LRG1 promotes proliferation and inhibits apoptosis in colorectal cancer cells via RUNX1 activation

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Abstract

Leucine-rich-alpha-2-glycoprotein 1 (LRG1) has been shown to be involved in various human malignancies. Whether it plays a role in colorectal cancer (CRC) development remains unclear. Here, we investigated whether and through what mechanism LRG1 functions in human CRC cells. The plasma level of LRG1 was significantly increased in CRC patients, but it was remarkably decreased in patients with resected colorectal cancers. Meanwhile, both mRNA and protein levels of LRG1 were remarkable overexpressed in CRC tissues than normal tissues. The knockdown of LRG1 significantly inhibited cell proliferation, induced cell cycle arrest at the G0/G1 phase, and promoted apoptosis in SW480 and HCT116 cells in vitro. In addition, LRG1 silencing led to the downregulation of the levels of key cell cycle factors, such as cyclin D1, B, and E and anti-apoptotic B-cell lymphoma-2(Bcl-2). However, it up-regulated the expression of pro-apoptotic Bax and cleaved caspase-3. Furthermore, RUNX1 could be induced by LRG1 in a concentration-dependent manner, while the knockdown of RUNX1 blocked the promotion of the proliferation and inhibition of apoptosis induced by LRG1. Collectively, these findings indicate that LRG1 plays a crucial role in the proliferation and apoptosis of CRC by regulating RUNX1 expression. Thus, LRG1 may be a potential detection biomarker as well as a marker for monitoring recurrence and therapeutic target for CRC.

Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-related deaths worldwide[1]. In view of its high morbidity and mortality, early detection methods and novel treatments are urgently needed. Currently, colonoscopies are extensively used as screening methods for its important role in diagnosis of early colorectal cancer throughout the world [2, 3]. However, a number of patients do not undergo colonoscopy at recommended intervals. CEA is typically a prognostic marker while it lacks the specificity and sensitivity to be a early detection marker for CRC[4], and the pathogenesis of CRC has not yet

submucosal dissection; LGN, low-grade neoplasia; LRG1, leucine-rich-alpha-2-glycoprotein 1; RUNX, runt-related transcription factors.

been fully elucidated. Therefore, it is important to determine the molecular mechanism for CRC development to recognize novel detection biomarkers and establish therapeutic targets for CRC.

Leucine-rich-alpha-2-glycoprotein1 (LRG1), a membrane-associated leucine-rich repeat (LRR) family member, was isolated from human serum by Haupt and Baudner in 1977[5]. LRG1 is induced by proinflammatory cytokines[6]. The expression of LRG1 is overexpressed in paediatric appendicitis, ulcerative colitis, inflammation, the immune response and neovascularization[7–10]. Moreover, LRG1 has been shown to be up-regulated in several types of carcinomas, such as hepatocellular carcinoma, gastric cancer, pancreatic cancer, leukaemia, ovarian cancer, bladder cancer and non-small cell lung cancer[11–17]. However, the biological function of LRG1 in the tumourigenesis and progression of colorectal cancer is not yet clear.

To investigate the downstream signalling of LRG1, we performed a gene microarray and found that the runt-related transcription factors (RUNX) were affected by the depletion of LRG1 in CRC cells. The RUNX family contains three members, RUNX1, RUNX2 and RUNX3. They function as either transcriptional repressors or activators, depending on the different genes and cell types; additionally, they have tissue-specific properties[18]. RUNX1 has been shown to play a role in haematopoiesis and haematopoietic function[19]. RUNX2 has been reported to be a key regulator required for osteogenic differentiation[20], while RUNX3 has been shown to be related to gastrointestinal tract development[21]. All three RUNX genes are associated with Smads and the TGF-β signalling pathway[22–24]. However, little is known about the relationship between LRG1 and RUNX genes in CRC.

In the present study, we explored the plasma and tissue levels of LRG1 in CRC patients and investigated the role of LRG1 in CRC cell proliferation and apoptosis. Additionally, this study aimed to determine which RUNX genes mediated by LRG1 act as downstream effectors in the proliferation and apoptosis progress of human CRC cells.

Materials and methods

Cell culture and LRG1 treatment

The human colorectal carcinoma cell lines SW480 and HCT116 were obtained from the China Center for Type Culture Collection (Beijing, China). Both cell lines were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS, Invitrogen, California, USA) in a humidified 5.0% CO_2 atmosphere at 37°C. Recombinant LRG1 was purchased from Biorbyt and added to the culture medium at concentrations of 50–1,000 ng/ml for the indicated time before harvesting.

Small interfering RNA silencing

Small interfering RNAs (siRNAs) were purchased from GenePharma (China) in addition to the oligos for LRG1 (#1: sense, 5′-GCAAUUAGAACGGCUACAUT T-3′ and antisense, 5′-AUGUAGCCGUUCUAAUUGCTT-3′; #2: sense, 5′-CCUCUAAGCUCCAAGAAUUTT-3′ and antisense, 5′-AAUUCUUGGAGCUUAGAGGTT-3′), RUNX-1 (sense, 5′-CCAGGUUGCAA GAUUUAAUTT-3′ and antisense, 5′-AUUAAAUCUUGCAACCUGGTT-3′), and a non-targeting control siRNA. Transfection of siRNAs was performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. The culture medium was exchanged 6 h after transfection, and the cells were harvested 24 h or 48 h later.

