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**Knockdown of RPL9 expression
inhibits colorectal carcinoma growth
via the inactivation of
Id-1/NF- κ B signaling axis**

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Department of Medical Science

The Graduate School, Yonsei University

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Directed by Professor Min-Goo Lee

The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

In Hye Baik

December 2016

This certifies that the Master's Thesis
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In Hye Baik

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ABSTRACT

Knockdown of RPL9 expression inhibits colorectal carcinoma growth via the inactivation of Id-1/NF- κ B signaling axis

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Ribosomal protein L9 (RPL9), a component of the 60S subunit for protein synthesis, is up-regulated in human colorectal cancer. Here we investigated whether RPL9 gained extraribosomal function during tumorigenesis and that targeting of RPL9 with small interfering (si) RNA could alter the course of colorectal cancer progression. Our results showed that siRNA knockdown of RPL9 suppresses colorectal cancer (CRC) cell growth and long-term colony formation through an increase in sub-G1 cell population and a strong induction of apoptotic cell death. To obtain insights into the molecular changes in

response to RPL9 knockdown, global changes in gene expression were examined using RNA sequencing. It revealed that RPL9-specific knockdown led to dysregulation of 918 genes in HCT116 and 3178 genes in HT29 cells. Among those, 296 genes showed same directional regulation (128 up- and 168 down-regulated genes) and were considered as a common RPL9 knockdown signature. Particularly, we found through a network analysis that Id-1, which is functionally associated with activation of NF- κ B and cell survival, was commonly down-regulated. Subsequent Western blot analysis affirmed that RPL9 silencing induced the decrease in the levels of Id-1 and phosphorylated I κ B α in both HCT116 and HT29 cells. Also, the same condition decreased the levels of PARP-1 and pro-caspase 3, accelerating apoptosis. Furthermore, inhibition of RPL9 expression significantly suppressed the growth of human CRC xenografts in nude mice. These findings indicate that the function of RPL9 is correlated with Id-1/NF- κ B signaling axis and suggest that targeting RPL9 could be an attractive option for molecular therapy of colorectal cancer.

Key words: RPL9, RNA interference, extraribosomal function, apoptosis

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I. INTRODUCTION

Colorectal cancer is the third most common cancer worldwide and the fourth leading cause of cancer-related death.¹ The patients diagnosed with colorectal cancer often develop colorectal metastases and about 80% to 90% of these patients are found with unresectable metastatic liver disease.² Colorectal cancer is associated with the progressive accumulation of mutations in oncogenes and tumor suppressor genes. These tumor-specific mutations are the key to understanding the cellular processes underlying tumorigenesis and could be

used as diagnostic and therapeutic target.³

Ribosomal proteins (RPs) are one of the components of ribosomes and there are approximately 80 RPs in eukaryotes.⁴ RPs are known to stabilize specific rRNA structures in mature ribosomal subunits and promote correct folding of rRNAs during ribosomal assembly. However, not only RPs have crucial role in protein biosynthesis, but recent studies have also associated them to human congenital disorders and cancers.⁵ For example, RPS4 has been implicated in Turner's syndrome,⁶ and mutant RPS19 was found in individuals with Diamond–Blackfan anemia.⁷ Hence, RPs' functions that are independent of protein biosynthesis are called extraribosomal functions. The extraribosomal functions include transcription and repair, RNA splicing and modification, cell growth and proliferation, regulation of apoptosis and development.⁸ Moreover, knockdown of many individual RPs resulted in p53 accumulation, cell death, and defective development.^{8,9} Various RPs have been found to be overexpressed in many cancer cells and are associated with the development and progression of malignant cancers.⁹ For instance, in gastric cancer cell line, RPL6 promoted cell cycle progression and cancer cell proliferation by up-regulating the expression of cyclin E.¹⁰ Also, phosphorylated RPS3 activated anti-apoptotic protein TRAF2 and enhanced radio-resistance in non-small cell lung cancer

cells.¹¹

Ribosomal protein L9 (RPL9) is a component of the 60S subunit that belongs to the L6P family of ribosomal proteins. From what we have gathered, RPL9's extraribosomal function is not known, but one of the studies have suggested that human RPL9 appears to be involved with the uncontrolled growth by promoting stress-mediated survival and that RPL9 is more like anti-apoptotic-encoding RP genes in yeast.¹² In addition, there have been reports that RPL9 gene is overexpressed in colorectal cancer compared to normal colon,^{5,13,14} which provides the possibility that RPL9 might be involved in tumorigenesis of colorectal cancer.

In the present study, our aim was to define the extraribosomal function of RPL9 in colorectal cancer progression. Using RNA interference (RNAi) techniques, we targeted RPL9 gene in colorectal cancer cells, HCT116 and HT29. We found that silencing of RPL9 inhibited colorectal cancer cell growth *in vitro* as well as *in vivo* and caused apoptotic cell death. The anti-proliferative effects were induced through a common subset of molecular alterations of 296 mRNA transcripts, including downregulation of Id-1 (inhibitor of DNA binding-1).¹⁵ Western blotting proved that the decrease in Id-1 protein level by RPL9

knockdown was accompanied with the reduction in I κ B α phosphorylation, which suggests that RPL9 is functionally associated with Id-1/NF- κ B signaling pathway and that the regulation of RPL9 expression may be a potential therapeutic target in colorectal cancer treatment.

II. MATERIALS AND METHODS

1. Cell culture and siRNA transfection

Human colorectal cancer cell lines, HCT116 and HT29, were purchased from the Korean Cell Line Bank (KCLB, Seoul, South Korea). Both HCT116 and HT29 cells were cultured in RPMI-1640 medium (Corning, VA, USA) supplemented with 10% of fetal bovine serum (HyClone, South Logan, UT, USA) and 1% of penicillin/streptomycin solution (HyClone), in a 37°C humidified incubator with a mixture of 95% air and 5% CO₂. The phenotypes of these cell lines have been authenticated by the KCLB. Cells were plated at 30% density 24 hr before transfection. 15 nM siRNA was mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in Opti-MEM (Invitrogen) and the medium was replaced 6 hr after transfection. RPL9 siRNA duplexes (si-RPL9) were chemically synthesized by Ambion (Austin, TX, USA, siRNA ID# s226955). The negative control siRNA (si-NC) that does not target any endogenous transcript was used for control experiments. The sequences of si-NC (Bioneer, Daejeon, South Korea) are as follows: 5'-ACGUGACACGUUCGGAGAA(UU)-3'(sense) and 5'-UUCUCCGAACGUGUCACGU-3'(antisense).

2. Cell proliferation assay

Cell growth was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Log phase cells were trypsinized into single cell suspension and HCT116 (2×10^3 cells) and HT29 (1×10^3 cells) were seeded into 96-well plates. After 24 hr, cells were transfected as described above and after Day 1, Day 2, Day 3, and Day 4 of cell culture, the CCK-8 solution was added into each well. After 2 hr, OD value was measured at a wavelength of 450 nm using a VersaMax microplate reader (Molecular Device, Sunnyvale, CA, USA).

