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Original Article

Retracted: Down-regulation of GPR137 expression inhibits proliferation of colon cancer cells

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G protein-coupled receptors (GPRs) are highly related to oncogenesis and cancer metastasis. G protein-coupled receptor 137 (GPR137) was initially reported as a novel orphan GPR about 10 years ago. Some orphan GPRs have been implicated in human cancers. The aim of this study is to investigate the role of GPR137 in human colon cancer. Expression levels of GRP137 were analyzed in different colon cancer cell lines by quantitative polymerase chain reaction and western blot analysis. Lentivirus-mediated short hairpin RNA was specifically designed to knock down GPR137 expression in colon cancer cells. Cell viability was measured by methylthiazoletetrazolium and colony formation assays. In addition, cell cycle characteristic was investigated by flow cytometry. GRP137 expression was observed in all seven colon cancer cell lines at different levels. The mRNA and protein levels of GPR137 were down-regulated in both HCT116 and RKO cells after lentivirus infection. Lentivirus-mediated silencing of GPR137 reduced the proliferation rate and colonies numbers. Knockdown of GPR137 in both cell lines led to cell cycle arrest in the G_0/G_1 phase. These results indicated that GPR137 plays an important role in colon cancer cell proliferation. A better understanding of GPR137's effects on signal transduction pathways in colon cancer cells may provide insights into the novel gene therapy of colon cancer.

Keywords GPR137; shRNA; cell proliferation; colon cancer; cell cycle arrest

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Introduction

Colorectal cancer is one of the major causes that are responsible for cancer death in both male and female population in the United States of America [1]. Through careful investigation, researchers estimated that there were up to 102,480 new cases and 50,830 deaths of colorectal cancer in the year of 2013 [2]. Although various therapeutic approaches such as surgical resection, chemotherapy, and radiotherapy have been applied in patients, there is still not much breakthrough in finding an effective and side effect free treatment for this deadly killer. But recently, gene therapy strategy is under heated research for it may be the key to fundamentally cure cancer by switching off or blocking the cancer-causing genes [3]. This might be a new way to cure colon cancer.

It has been proved that colorectal cancer development is closely associated with the accumulation of genetic mutations [4]. The causes for genetic mutation can be various. It can be triggered by virus infection, exposure to cancerogenic chemical or physical factors, family hereditary factors, and duplication error during DNA replication, etc. [5]. These factors impair self-repairing capability of DNA, suppress anti-tumor gene and activate oncogenes [6,7]. These genetic mutations accumulate, and then lead to malfunction of signal transduction pathways. As a consequence, cell proliferation is altered and normal cell apoptosis stops, which directly underlies tumorigenesis [8].

G protein-coupled receptor (GPR) is one of the most important signal transduction mediators that are related to physiological and pathological condition of a cell [9]. The expression of GPR is abundant in endocrine, cardiovascular, gastroenteric, immune, reproductive tissue and organs [10], and has impact on cancer progress. To investigate the relationship between GPR expression and cancer incidence, morbidity and mortality rate, much research work has been done [11–14]. According to the results of our survey, orphan GPR49 has been identified in both colon and ovarian cancers. Furthermore, its over-expression was related to later stage of colon and ovarian primary tumors, for knock-down of GPR49 gene could induce cell apoptosis [15]. Orphan GPR55 has been implicated in the tumorigenesis of several cancers, such as cholangiocarcinoma, breast cancer, glioblastoma, prostate and ovarian cancer, which suggests that GPR55 may be a potential target for cancer therapy [16]. These studies inspire us to explore the possible existence of another orphan GPR that could influence cancer cell growth. GPR137 was initially identified as a novel orphan GPR in

various neural system tissues a decade ago [17] and it shares identity with a prostate-specific odorant-like GPCR-encoding gene (PSGR). However, little is known about the biological role of GPR137 in human diseases, particularly in cancers.

In this study, to explore the role of GPR137 in human colon cancer, its expression was first detected in multiple human colon cancer cell lines. It was found that GPR137 was ubiquitously expressed in different colon cancer cell lines. Among them, HCT116 and RKO colon cell lines were chosen as our research targets. Specific knockdown of GPR137 by RNA interference (RNAi) remarkably inhibited colon cancer cell growth and cell cycle progression. Our results suggested that silencing of GPR137 might be a novel therapeutic method for colon cancer in the future.