RNA extraction and quantitative real-time PCR

The total RNA was extracted using TRIzol reagent (Invitrogen, USA), and cDNA was synthesized using the PrimeScriptTM RT Reagent Kit (Perfect Real Time, TaKaRa, Japan). Quantitative real-time PCR was performed in a total volume of 10 μl containing SYBR Green (SYBR® Premix Ex TaqTM II, TaKaRa, Japan) on an Applied Biosystems 7900 quantitative PCR system. After normalization to the β-actin gene, the relative expression of each target gene was determined according to the 2−ΔΔCt method. The data are presented as the means ± standard deviation (SD) from three independent experiments.

Protein extraction and western blot analysis

Whole cellular proteins were isolated from fresh cells with lysis buffer containing RAPI PMSF. Protein concentrations in the supernatants were measured using a BCA protein assay. Equal amounts of total protein were loaded onto a 10% SDS-PAGE gel, transferred onto polyvinylidene difluoride membranes, and blocked with 5% fat-free milk for 2 h at room temperature. The membranes were incubated overnight at 4˚C with a primary antibody, and this step was followed by treatment with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunodetection was performed using the Super Signal West Femto Maximum Sensitivity Substrate (Thermo Fisher, Massachusetts, USA). The antibody for LRG1 was purchased from Sigma (HPA001888, rabbit polyclonal), and the other antibodies were all purchased from Cell Signaling Technology, Inc. (USA). The intensity of the target protein bands was determined using the ImageJ software and normalized to that of β-actin.

Evaluation of cell proliferation

To evaluate cell proliferation, SW480 and HCT116 cells were seeded in 96-well microplates at a density of 3×10^3 cells/well. At the indicated time points (0, 24, 48, 72, or 96 h), 10 μL of CCK-8 solution (DOJINDO, Japan) was added to 100 μL of culture medium. After 2 h of incubation at 37˚C, the absorbance of the culture medium was measured at 450 nm (A450) using a scanning microplate spectrophotometer (Multiscan GO, Thermo Fisher Scientific, Waltham, MA, USA).

Evaluation of cell cycle

Forty-eight hours after transfection, the cells were collected, washed with ice-cold phosphate buffer solution (PBS), and fixed in 75% ethanol at -20˚C overnight. Prior to staining, the cells were washed with PBS. The cells were treated with 10 μL RNase A (10 mg/mL, TaKaRa, Japan) for 30 min at 37˚C and then stained with 50 μL of a propidium iodide (PI) (250 μg/mL) solution. In total, 30,000–50,000 cells were analysed on a FACScan flow cytometer (Becton–Dickinson LSRFortessa, San Jose, CA, USA) and analysed with FlowJo V10 software.

Evaluation of cell apoptosis

Cell apoptosis was probed using the FITC Annexin V/PI Apoptosis Detection Kit (BD Pharmingen, USA) according to the manufacturer's instructions. In brief, cells were collected, washed with Binding Buffer at 4˚C, and resuspended in 200 μL of Binding Buffer. A total of 1×10^6 cells were stained with 2.5 μL of Annexin V-FITC and 5 μL of PI. Cells were then incubated with the staining solution at room temperature in the dark. In total, 30,000–50,000 cells were analysed on a FACScan flow cytometer (Becton–Dickinson LSRFortessa, San Jose, CA, USA) and analysed with FlowJo V10 software.

Clinical specimens and enzyme-linked immunosorbent assay

Thirty patients with colorectal cancer, 30 patients with adenoma or low-grade neoplasia (LGN), and 30 matched normal patients, totally 90 patients were prospectively enrolled at Shanghai Renji Hospital. All cases were individually matched based on age (within 3 years) and gender. Plasma samples were obtained from the above patients when they underwent endoscopic examination or endoscopic resection or surgery. On the other hand, plasma samples were obtained again from the 30 patients with colorectal cancer half a year post surgery, at that time those 30 patients didn't find any recurrent or metastatic tumor. Patients with other system diseases were excluded in this study. Sixty human CRC tissues and adjacent non-cancerous tissues were obtained from patients who underwent surgery at Renji Hospital. Histological diagnoses were performed by expert pathologists. The study was approved by the Ethics Committee of Renji Hospital with written informed consent from all patients included in this study. Human LRG1 was quantified using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Europe, UK) according to the manufacturer's instructions.

Statistical analysis

The data and figures were processed using the GraphPad Prism 5.0 software (San Diego, CA, USA). Quantitative data were expressed as the means ± SEM, and comparisons between two groups were performed using Student's t test or a paired t test. P-values <0.05 were considered statistically significant.

Results

Increased plasma and tissue level of LRG1 was related to human CRCs

To investigate the correlation between the plasma level of LRG1 and human CRCs, we collected plasma samples from patients who underwent coloscopy examination or endoscopic resection or surgery at Renji Hospital. Fig 1a shows that the plasma level of LRG1 was increased in adenomas (LGN) and CRCs, and the concentration of LRG1 was significantly increased in human CRCs compared with adenomas and controls. Among 30 CRC patients, 11 patients were at Duke' s stage A (T1N0M0+T2N0M0),13 patients were at stage B(T3N0M0+T4N0M0), 6 patients were at stage C (patients with lymph node metastasis), while all patients didn't have distant metastasis. Furthermore, the plasma level of LRG1 was remarkably decreased in patients who had resected colorectal cancers, which was consistent with normal patients.

To investigate the mRNA expression level of LRG1 in CRC, we performed real-time RT-PCR and Western blot to assess LRG1 expression in 60 cases of CRC tissues and matched normal tissues. The result revealed that both mRNA and protein levels of LRG1 were remarkable overexpressed in CRC tissues than normal tissues (P < 0.001, Fig 1b).