3. Quantitative real-time RT-PCR (RT-qPCR)

The changes in target gene expression were detected using RT-qPCR. Total RNA was isolated using TRIzol (Ambion) and synthesized into cDNA by using 1st strand cDNA Synthesis Kit (Takara Biotech, Kusatsu, Shiga, Japan) according to the manufacturer's instruction. cDNAs were then amplified using corresponding pair of primers (RPL9 forward, 5'-GCACAGTTATCGTGAAGG GC-3'; RPL9 reverse, 5'-TTACCCCACCATTTGTCAACC-3' and GAPDH forward, 5'-GGGAGCCAAAAGGGTCATCATCTC-3'; GAPDH reverse, 5'-CATGCCAGTGAGCTTCCCGTTC-3') synthesized by Macrogen (Seoul,

South Korea). The relative quantification of mRNA was measured by LightCycler 96 (Roche, Basel, Switzerland) according to the manufacturer's instructions and quantified using LightCycler 96 software version 1.1, comparing with the Ct (threshold cycle) values of each target gene. The mRNA levels of GAPDH were used for normalization.

4. Clonogenic assay

siRNA transfected cells were seeded into three independent wells of 6-well culture plates (1×10^3 cells per well) and cultured at 37 °C in 5% CO₂. Cells were maintained without medium change to let the viable cells propagate to sizable colonies for quantification. The colonies were fixed with methanol and then stained with 0.5% crystal violet for 30 min at room temperature. The number of colonies formed in each well was counted under the microscope and statistically analyzed.

5. Cell cycle analysis

Cells were cultured in 60 mm culture dishes and harvested at 72 hr after siRNA transfection. Cells were washed with cold PBS, and then fixed 24 hr with 70% cold ethanol at -20 °C. Cells were washed with cold PBS again and incubated in

a dark condition with propidium iodide (PI) staining solution containing RNase A (BD Biosciences, San Diego, CA, USA) for 30 min at room temperature. The cell cycle was measured by FACSVerse flow cytometry (BD Bioscience) according to the manufacturer's instructions and quantified using FlowJo software program.

6. Detection of apoptosis

Cells were cultured in 60 mm culture dishes and harvested at 48 hr and 72 hr after siRNA transfection. Apoptotic cells were stained with Annexin V and PI using FITC Annexin V Apoptosis Detection Kit I (BD Bioscience) following the manufacturer's instruction. The cell death was measured by FACSVerse flow cytometry (BD Bioscience) and quantified using FlowJo software program.

7. RNA sequencing

Total RNA was extracted 48 hr after siRNA transfection using the RNeasy mini kit (Qiagen, Valencia, CA, USA). The quantity of the total RNA was evaluated using RNA electropherograms (Bio-Rad Experion, Hercules, CA, USA); RNA quality was assessed based on the RNA quality indicator (RQI). The total RNA

from each sample with a RQI value of 8.0 or higher was used. The resulting mRNA samples were processed for sequencing libraries using the Illumina TruSeq Stranded mRNA sample preparation kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocols. RNA sequencing was performed using the Illumina HiSeq 2500 to generate non-directional, paired-end 100-base-pair reads. Quality-filtered reads were mapped to the human reference genome sequence hg19 (UCSC Genome Bioinformatics, <https://genome.ucsc.edu>) using tophat2 (<http://ccb.jhu.edu/software/tophat>). The relative transcript abundance was estimated by counting the fragments per kilobase of the exon model per million mapped sequence reads (FPKM), and differentially expressed genes were evaluated using the cufflinks package (<http://cole-trapnell-lab.github.io/cufflinks>). The significantly overlapping pathways and Gene Ontology categories with differentially expressed genes were analyzed using DAVID (<http://david.abcc.ncifcrf.gov>) and IPA (ingenuity pathway analysis, www.ingenuity.com).

8. Western blot analysis

Cells were suspended in RIPA buffer (Thermo Scientific, Rockford, IL, USA) containing 0.01 % of a protease and phosphatase inhibitor cocktail (Thermo

Scientific) at 48 hr after siRNA transfection. The amount of protein was quantified by using the Pierce BCA Protein Assay Kit (Thermo Scientific). Nuclear–cytoplasmic fractionation was conducted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific) according to the manufacturer’s protocol. Equal amounts of total proteins were fractionated by SDS-PAGE on a 10% gel and transferred to PVDF membranes (Roche, Basel, Switzerland). 0.1% Naphthol Blue Black (NBB) (Sigma-Aldrich Korea, Seoul, Korea) was used to stain PVDF membrane to confirm equal sample loading. The membrane was blocked with 5% milk/Tris-buffered saline plus Tween 20 (TBST) and incubated with primary antibodies against human Id-1 (sc-133104), PARP-1 (sc-8007), NF- κ B p65 (sc-372), Lamin B1 (sc-30264), β -actin (sc-47778) (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase-3 (#9662, Cell Signaling, Danvers, MA, USA), Phospho-I κ B α (Ser32/36) (#9246, Cell Signaling), GAPDH (NB600-502, Novus Biologicals, Littleton, CO, USA), RPL9 (ab182556, Abcam, Cambridge, MA, USA). HRP goat anti-mouse IgG, HRP goat anti-rabbit IgG and HRP rabbit anti-goat IgG (Santa Cruz) were used as the secondary antibodies. Immunoreactive bands were visualized with an LAS-3000 Imager (Fujifilm Corporation, Tokyo, Japan).

9. Tumorigenic assay in nude mice

Tumor formation was assessed to define the effects of RPL9 silencing on tumorigenicity *in vivo*. HCT116 cells were transfected with si-NC or si-RPL9 using Lipofectamine 2000. 24 hr after transfection, cells were harvested by trypsinization and then they were washed and resuspended in RPMI mixed with Matrigel (Corning). 1×10^6 cells transfected with si-NC and si-RPL9 were injected subcutaneously into the left and right flank, respectively, of four-week old male BALB/c nude mice (Orientbio, Seongnam, Korea). Tumor formation was monitored in two day interval for 14 days after tumor injection. The tumor size was measured using a vernier caliper and calculated as $(\text{width}^2 \times \text{length} \times 1/2)$. The mice were then sacrificed and tumor weight of each mouse was evaluated. All the animal experiments were performed in accordance with the guidelines of IACUC (Institutional Animal Care and Use Committee) in Yonsei University Health System with the approval number 2015-0066.

10. Statistical analysis

All the statistical analysis was performed using Student's t-test, except for *in vivo* experiment data which was applied with Bootstrap t-test with 10,000 random repetitions. All data are shown as means \pm SEM. *P* values $\leq 0.05(*)$, \leq

0.01(**), and ≤ 0.001 (***) were considered as statistically significant.

III. RESULTS

1. RPL9 knockdown inhibits colorectal cancer cell proliferation and long-term colony formation

To test the effect of RPL9 knockdown on colorectal cancer cell survival, we transfected HCT116 and HT29 cells with si-NC or si-RPL9. After 96 hr, we found from microscopic observation that the cell viability of RPL9 knockdown cells had decreased notably (Figure 1A). In addition, we observed the maximum growth suppression of approximately 60% - 80% in both HCT116 and HT29 cells after 4 days of treatment (Figure 1B). Concordance with the phenotypic assay result, si-RPL9 effectively silenced target gene expression in both of the examined tumor cell lines (Figure 1C). Consistent with the short-term results, RPL9 knockdown effectively suppressed the long-term colony formation of HCT116 and HT29 cells at Day 7 and Day 10, respectively, by about 60% inhibition in both cell lines (Figure 2). These data indicate that RPL9 is functionally involved in colorectal cell growth.

