BMP4 derived from human gastrointestinal carcinoma cells impairs myogenic differentiation: A possible *in vitro* mechanism of cancer-induced cachexia

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Abstract. Gastrointestinal cancer (GIC) frequently causes cancer cachexia, the major feature of which is the loss of skeletal muscle mass. The degradation of muscular proteins by cancer-derived factors in the major pathogenesis of cancer-induced muscle wasting is a known phenomenon. However, this mechanism has mainly been demonstrated using rodent cancer cells, and it may not always be applicable to human cancer types. Impaired skeletal muscle differentiation and regeneration have attracted attention as alternative inducers of cancer cachexia. The present study revealed that conditioned medium from four human GIC cell lines inhibited C2C12 myoblast differentiation by inducing the expression of the inhibitor of DNA binding (Id) proteins Id1 and Id3, which mediated via bone morphogenetic protein (BMP)-Smad signaling. The results suggested that BMP-Smad1/5/8-Id signaling inhibited the expression of a MRF member, myogenin and its downstream myogenic genes, thus leading to unsuccessful differentiation into myotubes. Furthermore, the present study identified high levels of BMP4 secretion from these four human GIC cell lines and demonstrated that an inhibitor of BMP receptor, dorsomorphin or abrogation of BMP4 by siRNA in the GIC cells restored myogenic differentiation in C2C12 cells. The present study uncovered, for the first time, that BMP4 derived from human GIC cells exogenously inhibited myoblast differentiation by activating the Smad1/5/8-Id signaling axis. In the future, this *in vitro* study may help to elucidate the complicated mechanisms underlying cancer-induced cachexia in humans.

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Abbreviations: GIC, gastrointestinal cancer; UPS, ubiquitin-protease system; ALS, autophagy-lysosome system; GM, growth medium; DM, differentiation medium; CM, conditioned medium; IFS, immunofluorescence staining; siRNA, small interfering RNA; siCtl, control siRNA; siBMP4, BMP4 siRNA

Key words: bone morphogenetic protein, cachexia, gastrointestinal cancer, inhibitor of DNA binding, myogenic differentiation

Introduction

Cancer cachexia is a complicated syndrome associated with tissue damage caused by multiple factors and is fatal in ~20% of patients with cancer (1,2). Patients with gastrointestinal types of cancer (GIC), such as pancreatic, gastroesophageal and colorectal cancer, frequently experience weight loss at diagnosis (3,4). A major feature of cancer cachexia is the loss of skeletal muscle mass (5). To date, previous studies have highlighted increased muscle protein catabolism and decreased protein synthesis as key mechanisms underlying cancer cachexia (6,7). In particular, cancer cachexia has been considered to be the degradation of muscle proteins. This process is accelerated via the ubiquitin-proteasome system (UPS) or autophagy-lysosome system (ALS) mediated by inflammation (cytokine and downstream IL1β/TNFα-NFκB and IL-6-JAK-STAT3 pathways) or TGF-β (myostatin/activinA-SMAD2/3) pathway (8). However, the majority of these studies were based on rodent models using mouse colon 26 (C26) and Lewis lung carcinoma (LLC) (8-11). Comparative studies have reported that cachexia induced by C26 and LLC cells did not fully reflect the cachectic features observed in patients with cancer (8,12-17). Therefore, the identification of alternative mechanisms that are distinct from those in rodent models may provide novel insights into the pathogenesis of cancer-induced cachexia in patients with cancer.

Impaired skeletal muscle differentiation and regeneration have also attracted attention as alternative inducers of cancer cachexia (18). A previous study has shown that defective myoblast differentiation and fusion result in the accumulation of muscle precursor cells in cancer-cachectic mouse muscles (18). Another study reported that patients with cancer display reduced expression of key myogenic factors in the cachectic muscles (16,18-20).

During the known process of postnatal myogenic differentiation and regeneration, Pax7-positive myogenic stem cells, called satellite cells, are first activated and express myogenic regulatory factors (MRFs), such as MyoD and/or Myf5. These activated cells proliferate to produce muscle precursor cells, known as myoblasts. These cells subsequently decrease Pax7, and instead elevate the expression of other MRFs, such as myogenin and MRF4, which drive further differentiation and promote myocyte fusion by upregulating downstream target genes associated with myogenesis, eventually increasing muscle fiber size (21-25).

Previous studies have reported the key role of bone morphogenetic protein (BMP) signaling in myogenic differentiation (26-31). The BMP family (comprising BMP 1-15), a member of TGF-β, binds to the BMP receptor and activates intracellular signaling pathways. This interaction phosphorylates the BMP receptor-regulated Smad (R-Smad), including Smad1, Smad5 and Smad8 (32-34). Phosphorylated (p) Smad1/5/8 further induces heteromeric assembly with common-partner Smad (co-Smad; Smad4) and translocates into the nucleus, upregulating the expression of target genes (35-38). Previously, the Smad1/5/8-Smad4 complex was reported to directly bind to the Smad-responsive DNA element within the inhibitor of DNA binding (Id)1 and Id3 gene promoters to upregulate their expression (39,40). BMP-Smad-induced Id has been reported to suppress myogenic differentiation by directly inhibiting the transcriptional activity of MRFs, especially MyoD (28,29). Other studies have demonstrated that hyperactivation of BMP signaling during muscle injury causes delayed muscle regeneration (26,27). By contrast, the proper activation of the BMP-Smad-Id signaling pathway is critically committed to muscle development and adult muscle regeneration (30,31). However, to the best of our knowledge, few studies have investigated whether cancer-derived BMPs suppress myoblast differentiation exogenously.

Therefore, the present study aimed to identify cancer-derived factors that impair myogenic differentiation using conditioned medium (CM) from 20 human GIC cell lines. In addition, we sought to explore the potential signaling pathways through which these factors may affect myogenic differentiation in C2C12 myoblasts.

Materials and methods

Cell culture and differentiation. Human colorectal cancer cell lines HT29 (cat. no. JCRB1383) and DLD1 (cat. no. JCRB1382) were purchased from the Japanese Collection of Research Bioresources Cell Bank, gastric cancer cell line KATO III (cat. no. RCB2088) was purchased from the RIKEN BioResource Center, 44As3 was obtained from Dr Kazuyoshi Yanagihara (National Cancer Center Hospital, Kashiwa, Japan), and pancreatic cancer cell line BxPC3 from Dr. Kenoki Ohuchida (Kyusyu University, Fukuoka, Japan). These cells were cultured in RPMI 1640 medium (Nacalai Tesque, Inc.) supplemented with 10% FBS (Nichirei Biosciences, Inc.) and 1% penicillin-streptomycin (Fujifilm Wako Pure Chemical Corporation). The details of the other human cancer cell lines used in the present study are shown in Table I. The murine colon cancer cell line C26 (cat. no. RCB2657) was obtained from the RIKEN Cell Bank and cultured in RPMI1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. Murine myoblasts C2C12 (cat. no. CRL-1772), obtained from the American Type Culture Collection and were grown in growth medium (GM) consisting of DMEM (Nacarai Tesque, Inc.) supplemented with 10% FBS and 1% penicillin-streptomycin. Myoblast differentiation was induced by replacing GM with differentiation medium (DM) consisting of DMEM supplemented with 2% horse serum (MillporeSigma) and 1% penicillin-streptomycin or conditioned medium (CM) prepared as described in *CM preparation*. All cell lines were incubated under standard culture conditions in a humidified 5% CO_2 incubator at 37˚C. The cell lines were confirmed to be free of *Mycoplasma* contamination for at least 6 months.

CM preparation. Cancer cells were seeded at a density of 1.5-2.0x10^6 cells in 100-mm culture dishes (Corning, Inc.) and cultured in growth medium for 2-3 days. When the cancer cells reached 50-60% confluence, they were washed with PBS and 10 ml of fresh DM was added. After 48 h, when the cells reached 80-90% confluence, the culture medium was collected and centrifuged at 1,000 x g for 10 min at room temperature, and stored at -80˚C until use. Finally, a CM consisting of 33% cancer cell culture medium and 66% fresh DM was prepared.