Materials and Methods

Cell culture

Human colon cancer cell lines (DLD-1, RKO, SW1116, SW480 and SW620) were maintained in RPMI-1640 (Hyclone, Logan, USA) supplemented with 10% fetal calf serum (FBS). Human colon cancer cell lines (HCT116 and HT-29) were maintained in MCCOYS′5A (Sigma, St Louis, USA) supplemented with 10% FBS. Human embryonic kidney cell line (HEK293T) was maintained in DMEM (Hyclone) with 10% FBS. All cells were cultured at 37°C in a humidified 5% CO_2/95% air atmosphere. All cell lines were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China).

Construction and infection of lentivirus vectors

The lentivirus vector corresponding to *GPR137* gene (NM_001170726.1) was designed as follows: short hairpin RNA (shRNA) targeting *GPR137* is sequenced as 5′-GA ACAAAGGCTACCTGGTATTCTCGAGAATACCAGGT AGCCTTTGTTCTTTTTT-3′; shRNA for control (non-silencing) is 5′-CTAGCCCGGCCAAGGAAGTGCAATT GCATACTCGAGTATGCAATTGCACTTCCTTGGTTTT TGTTAAT-3′. Then these oligonucleotides were inserted into the pFH-L plasmid (Shanghai Hollybio, Shanghai, China) respectively. These lentiviral vectors expressing shRNA were all confirmed by DNA sequencing before being transfected into HEK293T cells together with virus-packaging-related vectors pVSVG-I and pCMVΔR8.92 plasmids [18,19]. Lentivirus was subsequently harvested at 72 h post-transfection, centrifuged to get supernatants, and then filtered before purification using ultracentrifugation. HCT116 cells (5×10^4 cells/well) were seeded in 6 well plates and infected with Lv-shGPR137 lentivirus or Lv-shCon lentivirus at multiplicity of infection (MOI) of 20. And RKO cells (5×10^4 cells/well) were infected with lentivirus at MOI of 15. After infection for 72 h, fluorescence microscopy was used to observe the infection efficiency.

Quantitative polymerase chain reaction

For both cell lines (HCT116 and RKO), 5 days after lentivirus infection, cells were lysed to extract RNA [20]. Quantitative polymerase chain reaction (qPCR) was performed on Bio-Rad Connet Real-Time PCR platform (Hercules, USA). The qPCR reaction system consisted of 10 μl of 2 × SYBR premix *ex Taq*, 0.4 μl forward and reverse primers, respectively (2.5 μM), 5 μl cDNA, and 4.2 μl ddH2O [21]. For *GPR137*, the primers are 5′-ACCTGGGGAACAAAGGCTAC-3′ (forward) and 5′-TAGGACCGAGAGGCAAAGAC-3′ (reverse) respectively. For *β-actin*, the primers are 5′-GTGGACATCCGC AAAGAC-3′ (forward) and 5′-AAAGGGTGTAACGCAA CTA-3′ (reverse). The qPCR procedure started with initial denaturation at 95°C for 1 min, and then denatured at 95°C for 5 s before annealing extension of 60°C for 20 s (a total of 40 cycles). The relative expression of *GPR137* was determined by cycle threshold (Ct) normalized with that of *β-actin* using $2^{-\Delta\Delta Ct}$ formula.

Western blot analysis

Lentivirus-transduced cells were lysed in 2 × sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl, pH 6.8, 10 mM EDTA, 4% SDS, and 10% Glycine) on ice for 30 min, and were then separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis by placing 30 μg protein in each lane of the gels at 50 V for 3 h. Then, the proteins were transferred onto PVDF membrane for 1.5 h with 300 mA before being incubated with indicated primary antibodies at 4°C overnight and horseradish peroxidase-conjugated secondary antibodies at room temperature for 2 h [22]. GAPDH protein was used as a loading control.

Methylthiazoletetrazolium assay

The methylthiazoletetrazolium (MTT) assay is a colorimetric assay for assessing cell viability. NAD(P)H-dependent cellular oxidoreductase enzymes are capable of reducing the tetrazolium dye MTT to its insoluble formazan, which has a purple color [23]. After infection for 96 h, HCT116 (3×10^3 cells/well) and RKO (2.5×10^3 cells/well) were seeded into 96 well plates, respectively. The number of viable cells was measured at indicated time points (1, 2, 3, 4, and 5 days), when 20 μl of 5 mg/ml MTT solution was added into each well. After incubation for 4 h, the MTT solution was aspirated off and replaced with 100 μl of acidic isopropanol (10% SDS, 5% isopropanol and 0.01 M HCl). The absorbance of each plate was measured at 595 nm using a spectrophotometer.