LRG1 influenced CRC cell proliferation and cell cycle arrest

The increased plasma level of LRG1 in CRCs suggested that LRG1 might play a role in its proliferation capability. To investigate the role of LRG1 in CRC cells, we knocked down LRG1 using the transfection of specific siRNA. Both the mRNA and protein levels of LRG1 were significantly down-regulated in SW480 and HCT116 cells (Fig 2a). According to the performed CCK-8 assays, the knockdown of LRG1 efficiently reduced the proliferation rates of SW480 and HCT116 cells (Fig 2b), implying that LRG1 silencing inhibited the growth of SW480 and HCT116 cells. Furthermore, the knockdown of LRG1 resulted in the accumulation of cells in the G0/G1 phase and decreased the number of cells in the S and G2/M phases, which was more significant in SW480 cells than HCT116 cells (Fig 2c and 2d). Next, we investigated the
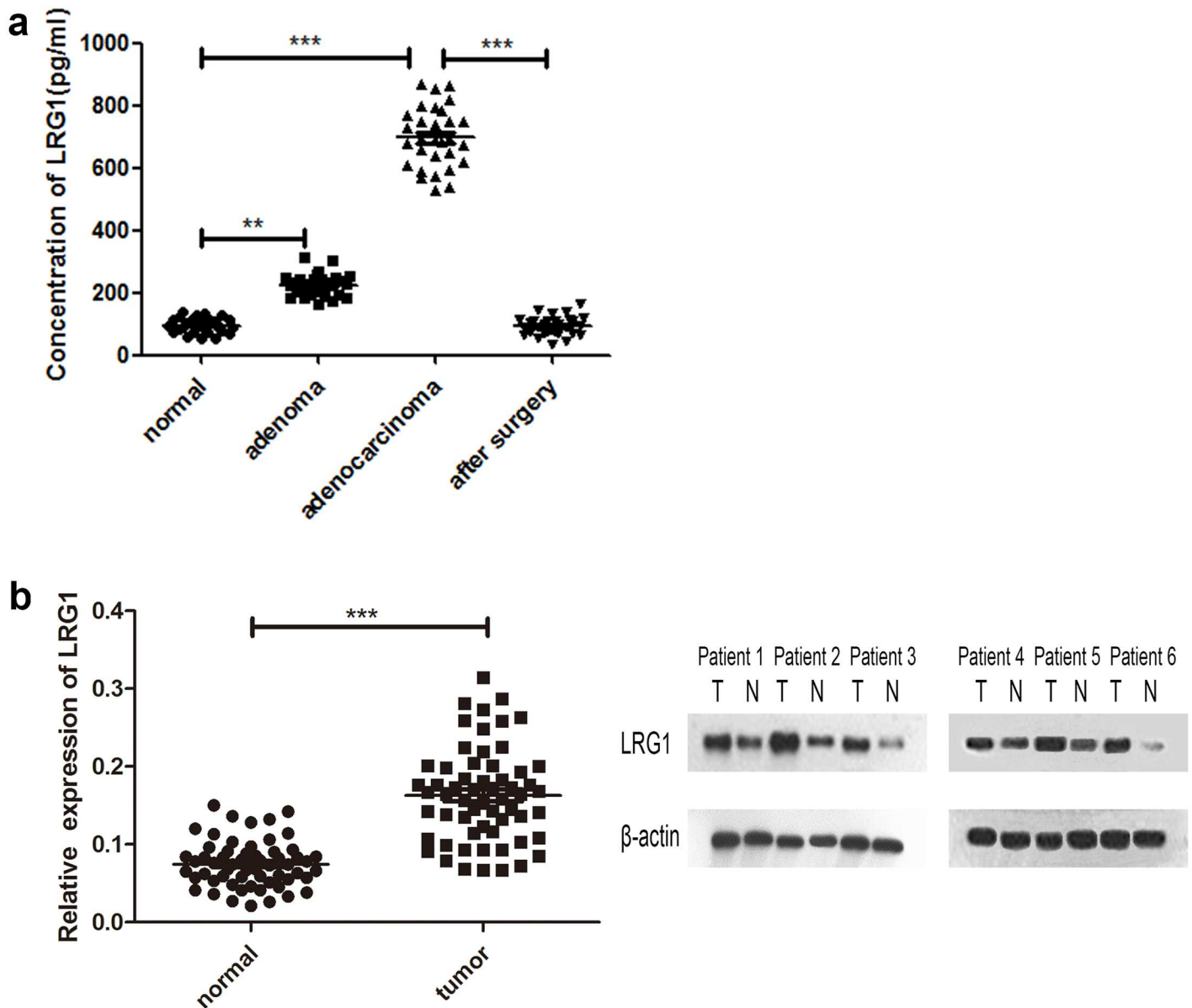
Fig 1. The plasma and tissue level of LRG1 was increased in human CRCs. (a) Plasma samples from 30 normal patients, 30 adenoma patients, 30 CRC patients and the same 30 CRC patients after surgery were obtained, and the secretion of LRG1 was quantified by an ELISA. **(b)** Real-time RT-PCR and western blot were performed to assess LRG1 expression in 60 cases of CRC tissues and matched normal tissues. **P<0.01, and ***P<0.001.

expression of cell cycle markers following the knockdown of LRG1. Real-time RT-PCR and Western blot analysis showed that the expression levels of cyclin D1, B, and E in LRG1-knock-down cells were significantly decreased compared with NC cells (Fig 2e and 2f). In summary, LRG1 silencing suppressed cell growth by inhibiting cell proliferation as well as inducing cell cycle arrest in SW480 and HCT116 cells.