Treatment with dorsomorphin. C2C12 cells were treated with 5 μM dorsomorphin (cat. no. S7840; Selleck Chemicals) at the time of inducing differentiation with DM or CM. After 1 h of treatment, the medium containing dorsomorphin was removed and fresh DM or CM was added. DMSO was used as a vehicle control at a final concentration of 0.05%.

Gene knockdown of BMP4 by small interfering RNA (siRNA). Transient transfection was performed in HT29 cells using 15 nM control siRNA (ON-TARGETplus Non-targeting siRNA; cat. no. D-001810-01-05; Revvity) or 15 nM BMP4 siRNA (ON-TARGETplus Human BMP4 siRNA SMARTpool; cat. no. L-11221-00-0005; Revvity). The BMP4 siRNA SMARTpool consists of four siRNA duplexes targeting human BMP4, with the following sequences (5' to 3'): GAGCCAUGCUAGUUUGAUA, UAGCAAGAGUGCCGUCAUU, CGACACUUCUGCAGAUGUU, and CAGGAUUAGCCGAUCGUUA. The non-targeting control siRNA, designed not to target any known human gene, has the following sequence (5' to 3'): UGGUUUACAUGUCGACUAA. Lipofectamine® RNAiMAX (Thermo Fisher Scientific, Inc.) was used as the transfection reagent according to the manufacturer's protocol. After 24 h of transfection at 37˚C in a humidified 5% CO_2 incubator, cells were washed with PBS and the medium was changed to DM. The culture medium was collected for CM and ELISA after 48 h of incubation.

Assessment of cell growth. C2C12 myoblasts were cultured in DM or CM for 5 days, and the number of viable cells was determined daily using the trypan blue exclusion test as described previously (41). Briefly, the cells were detached using 0.05% trypsin-EDTA, collected after neutralization with growth medium, and resuspended to single-cell suspension for counting. An aliquot (10 μl) of the cell suspension was mixed with 0.4% trypan blue (cat. no. T8154; Merck KGaA) and

Table I. Summary of human cancer cell lines used in the present study.

Tissue	Cell line	Medium	Provided by
Esophagus	KYSE30	RPMI1640	JCRB
	KYSE150	RPMI1640	JCRB
	TE-1	RPMI1640	RIKEN BioResource Center
	TE-5	RPMI1640	RIKEN BioResource Center
	TE-6	RPMI1640	RIKEN BioResource Center
Stomach	44As3	RPMI1640	Dr. K. Yanagihara
	58As9	RPMI1640	Dr. K. Yanagihara
	KATO III	RPMI1640	RIKEN BioResource Center
	MKN45	RPMI1640	RIKEN BioResource Center
	MKN74	RPMI1640	RIKEN BioResource Center
Colon	SW480	RPMI1640	American Type Culture Collection
	HT29	RPMI1640	JCRB
	DLD1	RPMI1640	JCRB
	HCT116	DMEM	RIKEN BioResource Center
	LoVo	HamF12	RIKEN BioResource Center
Pancreas	BxPC3	RPMI1640	Dr. K. Ohuchida
	SUIT-2	RPMI1640	Dr. K. Ohuchida
	AsPC-1	RPMI1640	Dr. K. Ohuchida
	Panc-1	RPMI1640	RIKEN BioResource Center
	MIA Paca2	DMEM	RIKEN BioResource Center

JCRB, Japanese Collection of Research Bioresources Cell Bank. All cancer cells grown in the indicated medium (DMEM, RPMI1640 or HamF12) supplemented with 10% FBS and 1% streptomycin-penicillin. The 44As3 and 58As9 cell lines were kindly provided by Dr. Kazuyoshi Yanagihara (National Cancer Center Hospital, Kashiwa, Japan). BxPC3, SUIT-2, and AsPC-1 cell lines were kindly provided by Dr. Kenoki Ohuchida (Kyusyu University, Fukuoka, Japan).

the number of living cells was determined using a TC20 cell counter (Bio-Rad Laboratories, Inc.). The number of cells was counted every day from day 0 to day 5.

Immunofluorescence staining (IFS). C2C12 myotubes on sterile glass coverslips were washed in PBS and fixed with 4% paraformaldehyde (cat. no 163-20145; FUJIFILM Wako Pure Chemical Corporation) for 15 min at room temperature followed by permeabilization with 0.2% Triton X-100 in PBS for 10 min at room temperature. Samples were blocked with 5% donkey serum (cat. no SIG-D9663; MilliporeSigma) and 1% BSA (cat. no 01-2030-2; MilliporeSigma) in PBS for 60 min at room temperature, and then incubated with primary MYH antibody (1:100; cat. no. sc376157; Santa Cruz Biotechnology, Inc.) overnight at 4˚C, followed by incubation with Donkey Anti-Mouse IgG H&L (Alexa Fluor 488) secondary antibody (1:200; cat. no. ab150105; Abcam) for 1 h at room temperature. Nuclei were stained with DAPI (cat. no. ab-104139; Abcam) for 5 min at room temperature. Images were captured using a Zeiss LSM 880 Fast Airyscan Confocal and analyzed using IMARIS (Oxford Instruments). The differentiation index was calculated as the percentage of nuclei expressing MYH cells relative to the total nuclei. The fusion index was calculated as the percentage of nuclei in multinucleated cells with two or more nuclei relative to the total number of nuclei. These indices were determined by randomly analyzing at least 10 images from each sample.

Western bolt analysis. Whole-cell lysates were extracted using lysis buffer (150 mM NaCl; 50 mM Tris-HCl; pH 7.5; 2 mM EDTA; 1% Triton X-100; 1% sodium deoxycholate and 2% sodium dodecyl sulfate) containing protease inhibitors (Roche Diagnostics) and phenylmethylsulfonyl fluoride (Roche Diagnostics). The total protein concentration was determined using Protein Assay Dye Reagent (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. The samples were dissolved in NuPage LDS sample buffer (Thermo Fisher Scientific Inc.) and 1 M dithiothreitol, and then heated for 5 min at 95˚C. Proteins (20-30 μg) were separated on 5-20% Bis-Tris gels (International Techno Center Co., Ltd.) and transferred to Hybond-ECL membranes (Cytiva). Membranes were blocked with 5% skim milk at room temperature for 60 min and then incubated overnight at 4˚C with the following primary antibodies: MYH (1:20,000; cat. no. sc-376157; Santa Cruz Biotechnology, Inc.), MyoD (1:500; cat. no. sc-377460; Santa Cruz Biotechnology, Inc.), myogenin (1:1,000; cat.no. sc-12732; Santa Cruz Biotechnology, Inc.), myomaker (1:1,000; cat. no. NBP2-34175; Novus Biologicals, LLC), myomixer (1:2,000; cat. no. AF4580; R&D systems, Inc.), Pax7 (1:1,000; cat. no. AB_528428; Developmental Studies Hybridoma Bank), Id1 (1:1,000; cat. no. 18475-1-AP, Proteintech Group Inc.), Id3 (1:1,000; cat. no. 10389-1-AP, Proteintech Group, Inc.), p-Smad1/5/8 (1:500; cat. no. 13820, Cell Signaling technology, Inc.), Smad1/5/8 (1:1,000; cat. no. NB600-962, Novus Biologicals, LLC), Smad4 (1:1,000; cat. no. 38454, Cell Signaling

Table II. Sequences of primer used for reverse transcription-quantitative PCR in the present study.