Colony formation assay

Lentivirus-transduced cells were seeded into a 6-well plate at a concentration of 300 cells/well and maintained at 37°C for 9 or 8 days (HCT116 and RKO, respectively). The culture media were changed every 2–3 days. When the colonies were formed, the plate was washed and fixed, stained with crystals purple for 20 min, and sequentially washed three times with ddH_2O. The number of colonies (>50 cells/colony) were counted.

Flow cytometry analysis

The relative proportions of cells in the G_1/G_0, S, and G_2/M phases of the cell cycle were determined by flow cytometry. In brief, cells were collected by centrifugation at 200 *g* for 5 min, washed with phosphate buffered saline (PBS), and fixed in 75% ethanol. Then the fixed cells were resuspended in propidium iodide/RNase/PBS buffer for incubation in dark (37°C, 30 min). The stained cells were analyzed by a FACs caliber II sorter and Cell Quest FACS system (BD Biosciences, San Diego, USA).

Statistical analysis

Data were presented as the mean ± standard deviations (SD) from at least three independent experiments performed in triplicate. Statistical significance was conducted with Student's *t*-test. $P < 0.05$ was considered statistically significant.

Ethical approval statement

All experimental research reported in the manuscript has been performed with the approval of Institutional Ethics Committee of China-Japan Union Hospital of Jilin University.

Results

The expression of GPR137 was ubiquitous in colon cancer cells

To examine the expression of GPR137 in colon cancer cells, seven common colon cancer cell lines (DLD-1, HCT116, HT-29, RKO, SW1116, SW480, and SW620) were first carefully cultured after which quantitative PCR and western blot analysis were performed. As shown in **Fig. 1A**, *GPR137* was widely expressed in all seven cell lines, in which RKO cell line was highlighted with the highest *GPR137* mRNA level. Western blot analysis further confirmed the ubiquitous expression of GPR137 protein in all seven cell lines (**Fig. 1B**). These results suggested that GPR137 expression may be related to colon tumorigenesis in some way. Therefore, we chose RKO cell line along with HCT116 cell line for the subsequent 'loss of function' investigation.

The GPR137 expression level was down-regulated by lentivirus-mediated RNAi in HCT116 and RKO cells

GPR137 was silenced by lentivirus-mediated method to investigate whether its expression was necessary for the normal viability of human colon cancer cells. A specific lentivirus-shRNA system (Lv-shGPR137) was designed and used to infect HCT116 and RKO cells, respectively. In addition, to avoid the non-specific gene-silencing effect of the lentivirus alone [24,25], a control shRNA (Lv-shCon) was constructed and used to infect both cell lines. As a result, a high expression of green fluorescence protein (GFP) (>80%) after infection for 72 h was observed under fluorescent microscope, whereas the non-infected control groups showed no green fluorescence at all (**Fig. 2A,D**), suggesting that the efficiency of lentivirus infection was stable and substantial. Next, to demonstrate the knockdown efficiency of the lentivirus system, quantitative PCR and western blot analysis were performed 5 days after infection. The mRNA levels of *GPR137* in Lv-shCon groups were almost the same as control (Con) groups in both cell types, whereas Lv-shGPR137 groups showed a dramatic 70.9% and 79.7% reduction in HCT116 and RKO cells, respectively, compared with Lv-shCon group ($P < 0.01$; **Fig. 2B,E**). As shown in **Fig. 2C,F**, the protein levels of GPR137 were also obviously