LRG1 influenced CRC cell apoptosis

The cell apoptotic status was analysed using an FITC annexin V/PI double staining assay. As shown in Fig 3a and 3b, the number of apoptotic cells was markedly increased in LRG1-
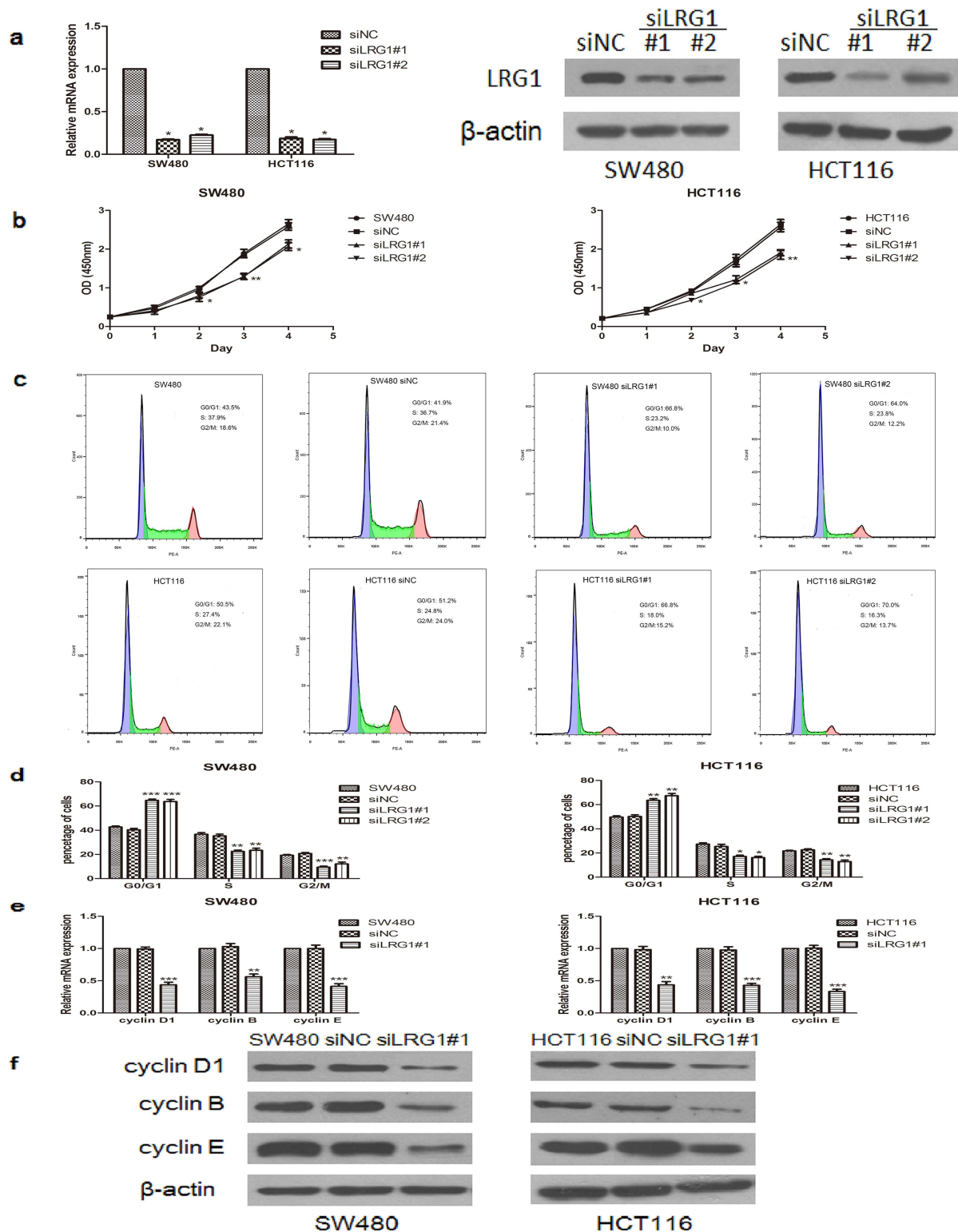
Fig 2. The knockdown of LRG1 inhibited CRC cell proliferation and induced cell cycle arrest. (a) RT-PCR and Western blot analyses showed that LRG1 was effectively down-regulated by siRNA transfection. **(b)** A CCK-8 assay was performed to evaluate cell proliferation in vitro. **(c-d)** SW480 and HCT116 cells were fixed, stained with PI, and subjected to flow cytometry analysis for cell cycle. **(e–f)** The expression levels of cyclin D1, B, and E in SW480 and HCT116 cells transfected with LRG1 siRNA or control siRNA were determined by RT-PCR and Western blot analysis. This figure shows representative images of repeated experiments, and the data are presented as the mean±standard deviation. Compared with NC, *P<0.05, **P<0.01, and ***P<0.001.
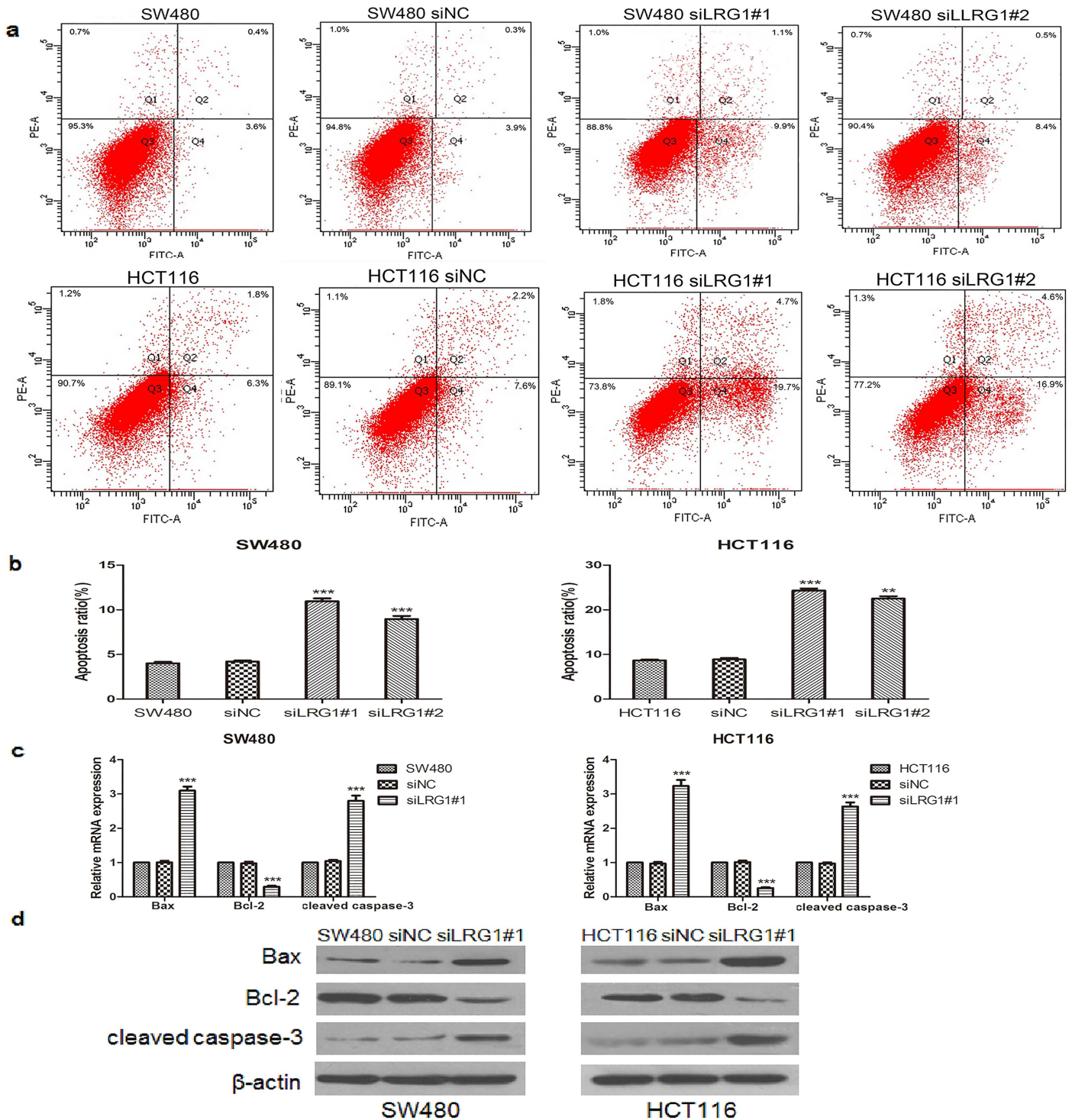
Fig 3. The knockdown of LRG1 promoted CRC cell apoptosis. (a-b) SW480 and HCT116 cells were double stained with an FITC-conjugated anti-Annexin V antibody and PI and then subjected to flow cytometry for cell apoptosis analysis. The cells in the upper right and lower right quadrants were considered late and early apoptotic cells, respectively. **(c-d)** The expression levels of Bcl-2, Bax, and cleaved caspase-3 in SW480 and HCT116 cells transfected with LRG1 siRNA or control siRNA were determined by RT-PCR and Western blot analysis. This figure shows representative images of repeated experiments, and the data are presented as the mean±standard deviation. Compared with NC, **P<0.01 and ***P<0.001.

