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Ribosomal Protein L9 Maintains Stemness of Colorectal Cancer via an ID-1 Dependent Mechanism

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The identification of therapeutic target genes that are functionally involved in stemness is crucial to effectively cure patients with metastatic carcinoma. We have previously reported that inhibition of ribosomal protein L9 (RPL9) expression suppresses the growth of colorectal cancer (CRC) cells by inactivating the inhibitor of DNA-binding 1 (ID-1) signaling axis, which is functionally associated with cancer cell survival. In addition to cell proliferation, ID-1 is also involved in the maintenance of cancer stemness. Thus, we aimed in this study to investigate whether the function of RPL9 could correlate with CRC stem cell-like properties. Here, we demonstrated that siRNA silencing of RPL9 reduced the invasiveness and migrative capabilities of HT29 and HCT116 parental cell populations and the capacity for sphere formation in the HT29 parental cell population. $CD133^+$ cancer stem cells (CSCs) were then separated from $CD133^-$ cancer cells of the HT29 parental cell culture and treated with RPL9-specific siRNAs to verify the effects of RPL9 targeting on stemness. As a result, knockdown of RPL9 significantly suppressed the proliferative potential of $CD133^+$ colorectal CSCs, accompanied by a reduction in CD133, ID-1, and p-IκBα levels. In line with these molecular alterations, targeting RPL9 inhibited the invasion, migration, and sphere-forming capacity of $CD133^+$ HT29 CSCs. Taken together, these findings suggest that RPL9 promotes CRC stemness via ID-1 and that RPL9 could be a potential therapeutic target for both primary CRC treatment and the prevention of metastasis and/or recurrence.

Key Words Colorectal cancer, Ribosomal protein L9, Stemness, Inhibitor of DNA-binding 1

INTRODUCTION

Colorectal cancer (CRC) accounts for 9.7% of all newly diagnosed cancer cases (the third most common cancer) and 10% of global cancer-related deaths (the second highest) [1]. In addition to the causative factors of CRC, including excessive meat eating, alcohol consumption, and smoking [2-8], several genetic factors are involved in CRC development [9,10]. Approximately 50%–60% of patients with CRC are diagnosed with metastatic lesions, and liver is the most common organ involved [11-13]. Even after various locoregional surgeries, radiation therapy, and conventional or targeted chemotherapy depending on tumor location and disease stage [14], 20%–34% of patients with CRC still develop synchronous liver metastases [15]. Emerging evidence suggests that colorectal cancer stem cells (CR-CSCs) are biologically involved in the initiation of primary CRC formation and the development of metastatic CRC [14]. Thus, there is an urgent need to identify other target genes that are functionally associated with CR-CSC-like properties to support the development of alternative treatment options and improve the CRC therapeutic index.

Ribosomal proteins (RPs) are the components of ribosomes that mediate numerous cellular functions such as protein synthesis and metabolic regulation, and approximately 80 types of RPs are present in eukaryotic cells [16]. Some RPs also perform non-canonical functions, including DNA repair, transcriptional regulation, and apoptosis [17]. Moreover, recent studies have shown that ribosome biogenesis itself plays an important role in cell division and that the upregulation of RPs can increase the risk of tumor development and stemness acquisition, which has been termed an extra-ribosomal function [18]. For example, we have reported that ribosomal protein L17 (RPL17) and RPL27 are upregulated and functionally involved in CRC progression and stemness via NEK2/β-catenin and PLK-1 signaling, respectively [19,20]. Other groups have also shown that upregulation of RPL23 increases the metastatic potential of lung adenocarcinoma

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cell lines [21] and that RPs induce plasticity and stemness in glioma cells [22].

RPL9, a component of the 60S subunit, belongs to the L6P family of RPs. It has been reported that the expression level of RPL9 is elevated in CRC tissues compared to that in a healthy colon [23-25], which raises the possibility that RPL9 may be a candidate target gene for CRC therapy. Supporting this hypothesis, we have previously demonstrated that the knockdown of RPL9 inhibits CRC growth in vitro and in vivo by disrupting the inhibitor of DNA-binding 1 (ID-1) signaling axis [26]. ID-1 is a helix-loop-helix (HLH) protein that forms heterodimers with members of the basic HLH family of transcription factors. ID-1 has no DNA-binding activity and can therefore inhibit the DNA-binding and transcriptional activation ability of the basic HLH proteins with which it interacts. It is also involved in cell growth, senescence, and differentiation. In addition to cell proliferation, ID-1 also plays a crucial role in maintaining cancer stemness. Silencing of ID-1 resulted in a decrease in CRC proliferation and suppression of hepatic metastasis [27]. Thus, we attempted to investigate whether the function of RPL9 correlates with CR-CSC-like properties. From what we have gathered, direct evidence connecting the function of RPL9 with cancer stemness is not yet reported. A recent study claimed that the recurrence of non-muscle invasive bladder cancer with BCG therapy could be predicted using RPL9 expression [28].

