



ORIGINAL ARTICLE

JNK pathway plays a critical role for expansion of human colorectal cancer in the context of BRG1 suppression

Takaaki Yoshikawa^{1,2}  | Akihisa Fukuda¹ | Mayuki Omatsu¹ | Mio Namikawa¹ | Makoto Sono¹ | Yuichi Fukunaga^{1,3} | Tomonori Masuda¹ | Osamu Araki¹ | Munemasa Nagao¹ | Satoshi Ogawa¹ | Kenji Masuo^{1,4} | Norihiro Goto¹ | Yukiko Hiramatsu¹ | Yu Muta¹ | Motoyuki Tsuda¹  | Takahisa Maruno¹ | Yuki Nakanishi¹ | Kenji Kawada⁵ | Shigeo Takaishi⁴ | Hiroshi Seno¹

¹Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan

²Department of Gastroenterology and Hepatology, Kitano Hospital, Tazuke Kofukai Medical Research Institute, Osaka, Japan

³Department of Drug Discovery Medicine, Medical Innovation Center, Kyoto University Graduate School of Medicine, Kyoto, Japan

⁴Laboratory for Malignancy Control Research (DSK Project), Medical Innovation Center, Kyoto University Graduate School of Medicine, Kyoto, Japan

⁵Department of Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan

Correspondence

Akihisa Fukuda, Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan.
Email: fukuda26@kuhp.kyoto-u.ac.jp

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Abstract

Tumor stem cells (TSCs), capable of self-renewal and continuous production of progeny cells, could be potential therapeutic targets. We have recently reported that chromatin remodeling regulator Brg1 is required for maintenance of murine intestinal TSCs and stemness feature of human colorectal cancer (CRC) cells by inhibiting apoptosis. However, it is still unclear how BRG1 suppression changes the underlying intracellular mechanisms of human CRC cells. We found that Brg1 suppression resulted in upregulation of the JNK signaling pathway in human CRC cells and murine intestinal TSCs. Simultaneous suppression of BRG1 and the JNK pathway, either by pharmacological inhibition or silencing of *c-JUN*, resulted in even stronger inhibition of the expansion of human CRC cells compared to Brg1 suppression alone. Consistently, high *c-JUN* expression correlated with worse prognosis for survival in human CRC patients with low *BRG1* expression. Therefore, the JNK pathway plays a critical role for expansion and stemness of human CRC cells in the context of BRG1 suppression, and thus a combined blockade of BRG1 and the JNK pathway could be a novel therapeutic approach against human CRC.

KEYWORDS

apoptosis, colorectal cancer, epigenetics, prognosis, tumor stem cell

Abbreviations: 4-OHT, 4-hydroxytamoxifen; BRG1, Brahma-related gene 1; BRM, Brahma; CRC, colorectal cancer; DA, *Dclk1*^{CreERT2-IRES-EGFP/+}; *Apc*^{Min/+}; *Brg1*^{flox/wt} or *Dclk1*^{CreERT2-IRES-EGFP/+}; *Apc*^{Min/+}; *Brg1*^{wt/wt}; DAB, *Dclk1*^{CreERT2-IRES-EGFP/+}; *Apc*^{Min/+}; *Brg1*^{flox/flox}; ER, endoplasmic reticulum; GSEA, Gene Set Enrichment Analysis; HAB, *Hnf1b*^{CreERT2/+}; *Apc*^{Min/+}; *Brg1*^{flox/flox}; hCRC, human colorectal cancer; IHC, immunohistochemical; qRT-PCR, quantitative real-time PCR; siBRG1, siRNA targeting BRG1; siJUN, siRNA targeting JUNs; TCGA, The Cancer Genome Atlas; TSC, tumor stem cell.

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1 | INTRODUCTION

Colorectal cancer is the third most common type of cancer worldwide and the second most common cause of cancer-related death.¹ Chemotherapy is a main therapeutic option against inoperable CRCs. However, they finally acquire resistance to conventional cytotoxic chemotherapy. Thus, there is an unmet need to invent a novel therapeutic approach. In this context, drug discovery related to epigenetic modifications represents a promising field for the treatment of chemoresistant CRC. Epigenetic modifications including methylation and histone modifications and chromatin remodeling play important roles in initiation and progression of CRC.^{2,3}

Switch/sucrose nonfermentable (SWI/SNF) chromatin remodeling complexes play important roles in transcriptional regulation, DNA replication, and damage repair in an ATP-dependent manner.^{4,5} They contain either one of the two mutually exclusive catalytic ATPase subunits: SMARCA4 (BRG1, also known as Brahma-related gene 1) or SMARCA2 (BRM, also known as Brahma).⁶ Although human BRG1 negative cancers exist and harbor malignant behavior,⁷⁻⁹ BRG1 has recently been shown to be overexpressed in many tumor types and is associated with tumor aggressiveness and poor prognosis, including hCRC.¹⁰⁻¹³ We previously showed that Brg1 is essential for acinar cell-derived pancreatic cancer formation by inhibiting apoptosis.¹⁴ Previous reports, including our report, have shown that hCRCs strongly express BRG1.^{12,15,16} As in other types of cancer, expression of BRG1 in hCRC is associated with recurrence, metastasis, and poor prognosis.^{12,13,16} We have recently reported that Brg1 plays an essential role for maintenance of murine intestinal TSCs and for cell survival and stemness features of hCRC cells.¹⁶ Continuous ablation of Brg1 in intestinal TSCs maintains suppression of intestinal tumors accompanied by increased apoptosis and loss of their capacity for self-renewal in mice. However, it is still unclear how BRG1 suppression changes the underlying intracellular mechanisms of hCRC cells. In this study, we investigated the underlying molecular mechanism by which BRG1 suppression affects stemness of hCRC cells to develop a novel therapeutic approach against hCRC.

2 | MATERIALS AND METHODS

2.1 | Animals

The following mouse strains were used. *Dclk1*^{CreERT2-IRES-EGFP} mice were generated by our group, as previously described.¹⁷ *Brg1*^{flox} mice were kindly gifted by David Reisman (University of Florida), with permission from Pierre Chambon (University of Strasbourg Institute for Advanced Study).¹⁸ *Hnf1b*^{CreERT2} mice were also kindly gifted by Jorge Ferrer (Imperial College).¹⁹ *Apc*^{Min} mice (JAX strain 002020) were obtained from the Jackson Laboratory. Mice were housed under specific pathogen-free conditions at the animal facilities

of Kyoto University. Mice were maintained against a C57BL/6 background. *Apc*^{Min} mice were maintained by breeding *Apc*^{Min} male mice to C57BL/6J female mice. For induction of CreER-mediated recombination in vivo, 200 μ l of 20 mg/ml tamoxifen (Sigma-Aldrich) in corn oil was intraperitoneally injected.