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silenced SW480 and HCT116 cells compared with NC cells (P<0.001). We further examined the mRNA and protein levels of the apoptotic suppressor B-cell lymphoma-2(Bcl-2) as well as its downstream apoptotic promoters cleaved caspase-3 and Bax by real-time PCR and Western blot analysis. The results showed that the knockdown of LRG1 significantly down-regulated the levels of Bcl-2, whereas it elevated the expression of Bax and cleaved caspase-3 in both SW480 and HCT116 cells (Fig 3c and 3d). The above findings provide evidence for the role of LRG1 silencing in enhanced apoptosis in SW480 and HCT 116 cells.

RUNX1 was essential for LRG1-mediated proliferation and apoptosis

The RUNX family is believed to play an essential role in carcinogenesis and normal development. To investigate whether LRG1 could regulate the expression of RUNXs, we analysed the relative mRNA expression of the RUNX family in LRG1-silenced CRC cells. Real-time RT-PCR data showed that RUNX1 expression was significantly decreased in LRG1-silenced CRC cells, while the knockdown of LRG1 had no effect on the levels of RUNX2 and RUNX3 (Fig 4a). To determine whether LRG1 could enhance RUNX1 expression, CRC cells were stimulated with rLRG1 for 24 h. The results showed that the mRNA expression of RUNX1 was elevated when treated with 100 ng/ml rLRG1 and reached a maximum level at 1,000 ng/ml rLRG1 (Fig 4b). LRG1 stimulation also resulted in the promotion of the growth of SW480 and HCT116 cells (Fig 4c). Moreover, the protein expression levels of RUNX1 as well as cyclin D1, B, E and Bcl-2 were also increased, while protein expression of Bax and cleaved caspase-3 were decreased in a concentration-dependent manner following stimulation with rLRG1 in SW480 cells (Fig 4d). Furthermore, we investigated the role of RUNX1 in LRG1-induced proliferation and apoptosis. Transfection with siRNA successfully downregulated the mRNA and protein levels of RUNX1 in SW480 cells (Fig 5a). As shown in Fig 5b–5f, silencing RUNX1 inhibited the enhanced proliferation and anti-apoptosis ability of CRC cells induced by rLRG1 and resulted in cell cycle arrest in SW480 cells. The knockdown of RUNX1 also reversed LRG1-induced expression of cell cycle- and apoptosis-associated proteins, increasing the expression of Bax and cleaved caspase-3 and decreasing the expression of cyclin D1, B, E and Bcl-2 (Fig 5g). The above results strongly suggest that RUNX1 is involved in LRG1-induced CRC cell proliferation, cell cycle and apoptosis.

Discussion

LRG1 has been reported to be up-regulated in several types of carcinomas, but its role in colorectal cancer remains unclear. In this study, we demonstrated that the plasma level of LRG1 was increased in adenomas (LGN) and CRCs, and the concentration of LRG1 was remarkably increased in human CRCs compared with adenomas and controls, which was consistent with data found in the literature[6, 25]. Moreover, we found that the plasma level of LRG1 was significantly decreased in patients who have undergone CRC resections. This result indicates that LRG1 might be a potential biomarker for early detection and following-up postoperative CRCs. Meanwhile, both RT-PCR and Western blot showed that LRG1 expression was significantly higher in CRC tissues compared with normal tissues, which confirmed our previous study[26]. The high plasma and tissue level of LRG1 in CRCs suggests that LRG1 may play role in the tumorigenesis of colorectal cancer.