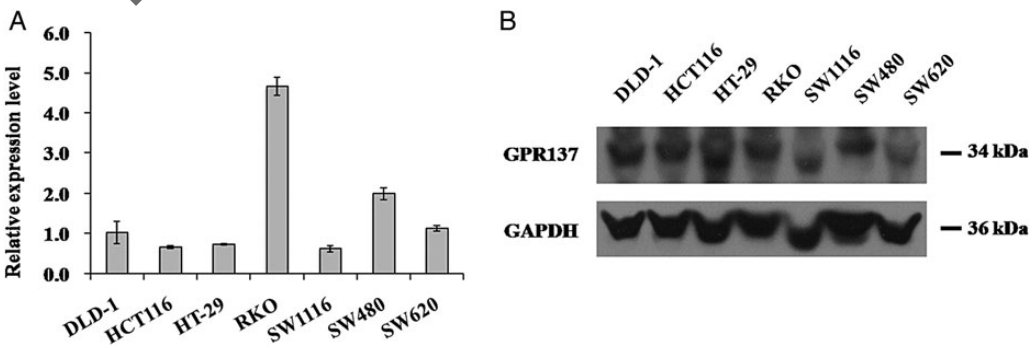

Figure 1. Expression levels of GPR137 in various colon cancer cell lines (A) qPCR analysis of *GPR137* mRNA in seven colon cancer cell lines. (B) Western blot analysis of GPR137 protein in seven colon cancer cell lines.