MATERIALS AND METHODS

Cell culture and siRNA transfection

The human CRC cell lines HT29 and HCT116 were obtained from the Korean Cell Line Bank (KCLB). Both the HT29 and HCT116 cells were incubated at 37ºC in a humidified incubator with 5% CO_2. RPMI-1640 culture medium was supplemented with 10% FBS and 1% penicillin/streptomycin (Welgene). CRC cells were transfected with 20 nM negative control (NC) siRNA or RPL9-specific siRNA (s226955; Ambion) for 5 hours using Lipofectamine 2000 (11668-019; Invitrogen) and Opti-MEM I (31985-070; Gibco) in a humidified incubator. The NC siRNA sequences designed are as follows: sense strand, 5′-ACGUGACACGUUCGGAGAA(UU)-3′; antisense strand, 5′-UUCUCCGAACGUGUCAC GU-3′, and synthesized by Bioneer.

MTT assay

Cell proliferation was assessed by using the MTT assay following the manufacturer's guidelines. HT29 and HCT116 cells were transfected with RPL9 siRNA at a concentration of 20 nM, as described above. After 96 hours of cell culture, 90 μL of plain medium and 10 μL of MTT were added to each well and incubated at 37ºC in a humidified incubator for 1 hour. Subsequently, MTT solution was aspirated, and 100 μL of DMSO was added. The absorbance was measured at 540 nm using an Asys UVM 340 microplate reader (Biochrom).

Invasion and migration assay

Invasion and migration assays were performed using 24-well Matrigel invasion chambers (354480; Corning) and a transwell migration assay kit (3422; Corning), respectively. Cells transfected with either NC siRNA or RPL9 siRNA for 24 hours were suspended at a density of 5×10^4 cells in 200 μL of medium in the upper chamber. In the lower chamber, 600 μL of medium containing 10% FBS was added. After 24 hours, cells were fixed with methanol for 15 minutes and stained with 0.5% crystal violet for 30 minutes. Images were captured at 100 × magnification using a light microscope.

Sphere forming assay

HT29 parental cells (3×10^3), HCT116 parental cells (5×10^3), and $CD133^+$ HT29 cancer stem cells (5×10^3) were suspended in serum-free DMEM/F-12 medium (11320-033; Gibco) containing 4 ng/mL insulin (Invitrogen), 2% B27 (Invitrogen), 20 ng/mL epidermal growth factor (Sigma Aldrich), and 10 ng/mL basic fibroblast growth factor (Invitrogen). The cell suspension was seeded into 24-well ultra-low-attachment plates (3473; Corning). After plating, single cells were transfected with either NC siRNA or RPL9 siRNA using lipofectamine RNAiMAX (13778-150; Invitrogen) following the manufacturer's protocol. The number of spheres was counted on day 9 or 15, when a substantial number of spheres had formed.

Protein extraction and Western blotting

$CD133^+$ HT29 cancer stem cells were suspended in RIPA buffer (89900; Thermo Fisher Scientific) containing 0.01% protease and phosphatase inhibitor cocktail (1861281; Thermo Fisher Scientific) in 48 hours of siRNA transfection. The amount of protein was quantified using a Pierce BCA Protein Assay Kit (23225; Thermo Fisher Scientific). Subsequently, total protein (50 μL) were separated using 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Roche). Following blocking with 5% skim milk in Tris-buffered saline plus Tween 20 (CBT3085; Dynebio), the membranes were incubated with primary antibodies against RPL9 (ab182556; Abcam) CD133 (64326; Cell Signaling Technology), ID-1 (sc-133104; Santa Cruz Biotechnology), p-IκBα (2859; Cell Signaling Technology), and β-actin (sc-47778; Santa Cruz Biotechnology). The following HRP-conjugated secondary antibodies were used for detection: goat anti-mouse IgG (115-035-062; Jackson ImmunoResearch Laboratories), rabbit anti-goat IgG-HRP (sc-2768; Santa Cruz Biotechnology), and goat anti-rabbit IgG (sc-2301; Santa Cruz Biotechnology). The cells were then incubated at room temperature for 1 hour. The immunoreactive bands were visualized using Fusion Fx7 imaging system (Vilber Lourmat).