Although LRG1 was shown to participate in a variety of benign and malignant diseases, the molecular mechanism of LRG1 has not been fully clarified thus far. Recent studies showed that LRG1 promotes pathogenic neovascularization, cell adhesion, cell migration, and cell invasion and is involved in the epithelial-to-mesenchymal transition (EMT) process[10, 26, 27]. As proliferation play an important role in the carcinogenesis of tumours, and the cell cycle of
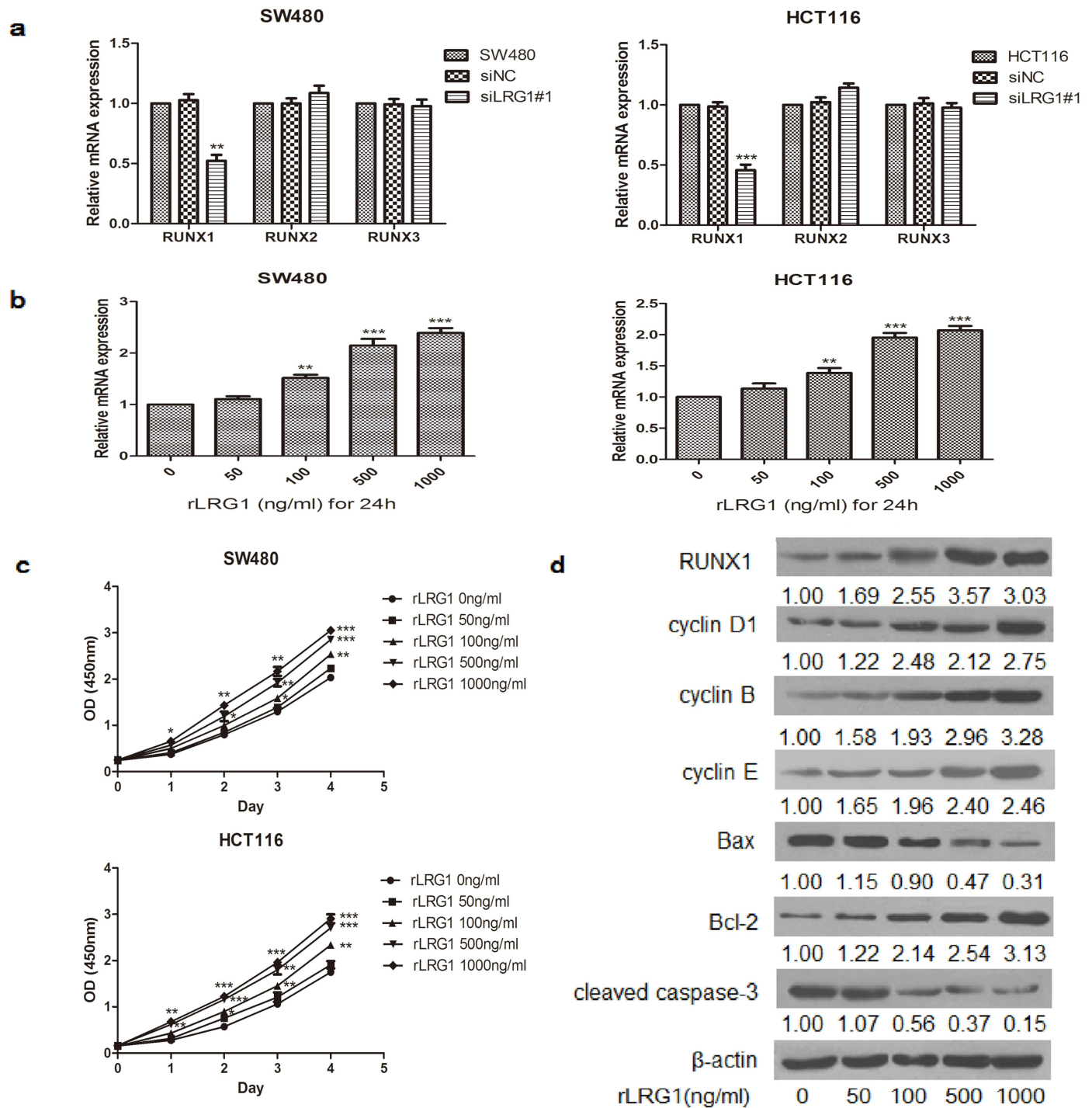
Fig 4. LRG1 promoted the expression of RUNX1 and CRC cell proliferation. (a) SW480 and HCT116 cells were transfection with LRG1 siRNA, and the expression of the tested RUNXs was quantified by RT-PCR. **(b)** Cells were stimulated with rLRG1 (0–1,000 ng/ml) for 24 h, and the RUNX1 expression was determined by RT-PCR. **(c)** Cells were stimulated with rLRG1 (0–1000 ng/ml), and a CCK-8 assay was performed to evaluate cell proliferation. **(d)** The protein levels of cyclin D1, B, E, Bcl-2, Bax, and cleaved caspase-3 in response to LRG1 treatment in SW480 cells were analysed