2.2 | Human subjects

Specimens of surgically resected CRC were obtained from patients at Kyoto University Hospital. Clinicopathologic data were collected from medical records and pathological reports of Kyoto University Hospital. The TNM classification was decided in accordance with the UICC 8th classification.

2.3 | Histological analysis

Mice tissues were fixed with 4% buffered paraformaldehyde solution overnight at 4°C. They were then paraffin-embedded and sectioned (5 μ m thickness). Sections were deparaffinized and rehydrated. Antigen retrieval for all primary Abs was achieved by boiling in 10 mM citrate buffer at pH 6.0 for 15 min. For immunohistochemistry, sections were incubated with primary Abs overnight at 4°C, followed by incubation with biotinylated secondary Ab for 1 h at room temperature. Immunoperoxidase labeling was undertaken with Vectastain ABC kit (Vector Laboratories; catalog no. PK-6102), and sections were then colored with diaminobenzidine substrate (Dako; catalog no. K3468) and counterstained with hematoxylin. The primary Abs used in this study were obtained from the indicated suppliers as follows: rabbit anti-p-c-Jun (1:200; 3270, Cell Signaling Technology) and rabbit anti-Brg1 (1:200; ab110641, Abcam).

2.4 | RNA extraction and qRT-PCR

Total RNA was extracted from tissues or cells using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the ReverTra Ace qPCR RT Kit (Toyobo). Quantitative RT-PCR was carried out using FastStart SYBR Green Master (Roche Applied Science) and Light Cycler 96 (Roche Applied Science). The Cq values were measured in triplicate. The expression levels were standardized by comparing to the levels of GAPDH. Primers were designed using Primerbank and are listed in Table S1.

2.5 | Spheroid establishment and culture

The extracted mice intestines were washed several times with PBS and the intestinal tumors were dissected. Tumor cells were dissociated using 2.5 mg collagenase from *Clostridium histolyticum*

(Sigma-Aldrich) with 2.5 ml advanced DMEM/F-12 (Thermo Fisher Scientific). After dissociation, tumor cells were collected by centrifugation and embedded in Matrigel (Corning). For spheroid culture of tumor cells, advanced DMEM/F-12 and 10% FBS (Sigma-Aldrich) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (FUJIFILM Wako Pure Chemical Corporation), and GlutaMAX (Thermo Fisher Scientific) was added to each well. To establish the spheroids (P0), 10 mmol/L Y-27632 (Tocris Bioscience) was added to the culture medium. For spheroid culture of intestinal tumors of *Dclk1^{CreERT2-IRES-EGFP/+}; Apc^{Min/+}; Brg1^{flox/flox}* spheroid, 20 ng/ml murine interleukin-13 (PeproTech) and 1 mM valproic acid (Fujifilm Wako Pure Chemical Corporation) was added to the culture medium.

For induction of Cre-mediated recombination *in vitro*, 1 µmol/L 4-OHT (Sigma-Aldrich) was added to the culture medium.

2.6 | Cell lines

DLD-1 (CCL-221) and HCT 116 (CCL-247) cells were obtained from ATCC. Culture medium was made from DMEM/F-12 (Fujifilm Wako Pure Chemical Corporation) and supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated at 37°C with 5% CO₂. Cells that had undergone 5–10 passages were used for experiments. Mycoplasma was confirmed negative using an e-Myco Mycoplasma PCR Detection Kit (iNtRON Biotechnology). SP600125 was purchased from Fujifilm Wako Pure Chemical Corporation.

2.7 | Small interfering RNA transfection

Human CRC cell lines were transfected with 10 nmol/L siRNA targeting BRG1 and JUN (SMARTpool: ON-TARGETplus SMARCA4 siRNA; Dharmacon), and siRNA nontargeting control (ON-TARGETplus nontargeting pool; Dharmacon) using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) and Opti-MEM (Thermo Fisher Scientific). We added this mixture into dishes or wells overnight and changed to fresh medium the next day. Due to unexpected toxicity, penicillin and streptomycin were excluded from the culture medium with siRNA.

2.8 | Crystal violet staining

Twenty thousand cells were seeded in 35-mm plates overnight prior to intervention. After removal of the culture medium of cell lines, cells were gently washed with PBS. They were fixed and stained with a mixture of 6.0% v/v glutaraldehyde (Sigma-Aldrich) and 0.4% w/v crystal violet (Merck) for 30 min. Plates were carefully washed with water so as not to tear off the fixed cells on the plates. Images were taken using an optical microscope.

2.9 | Western blot analysis

Cell lines were homogenized in Cell Lysis Buffer (Cell Signaling Technology). We measured the protein concentration using a Bio-Rad protein assay (Bio-Rad) and added sample buffer solution with reducing reagent (6x) for SDS-PAGE (Nacalai Tesque) to each sample after matching the concentration. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with blocking buffer (Nacalai Tesque), membranes were incubated with primary Abs overnight at 4°C. The next day, membranes were incubated with HRP-conjugated secondary Abs (1:5000 anti-rabbit, 7074; 1:5000 anti-mouse, 7076; both Cell Signaling Technology). The immobilized peroxidase activity was detected using SuperSignal West Pico PLUS (Thermo Fisher Scientific). The primary Abs used in this study were obtained from the indicated suppliers as follows: rabbit anti-p-c-Jun (1:500, #3270; Cell Signaling Technology), rabbit anti-c-Jun (1:1000, #9165; Cell Signaling Technology), and mouse anti-β-actin (1:10,000, A1978; Sigma-Aldrich).

2.10 | Cell proliferation assay

Cells were seeded in 96-well plates (1000 cells/well) overnight prior to intervention and were then maintained in the presence of 100 µl culture medium. We added 20 µl CellTiter 96 Aqueous One Solution (Promega) per well. Plates were incubated for 1 h before absorbance was measured at 490 nm with a Sunrise microplate reader (Tecan).

2.11 | Microarray analysis

We collected three samples with Control and three samples with siBRG1 per cell line. We used two cell lines (DLD-1 and HCT 116). The quality of RNA extracted from cell lines was examined using a Nanodrop (Thermo Fisher Scientific). The RNA samples were hybridized using a Clariom S Assay (Thermo Fisher Scientific). Normalization was undertaken using Affymetrix Power Tools Software (Thermo Fisher Scientific). The gene expression data of the cell line was analyzed using GSEA software and the Molecular Signature Database, which was provided by the Broad Institute of MIT and Harvard. A gene set was enrolled at the Broad Institute. All original microarray data were deposited in the Gene Expression Omnibus at NCBI (accession number: GSE 169627).