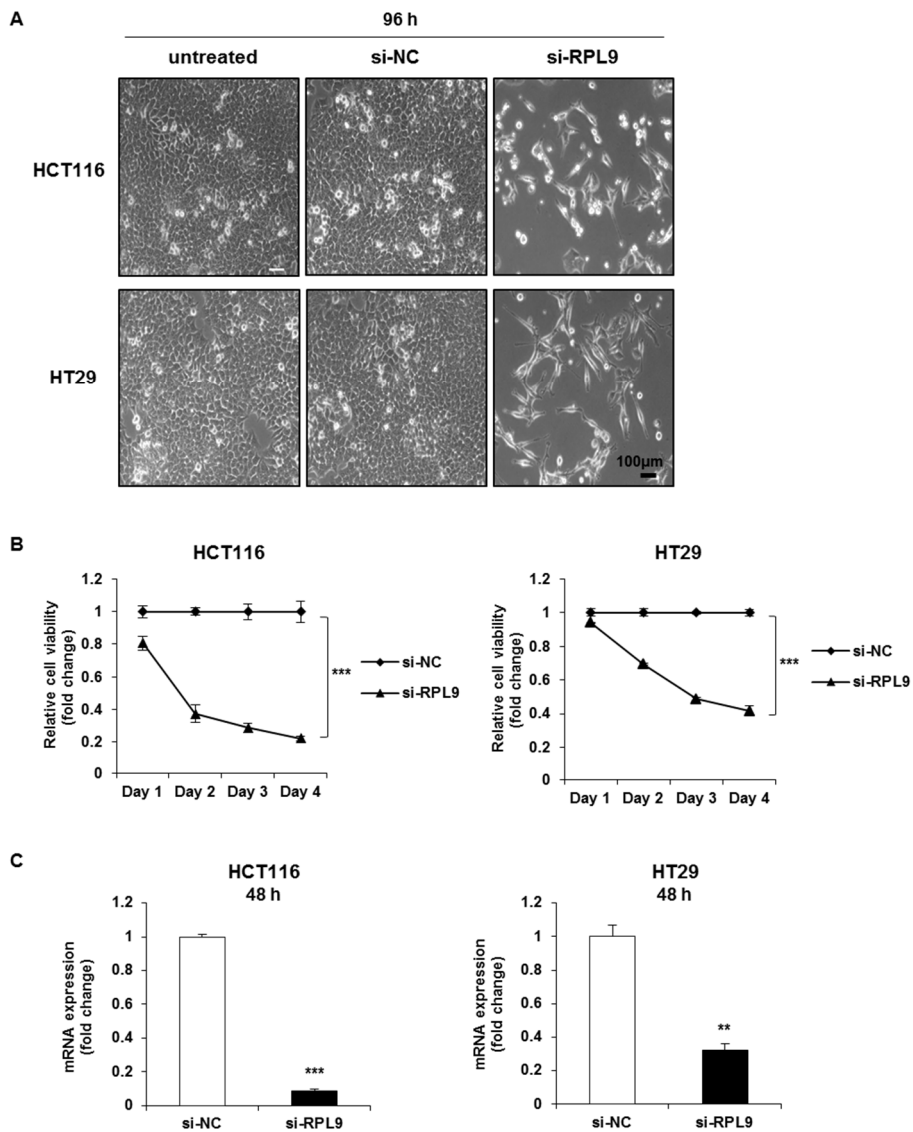


Figure 1. siRNA knockdown of RPL9 inhibits growth of colorectal cancer cells. (A) Representative light microscopy images of HCT116 and HT29 cells

96 hr after transfection. Scale bar, 100 μ m. (B) Detection of cell viability on Day 1, Day 2, Day 3, and Day 4 after transfection in HCT116 and HT29 cells. It is expressed as fold changes relative to *si-NC*. Data represent three independent experiments (***, $P < 0.001$ by Student's t-test). (C) Detection of RPL9 mRNA expression in HCT116 and HT29 cells at 48 hr after transfection. The data are shown as means \pm SEM of triplicate experiments (**, $P < 0.01$; ***, $P < 0.001$ by Student's t-test). *si-NC*, negative control siRNA; *si-RPL9*, RPL9 specific siRNA.

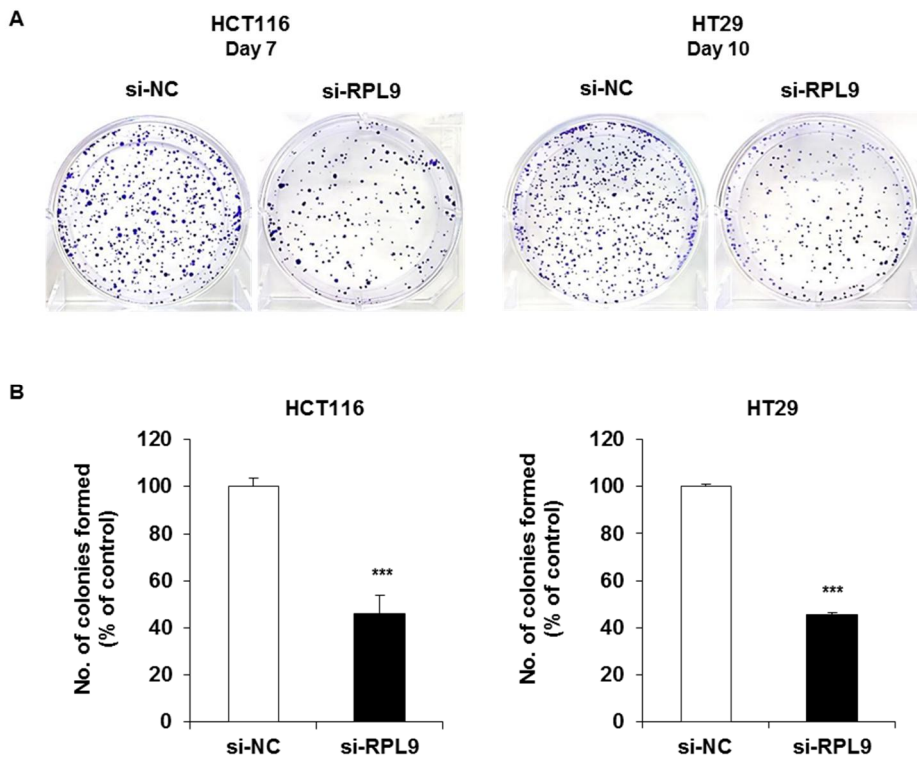


Figure 2. Knockdown of RPL9 reduces long-term colony formation in colorectal cancer cells. (A) Observation of long-term colony formation by clonogenic assay at 7 days and 10 days after transfection in HCT116 and HT29 cells, respectively. (B) The number of colonies was counted and graphed to compare effectively. The data represent three independent experiments (***, $P < 0.001$ by Student's t-test).