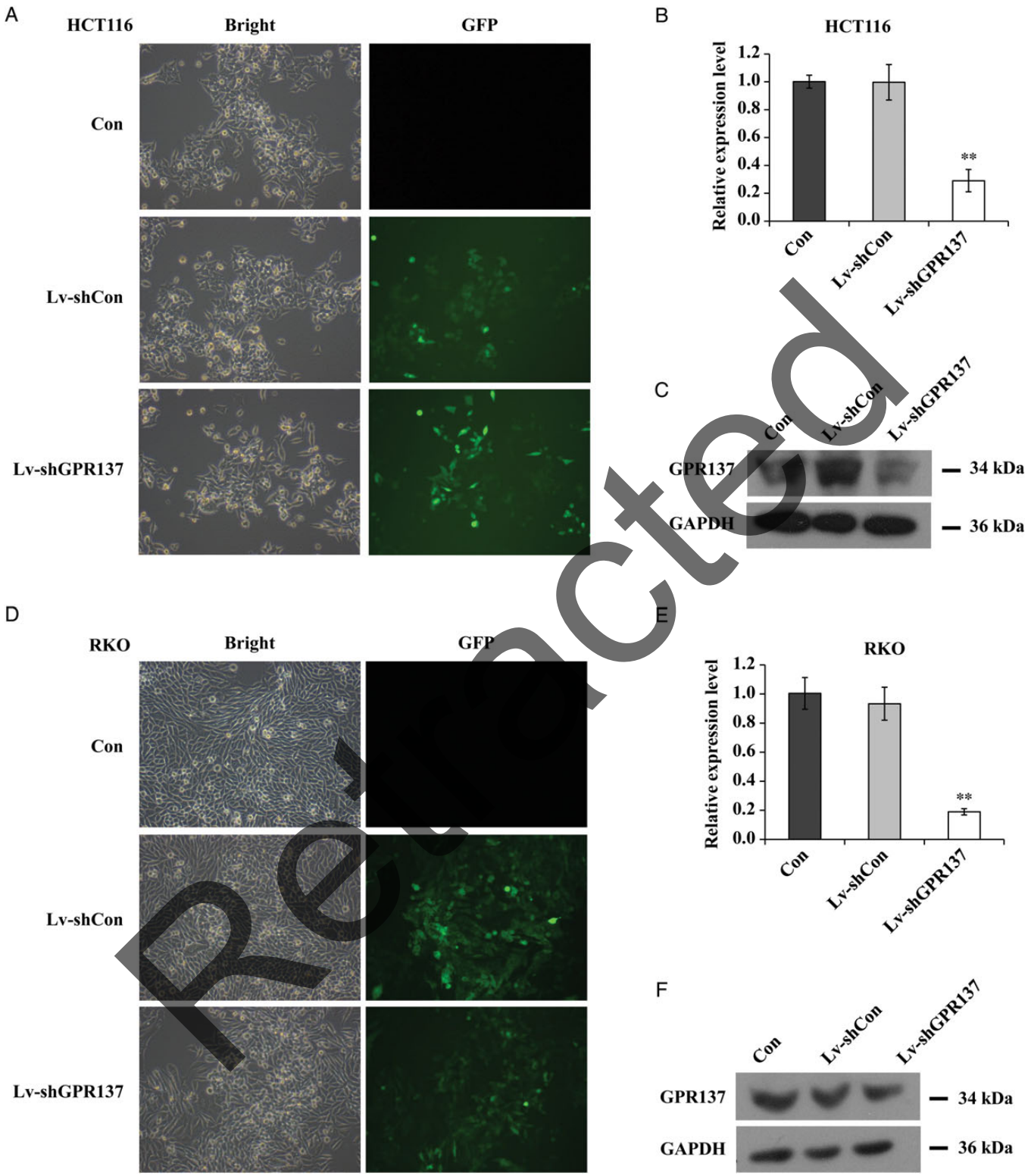

Figure 2. Lentiviral infection efficiency and knockdown efficiency Representative images of HCT116 (A) and RKO (D) cells after lentiviral infection, respectively. qPCR analysis of *GPR137* mRNA in HCT116 (B) and RKO (E) cells after lentiviral infection, respectively. Western blot analysis of GPR137 protein in HCT116 (C) and RKO (F) cells after lentiviral infection, respectively. $^{**}P < 0.01$, compared with Lv-shCon.

down-regulated in both cell types after GPR137 knockdown. These results suggested that the lentivirus-mediated knockdown system is highly effective to shut down GPR137 expression in colon cancer cells. Therefore, it is feasible to use Lv-shGPR137 as a down-regulator to examine its role in colon cancer cells.

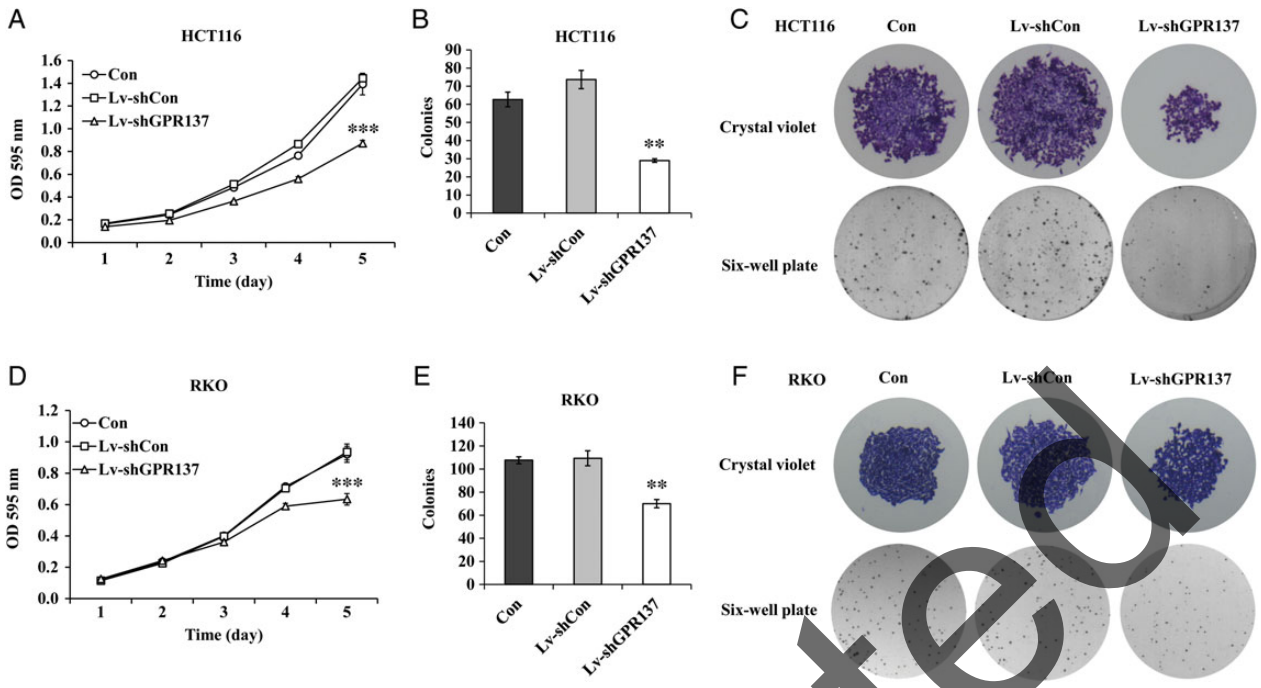

Figure 3. Effect of GPR137 silencing on the proliferation and colony formation ability of colon cells Optical density at 595 nm of HCT116 (A) and RKO (D) cells with three different treatments (Con, Lv-shCon and Lv-shGPR137), respectively. The number of colonies formed in HCT116 (B) and RKO (E) cells with three different treatments (Con, Lv-shCon, and Lv-shGPR137), respectively. Representative images of single colony and total colonies in HCT116 (C) and RKO (F) cells with three different treatments (Con, Lv-shCon, and Lv-shGPR137), respectively. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with Lv-shCon.