Gene name	Species	Forward primer (5' to 3')	Reverse primer (5' to 3')
Pax7	Mouse	GTGCCCTCAGTGAGTTCGAT	CGGGTTCTGATTCCACATCT
MyoD	Mouse	AGTGAATGAGGCCTTCGAGA	GCATCTGAGTCGCCACTGTA
myogenin	Mouse	CTACAGGCCTTGCTCAGCTC	ATGGACGTAAGGGAGTGCAG
myomaker	Mouse	GGCCTTTACCACCTTCTCC	AAGCACAGCACAGACAAACC
myomixier	Mouse	AGTGAACTCCTTAACCAGCTTTC	CACCTCTGTACTCCCCAGTTT
BMP4	Human	GGAAGCTAGGTGAGTGTGGC	CTACGGAATGGCTCCATAGGTC
BMP2	Human	AGAATGCAAGCAGGTGGGAA	CCACTTCCACCACGAATCCA
BMP6	Human	TCAACCGCAAGAGCCTTC	TTGTCGTACTCCACCAGGTT
BMP7	Human	CTCTGGCCAGCCTGCAAGATA	CCGGAACTCTCGATGGTGGA
Id1	Mouse	GGTACTTGGTCTGTCGGAGC	GCAGGTCCCTGATGTAGTCG
Id3	Mouse	ACTTACCCTGAACTCAACGCC	CAGGCCACCCAAGTTCAGTC
IL-6	Human	TACCCCCAGGAGAAGATTCC	TTTTCTGCCAGTGCCTCTTT
GAPDH	Mouse	CAGGGCAAATTCAACGGCACAGTCAA	GTTCACACCCATCACAAACATGG
ACTB	Human	ACGCCTCTGGCCGTACCACT	TAATGTCACGCACGATTTCCC

Technology, Inc.), BMP4 (1:2,000; cat. no. ab39973; Abcam), MuRF-1 (1:100; cat. no. sc-398608, Santa Cruz Biotechnology, Inc.), LC3B (1:5,000; cat. no. 2775; Cell Signaling Technology, Inc.), GAPDH (1:20,000; cat. no. 60004-1-ig; Proteintech Group Inc.), ACTB (1:1,000; cat. no A1978; Merck KGaA). Membranes were then washed and incubated with the corresponding HRP-conjugated secondary antibodies [goat anti-rabbit IgG (1:3,000; cat. no. 4050-05; SouthernBiotech), goat anti-mouse IgG (1:3,000; cat. no. 1031-05; SouthernBiotech) and donkey anti-sheep IgG (1:1,000; cat. no. HAF016, R&D Systems, Inc.)] for 60 min at room temperature. The signals were detected using the ECL Prime Western Blotting Detection Reagent (Cytiva) and images were acquired using a FUSION-FX7 imaging system (Vilber-Lourmat).

Isolation of RNA and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using Isogen II (Nippon Gene Co., Ltd.,) and 1 μg aliquots were reverse-transcribed to cDNA using ReverTra Ace qPCR RT Master Mix (cat. no. FSQ-201; Toyobo Co., Ltd.). qPCR was performed using the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) with TB Green Premix Ex Taq™ II Fast qPCR (cat. no. RR830A; Takara Bio, Inc.) according to the manufacturer's protocol. After performing a denaturation step at 95˚C for 3 min, PCR amplification was conducted using 50 cycles of 15 sec of denaturation at 95˚C, 5 sec, annealing at 60˚C and 10 sec of extension at 72˚C. Quantitative values were calculated using the $2^{-\Delta\Delta Cq}$ (42) method and normalized to the expression of ACTB and GAPDH. RT-qPCR primers were designed for either human or mouse genes depending on the experimental system. Primers for MyoD, myogenin, myomaker, myomixer, Id1, Id3, Pax7 and GAPDH were specific for mouse genes and used for C2C12 cells, whereas primers for BMP family genes and IL-6 were designed for human genes. The primers are listed in Table II.

ELISA. BMP4 concentration in the culture supernatant from cancer cells was determined in triplicate using a Human BMP4 Quantikine ELISA kit (cat. no DBP400; R&D Systems, Inc.) according to the manufacturer's protocol.

Statistical analysis. The data were analyzed using JMP Pro 14 (SAS Institute, Inc.). To compare two groups, differences in mean values were evaluated using a two-tailed unpaired Student's t-test. For comparisons of three or more groups, ANOVA followed by Dunnett's or Tukey's post hoc tests was performed. $P<0.05$ was considered to indicate a statistically significant difference and all results were expressed as the means ± SD.

Results

CM from several human GIC cells inhibit myoblast differentiation in C2C12. The present study first investigated the morphological changes in C2C12 cells cultured in DM or CM from cancer cells for 5 days. From the beginning of C2C12 differentiation induction in DM or CM (designated as day 0), the medium was changed every 24 h (Fig. 1A). C2C12 myoblasts cultured in DM fused with each other and transformed into myotubes with multiple nuclei on days 3 and 5 (Fig. 1B). By contrast, C2C12 cells cultured with CM from C26, formed fewer myotubes on day 5 (Fig. 1B). Next, the inhibitory effects of CM from 20 human GIC cell lines on myotube formation in C2C12 cells was explored. As shown in Fig. 1C, CM from the colorectal cancer cell lines HT29 and DLD1, the pancreatic cancer cell lines BxPC3 and the gastric cancer cell lines KATOIII and 44As3, inhibited the myogenic differentiation of C2C12 cells. By contrast, CMs from the remaining GIC cell lines exhibited either minimal or weak inhibitory effects on myoblast differentiation (Fig. S1).

GIC CM inhibits myoblast differentiation into myotubes and suppresses the expression of myogenic factors in C2C12. To investigate the mechanism underlying the inhibitory effect of CM from GIC on myoblast differentiation, subsequent analysis focused on HT29 and DLD1 cell lines, which exhibited the