2. Silencing of RPL9 induces apoptosis in colorectal cancer cells.

To further investigate the role of RPL9 in colorectal cancer growth, the effect of RPL9 silencing on cell cycle progression of HCT116 and HT29 cells were analyzed with flow cytometry (Figure 3A). It revealed that, when compared to the control treatment, RPL9 knockdown cells showed increased percentage of sub-G1 population and a concomitant decrease in the G1-phase cells in both HCT116 and HT29 cells (Figure 3B). Since sub-G1 population represents apoptotic cells, we then confirmed the phenomenon by staining CRC cells with the apoptotic marker Annexin V using FACS. Consequently, we affirmed that siRNA knockdown of RPL9 significantly induced the apoptotic cell death at 72 hr after transfection (Figure 4A). Moreover, the percentages of early-apoptotic cell populations (Q3 region) plus late-apoptotic cell populations (Q2 region) in HCT116 and HT29 cells have increased in both 48 hr and 72 hr after transfection (Figure 4B). Early apoptotic cells are considered as cells that have intact plasma membrane which expose phosphatidylserine (PS) on the surface and late apoptotic cells have permeabilized plasma membrane.¹⁶ These results suggest that RPL9 knockdown induces apoptosis in colorectal cancer cells.

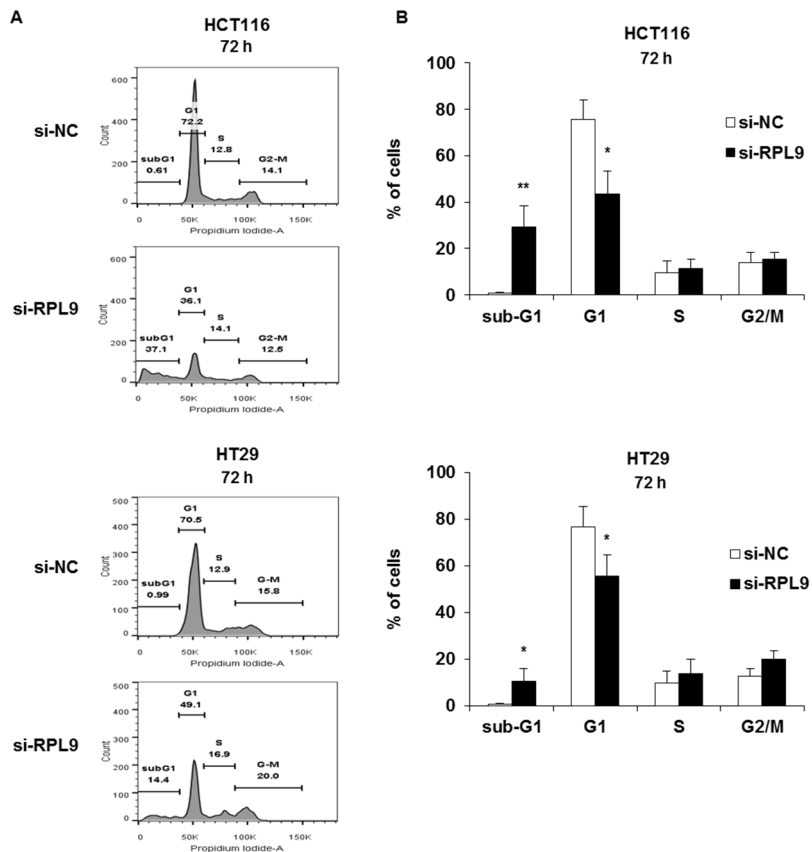


Figure 3. Silencing of RPL9 increases the sub-G1 population in HCT116 and HT29 cells. (A) FACS analysis of cell cycle progression at 72 hr after transfection with si-NC and si-RPL9 is shown in histogram. (B) For each phase of cell cycle, percentage of cells is shown in bar graph. The data are shown as means \pm SEM of triplicate experiments (*, $P < 0.05$; **, $P < 0.01$ by Student's t-test).

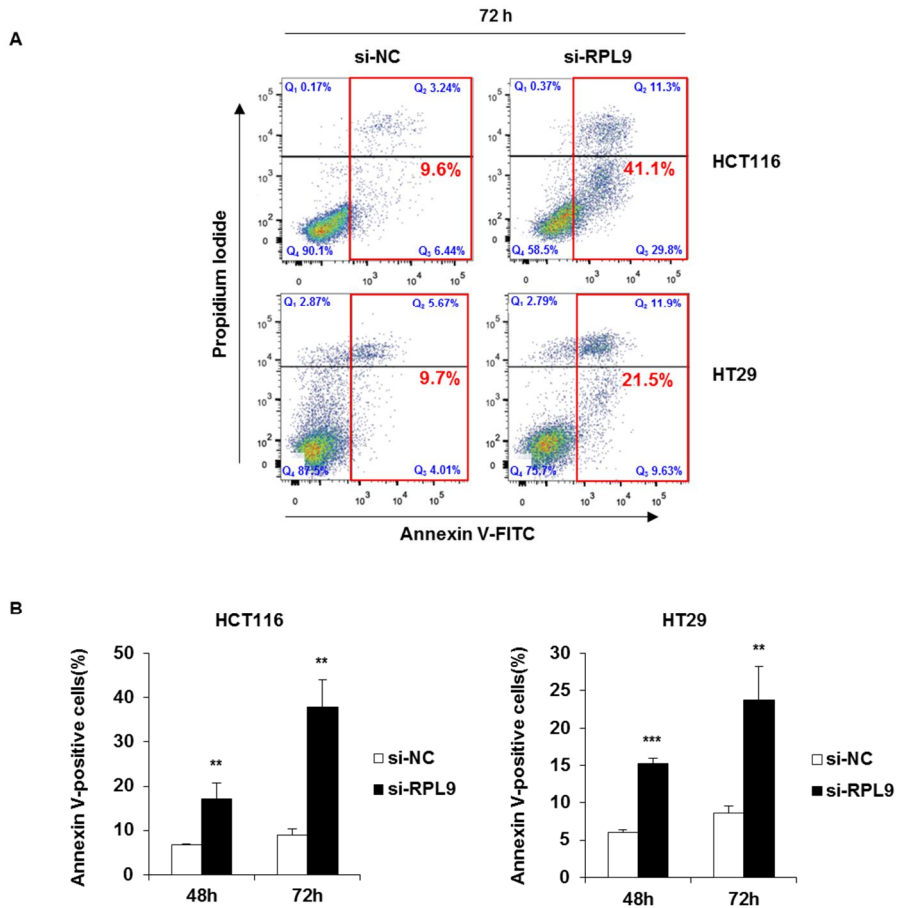


Figure 4. RPL9 silencing induces apoptosis in colorectal cancer cells. (A) Detection of the apoptotic cell population through FACS analysis with the intensity of apoptotic marker Annexin V in HCT116 and HT29 cells at 72 hr after transfection. (B) Number of Annexin V positive cells at 48 hr and 72 hr after transfection. The data represent three independent experiments (**, $P < 0.01$;