RNA isolation and real-time quantitative reverse transcription-PCR

Alterations in gene expression at the mRNA level were assessed by real-time quantitative reverse transcription-PCR. Total RNA was extracted using the RNeasy Plus Mini kit (74134; Qiagen GmbH), and cDNA was synthesized using the PrimeScript II 1st strand cDNA Synthesis kit (Takara Bio) following the manufacturer's instructions. Amplification of the target genes was performed using the respective pairs of primers (RPL9 forward, 5'-GCACAGTTATCGTG AAGGGC-3', and RPL9 reverse, 5'-TTACCCCACCATTTGT-CAACC-3'; CD133 forward, 5'-AGTCGGAAACTGGCAGA-TAGC-3', and CD133 reverse, 5'-GGTAGTGTTGTACTG GG-CCAA T-3'; ID-1 forward, 5'-CCAGCACGTCATCGACTAC-3', and ID-1 reverse, 5'-GCTTCAGCGACACAAGATG-3'; GAP-DH forward, 5'-ACATCAAGAAGGTGGTGAAG-3', and GAP-DH reverse, 5'-GGTGTCGCTGTTGAAGTC-3') synthesized by Genotech, in conjunction with TB Green (Takara Bio). Relative mRNA expression was evaluated and quantified using a LightCyclear 96 system (04729692001; Roche) according to the manufacturer's instructions. The mRNA levels were normalized to GAPDH expression.