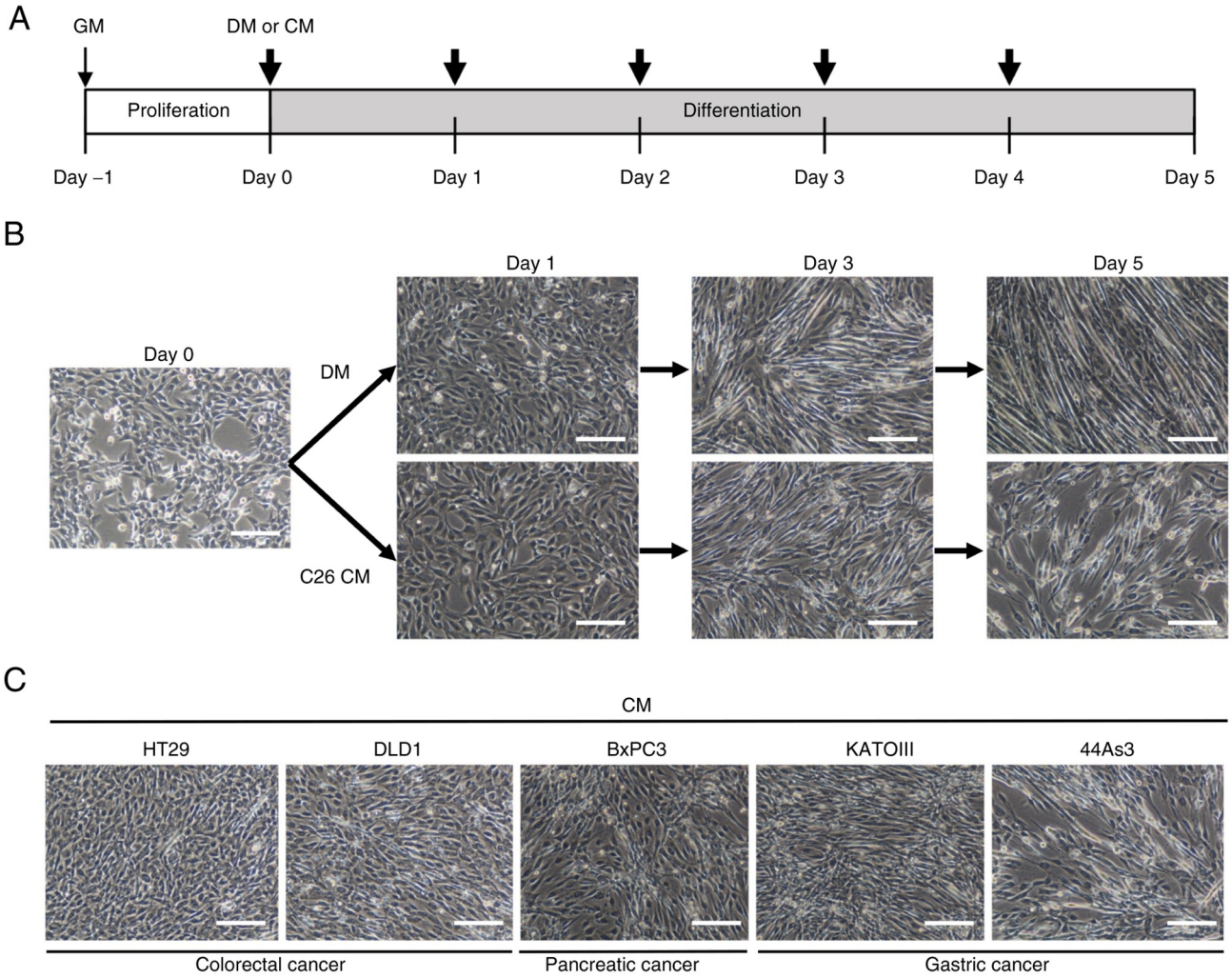

Figure 1. Morphological changes in C2C12 cells cultured with DM and CM from five human GIC cell lines or mouse colon cancer C26. (A) The experimental schedule. After C2C12 cells were cultured in GM for 24 h (shown by fine arrow), myogenic differentiation was induced by DM or CM from cancer cells (designated as day 0), and the medium was changed every 24 h by day 4 (shown by bold arrow). The morphological changes of C2C12 were assessed using phase-contrast microscope. (B) Representative images of C2C12, which was cultured with DM or CM from mouse C26 cells from day 1 to day 5. (C) Representative images of C2C12 cells with CM from five human GIC cells on day 5. Scale bars, 200 μm. DM, differentiation medium; CM, conditioned medium; GM, growth medium; GIC, gastrointestinal cancer.

most pronounced inhibitory effects on C2C12 morphology based on visual assessment in the initial screening (Figs. 1C and S1). The 58As9 cell line, which did not inhibit the differentiation, was used as a negative control.

In C2C12 cells cultured with DM and 58As9 CM, cell growth was markedly suppressed, and mature myotubes with MYH-positive staining appeared on day 5 (Fig. 2A and B). Differentiation and fusion indices were estimated to be >50% (Fig. 2C). By contrast, C2C12 cells cultured with HT29 and DLD1 CMs proliferated with time dependency by day 5 (Fig. 2A). The formation of MYH-positive myotubes (Fig. 2B), and differentiation and fusion indices were significantly suppressed compared with cells cultured in DM and 58As9 CM (Fig. 2C).

Next, the present study evaluated the expression of myogenic genes related to differentiation and cell fusion. In C2C12 cells with controls, the protein expression level of Pax7, which is a key marker for satellite cells and myoblasts, visibly decreased on day 3, whereas the expression of the MRF member MyoD showed a slight reduction. Conversely, the mRNA and protein expression levels of another MRF member, myogenin and its downstream targets, myomaker and myomixer, increased over time (days 1-3) under control conditions (Fig. 2D and E). By contrast, in C2C12 cells cultured with HT29 and DLD1 CM, Pax7 mRNA expression significantly increased, and its protein expression level was preserved on day 3. The expression of MyoD did not show a significant difference when compared with cells cultured in DM and 58As9 CM. However, the expression of myogenin and its downstream targets was remarkably suppressed compared with cells cultured in DM and 58As9 CM (Fig. 2D and E). These results suggest that CMs from HT29 and DLD1 inhibited C2C12 differentiation from myoblasts to myotubes by decreasing the expression of myogenin and its downstream factors. At this point, we hypothesized that some secreted factor from HT29 and DLD1 may exogenously inhibit myoblast differentiation in C2C12 cells by activating the intrinsic signaling pathway.

HT29 and DLD1 cells secrete BMP4, which activates Smad-Id signaling in C2C12 myoblasts. The present study focused on

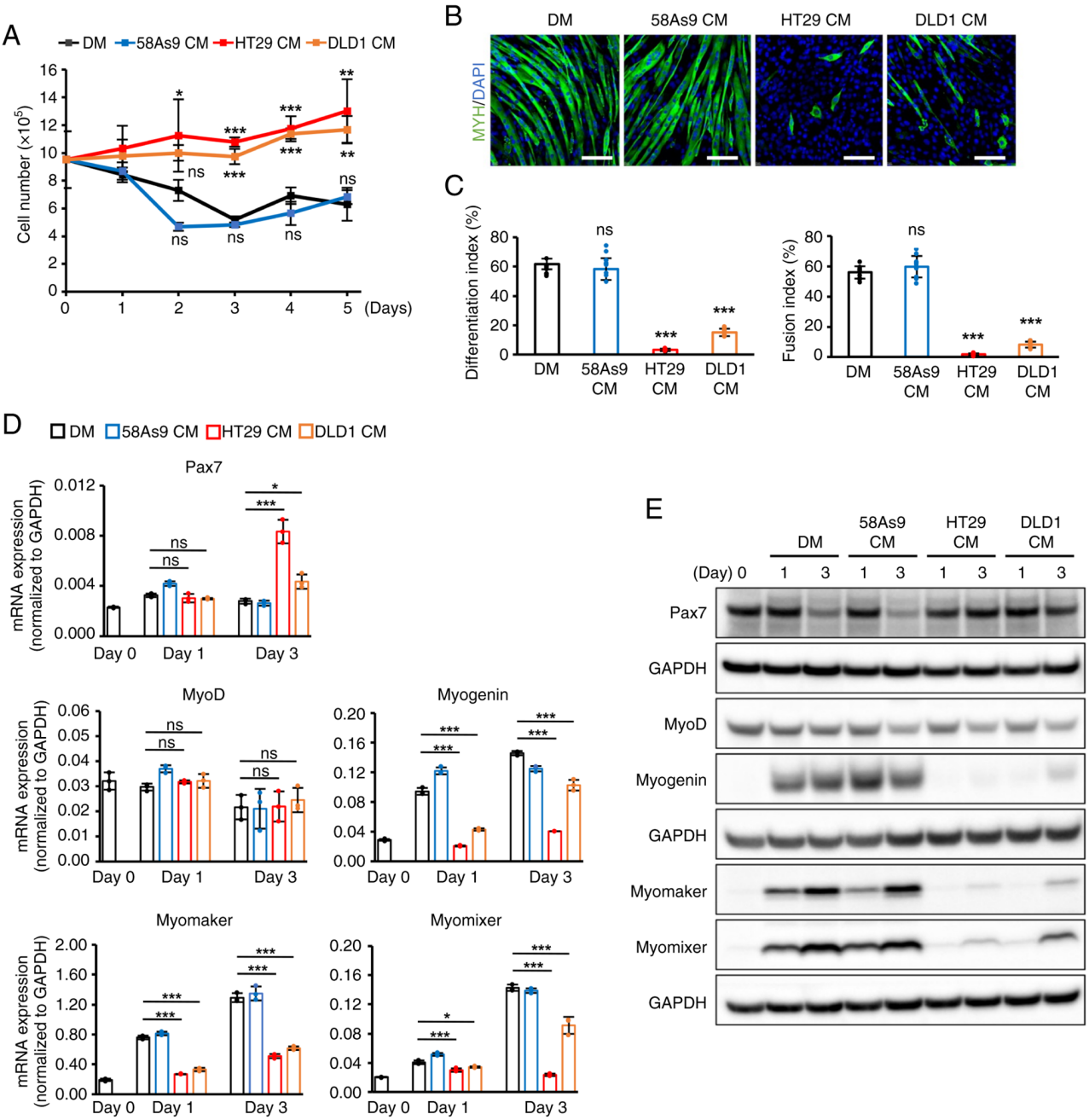

Figure 2. Analysis of gene expression associated with myogenic differentiation in C2C12 cells which cultured with DM or CM from control 58As9, HT29 and DLD1 cells. (A) Cell proliferation of C2C12 myoblast cultured with DM or three GIC CMs for 5 days (n=3). (B) Immunofluorescence staining of MYH in C2C12 cells with DM or CMs from three GIC cells on day 5. Representative images are shown (green, MYH; blue, DAPI). Scale bar, 100 μm. (C) Differentiation and the fusion indices that were quantitatively estimated in C2C12 cells with DM or CM from three GIC cells (n=10). (D) Reverse transcription-quantitative PCR (n=3) and (E) western blot analysis of Pax7, MyoD, myogenin, myomaker and myomixer in C2C12 cells with DM or three GIC CMs (on day 0, day 1 and day 3). All experiments were independently repeated at least three times. Data are presented as mean ± SD. Statistical significance was determined by comparison with control (DM) on each day. ns not significant, *P<0.05, **P<0.01, ***P<0.001. DM, differentiation medium; CM, conditioned medium.