***, $P < 0.001$ by Student's t-test).

3. The function of RPL9 correlates with Id-1/NF- κ B signaling.

To explore the molecular basis of the growth inhibition caused by RPL9 silencing, we compared the global gene expression profiles of RPL9 knockdown HCT116 and HT29 cells to those of control cells transfected with si-NC using next-generation RNA sequencing. The global gene expression analysis, defined as at least a 2.0-fold change, revealed that RPL9-specific knockdown resulted in the up- and down-regulation of 918 RNA transcripts in HCT116 and 3,178 transcripts in HT29 cells (Figure 5A). Comparing these two gene sets generated a statistically significant overlap of 296 genes (128 up- and 168 down-regulated genes) which are considered as a common RPL9 knockdown signature (Figure 5B). Subsequent IPA showed that the 269 mRNA transcripts were functionally enriched in the top five networks (Table 1). In particular, as shown in (Figure 5C), we found that the expression level of Id-1, which is involved in carcinogenesis¹⁷ and NF- κ B activation,¹⁸ was down-regulated in mRNA level by RPL9 silencing. Consistent with our observation, it was previously demonstrated that Id-1 silenced colorectal cancer cells resulted in decreased proliferation and increased in apoptotic rate.¹⁵

Subsequent western blot analysis confirmed that the protein level of Id-1 was decreased in RPL9 knockdown HCT116 and HT29 cells when compared to the control (Figure 6A). In addition, a concomitant decrease in pro-caspase 3, which activate apoptotic signaling, and PARP-1, a nuclear enzyme essential for genomic stability, were observed (Figure 6A). Meanwhile, I κ B α is an inhibitory protein that binds with NF- κ B in the cytosol making NF- κ B as inactivated state. However, when I κ B α becomes phosphorylated it dissociates from NF- κ B. Then the activated NF- κ B translocates into the nucleus where it binds to the specific sequences of DNA causing changes in cell function such as promoting cell survival.¹⁹ We observed that the protein level of p-I κ B α was decreased by RPL9 silencing (Figure 6A), and NF- κ B protein level also decreased in nucleic fraction of the RPL9 knockdown colorectal cancer cells (Figure 6B). Since the expression level of housekeeping proteins may vary depending on the experimental condition,^{20,21} equal sample loading was confirmed by Naphthol Blue Black staining of the Western blot membrane (Figure 6B). In summary, RPL9 silencing decreased Id-1 expression and inactivated NF- κ B signaling pathway resulting in apoptosis (Figure 6C) of colorectal cancer cells.

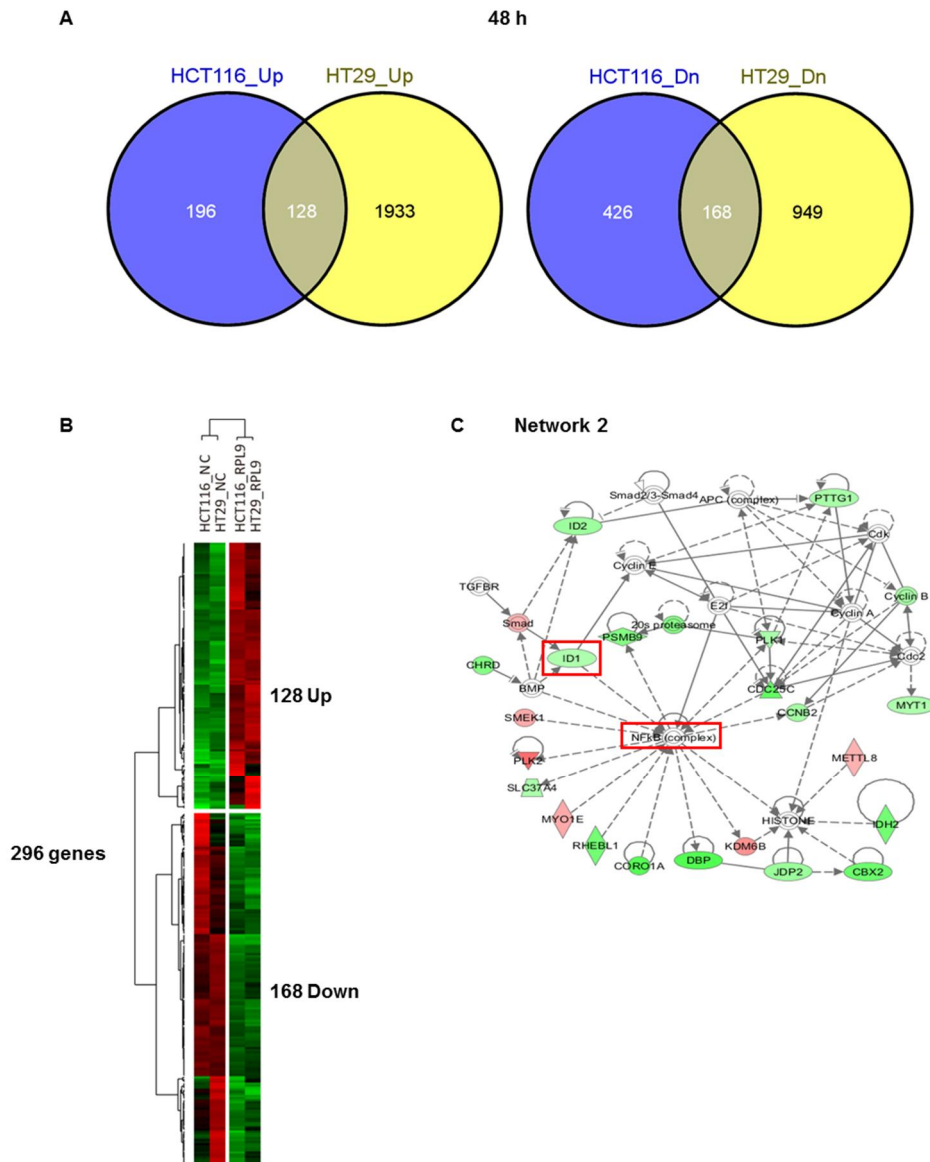


Figure 5. Transcriptomic analysis of RPL9 knockdown gene expression by RNA sequencing. (A) Venn diagrams represent the number of mRNA