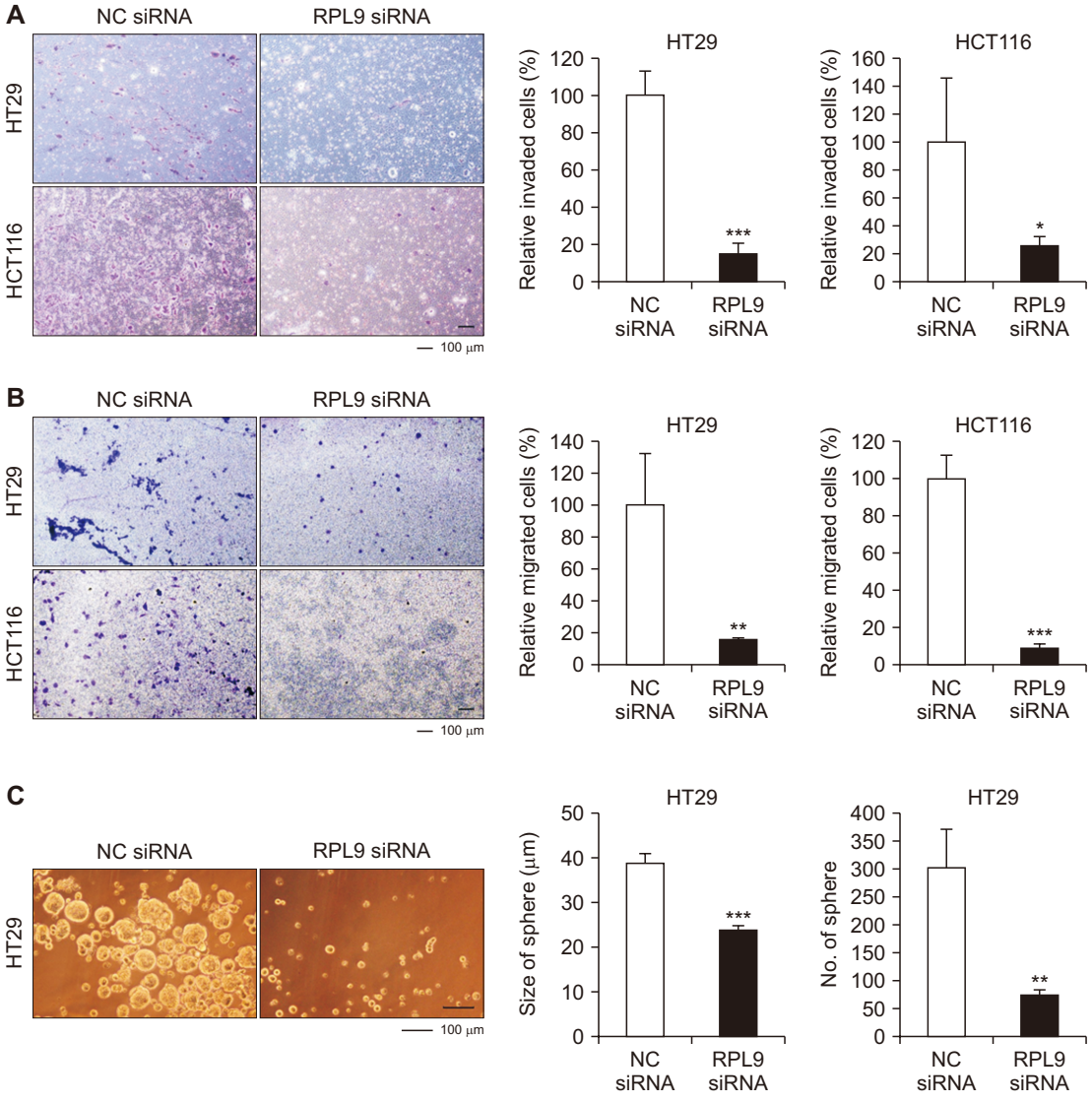

Figure 1. Transfection of RPL9 siRNA reduces the metastatic potential of parental CRC cell population. (A, B) Representative light microscopy images and relative percentage of invasive (A) and migratory (B) HT29 and HCT116 cells treated with NC siRNA or RPL9 siRNA. Numbers of invaded and migrated cells were stained with crystal violet, counted, and graphed. Scale bar, 100 μm. (C) Detection of sphere formation in HT29 cells treated with NC siRNA or RPL9 siRNA. The differences in sphere forming capacity were compared by calculating both size and number of spheres. NC siRNA, negative control siRNA; RPL9 siRNA, RPL9-specific siRNA. Statistical significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. NC siRNA treatment. RPL9, ribosomal protein L9; CRC, colorectal cancer; NC, negative control.

Magnetic cell sorting and flow cytometry

To separate colorectal CSCs from CRC cells, the CD133 CSC marker was used in this study. HT29 parental cell population was trypsinized and resuspended in DPBS with FcR Blocking Reagent and CD133 microbeads (Miltenyi Biotec) and incubated in the dark at 4ºC for 15 minutes. After washing with DPBS, the cells labeled by CD133 microbeads were separated using an autoMACS Pro system (Miltenyi Biotec). Magnetically labeled $CD133^+$ and unlabeled $CD133^-$ cells were separately incubated for 30 minutes along with either isotype control antibody (130122932, mouse IgG2b APC conjugate; Miltenyi Biotec) or CD133/2 antibody (130113746, anti-human APC conjugate; Miltenyi Biotec). Separation efficiency was quantified using a FACSCanto II flow cytometer (BD Bioscience).

Statistical analysis

All data were subjected to statistical analyses using Microsoft Excel 2016 (Microsoft). Each experiment was conducted using three replicas, and statistical evaluations were performed using the Student's *t*-test. The results are displayed as mean ± SEM. Statistically significant differences were denoted by *P*-values, i.e., $P < 0.05$, $P < 0.01$, and $P < 0.001$.

RESULTS AND DISCUSSION

Silencing of RPL9 inhibits metastatic potential of parental CRC cell population

For this study, we chose the same RPL9-specific siRNA sequence and transfection conditions that were used in a previous study [26] and confirmed its reproducibility in terms of CRC cell growth inhibition and efficient target gene silencing (Fig. S1). As mentioned previously, targeting RPL9 suppresses CRC growth by downregulating ID-1 which is involved in both cancer cell growth and stemness [27]. Given the functional involvement of ID-1 as a metastatic factor, prior to isolating CR-CSCs, we verified whether RPL9 knockdown could substantially inhibit the metastatic abilities of the parental CRC cell population. To accomplish this, invasion, migration, and sphere formation assays were performed after transfection of RPL9 siRNA to HT29 or HCT116 CRC cell culture. As expected, treatment with 20 nM RPL9 siRNA suppressed the invasion of HT29 and HCT116 parental cell populations by approximately 80% and 70%, respectively, compared to that of NC siRNA treatment (Fig. 1A). Similarly, the treatment induced a reduction in the migration ability by more than 80% in both cell lines (Fig. 1B).