BMP signaling as a possible mechanism underlying impaired differentiation in C2C12 cells treated with HT29 and DLD1 CMs. First, the mRNA expression levels of BMP family members BMP2, BMP4, BMP6 and BMP7 in control 58As9, HT29 and DLD1 cells were investigated. BMP4 mRNA expression was significantly higher in HT29 and DLD1 when compared with that in 58As9 cells (Fig. 3A). Higher expression of BMP4 protein expression was also observed in cell lysates and culture supernatants from HT29 and DLD1 cells when compared with that in 58As9 cells (Fig. 3B and C). In addition, mRNA expression of the other BMPs was not commonly expressed in HT29 and DLD1 cells (Fig. S2). Next, the present study analyzed whether BMP downstream Smad-Id signaling was activated in C2C12 cells treated with HT29 and DLD1 CM. Expression of p-Smad1/5/8 (pSmad1/5/8), which is the activated form of Smad1/5/8, was visibly higher in C2C12 cells treated with HT29 and DLD1 CMs when compared with the other two controls during differentiation (Fig. 3D). There was no apparent difference in the expression levels of total Smad1/5/8 or Smad4 among all four groups (Fig. 3D).

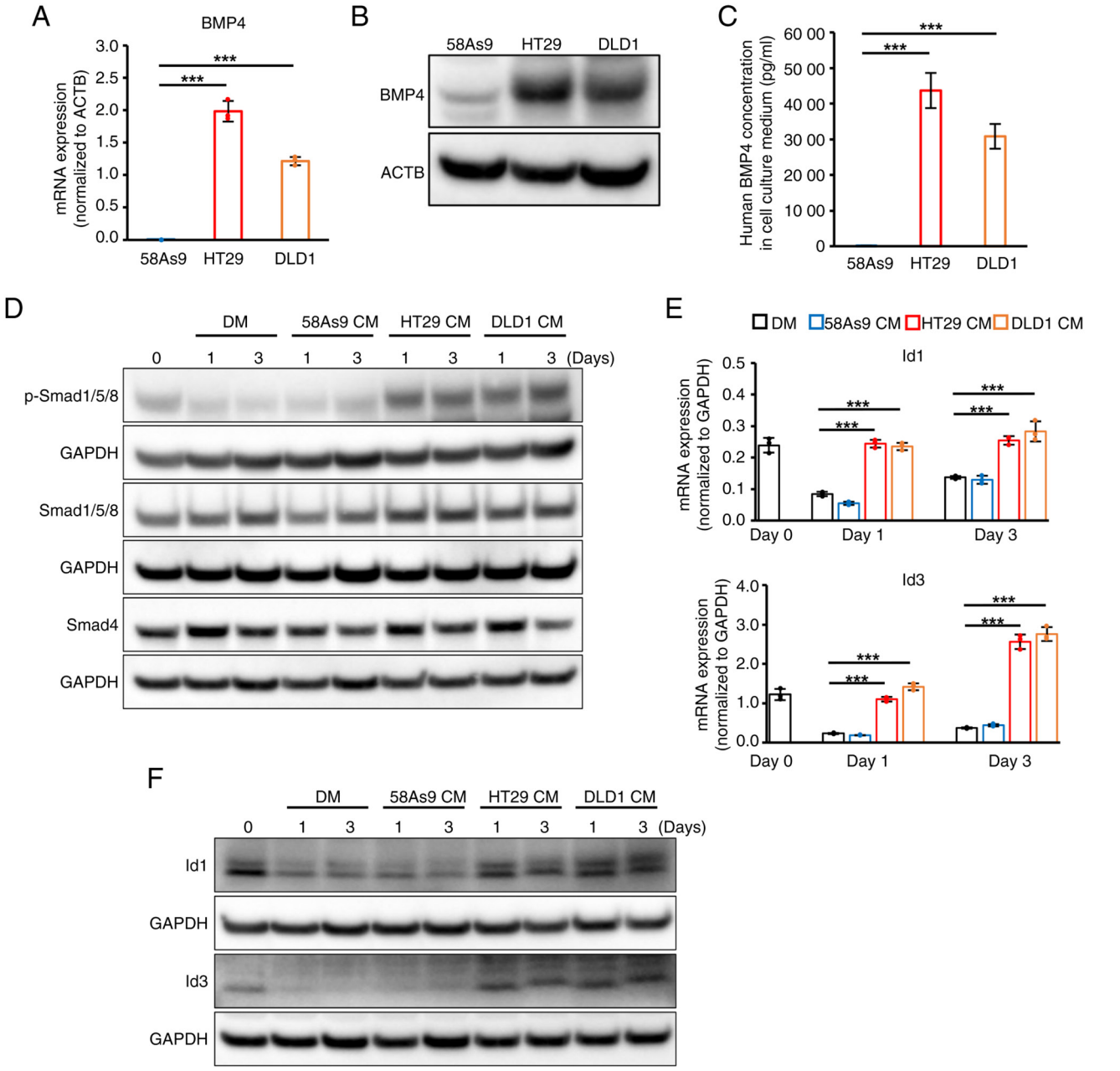

Figure 3. Analysis of BMP4 secretion from HT29 and DLD1 cells and BMP-Smad-Id signaling in C2C12 cells. (A) RT-qPCR (n=3) and (B) WB of BMP4 expression in three GIC cells. (C) ELISA of human BMP4 in the cell culture supernatants from three GIC cells cultured for 48 h (n=6). (D) WB of the p-Smad1/5/8, Smad1/5/8 and Smad4 expressions in C2C12 cells cultured with DM or CMs from three GIC cells on day 0, 1 and 3. (E) RT-qPCR (n=3) and (F) WB analysis of Id1 and Id3 expressions in C2C12 cells with DM or CMs from three GIC CMs. All experiments were independently repeated at least three times. Data are presented as mean ± SD, and statistical significance was determined by comparison with control (58As9 or DM) on each day. ***P<0.001. WB, western blotting; p, phosphorylation; RT-qPCR, reverse transcription-quantitative PCR; DM, differentiation medium; CM, conditioned medium; GIC, gastrointestinal cancer.

With respect to the Id family, mRNA expression of Id1 and Id3 was observed in C2C12 cells on day 0. Expression of these mRNAs declined in C2C12 cells treated with DM and 58As9 CM on day 1 and 3; however, high expression of Id1 and Id3 was sustained in C2C12 cells treated with HT29 and DLD1 CMs (Fig. 3E). Furthermore, western blotting analysis demonstrated findings consistent with RT-qPCR results for the protein levels of Id1 and Id3 (Fig. 3F). These results indicate that exogenous BMP4, which is secreted by HT29 and DLD1, may inhibit C2C12 differentiation by activating the Smad-Id pathway.