2.12 | Kaplan–Meier curve

For Figure 5A,B, the RNA sequencing dataset of colon adenocarcinomas combining data ($n = 380$) in the TCGA dataset was downloaded from cBioportal. For Figure 5A, the highest 25% and the lowest 25% of *c-JUN* expression data with colon adenocarcinomas

were extracted from the TCGA dataset ($n = 95$). For Figure 5B, the high 50% and the low 50% of *c-JUN* expression data from colon adenocarcinomas with lower *BRG1* expression ($n = 95$) was extracted from the TCGA dataset ($n = 47$, respectively). The analysis including the log-rank test was carried out using EZR version 1.51.

2.13 | Statistics

All values are presented as mean \pm SEM, unless otherwise described. A two-tailed unpaired Student's *t*-test after F-test was used for statistical analysis of continuous data. The χ^2 -test was used for statistical analysis of categorical data. All statistical analyses were

undertaken using GraphPad Prism version 6.07 for Windows (GraphPad Software). Those p values <0.05 , <0.01 , and <0.001 were considered statistically significant.

2.14 | Study approval

All mouse experiments were approved by the animal research committee of Kyoto University (180260) and carried out in accordance with Japanese government regulations. Analyses for human subjects were approved by the ethical committee of Kyoto University Hospital (#G1200-1, R2904) and carried out in accordance with the Declaration of Helsinki.

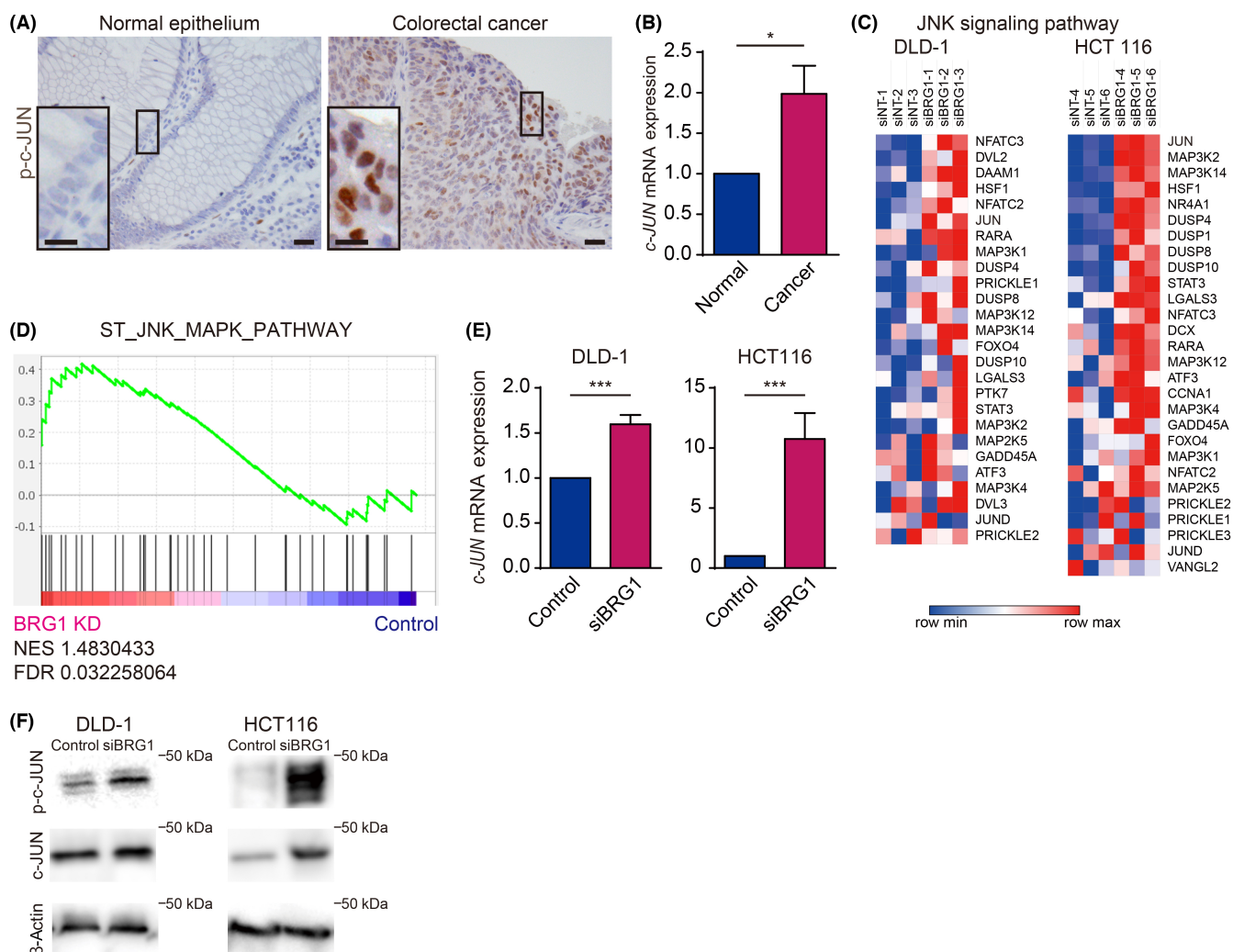


FIGURE 1 Activation of the JNK pathway in murine intestinal tumors and human colorectal cancer (hCRC) was stimulated by BRG1 suppression. (A) Immunostaining for BRG1 in human colon epithelium and CRC. Scale bars = 50 μ m and 20 μ m. (B) Quantitative real-time (qRT)-PCR analysis of *BRG1* in hCRC and surrounding normal epithelium ($p = 0.0255$, $n = 8$). Means \pm SEM are shown. (C) Heatmap of microarray analysis of JNK signaling pathway in DLD-1 (left) and HCT 116 (right) cells silenced with si nontargeting RNA or siBRG1 through Morpheus (<https://software.broadinstitute.org/morpheus>) ($n = 3$ independent experiments). (D) Gene Set Enrichment Analysis of JNK pathway in hCRC cells silenced with control and siBRG1. FDR, false discovery rate; NES, normalized enrichment score. (E) qRT-PCR analysis of *c-JUN* expression in DLD-1 and HCT 116 cells silenced with control or siBRG1 (DLD-1, $p = 0.0045$; HCT 116, $p = 0.0041$; $n = 3-4$). (F) Western blot analysis for p-c-JUN and c-JUN expression in DLD-1 and HCT 116 cells silenced with control or siBRG1. Analyzed by Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3 | RESULTS

3.1 | Activation of the JNK pathway augmented by BRG1 suppression in hCRC cells

We investigated the expression pattern of p-c-JUN by IHC analysis in hCRC. p-c-JUN is the activated form of c-JUN by N-terminal phosphorylation, and the most popular marker of the JNK pathway activation.²⁰ The IHC analysis of hCRC showed that p-c-JUN expression was not observed in the normal colon epithelium (Figure 1A, left), whereas hCRC specimen showed positive expression of p-c-JUN (Figure 1A, right). Quantitative RT-PCR analysis showed that the expression level of *c-JUN* mRNA was significantly higher in hCRCs than in the surrounding normal colorectal epithelium (Figure 1B).