by Western blot. This figure shows representative images of repeated experiments, and the data are presented as the mean±standard deviation. Compared with NC, *P<0.05, **P<0.01, and ***P<0.001.
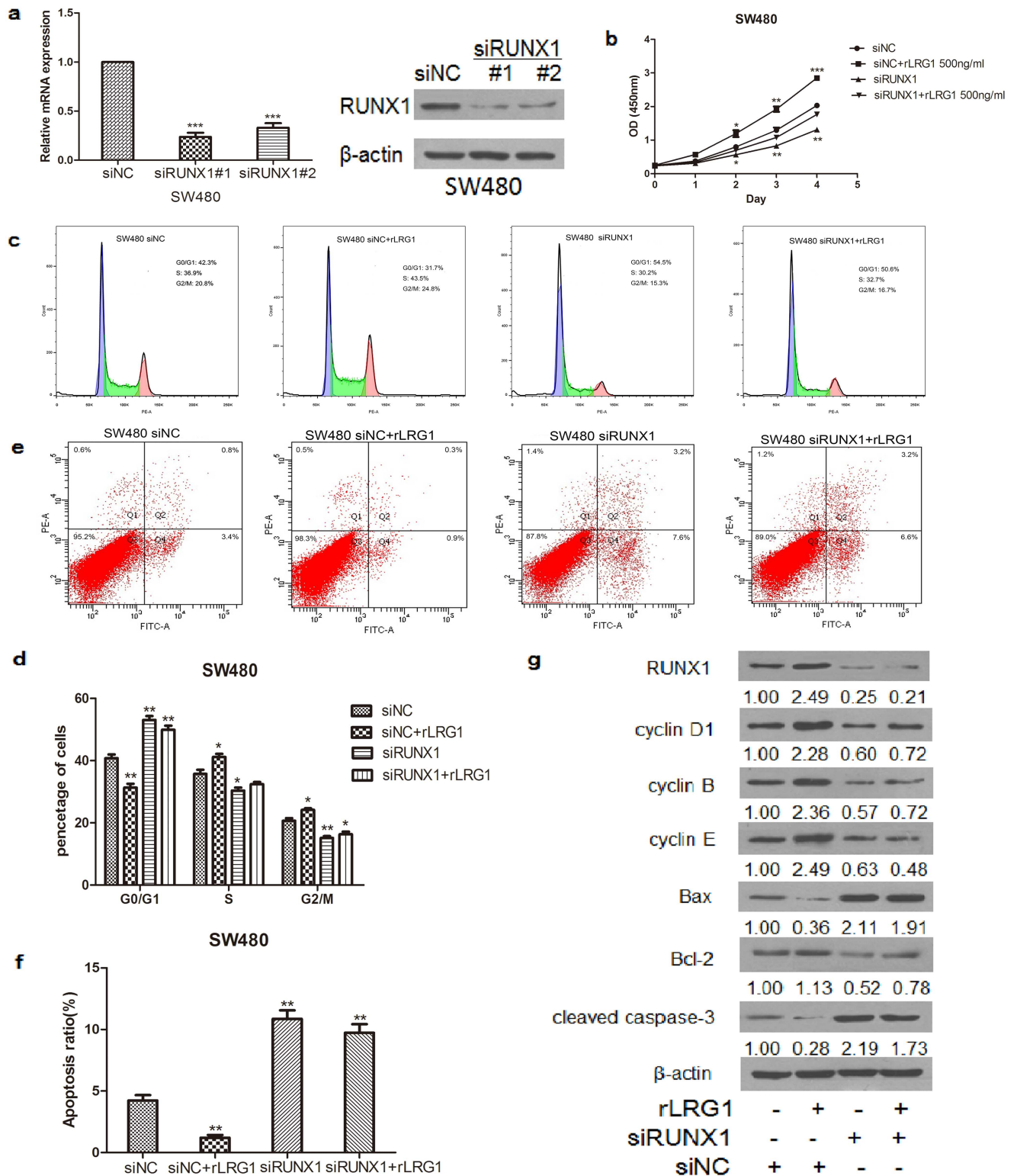
Fig 5. The role of RUNX1 in LRG1-induced cell proliferation and anti-apoptosis. (a) RUNX1 expression was knocked down in siRNA-transfected SW480 cells at both the mRNA and protein levels. **(b)** SW480 cells were transfected with RUNX1 or control siRNA with or without rLRG1 (500 ng/ml) stimulation, and a CCK-8 assay was performed to evaluate cell proliferation. **(c-d)** SW480 cells were transfected with RUNX1 or control siRNA for 24 h and treated with or without rLRG1 (500 ng/ml) for an additional 24 h. Then, the SW480 cells were fixed, stained with PI, and subjected to flow cytometry analysis to probe the cell cycle. **(e-f)** SW480 and HCT116 cells were harvested and double stained with an FITC-conjugated anti-Annexin V antibody and PI. Then, the cells were subjected to flow cytometry for cell apoptosis analysis. **(g)** The protein