CSCs represent a subpopulation of tumorigenic cells, comprising a very small portion of about 0.01%–2% of the total tumor cell number, and the formation of multicellular three-dimensional spheres in nonadherent serum-free conditions is a typical characteristic of CSCs for self-renewal and propagation [29,30]. Furthermore, it was previously demonstrated that CR-CSCs were successfully enriched using a CD133 surface marker [31,32] and that $CD133^+$ CR-CSCs could induce tumor formation in vivo, whereas $CD133^-$ cancer cells failed [32]. Of the two CRC cell lines, we decided to use HT29 for all subsequent experiments because the level of CD133 expression in HT29 cells was significantly higher than that in HCT116 cells [33], allowing us to obtain a sufficient number of CSCs suitable for analysis. We observed that HT29 cells formed spheres more efficiently than did HCT116 cells (data not shown), and transfection of RPL9 siRNA into the HT29 parental cell population effectively reduced both the size and the number of spheres after nine days of treatment (Fig. 1C). These results indicate that RPL9 is involved in the maintenance of CRC stemness.

RPL9 knockdown suppresses the proliferation of CR-CSCs through reduction of ID-1 and p-IκBα protein levels

We sorted the HT29 CSC population from cancer cells by incubating the HT29 parental cell culture with CD133 microbeads. As displayed in Fig. 2A, $CD133^+$ and $CD133^-$ cell populations were efficiently separated, showing a more than 3-fold difference in the level of CD133 mean fluorescence. To affirm the successful isolation, we have also evaluated the expression of CD133 on mRNA and protein levels and com-

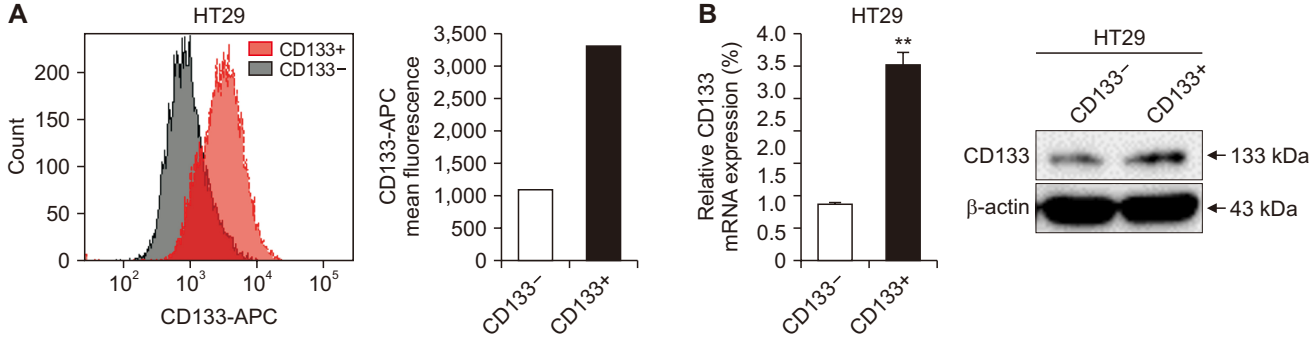

Figure 2. Use of CD133 CSC marker effectively isolates CR-CSCs. (A) Separation of HT29 CSC population from cancer cell population using CD133 antibody. (B) Relative levels of CD133 mRNA and CD133 protein in $CD133^-$ and $CD133^+$ fractions. $^{**}P < 0.01$ vs. NC siRNA treatment. CR-CSC, colorectal cancer stem cell; NC, negative control.

pared the results with those in CD133$^+$ and CD133$^-$ cancer cell populations. Both mRNA and protein levels of CD133 in the CD133$^+$ population were higher than that in the CD133$^-$ population by about 3.5-fold and 1.6-fold, respectively (Fig. 2B).

Next, RPL9 siRNA was transfected into isolated CD133$^+$ HT29 CSCs and assayed for growth inhibition and changes in the expression of key molecules. Silencing of RPL9 suppressed the growth of HT29 CSCs by more than 85% (Fig. 3A), accompanied by the downregulation of CD133 and ID-1 at both the mRNA and protein levels (Fig. 3B and Fig. 3C). Reduction of ID-1 protein led to the decrease of phosphorylated IκBα, which is known as one of downstream effectors of ID-1 signaling (Fig. 3C).