Our recent work indicated that the expression of *BRG1* is suppressed by siRNA technique at nearly 10% in hCRC cells (DLD-1 and HCT 116) and that *BRG1* silencing results in impaired cell growth, loss of stemness feature, and increased apoptosis in hCRC cells.¹⁶ In the report, we undertook a microarray analysis of control and *BRG1*-silenced hCRC cells (GSE 169627). The heatmap showed that expression of the JNK pathway genes was upregulated in *BRG1*-silenced DLD-1 and HCT 116 cells (Figure 1C). Our Kyoto Encyclopedia of Genes and Genomes pathway analysis also showed that *Brg1* suppression affected gene expression patterns of the ER and MAPK signaling pathways.¹⁶ Of the 50 most upregulated genes in *BRG1*-silenced hCRC cells, we observed *JUN* (*c-JUN*) and *MAP3K14*, which were known to be JNK pathway-related genes, and *DDIT3* (*CHOP*) and *HMOX1*, which were known to be ER stress-related genes.¹⁶ Gene Set Enrichment Analysis revealed that there was a positive enrichment of gene sets related to the JNK pathway ("ST_JNK_MAPK_PATHWAY") in *BRG1*-silenced cells (Figure 1D). These findings indicate that the JNK pathway was upregulated in the context of *BRG1* suppression in hCRC cells. The qRT-PCR analysis verified the upregulated expression of *c-JUN* in *BRG1*-silenced hCRC cells (Figure 1E). We found that the activated level of p-c-JUN was significantly increased in *BRG1*-silenced DLD-1 and HCT 116 cells (Figure 1F). Protein level of c-JUN was elevated in *BRG1*-silenced DLD-1 and HCT 116 cells (Figure 1F).

Therefore, these findings indicate that the JNK pathway was activated to some extent at the basal level in hCRCs and the activation of the JNK pathway was further reinforced by *BRG1* suppression.

3.2 | Activation of the JNK pathway reinforced by Brg1 ablation in murine intestinal tumors

We next investigated whether activation of the JNK pathway was augmented by *Brg1* ablation in murine intestinal tumors. *Hnf1b* is expressed in intestinal tumor cells and spheroids generated from intestinal tumors in *Apc^{Min}* mice.¹⁶ Therefore, we first generated *HAB* mice and established spheroids from intestinal tumors of *HAB* mice

(Figure 2A,B). Through this model, we could ablate *Brg1* in tumor cells of spheroids by adding 4-OHT in the medium. Regarding *Brg1* KO efficacy in *HAB* spheroids, we had verified the low expression of *Brg1*, which was shown in our previous report.¹⁶ We determined the expression level of *c-Jun* mRNA in spheroids from intestinal tumors from *HAB* mice compared to controls. Expression of *c-Jun* was significantly upregulated in *Brg1* KO spheroids from *HAB* mice compared to controls (Figure 2C). Immunohistochemistry for p-c-JUN also revealed that the JNK pathway was upregulated in *Brg1* KO spheroids from *HAB* mice (Figure 2D).

To further determine whether activation of the JNK pathway was reinforced by *Brg1* ablation in murine intestinal tumors in mice, we next generated *DAB* mice along with *Dclk1^{CreERT2-IRES-EGFP/+}; Apc^{Min/+}; Brg1^{wt/wt}* or *Dclk1^{CreERT2-IRES-EGFP/+}; Apc^{Min/+}; Brg1^{wt/wt}* (*DA*) mice (Figure 2E). Because we previously reported that *Dclk1* is an intestinal TSC marker,¹⁷ we could ablate *Brg1* specifically in intestinal TSCs by injecting tamoxifen intraperitoneally.¹⁶ We previously reported that most intestinal tumor cells were *Brg1* negative in *DAB* mice on day 5 after tamoxifen injection, resulting in the collapse of intestinal tumors.¹⁶ Immunostaining for p-c-Jun revealed that p-c-Jun was highly expressed in intestinal tumor cells in *DAB* mice on day 5 after tamoxifen injection, whereas p-c-Jun expression was rarely detected in intestinal tumor cells in *DA* mice (Figure 2F). *Brg1* negative intestinal tumor clusters were positive for p-c-Jun expression in *DAB* mice (Figure 2G).

These findings suggest that the JNK pathway is activated by *Brg1* ablation also in murine intestinal tumors.

3.3 | JNK pathway crucial for expansion and stemness of BRG1-silenced hCRC cells

Given that the JNK pathway was upregulated in the context of *BRG1* suppression in hCRC cells, we sought to clarify its role in *BRG1*-suppressed hCRC cells in the context of *BRG1* suppression. To this end, we first examined whether treatment with SP600125, a selective JNK pathway inhibitor,²¹ affects the expansion of siRNA-mediated *BRG1*-silenced hCRC cells. We validated the efficiency of si*BRG1* by qRT-PCR (Figure S1). Western blot analysis showed that c-JUN activation was sufficiently inhibited by SP600125 treatment (Figure 3A). We carried out an MTS assay and found that SP600125 does not influence cell viability or proliferation, or expression of stemness-related genes in hCRC cells without treatment of si*BRG1* (Figure 3B,C). We next incubated hCRC cells with si*BRG1* and SP600125 and compared them with si*BRG1* and vehicle control. Notably, cell expansion was significantly suppressed in the si*BRG1* and SP600125 group compared to the si*BRG1* and vehicle control group (Figure 3D,E). Expression of stem cell markers was significantly downregulated in the si*BRG1* and SP600125 group compared to the si*BRG1* and vehicle control group (Figure 3F). These results indicate that the JNK pathway is crucial for the expansion of *BRG1*-silenced hCRC cells.