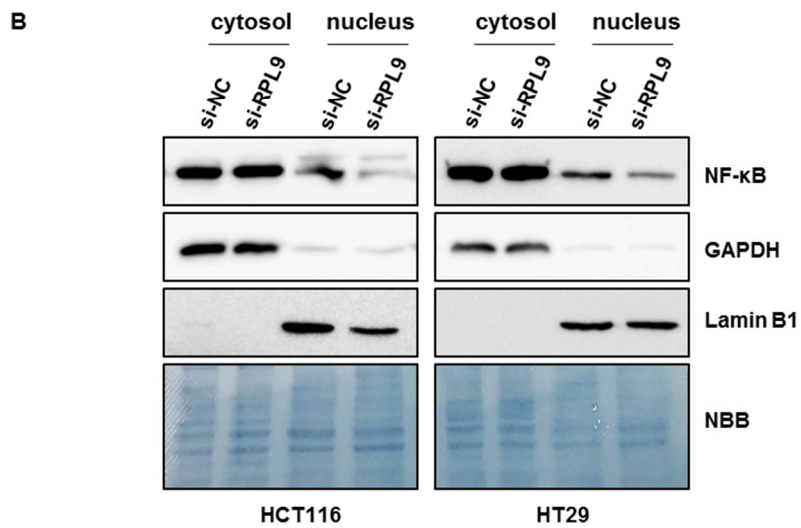
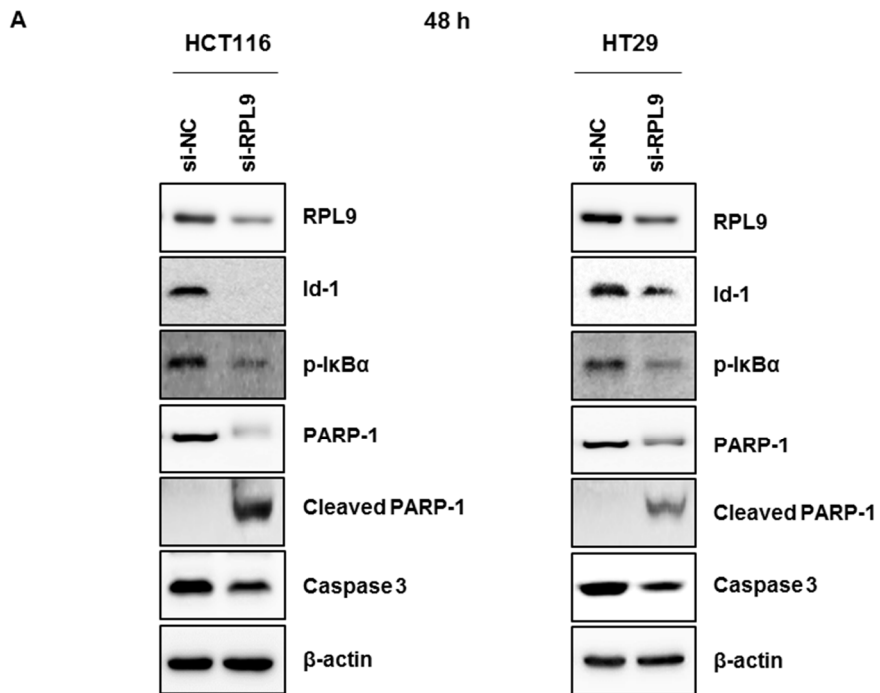
transcripts up- or down-regulated at 48 hr after transfection in HCT116 or HT29 cells. (B) A heat map of the 296 commonly deregulated genes in HCT116 and HT29 cells after normalization to the corresponding sham-treated cells (a threshold cut-off of 2-fold change, red (induced) and green (repressed), log₂-based scale). (C) IPA top network 2 associated with Id-1 and NF- κ B pathway. Up- and down-regulated genes by RPL9 silencing are shown in red and green, respectively. The genes shown in gray are associated with the regulated genes.

Table 1. Top 5 gene networks from Ingenuity Pathway Analysis

Network ID	Score	# of focus genes	Genes in network and their top functions
1	54	30	FBLN1↓, LAMC2↑, LAMB3↑, LAMA3↑, ERK1/2, DGKA↑, GLIPR2↓, NUCB2↓, TRIB1↑, VLDL-cholesterol, ACAT2↓, RAB26↓, ITGA7↓, SHB↑, AKAP12↑, FHL1↓, SMAD1/5, BCAR3↑, atypical protein kinase C, Sos, TIPARP↑, DAP↑, DAPK2↓, CA2↓, WWC1↑, AURK↓, AURKB↓, KLHL21↑, KIF20A↓, MAD2L1↓, KIF2C↓, NDC80↓, SPC24↓, NUF2↓, SPC25↓ Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair
2	33	24	TGFBR, Smad↑, ID1↓, Nfkb (complex), CHRD↓, BMP, ID2↓, Cyclin E, SMEK1↑, PLK2↑, SLC37A4↓, MYO1E↑, RHEBL1↓, CORO1A↓, DBP↓, JDP2↓, CBX2↓, KDM6B↑, HISTONE, IDH2↓, METTL8↑, CCNB2↓, CDC25C↓, PLK1↓, 20s proteasome↓, PSMB9↓, E2f, Smad2/3-Smad4, APC (complex), PTTG1↓, Cdk, Cyclin B↓, Cyclin A, Cdc2, MYT1↓ Cell Cycle, DNA Replication, Recombination, and Repair, Organismal Survival
3	30	23	IFRD1↑, DES↓, Pak, ARHGEF6↓, Dynamin, ERK, IgG2a, Alpha tubulin, KIF22↓, TMEM173↓, STMN1↓, ZFP36L1↑, CHAD↓, LIMAI↑, G-Actin↑, MKNK2↑, CaMKII, NFAT (complex), SDC4↑, Nfat (family), KLF6↑, Fcer1, Calcineurin protein(s), DUSP4↑, Cg, MAP2K1/2, PDGFA↑, CYR61↑, GUSB↓, HERPUD1↓, CTSV↓, DNASE2↓, H2AFZ↓, HPSE↓, CENPA↓ Organismal Functions, Organismal Injury and Abnormalities, Tissue Morphology
4	27	22	NRGN↓, thymidine kinase↓, Rxr, FABP6↓, Rar, ITGB3BP↓, CRABP2↓, T3-TR-RXR, SLC01B3↑, Nr1h, PEPCK↓, PCK2↓, C/ebp, GOT, ANGPTL4↑, Growth hormone, PPP1R15A↑, STAT5a/b, JINK1/2, thyroid hormone receptor, UCP2↓, Cbp/p300, PTPase↓, SLC22A17↓, ACAP1↑, Akt, ATF3↑, LOC102724788/PRODH↓, SEMA7A↑, PLAGL1↑, death receptor↑, TNFRSF10B↑, TP53I3↑, TNFRSF1D↑ HDL-cholesterol, Cell Death and Survival, Lipid Metabolism, Molecular Transport
5	25	22	MMP17↓, Fibrinogen, Ck2, LDL↓, PSRC1↓, PCSK9↓, Pdgf (complex)↑, Apl1, F3↑, Creb, PTPN6↓, phosphatase↓, RNASEH2A↓, PTPRM↑, EPHX2↓, PPFI4↓, Gsk3, Creb, Apl1, FSH, DUSP14↑, CFLAR↑, Pkc(s), Pka, Insulin, ATP2A3↓, 14-3-3, PP2A, MIDN↑, Histone H1↓, H1FX↓, PLC↓, ADRB, Lh, PTP4A1↑, LOC81691↓, CRYM↓ Neurological Disease, Psychological Disorders, Post-Translational Modification

¹The score is a numerical value used to rank networks according to how relevant they are to the genes in the input dataset (296 genes). The score takes into account the number of genes in network and the size of the network to approximate how relevant this network is to input gene list.

²Up- and down regulated genes are indicated with red and green arrow, respectively.