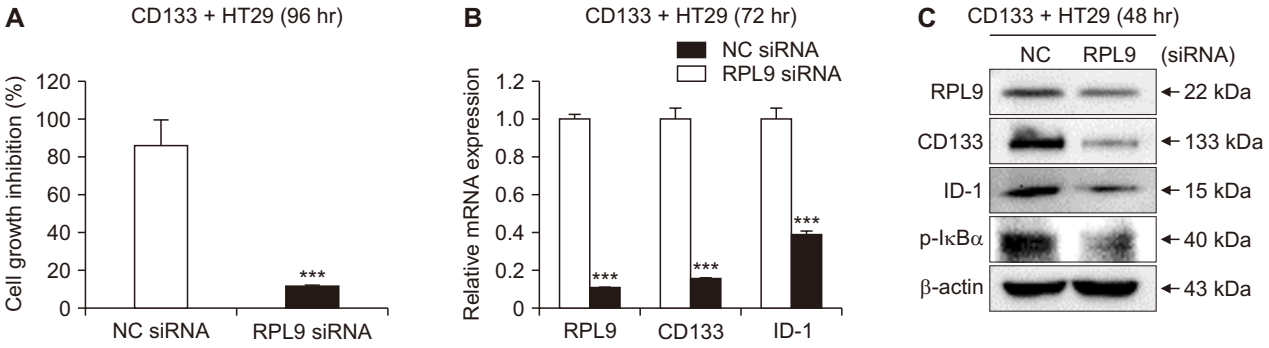

Figure 3. Targeting RPL9 inhibits the growth of CR-CSCs via disruption of ID-1 signaling axis. (A) Inhibition of growth of CD133$^+$ HT29 CSCs by RPL9 silencing. (B) Reduction of CD133 and ID-1 mRNA expression in RPL9-silenced CD133$^+$ HT29 CSCs. (C) Reduction of CD133, ID-1, and p-IκBα proteins in RPL9-silenced CD133$^+$ HT29 CSCs. β-Actin was used as a loading control. ***P < 0.001 vs. NC siRNA treatment. RPL9, ribosomal protein L9; CR-CSC, colorectal cancer stem cell; ID-1, inhibitor of DNA-binding 1; NC, negative control.