Next, we undertook dual silencing of *BRG1* and *c-JUN* in hCRC cells by siRNA. The qRT-PCR analysis validated that *c-JUN*

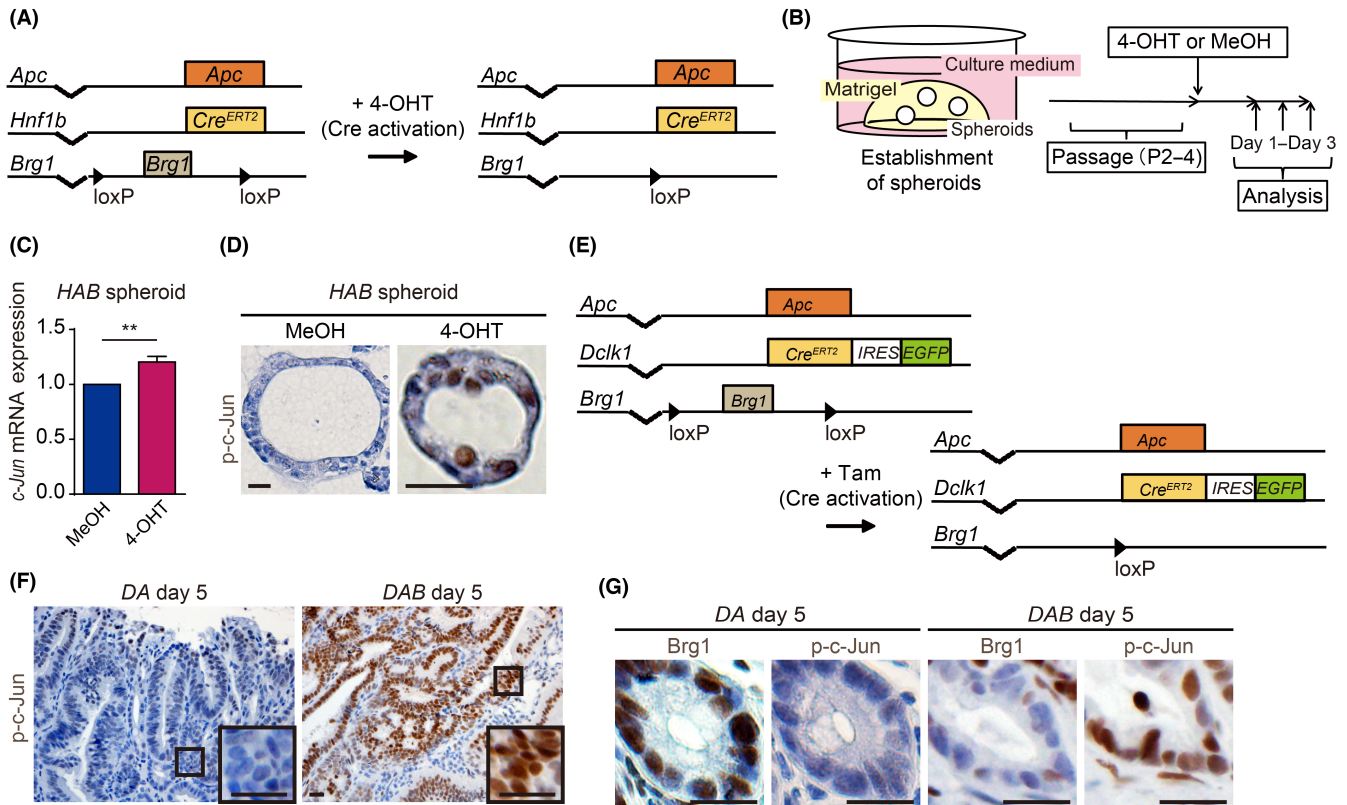


FIGURE 2 Increased activation of JNK pathway in murine intestinal tumors by Brg1 suppression. (A) Genetic strategy for Brg1 ablation in intestinal tumor cells following tamoxifen (4-OHT) induction in *Hnf1b*^{CreERT2/+}; *Apc*^{Min/+}; *Brg1*^{flox/flox} (HAB) mice. (B) Experimental schedule for establishment of HAB spheroids and analysis. (C) Quantitative real-time PCR analysis of *c-Jun* expression in HAB spheroids treated with MeOH or 4-OHT ($p = 0.0080$; $n = 4$). (D) Immunohistochemistry for p-c-JUN in HAB spheroids treated with MeOH or 4-OHT. Scale bars = 20 μ m. (E) Genetic strategy for *Dclk1*⁺ intestinal tumor cells specific Brg1 deletion following tamoxifen (Tam) induction in *Dclk1*^{CreERT2-IRES-EGFP/+}; *Apc*^{Min/+}; *Brg1*^{flox/flox} (DAB) mice. (F) Immunohistochemistry for p-c-JUN in intestinal tumors of *Dclk1*^{CreERT2-IRES-EGFP/+}; *Apc*^{Min/+}; *Brg1*^{flox/wt} or *Dclk1*^{CreERT2-IRES-EGFP/+}; *Apc*^{Min/+}; *Brg1*^{wt/wt} (DA) and DAB mice 5 days after the last Tam injection. Scale bars = 100 μ m and 20 μ m. (G) Immunohistochemistry for Brg1 and p-c-Jun in serial sections of intestinal tumors in DA and DAB mice at day 5 after the last Tam injection. Scale bars = 20 μ m. Analyzed by Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

expression was silenced to approximately 20% in DLD-1 and HCT 116 cells compared with controls (Figure 4A). Silencing of *c-JUN* alone did not affect cell expansion or expression of stemness-related genes in hCRC cells (Figure 4B,C). Dual silencing of *BRG1* and *c-JUN* inhibited the expansion of DLD-1 and HCT 116 cells compared with silencing of *BRG1* alone (Figure 4D,E). Moreover, the expression of stem cell markers was significantly downregulated in *BRG1* and *c-JUN* dual-silenced hCRC cells compared to *BRG1*-silenced CRC cells (Figure 4F). These results further indicated that the JNK pathway is crucial for the expansion and stemness of hCRC cells in the absence, but not in the presence, of *BRG1*.

3.4 | High *C-JUN* expression correlated with worse prognosis for survival in CRC patients with low *BRG1* expression

We next investigated the correlative relationship between *c-JUN* expression and prognosis for survival in CRC patients with low *BRG1* expression using the TCGA dataset. Consistent with our data

of hCRC cells, there was no significant difference in prognosis for survival in total CRC patients between the group with high *c-JUN* expression and the group with low *c-JUN* expression (Figure 5A). Next, patients were classified according to the expression of *BRG1*, and only patient cohort with low *BRG1* expression was further analyzed. Interestingly, among the patients with low *BRG1* expression, the high *c-JUN* expression group had a significantly worse prognosis for survival compared to the low *c-JUN* expression group (Figure 5B). These findings further support our notion that the JNK pathway is crucial for the expansion of hCRC cells when *BRG1* expression is low.