Dorsomorphin ameliorates myogenic differentiation by suppressing Smad-Id signaling in C2C12 with HT29 and DLD1 CMs. To clarify whether the inhibitory effect of HT29 and DLD1 CMs on C2C12 differentiation is caused by the activation of BMP-Smad signaling, the present study analyzed the reverse effect of an inhibitor of the BMP type I receptor, dorsomorphin. C2C12 cells cultured in HT29 and DLD1 CMs were treated with or without 5 μM dorsomorphin. IFS analysis showed that dorsomorphin markedly increased the number of MYH-positive myotubes on day 5 (Fig. 4A). The differentiation and fusion indices were also increased by

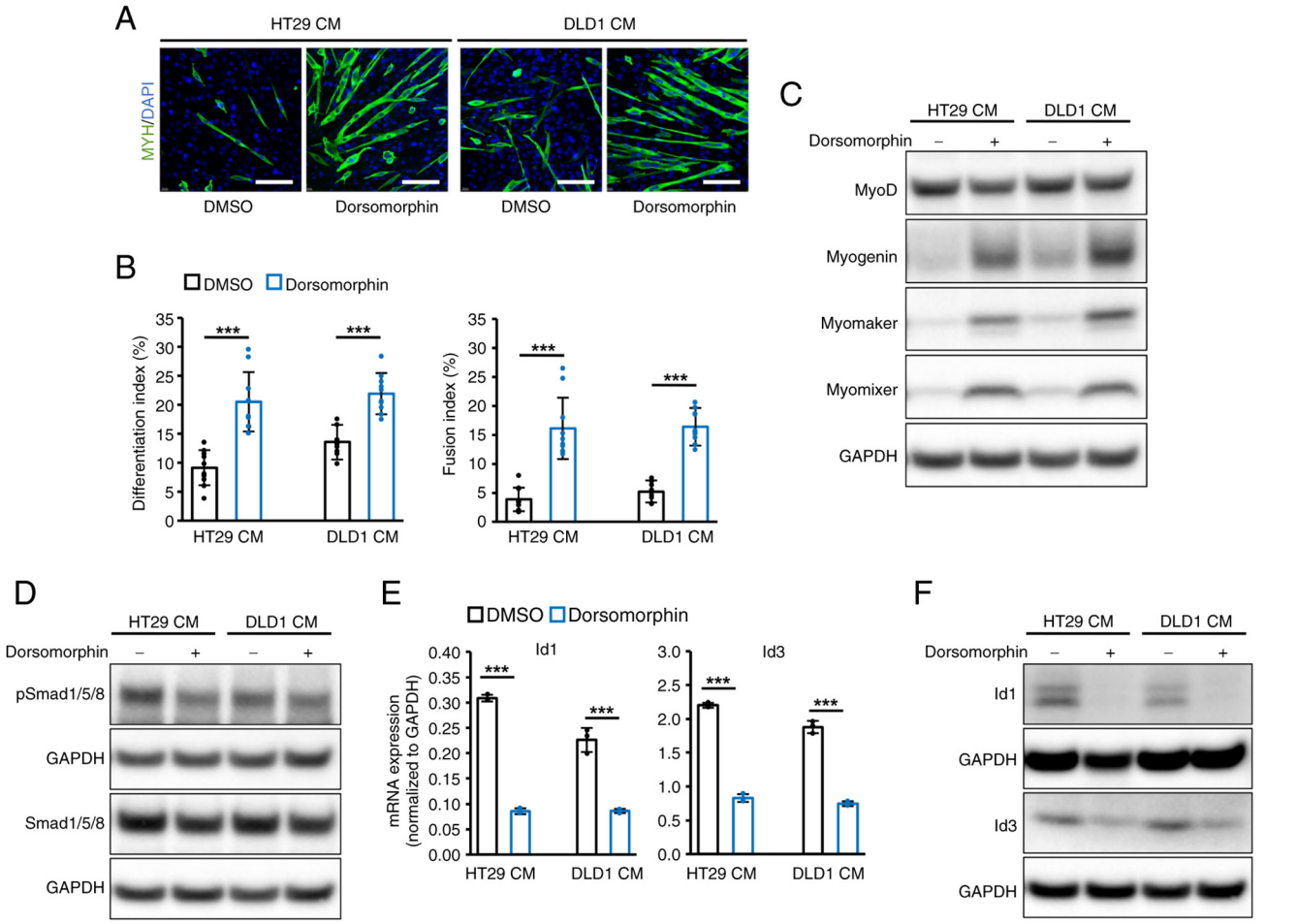

Figure 4. Dorsomorphin ameliorated the inhibitory effect of HT29 and DLD1 CMs on myogenic differentiation in C2C12 cells. (A) Immunofluorescence staining of MYH in C2C12 cells cultured with CM from HT29 and DLD1 on day 5 with or without Dorsomorphin treatment. Representative images were shown (green, MYH; blue, DAPI). Scale bar, 100 μm. (B) Differentiation and fusion indices were quantitatively assessed in C2C12 cells with HT29 and DLD1 CMs with or without Dorsomorphin (n=10). (C) The protein expression of MyoD, myogenin, myomaker and myomixer in C2C12 cells with HT29 and DLD1 CMs with or without Dorsomorphin on day 1. (D) WB analysis of Smad1/5/8 and p-Smad1/5/8 in C2C12 cells with HT29 and DLD1 CMs with or without Dorsomorphin on day 1. (E) Reverse transcription-quantitative PCR (n=3) and (F) WB analysis of Id1 and Id3 in C2C12 cells with HT29 and DLD1 CMs with or without Dorsomorphin on day 1. For panels (D) Smad1/5/8 and (F) Id3, the corresponding GAPDH loading controls were derived from the same membrane processed on the same experimental day; different exposure times were used for visualization. All experiments were independently repeated at least three times, and data are presented as mean ± SD. Statistical significance was determined by comparisons between the dorsomorphin-treated and -untreated C2C12 cells with HT29 and DLD1 CMs. ***P<0.001. WB, western blotting; DM, differentiation medium; CM, conditioned medium.

this treatment (Fig. 4B). Moreover, the expression levels of myogenin, myomaker and myomixer were markedly restored by dorsomorphin, whereas MyoD expression was slightly decreased (Fig. 4C). The present study further confirmed that the drug treatment decreased the expression of p-Smad1/5/8, Id1 and Id3 in C2C12 cells treated with both HT29 and DLD1 CMs (Fig. 4D-F). These results demonstrated that attenuation of BMP-Smad signaling by dorsomorphin reversed the inhibitory effect of HT29 and DLD1 CM on C2C12 differentiation.

Assessment of BMP-Smad signal in other GIC cell lines exhibiting the inhibitory effect on C2C12 differentiation. Whether the inhibition of C2C12 differentiation by CM from other human GIC cells (KATOIII, 44As3 and BxPC3) or mouse C26 cells is mediated by the BMP-Smad-Id signaling pathway was next investigated. The morphological changes with or without dorsomorphin in C2C12 cells cultured with CMs from these cells were first analyzed. Dorsomorphin treatment significantly restored MYH-positive myotube formation, along with increased differentiation and fusion indices, in C2C12 cells treated with KATOIII and BxPC3 CMs, as observed in HT29 and DLD1 CMs (Fig. 5A and B). However, this treatment did not affect the inhibitory effects of 44As3 or C26 CMs (Fig. 5A and B). Furthermore, CM from KATOIII and BxPC3, in addition to HT29 and DLD1, significantly increased the expression of Id1 and Id3 mRNAs in C2C12 cells compared to DM or CMs from other remaining cells (Fig. 5C). Finally, BMP4 was highly expressed and secreted not only in HT29 and DLD1 but also in KATOIII and BxPC3 cells, compared with control 58As9 cells. By contrast, 44As3 cells did not express or secrete BMP4 (Fig. 5D and E). These results suggest that the inhibitory effect of KATOIII and BxPC3 CMs on C2C12 differentiation was induced via the BMP4-Smad-Id signaling pathway.