levels of Runx1,cyclin D1, B, E, Bcl-2, Bax, and cleaved caspase-3 in response to RUNX1 or control siRNA with or without rLRG1 treatment in SW480 cells, which were analysed by Western blot. This figure shows representative images of repeated experiments, and the data are presented as the mean±standard deviation. Compared with NC, *P<0.05, **P<0.01, and ***P<0.001.

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eukaryotic cells is the essential process of cell proliferation[28], we investigated the role of LRG1 in the proliferation and the cell cycle of CRC cells. Our study demonstrated that the silencing of LRG1 expression significantly inhibited the growth of colorectal carcinoma cells; meanwhile, stimulation with recombinant LRG1 enhanced cell proliferation. What's more, the knockdown of LRG1 by siRNA resulted in colorectal cancer cell cycle arrest with the accumulation of cells in the G0/G1 phase and reduced the cell numbers in the S and G2/M phases, while stimulation with recombinant LRG1 had an inverse effect. Moreover, we demonstrated that silencing LRG1 expression led to the significant down-regulation of cell cycle genes, including cyclin D1, B, and E, which have been reported to play crucial roles in the transition of the G1/S phase during cell proliferation and the cell cycle[29] and the over-expression of these genes lead to a shortened G1 phase and accelerated tumour formation [30]. The results imply that LRG1 modulates the cell cycle and cell proliferation of colorectal cancer by regulating the expression of cyclin D1, B, and E.

The imbalance of cell apoptosis also leads to tumourigenesis. The stable knockdown of LRG1 has been reported to promote the apoptosis of glioblastoma cells[31]. However, the role of LRG1 in colorectal cancer cell apoptosis has not been addressed. In this study, the knockdown of LRG1 by siRNA significantly promoted the apoptosis of both SW480 cells and HCT116 cells, while stimulation with recombinant LRG1 remarkably inhibited SW480 cell apoptosis. Furthermore, LRG1 silencing significantly down-regulated both the mRNA and protein expression of the anti-apoptotic protein Bcl-2 but up-regulated both the mRNA and protein expression of pro-apoptotic protein Bax and cleaved caspase-3. The results presented above suggest that LRG1 may be involved in the apoptosis of colorectal cancer cells through these classic apoptotic proteins.

The RUNXs are believed to play an essential role in carcinogenesis in addition to their role in normal development[32]. Among the three RUNXs, gene polymorphisms and hypermethylation in RUNX3 have been reported to be related to colorectal cancer[33, 34]. However, as all three RUNX genes are involved in signalling cascades mediated by bone morphogenetic protein (BMP) and TGF-β, they all have the potential to participate in CRC aetiology[35, 36]. On the other hand, RUNX1 is vital for proliferation and serves as an oncogene in some tumors[37, 38]. Recent studies showed that the expression of RUNX1 was markedly up-regulated in CRC tissues and genetic variation in RUNX1 was associated with high risk for colon and rectal cancers [39, 40]. Our result revealed that LRG1 silencing significantly down-regulated the expression of RUNX1 rather than RUNX2 and RUNX3. Activation of different signaling pathway may account for different RUNXs' change induced by LRG1. Additionally, the mRNA and protein expression level of RUNX1 was increased in a concentration-dependent manner following stimulation with rLRG1 in CRC cells. Additionally, our results indicated that RUNX1 is involved in LRG1-induced proliferation and apoptosis since the knockdown of RUNX1 blocked the promotion of the proliferation and inhibition of apoptosis induced by LRG1. Moreover, RUNX1 knockdown reversed the LRG1-induced up-regulation of cyclin D1, B, E and Bcl-2 and the induced down-regulation of Bax and cleaved caspase-3, which act as key factors in proliferation and apoptosis. These data suggest that LRG1 may target the RUNX1 pathway to induce CRC cell proliferation and apoptosis. As both LRG1and RUNX1 are involved in the TGF-β signalling pathway[10, 41], it is not clear whether LRG1 activates RUNX1 transcription directly or via the TGF-β pathway.

In summary, our results provide evidence for the use of LRG1 as a novel detection biomarker for the prediction of CRCs as well as postoperative following-up for CRCs. We demonstrated that LRG1 promotes cell proliferation, while inhibiting apoptosis, in CRC cells through the regulation of RUNX1 expression. Thus, LRG1 may act as a potential detection biomarker and a new therapeutic target for colorectal cancer.

Supporting information

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Writing – review & editing: YZ XBL.

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