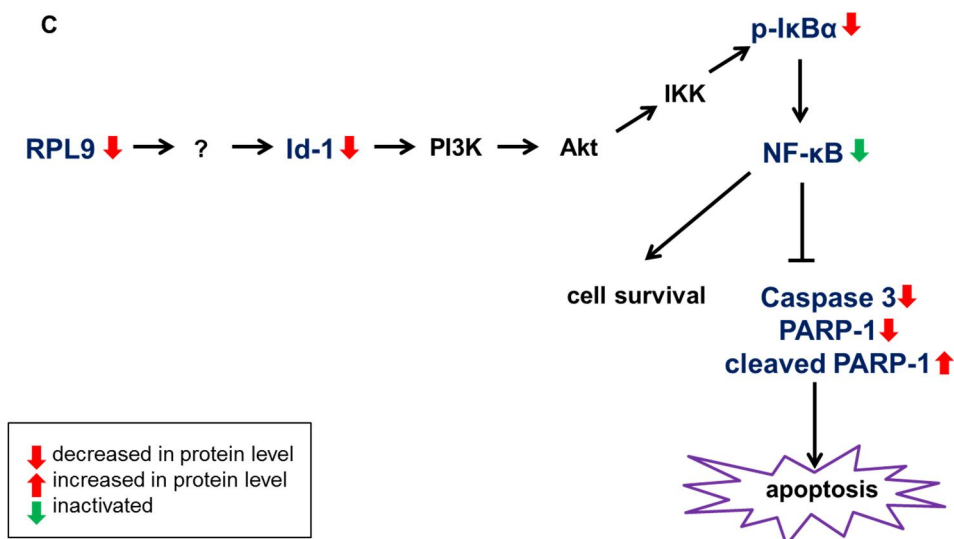


Figure 6. Downregulation of RPL9 causes apoptosis through inactivation of Id-1 and NF-κB. (A) The expression of RPL9, Id-1, p-IkBα (Ser32/36), PARP-1, cleaved PARP-1, caspase 3 in HCT116 and HT29 cells with RPL9 depletion was determined by Western blot using the corresponding antibodies. β-actin was detected as a loading control. (B) Proteins were extracted from both cytosol and nuclei fraction in order to examine localization of NF-κB p65. Lamin B1 and GAPDH were used as nucleic and cytosolic marker, respectively. Equal sample loading was confirmed by Naphthol Blue Black (NBB) staining of the Western blot membrane. (C) Schematic diagram of molecular mechanism involved in apoptosis of RPL9 silenced colorectal cancer cells.

4. Silencing of RPL9 delays tumor growth kinetics *in vivo*.

To further validate the effects of RPL9 knockdown on tumorigenesis of colorectal cancer, tumor formation assay was performed in BALB/c nude mice. si-NC and si-RPL9 transfected HCT116 cells were subcutaneously inoculated into the left and right flank, respectively. Tumor sizes were measured in two day interval for 14 days. 8 days after the inoculation, tumor sizes between the control and RPL9 knock-down showed definite difference and that the control tumor sizes were larger ($P<0.05$) (Figure 7A). After 14 days, a significant difference ($P<0.001$) was apparent in size and weight between the control and RPL9 knock-down (Figure 7). The results show that silencing of RPL9 significantly suppresses the growth of human CRC xenografts *in vivo*.

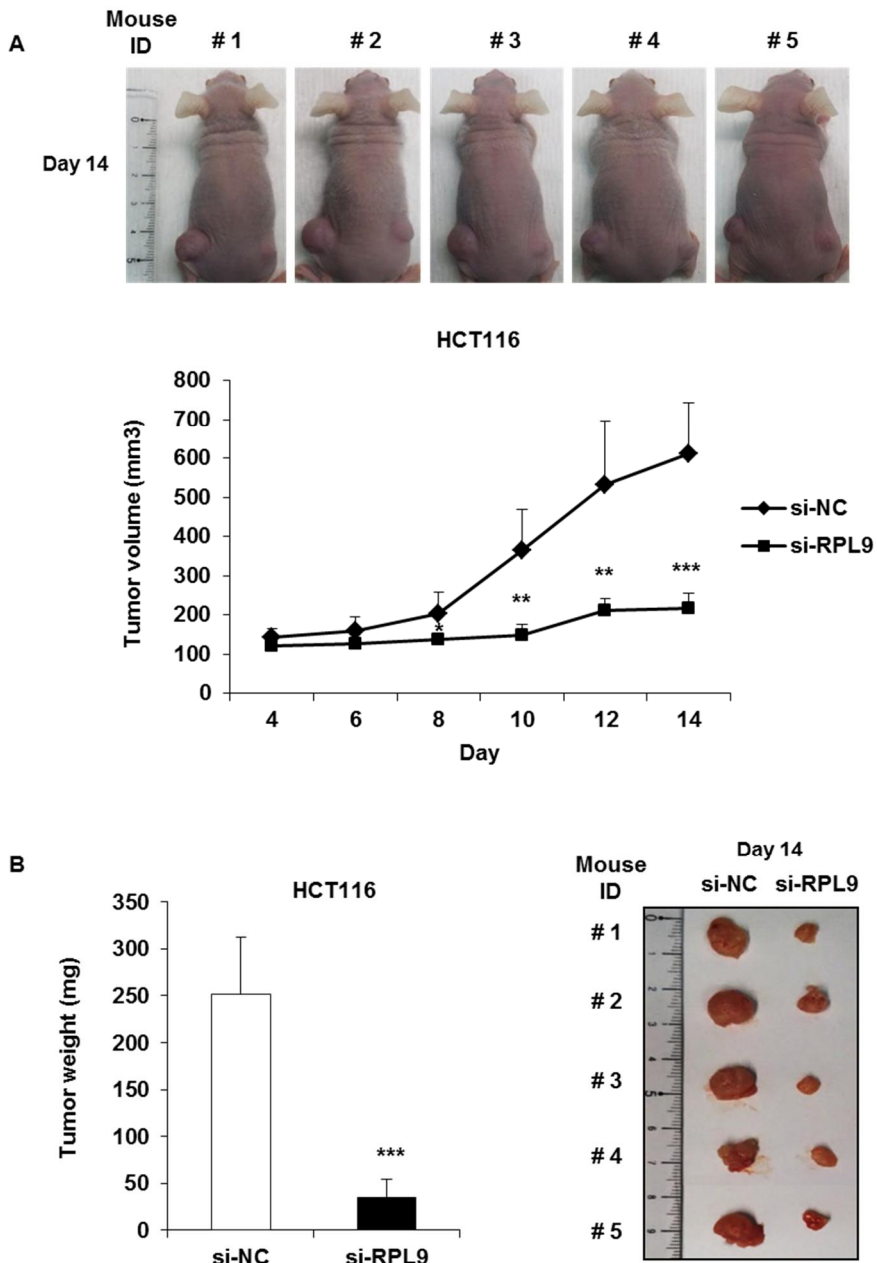


Figure 7. RPL9 silencing suppresses tumor growth *in vivo*. (A) Observation of tumor sizes between si-NC and si-RPL9 transfected xenograft. Tumor volumes were measured in two day interval for 14 days. The data represent five independent experiments. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ by Bootstrap t -test. (B) Measurement of the control and RPL9 knockdown tumor weight. The data represent five independent experiments. ***, $P<0.001$ by Student's t -test.