Cell growth was suppressed in colon cancer cells after GRP137 knockdown

To see if the lentivirus-mediated GPR137 silencing has potential inhibitory effect on cell growth and survival, both infected cell lines were routinely observed *in vitro* using MTT assay and colony formation assay. First, the proliferation rates of HCT116 and RKO cells in all groups were measured at indicated time points after infection (**Fig. 3A,D**). On day 4, the OD value at 595 nm of HCT116 cells in the Lv-shCon group (0.866 ± 0.024) was close to that of the Con group (0.763 ± 0.025), while that of the Lv-shGPR137 group (0.560 ± 0.020) had a 26.6% drop ($P < 0.001$). For RKO cells, the results are largely similar to those of HCT116 cells, where the OD values of non-infected, Lv-shCon and Lv-shGPR137 groups were 0.715 ± 0.015, 0.703 ± 0.019, and 0.590 ± 0.018, respectively. On day 5, the difference between GPR137-silenced cells and non-infected cells was more remarkable. This indicated that silencing of GPR137 had a general growth-suppressing effect on both HCT116 and RKO colon cancer cells, which further suggested that both GPR137 high-expression and low-expression cell lines could strongly react to the knockdown of GPR137, marking its essential role in maintaining the normal viability of human colon cancer cells.

In addition, it was also shown in the result of colony formation assay. Compared with the Lv-shCon infected group, the GPR137-silenced group of HCT116 cells showed an obvious 60.7% shrink in colony numbers ($P < 0.01$; **Fig. 3B,C**), while the colony numbers of RKO cells shrunk by about 36.0% (Con: 107.7 ± 3.1, Lv-shCon: 109.3 ± 6.5, Lv-shGPR137: 70.0 ± 3.6) ($P < 0.01$; **Fig. 3E,F**). The significant drop of survival rate in both cell lines indicated that inhibition of GPR137 could cause the suppression of growth in colon cancer cells, which further suggested that GPR137 might be indispensable for colon cancer growth and underscored the potential therapy of preventing the abnormal growth of these tumorigenic cells.

GPR137-silencing inhibited cell growth by arresting cell cycle progression

Flow cytometry method was employed to distinguish cells from different stages of the cell cycle. As shown in **Fig. 4A**, the cell population of G_0/G_1 phase in HCT116 cells infected with specific GPR137-targeted lentivirus ($63.76\% \pm 0.59\%$) was markedly increased compared with those of the non-infected ($55.16\% \pm 0.75\%$) and Lv-shCon infected cells ($54.79\% \pm 0.73\%$; $P < 0.001$), whereas the cell percentages of S phase and G_2/M phase were significantly decreased in HCT116 cells after GPR137 knockdown ($P < 0.01$). For RKO cells, the results are largely similar to those of HCT116 cells, where the cell population of G_0/G_1 phase was significantly increased after GPR137 knockdown, and the cell numbers in S phase and G_2/M phase were