Finally, we undertook IHC analysis for p-c-JUN in resected hCRC specimens in our institution. Sixty-eight percent (34/50) of hCRC specimens were positive for p-c-JUN (Figure 1B). There were no significant differences in clinicopathologic features between p-c-JUN positive hCRC and p-c-JUN negative hCRC (Table S2). Overall survival did not significantly differ between p-c-JUN positive hCRC and p-c-JUN negative hCRC ($p = 0.825$; Figure 5C). These results also suggested that activation of the JNK pathway alone is not a prognostic factor for hCRC.

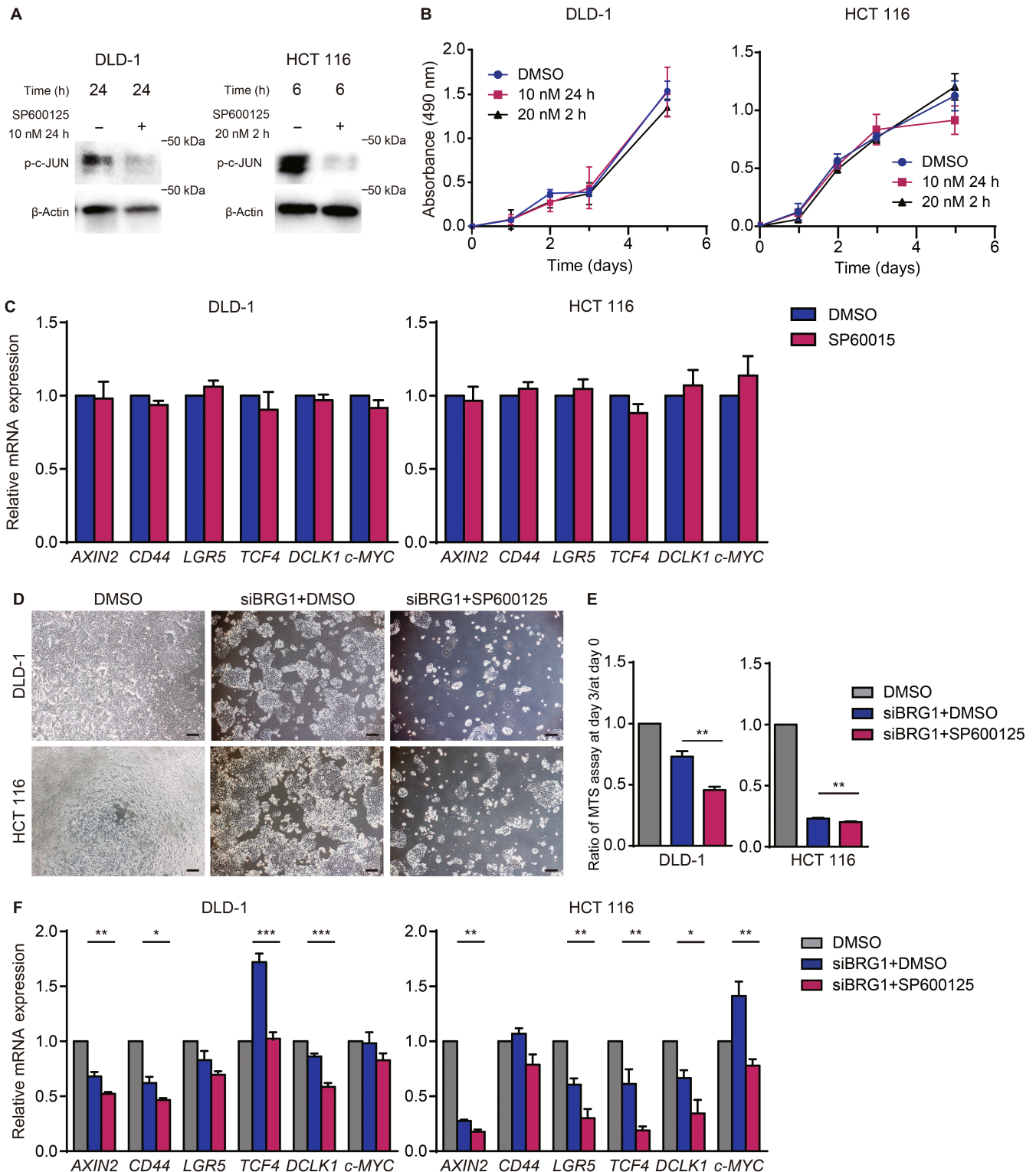


FIGURE 3 JNK pathway is crucial for expansion and stemness of human colorectal cancer cells in the context of BRG1 suppression. (A) Western blot analysis for p-c-JUN expression in DLD-1 and HCT 116 cells treated with DMSO or SP600125 at 10 nM for 24 h and at 20 nM for 2 h. (B) MTS assay of DLD-1 and HCT 116 cells treated with DMSO or SP600125 at 10 nM for 24 h and at 20 nM for 2 h ($n = 3, 4$). Means \pm SEM are shown. (C) Quantitative real-time (qRT)-PCR analysis of DLD-1 and HCT 116 cells 2 days after treatment with DMSO or SP600125 at 10 nM for 24 h ($n = 4$). Means \pm SEM are shown. (D) Images of DLD-1 and HCT 116 cells treated with siBRG1 + DMSO or siBRG1 + SP600125 on day 3 after siRNA treatment. Scale bars = 100 μ m. (E) Ratio of MTS assay on day 3 / day 0 of DLD-1 and HCT 116 cells silenced with DMSO, siBRG1 + DMSO, and siBRG1 + SP600125 at 10 nM for 24 h (DLD-1, $p = 0.007$; HCT 116, $p = 0.002$; $n = 5$). (F) qRT-PCR analysis of DLD-1 and HCT 116 cells silenced with DMSO, siBRG1 + DMSO, and siBRG1 + SP600125 at 10 nM for 24 h on day 2 after siRNA treatment ($n = 5$). Analyzed by Student's t -test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