IV. DISCUSSIONS

Ribosomal proteins are known as the components of ribosomes which play a role in protein biosynthesis. However, in many types of human cancer cells, some of these ribosomal proteins are ectopically expressed compared to normal cells. For example, the mRNA expression of S8, L12, L23a, L27 and L30 ribosomal proteins was up-regulated in human hepatocellular carcinoma.²² RPL34 was overexpressed in non-small cell lung cancer cells,²³ and RPS13 was found to promote the growth and cell cycle progression of gastric cancer cells through inhibiting p27^{Kip1} expression.⁹ Reversely, it was demonstrated that RPS29 induces apoptosis in non-small cell lung cancer cells by downregulating Bcl-2, Bcl-XL and survivin, and upregulating p53 and Bax.²⁴ These recent evidences suggest that ribosomal proteins are not only involved in the protein synthesis but also in the development of cancer, and it is likely that these proteins exhibit secondary functions called extraribosomal functions.²⁵

In colorectal cancer cells, the overexpression of ribosomal protein L9 was found,^{5,13,14} yet there is no report on the function of RPL9 in human cancers. In this study we aimed at finding the extraribosomal function of RPL9 in colorectal cancer cells. We used RNAi system to knockdown RPL9 and observed strong inhibition in cell proliferation and colony formation of HCT116

and HT29, which demonstrates that RPL9 is important for the growth of colorectal cancer. Also, we found an increased sub-G1 population in cell cycle analysis and likewise, a significant increase of apoptotic cells. Furthermore, *in vivo* mouse model showed that silencing of RPL9 delayed tumor growth. The global gene expression profiles of RPL9 knockdown HCT116 and HT29 cells to those of control cells by next-generation RNA sequencing showed common knockdown signature of 296 genes. These data suggest that RPL9 is differentially involved in protein synthesis, but does not influence entire cellular protein synthesis in a non-specific manner. Therefore, silencing of RPL9 does not end up down-regulating the protein synthesis as a whole but it is rather gene-specific and protein-specific.²⁶

Of all the genes that were dysregulated by RPL9 knockdown, we focused on finding the particular molecule that might be involved in cancer cell survival in colorectal cancer cells. Thus in network 2 of the IPA analysis, we found an oncogenic Id-1¹⁵ was down-regulated. Id-1, a helix-loop-helix (HLH) protein, is known to functionally involve in cell growth, senescence, and differentiation. Increased Id-1 expression has been found in over 20 types of human cancers including colorectal,²⁷ breast,²⁸ prostate,²⁹ and cervical cancers.³⁰ Many studies have suggested that Id-1 plays a key role in cancer progression and associates it

as a prognostic marker. According to a study, Id-1 knockdown colorectal cancer cells resulted decrease in proliferation, migration and invasion, and increased in apoptosis rate.¹⁵ Therefore, overexpression of Id-1 protects cancer cells against apoptosis, but decreasing the Id-1 expression would lead cancer cells to apoptosis. We found by western blotting that the protein level of Id-1 was decreased in RPL9 knockdown HCT116 and HT29 cells compared to the control. This finding shows that silencing of RPL9 decreased Id-1 protein expression in a mechanism we have not explored and may have induced apoptosis in CRC cells. In addition, Id-1 has been shown to promote cell survival by activating PI3K/Akt/NF- κ B signaling pathway in esophageal cancer and has been suggested that it may be one of the upstream regulators of NF- κ B.^{18,31} NF- κ B is a transcription factor that translocates the nucleus and mediates the transcription of a vast array of proteins involved in cell survival and proliferation, and anti-apoptotic factors.³² Hence, we investigated the change in NF- κ B activity in RPL9 knockdown HCT116 and HT29 cells and observed that protein level of p-I κ B α has decreased and NF- κ B protein level have decreased in the nuclear fraction of RPL9 silenced cells. These results reveal that the down-regulation of RPL9 cause inactivation of Id-1 and NF- κ B which leads to apoptosis of colorectal cancer cells. However, the downstream

mechanism of RPL9 silencing and the connection with Id-1 inactivation need to be further studied. Also, a better understanding of the effect of RPL9 silencing on the translation level might help clearly explain RPL9's extraribosomal function.

In Figure 6B, Lamin B1, a nuclear loading control, shows slight decrease in RPL9 knockdown HCT116 cell. The reason for this may be that Lamin B1 is involved in apoptosis and often cleaved by caspases or becomes degraded.^{33,34} Depending on the experimental conditions, the expression level of a particular housekeeping gene may change. Hence, there are studies that argue that reversible protein staining dye can be used advantageously over housekeeping protein detection for quality or equal loading control in Western blotting.^{20,21} Therefore, we stained the transferred membrane with Naphthol Blue Black and confirmed equal sample loading of Western blotting.

Here we demonstrated for the first time that silencing of RPL9 by RNAi suppresses the proliferation and induces apoptosis in colorectal cancer cells by *in vitro* and *in vivo* assays. Also, we found that the down-regulation of RPL9 induces the inactivation of Id-1/NF- κ B signaling axis.

V. CONCLUSION

Taken together, our findings suggest that RPL9 may play an important role in promoting the malignant growth of colorectal cancer cells and that targeting of RPL9 might be an attractive option for exploiting a next-line molecular therapy of colorectal cancer.

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ABSTRACT (IN KOREAN)

Id-1/NF- κ B 신호전달축의 불활성화로 인한 RPL9 표적의 대장암세포 성장 억제

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리보솜 단백질 L9 (RPL9)은 단백질 합성에 관여하는 60S 소단위의 구성요소로 인간 대장암에서 과발현한다. 본 연구에서는 종양 형성에 서 RPL9이 extraribosomal 기능을 획득하는지 여부와 RPL9을 짧은 간섭 RNA로 표적 하였을 때 대장암 진행에 미치는 영향을 조사 하였다. 그 결과, RNA 간섭현상을 이용한 RPL9 유전자의 발현 저해는 sub-G1 세포수의 증가와 강한 세포사멸의 유도를 통해 대장암 세포의 성장과 장기 군체 형성을 억제하였음을 확인하였다. RPL9 발현 저해로 인한 분자적 변화를 확인하기 위해 RNA 염기서열분석을 사용하

여 유전자 발현의 글로벌 변화를 검토하였다. RPL9-특정 발현 저해는 HCT116 세포에서 918개 유전자, HT29 세포에서 3178개 유전자가 이상조절 되었음을 나타내었다. 그 중, 296개의 유전자는 같은 방향조절(128개 상향- 그리고 168개 하향 조절된 유전자)을 보였으며 이들 유전자는 RPL9 발현 저해의 공통적인 징후이다. 특히, 유전자 네트워크 분석을 통해 NF- κ B의 활성화와 세포생존과 기능적으로 관련된 Id-1이 공통적으로 하향 조절되었음을 볼 수 있었다. 그 다음 웨스턴 블롯 분석을 통해 RPL9 침묵으로 HCT116과 HT29 세포 모두에서 Id-1과 인산화된 I κ B α 의 수준 감소가 유도됨을 확인하였다. 또한, 이와 같은 조건에서 PARP-1과 pro-caspase 3의 단백질 수준의 감소를 통해 세포사멸이 가속화되었음을 알 수 있었다. 뿐만 아니라, RPL9 발현 억제는 누드 마우스에 이종 이식한 인간 대장암의 성장을 현저히 저해하였다. 이러한 결과를 토대로 RPL9의 기능은 Id-1/NF- κ B 신호전달축과 연관성이 있음을 나타내고 RPL9의 표적은 대장암의 분자적 치료의 좋은 옵션이 될 것이라 생각된다.

핵심되는 말: RPL9, RNA 간섭, extraribosomal 기능, 세포사멸

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