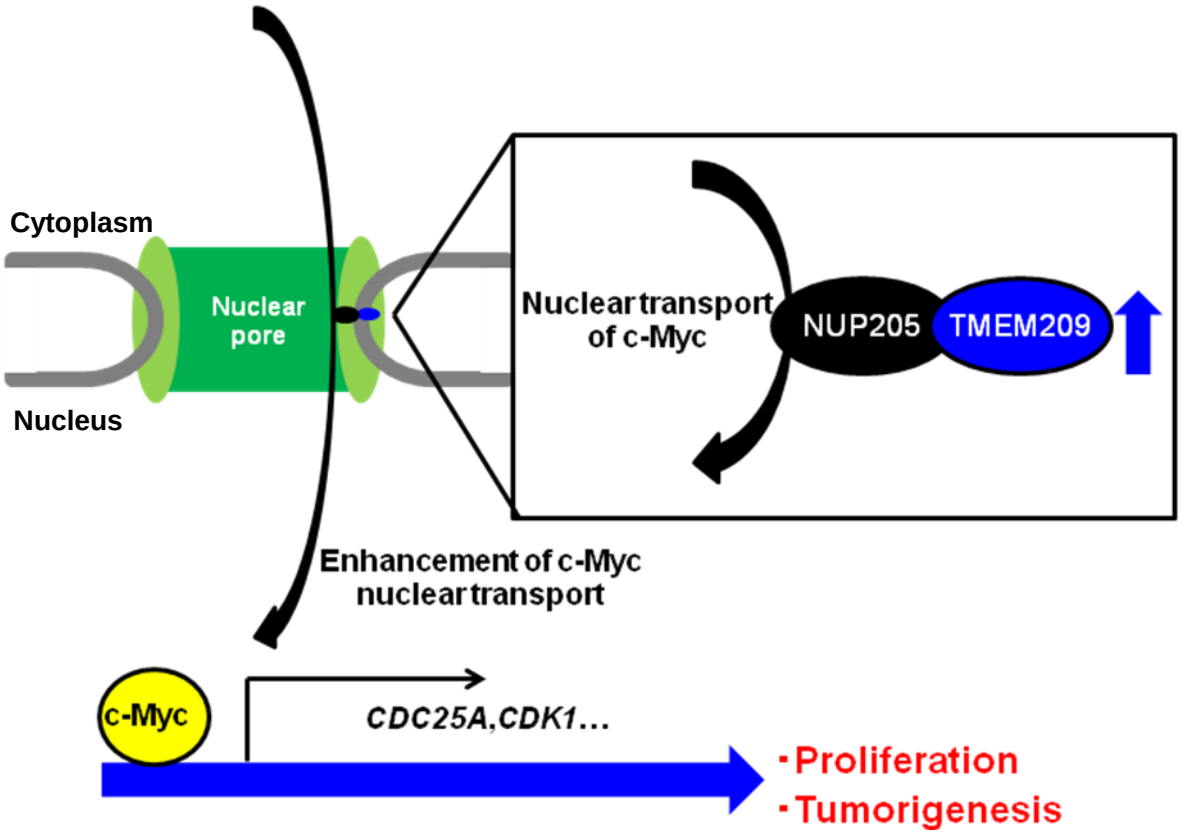

Figure. 20 Hypothesis of tumorigenesis by TMEM209.

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