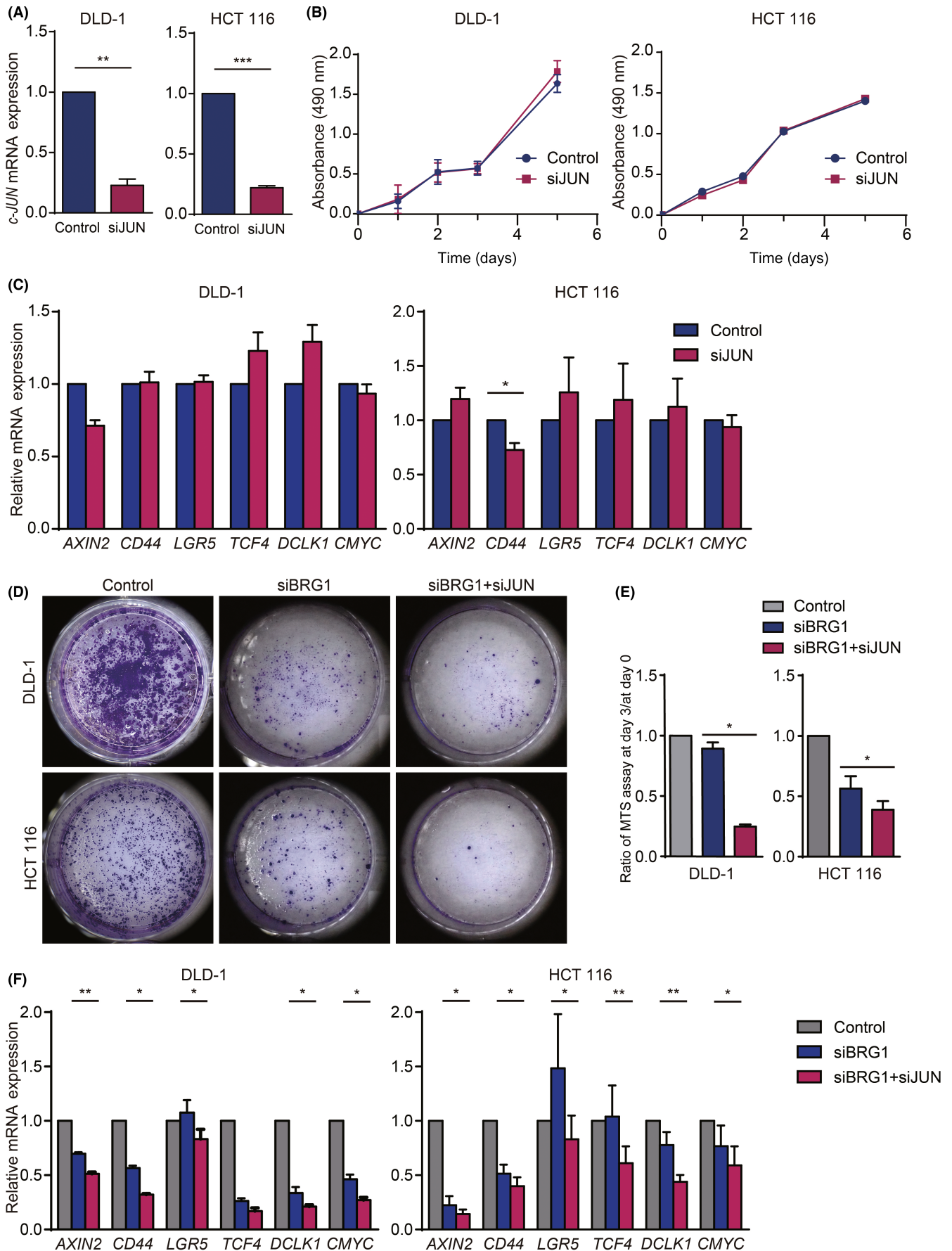


FIGURE 4 JNK pathway was crucial for expansion and stemness of human colorectal cancer cells in the context of BRG1 suppression. (A) Quantitative real-time (qRT)-PCR analysis of *c-JUN* expression in DLD-1 and HCT 116 cells silenced with control (si nontargeting RNA) or siJUN (DLD-1, $p = 0.0046$; HCT 116, $p < 0.0001$; $n = 3, 4$). Means \pm SEM are shown. (B) MTS assay of DLD-1 and HCT 116 cells treated with control or siJUN ($n = 4, 3$). Means \pm SEM are shown (HCT116 control: day 1, 0.288 ± 0.002 , day 2, 0.477 ± 0.009 , day 3, 1.027 ± 0.003 , day 5, 1.403 ± 0.001 ; siJUN: day 1, 0.244 ± 0.005 , day 2, 0.430 ± 0.005 , day 3, 1.037 ± 0.004 , day 5, 1.429 ± 0.011). (C) qRT-PCR analysis of HCT 116 cells silenced with control and siJUN on day 2 after siRNA treatment ($n = 5$). Means \pm SEM are shown. (D) Crystal violet staining of DLD-1 and HCT 116 cells silenced with control alone, siBRG1 alone, and siBRG1 + siJUN on day 3 after siRNA treatment. (E) Ratio of MTS assay on day 3 / day 0 of DLD-1 and HCT 116 cells silenced with control alone, siBRG1 alone, and siBRG1 + siJUN (DLD-1, $p = 0.0104$; HCT 116, $p = 0.0117$; $n = 3-4$). (F) qRT-PCR analysis of DLD-1 and HCT 116 cells silenced with control alone, siBRG1 alone, and siBRG1 + siJUN on day 2 after siRNA treatment ($n = 3$). Analyzed by Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