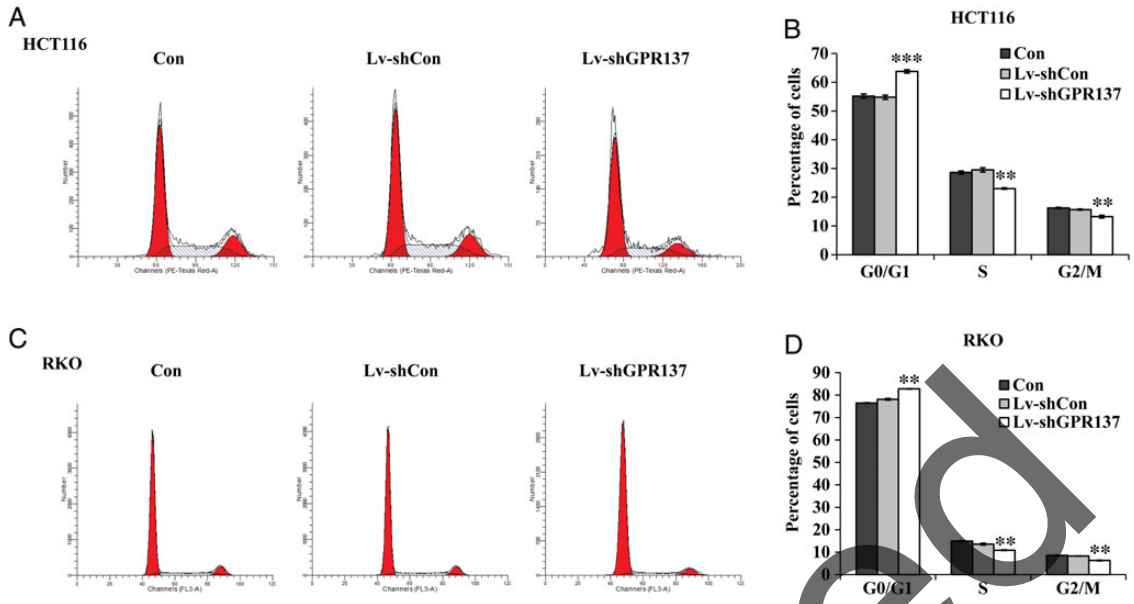

Figure 4. Effect of GPR137 silencing on the cell cycle progression Cell cycle distribution of HCT116 (A) and RKO (C) cells were analyzed by flow cytometry. Percentages of HCT116 (B) and RKO (D) cells under G_0/G_1, S, G_2/M phase in three groups (Con, Lv-shCon, and Lv-shGPR137) were shown. $^{**}P < 0.01$, $^{***}P < 0.001$, compared with Lv-shCon.

concomitantly decreased ($P < 0.01$). These results suggested that knockdown of GPR137 might cause cell arrest at G_0/G_1 phase, subsequently contributing to the suppression of cell proliferation. This evidence demonstrates the function of GPR137 in regulating colon cancer cell proliferation by stopping cells to 'go further on' to the S phage.

Discussion

Colorectal cancer, as mentioned above, is acknowledged as one of the most fatal diseases in the cancer category or even among all diseases. Though therapeutic protocols have been developed, the survival rate of colon cancer patient remains low due to its high metastasis. It has been found that tumor-inducing mutation of concerned genes was accumulated. Thus, a novel gene-therapy target for the cure of colon cancer is a heated and prospective research topic.

GPRs belong to the largest family of transmembrane receptors responsible for signal transduction including GPR40, GPR125, etc [26]. Recent studies demonstrated that many GPRs play a role in the tumorigenesis and metastasis of multiple human cancers [27]. Among them, GPR137 was found to behave as a biomarker in breast cancer [28]. In addition, the expression level of GPRs was shown to play a regulating role in the metastasis stage or level of cancer. For example, in the case of GPR49, its expression was revealed to have a positive correlation with the stage of colon and ovarian cancer: the higher the expression is, the later the stage of the cancer is [15]. In this study, we first investigated whether GPR137 is a commonly expressed gene in various colorectal cancer cells. Our data indicated that, in all the cell lines we examined, the expression of GPR137 is ubiquitous. In addition, lentivirus-based shRNA expression systems were employed to knockdown GPR137 in two different colon cancer cell lines (HCT116 and RKO) in order to further investigate the possibility of GPR137 being a therapeutic target. Our results suggested that the lentivirus-mediated knockdown system we constructed is effective in silencing GPR137 at both the gene and protein levels, which is also useful to examine its role in the tumorigenesis of colorectal cancer. After the specific knockdown of GPR137, the viability and colony-formation ability of both two colon cancer cell lines were considerably down-regulated, which suggested that GPR137 plays an essential role in colon cancer growth. Furthermore, results of cell cycle analysis revealed more about how GPR137 influenced cell survival rate. After GPR137 silencing, both HCT116 and RKO cells were substantially arrested in the G_0/G_1 phage of the cell cycle. And, the populations of cells in S phase and G_2/M phase were concomitantly reduced. Since S phage is the most important step in cell mitosis, these GPR137-silenced cells could not continue their proliferation, and thus the viability was down-regulated. This could be a hint for researchers to investigate the molecular pathways of the inhibition process.

In conclusion, our work first revealed that GPR137 is highly expressed in several types of colorectal cancer cells, and we successfully constructed a specific GPR137-targeted lentivirus system to knockdown this gene and found that GPR137 silencing remarkably inhibited cell growth along with G_0/G_1-phage arrest. Knocking down *GPR137* gene is highly efficient to suppress the abnormally fast growth of

colon cancer cells and may be a promising therapeutic approach for colorectal cancer.

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