By contrast, C26 cells are known to induce muscle atrophy via UPS and ALS, which are activated by proinflammatory cytokines, including IL-6 and TNFα (8-11). Thus, an experiment to analyze whether CM from 44As3 and C26 caused

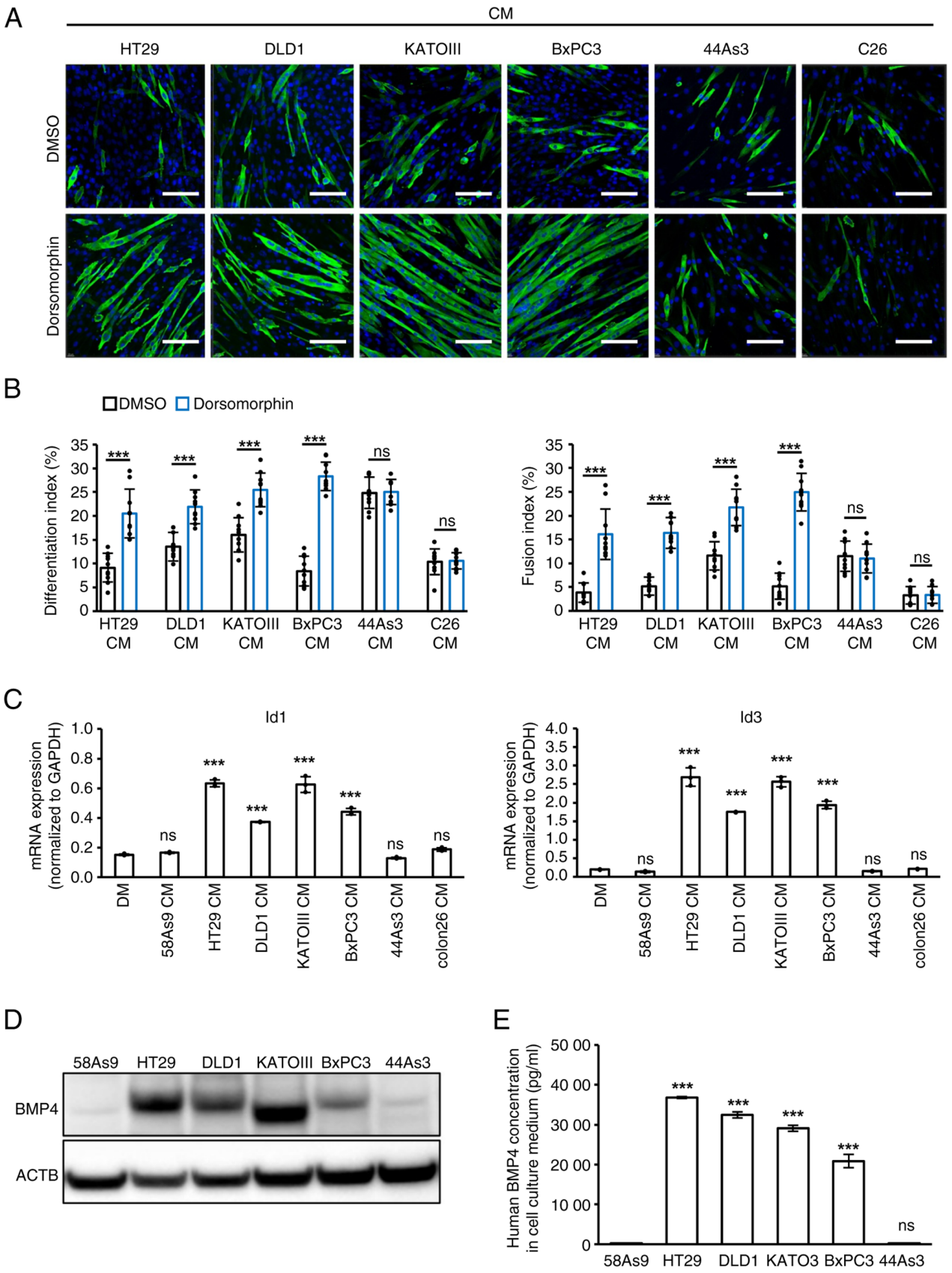

Figure 5. GIC KATOIII and BxPC3 cells inhibited myoblast differentiation in C2C12 cells via BMP4-Smad-Id signaling axis. (A) Representative images and (B) quantification of reversed effect of Dorsomorphin on the inhibited C2C12 differentiation by KATOIII, BxPC3, 44As3 and C26 CMs. Immunofluorescence staining of MYH in C2C12 cells on day 5, which were incubated in CMs of six cancer cells with or without Dorsomorphin. Scale bar, 100 μm. Differentiation and fusion indices are shown (n=10). Data are presented as mean ± SD. Statistical significance was determined by comparison between dorsomorphin-treated and -untreated C2C12 cells with CM from each cell lines (B). (C) mRNA expression of Id1 and Id3 in C2C12 cells with DM or CM from each of the cancer cell lines on day 1 (n=3). Statistical significance was analyzed compared with control DM. BMP4 protein levels in the (D) cell lysates and (E) the culture medium (n=3) from the indicated cancer cell lines. All experiments were independently repeated at least three times. Data are presented as mean ± SD, and statistical significance was determined by comparison with control (DM or 58As9). ns not significant, ***P<0.001. DM, differentiation medium; CM, conditioned medium.

atrophy in myotubes differentiated from C2C12 cells was conducted (Fig. S3A). Analysis revealed that CM from 44As3 and C26, but not HT29 or DLD1, induced visible atrophy in myotubes, with a significant decrease in myotube diameter (Fig. S3B and C). Higher expression levels of UPS (associated with MuRF-1) and ALS (associated with LC3B-II) were observed in myotubes cultured with 44As3 and C26 CMs than with HT29 and DLD1 CMs (Fig. S3D). In addition, 44As3 cells expressed higher levels of IL-6 mRNA compared with 4 BMP4-expressing GIC (Fig. S3E). These findings suggest that 44As3- and C26-derived IL-6 not only inhibited myogenic differentiation of C2C12 cells but also accelerated UPS- and ALS-dependent atrophy in myotubes.

BMP4 gene silencing by siRNA in HT29 cells rescues myogenic differentiation. The present study attempted to confirm whether cancer-derived BMP4 inhibits myogenic differentiation by activating Smad-Id signaling in C2C12 cells. A knockdown analysis was performed in HT29 cells using siBMP4. After siBMP4 transfection, BMP4 expression and secretion were effectively inhibited in siBMP4-HT29 cells, compared with siCtl (Fig. 6A-C). When C2C12 cells were cultured with CM from siBMP4-HT29, myotube formation with multinuclei was remarkably restored relative to siCtl-HT29, and significantly higher indices of differentiation and fusion were observed (Fig. 6D and E). Moreover, the expression levels of myogenin, myomaker and myomixer were apparently increased in C2C12 cells with CM from siBMP4-HT29 (Fig. 6F). Finally, siBMP4-HT29 CM did not elevate the expression of p-Smad1/5/8, Id1 and Id3 in C2C12 cells relative to siCtl-HT29 CM (Fig. 6G and H). Taken together, these results suggest that HT29-derived BMP4 itself inhibited C2C12 differentiation into myotubes by activating the Smad1/5/8-Id1 and -Id3 signaling pathways.

Discussion

The present study found that CM treatment from the five human GIC cell lines and mouse C26 cells morphologically inhibited myotube formation in C2C12 cells. Among the five GIC cell lines, HT29 and DLD1 cells were subjected to subsequent analyses. C2C12 cells cultured with CMs from these cells proliferated with time dependency and failed to fuse with each other. Furthermore, the expression of myogenin and its downstream targets was markedly suppressed, whereas Pax7 expression was sustained. In differentiating fetal myoblasts, Pax7 is co-expressed with MyoD, but is absent in myogenin-expressing myotubes (43). Thus, we hypothesized that a factor secreted by HT29 and DLD1 cells may inhibit the switching of gene expression from Pax7 to myogenin and drive C2C12 myoblasts out of the normal process of myogenic differentiation.