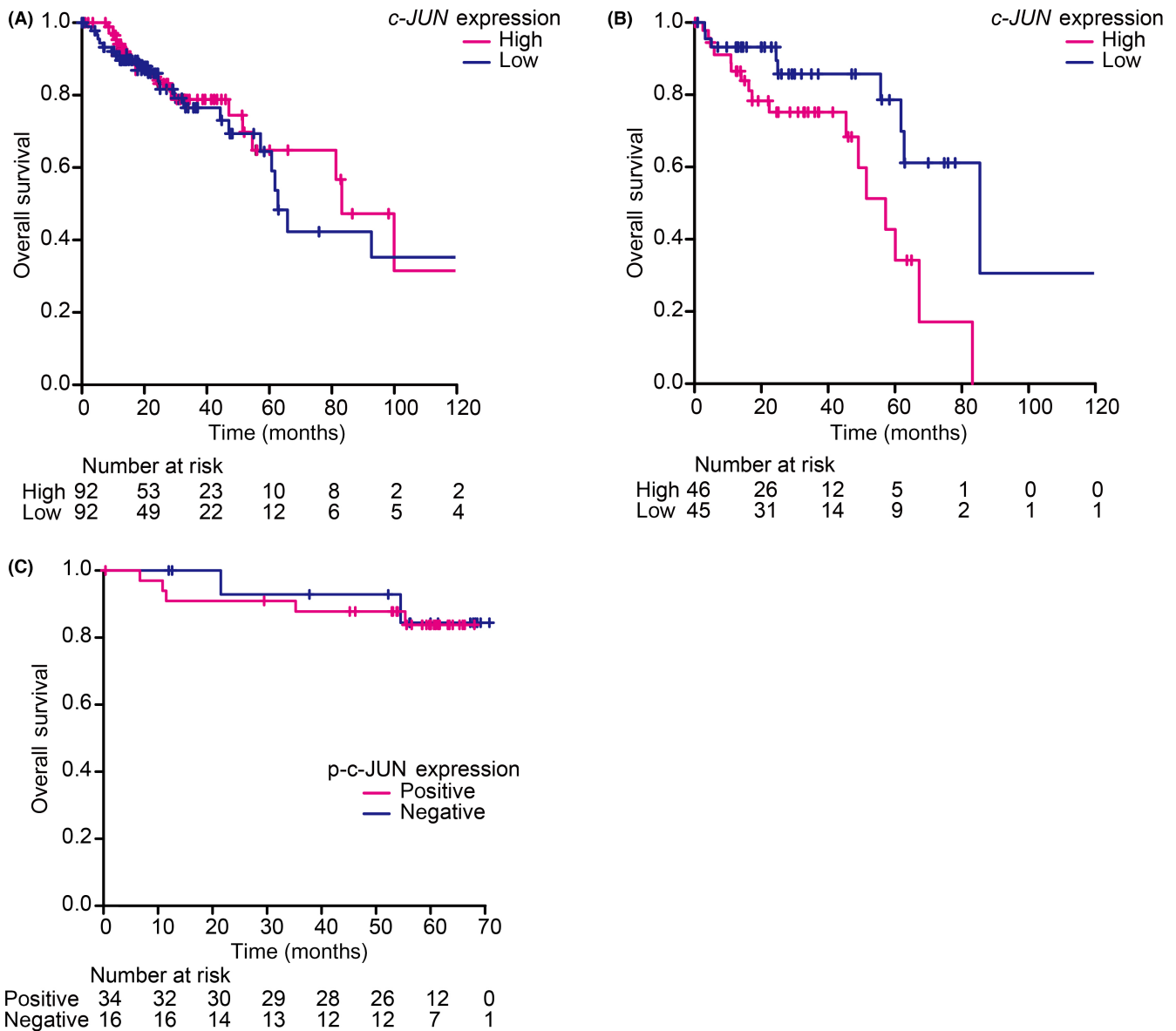


FIGURE 5 JNK pathway is crucial for expansion and stemness of human colorectal cancer (CRC) cells in the context of BRG1 suppression. (A) Kaplan–Meier analysis of CRC patients between the *c-JUN* high and low expression groups ($p = 0.653$). (B) Kaplan–Meier analysis of CRC patients with low *BRG1* expression between the *c-JUN* high and low expression groups ($p = 0.0131$). (C) Kaplan–Meier analysis of CRC patients between p-*c-JUN* positive and p-*c-JUN* negative expression groups ($p = 0.825$). Analyzed by log-rank test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

These data further strengthen the human relevance of our data, providing evidence that the JNK pathway plays a crucial role for CRC in the context of BRG1 suppression. Therefore, a combined blockade of BRG1 and the JNK pathway could be a novel therapeutic approach against hCRC.

4 | DISCUSSION

In this study, we found that the JNK pathway was upregulated in both hCRC cells and murine intestinal tumors by BRG1 suppression. Furthermore, we showed for the first time that the JNK pathway plays a critical role in the expansion and stemness of hCRC cells in the absence, but not in the presence, of BRG1. It is notable that suppression of BRG1 combined with the inhibition of the JNK pathway led to even stronger inhibition of expansion of hCRC cells than did Brg1 suppression alone. Therefore, our findings suggest that a combined blockade of BRG1 and the JNK pathway could be a novel therapeutic approach for hCRC.

Our data showed that JNK pathway blockade even strengthened the phenotype of BRG1-suppressed hCRC cells, which includes loss of cell expansion and stemness features. The JNK pathway has been shown to regulate stemness in normal murine intestinal epithelial cells and human pancreatic cancer cells.^{22,23} In this study, we showed that simultaneous suppression of BRG1 and the JNK pathway resulted in downregulated expression of stemness-related genes in hCRC cells compared to Brg1 suppression alone, whereas JNK inhibition alone did not affect the expansion of hCRC cells or the expression of stemness-related genes. This suggests that increased expression of JNK pathway genes is a compensatory mechanism to survive the fatal phenotypes (i.e., loss of expansion and stemness) induced by BRG1 suppression.

In this study, our findings provide important clinical relevance for the development of therapeutic strategies for hCRC. We showed that suppression of BRG1, combined with the inhibition of the JNK pathway, led to even stronger inhibition of the expansion of hCRC cells compared to Brg1 suppression alone. Consistently, it is notable that high *c-JUN* expression was correlated with worse prognosis for survival in hCRC patients with low BRG1 expression. Therefore, enhancing the antitumor effect of BRG1 inhibition through the use of combination therapy involving both BRG1 suppression and JNK inhibitors could be a new therapeutic strategy for hCRC. In particular, inhibition of the JNK pathway could be effective for hCRC patients with low BRG1 expression.

In conclusion, activation of the JNK signaling was augmented by BRG1 suppression in hCRC cells and murine intestinal tumors. The JNK pathway is critical for the expansion and stemness of hCRC cells in the context of BRG1 suppression. Consistently, high *c-JUN* expression correlated with worse prognosis for survival in hCRC patients with low BRG1 expression. Therefore, a combined blockade of BRG1 and the JNK pathway could be a novel therapeutic strategy against hCRC.

AUTHOR CONTRIBUTIONS

T.Y. and A.F. conceived and designed the study. M.O., M.N., M.S., Y.F., T.M., O.A., M.N., S.O., K.M., N.G., Y.H., Y.M., M.T., T. M., and

Y.N. conducted the experiments and analyzed the data. K.K. and S.T. contributed reagents, materials, and analytic tools. T.Y. wrote the manuscript, and A.F. and H.S. revised the manuscript.

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DISCLOSURE

Y.F. is an employee of Sumitomo Dainippon Pharma. S.T. is partially supported by Sumitomo Dainippon Pharma. The other authors have no conflict of interest.

ETHICS STATEMENT

Approval of the research protocol by an institutional review board: Analyses for human subjects were approved by the ethical committee of Kyoto University Hospital (#G1200-1, R2904) and conducted in accordance with the Declaration of Helsinki.

INFORMED CONSENT

N/A.

REGISTRY AND THE REGISTRATION OF THE STUDY

N/A.

ANIMAL STUDIES

All mouse experiments were approved by the animal research committee of Kyoto University (180260) and performed in accordance with Japanese government regulations.

ORCID

Takaaki Yoshikawa  <https://orcid.org/0000-0002-3173-4360>

Motoyuki Tsuda  <https://orcid.org/0000-0003-2873-1449>

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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