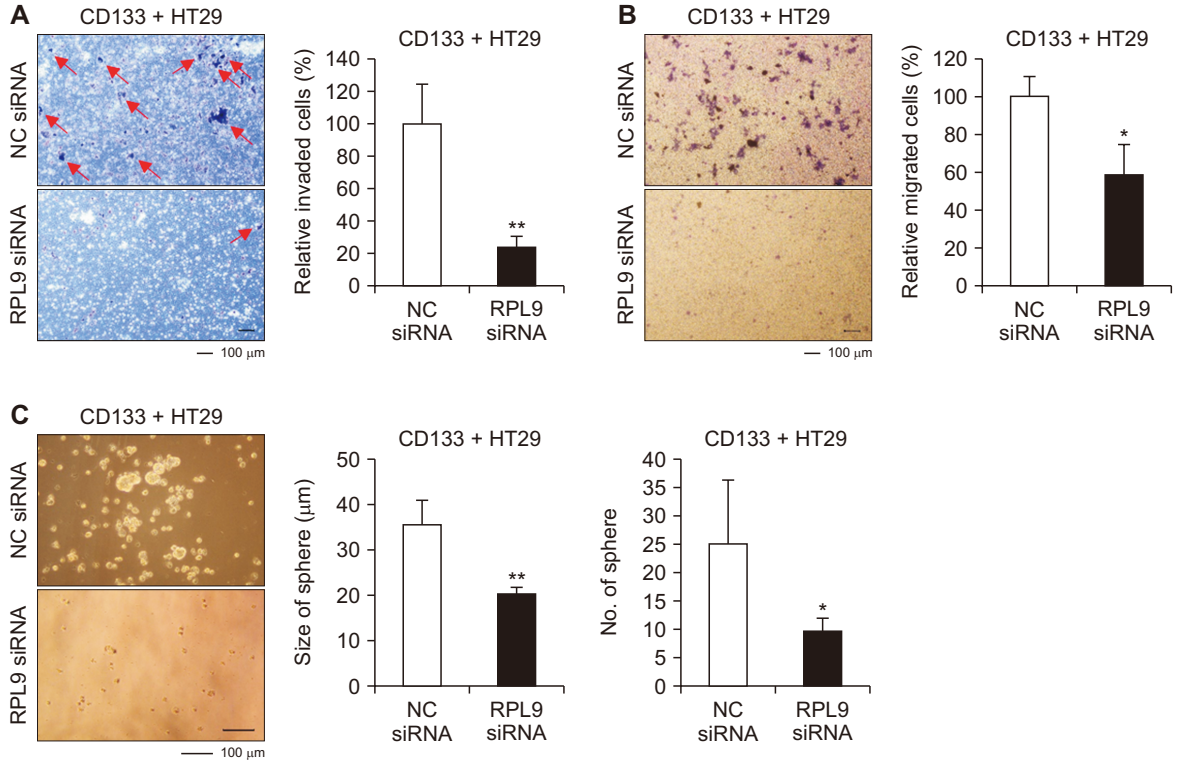

Figure 4. Treatment with RPL9 siRNA suppresses the abilities of invasion, migration and sphere formation in CR-CSCs. (A, B) Representative light microscopy images and relative percentage of invasive (A) and migratory (B) CD133$^+$ HT29 CSCs treated with NC siRNA or RPL9 siRNA. The invaded cells were marked with red arrows in (A). Scale bar, 100 μm. (C) Representative light microscopy images and relative efficiency of sphere formation in CD133$^+$ HT29 CSCs treated with NC siRNA or RPL9 siRNA. Scale bar, 100 μm. The difference in sphere forming capacity was compared by calculating size and quantifying number of spheres. *P < 0.05; **P < 0.01 vs. NC siRNA treatment. RPL9, ribosomal protein L9; CR-CSC, colorectal cancer stem cell; NC, negative control.

Overexpression of ID-1 has been observed in over 20 types of human malignancies, including colorectal [34], breast [35], prostate [36], and cervical [37] cancer, suggesting that ID-1 plays an important role in cancer development. Furthermore, knockdown of ID-1 expression results in the inhibition of CRC metastasis in vivo [27]. Consistent with the genetic profiling of RPL9 knockdown parental CRC cells [26], we found that the level of ID-1 was reduced in CR-CSCs as well, suggesting that RPL9 is functionally correlated with the ID-1 signaling axis to promote both proliferation and stemness of CR-CSCs.

Targeting RPL9 inhibits the stemness of CR-CSCs

Generally, almost 80% of patients with cancer die mainly due to the occurrence of metastasis, showing only 10 months of the median survival time [38]. In the case of CRC, about 25% of patients are diagnosed at an advanced stage with a higher chance of representing metastatic lesions, and even the patients in early stages have up to 50% of chance of developing metastases [11,12]. Biologically, it is well known that CR-CSCs play a critical role in developing metastatic CRC [14,31]. Thus, we investigated whether inhibition of RPL9 expression could substantially suppress the stemness of CR-CSCs. To accomplish this, the expression of RPL9 in $CD133^+$ HT29 CSCs was blocked by RPL9 siRNA and then changes in invasion and migration abilities were compared with that in the control group. As expected, the invasive and migratory abilities of RPL9-silenced CSCs were reduced by approximately 80% and 40%, respectively (Fig. 4A and 4B).

Finally, the change in sphere-forming capacity was assessed under the same conditions. As shown in Fig. 4C, treatment with RPL9 siRNA significantly suppressed sphere formation by decreasing both the size and number of spheres. For the first time, we report that the function of RPL9 is correlated with CRC stemness. Throughout our successive studies, a decrease in ID-1 expression was observed in all experimental CRC environments of the parental cell culture and in isolated $CD133^+$ CSCs with RPL9 depletion, indicating its potential therapeutic efficacy.

Taken together, our findings suggest that RPL9 is a potential therapeutic target for primary CRC treatment and prevention of metastasis. Our study has certain limitations; we could not obtain sufficient experimental evidence to fully elucidate how RPL9 silencing could disrupt ID1 signaling. Future studies should focus on addressing these questions. First, the molecular mechanisms by which RPL9 directly regulates the expression of ID-1 in CR-CSCs must be elucidated. Second, it would be interesting to elucidate the different mechanisms by which RPL9 is involved throughout the stages of CRC development.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.15430/JCP.24.004.

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