Previous studies have demonstrated that the number of muscle precursor cells increases under various muscle atrophy conditions, including cancer cachexia (16,44,45). This accumulation of muscle precursor cells in atrophying muscles may be the result of a fusion defect that inhibits myoblast differentiation and regeneration (16). At this point, both sustained proliferation with Pax7 expression and unsuccessful cell fusion, which were observed in C2C12 cells cultured with HT29 and DLD1, may be consistent with the accumulation of muscle precursor cells as reported in cancer-induced muscle atrophy (16,44,45).

Next, the BMP signaling pathway was investigated. We analyzed the mRNA expression of BMP-2, 4, 6 and 7, as studies have reported that these BMP members are expressed in human cancer cells and tissues (46-50). HT29 and DLD1 cells commonly expressed BMP4 mRNA, but not the mRNAs of the other three BMPs. The secretion of BMP4 protein was also confirmed. Furthermore, the present study showed that CMs from these cells inhibited C2C12 differentiation through activation of the Smad-Id signaling and blocked BMP4-Smad1/5/8 signaling with dorsomorphin, restoring myoblast differentiation. Finally, abrogation of BMP4 by siRNA verified that HT29-derived BMP4 was key for inhibiting myogenic differentiation in C2C12. Taken together, these results suggest that HT29- and DLD1-derived BMP4 triggered the impairment of C2C12 differentiation by activating the Smad1/5/8-Id1 and -Id3 signaling axis.

The present study also revealed that CMs from HT29 and DLD1 suppressed the expression of MRF myogenin and its downstream targets, but not that of MRF MyoD in C2C12 cells. MRFs are muscle-specific basic helix-loop-helix (bHLH) transcriptional factors that function as transcriptional activators via heterodimerization with a subfamily of bHLH member E-protein (51,52). In particular, the MyoD/E-protein complex transactivates another bHLH gene, myogenin, by binding to the E-box DNA element within the myogenin promoter, which cooperatively interacts with homeodomain transcription factors, such as Pbx and Meis (22,53-57). These proteins form a higher-order transcriptional complex that facilitates chromatin remodeling at the myogenin locus and allows the recruitment of additional transcriptional regulators (56,57). By contrast, Id proteins prevent MyoD activity by forming antagonistic dimers with E-protein (28,29). This sequestration of E-proteins by Id proteins prevents the formation of the MyoD/E-protein complex, thereby inhibiting the recruitment of MyoD to the myogenin promoter and impairing chromatin remodeling required for transcriptional activation (28,29). Given these results, the induction of Id1 and Id3 via BMP4-Smad signaling may suppress myogenin transcription by forming E-protein/Id1 and/or Id3 complexes, instead of E-protein/MyoD. A proposed scheme of the inhibitory effect of human GIC-derived BMP4 on myoblast differentiation is shown in Fig. 7.

Previous studies have reported that BMP signaling modulates myogenesis-related microRNAs, including miR-1, miR-133 and miR-206, which regulate the balance between myoblast proliferation and differentiation (58,59). BMP-Smad signaling may also influence the expression of these post-transcriptional regulators, which are essential for myogenic differentiation. Moreover, epigenetic mechanisms, such as chromatin remodeling and histone modifications, may contribute to the regulation of myogenic gene expression downstream of BMP signaling (60). These alternative mechanisms may cooperate with the Id-mediated inhibition of the MyoD activity to suppress the transcriptional activation of myogenic genes, such as myogenin.

Previously, Ono *et al* (61) demonstrated that in satellite cells isolated from mouse skeletal muscle, myogenic differentiation

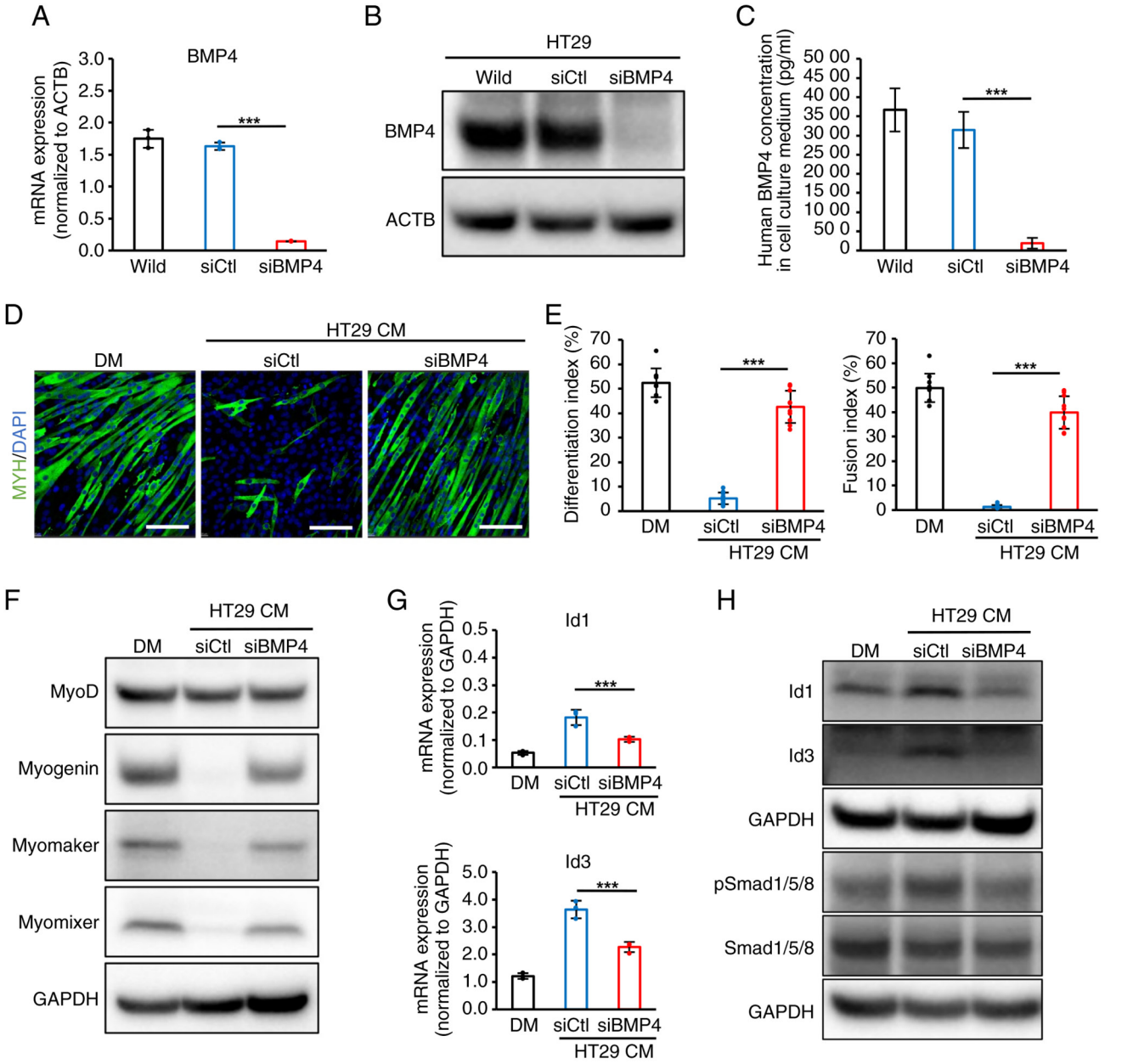

Figure 6. Abrogation of BMP4 by siRNA reversed the inhibitory effect of HT29 CM on myogenic differentiation in C2C12 cells. (A) The efficiency of BMP4 knockdown in HT29 cells was evaluated by (A) RT-qPCR (n=3) and (B) WB analysis. (C) BMP4 secretion from HT29 cell was evaluated by ELISA in triplicate. A total of two independent experiments were performed. (D) Representative images and (E) quantification of the effects of siBMP4 transfection to HT29 cells on C2C12 differentiation. Immunofluorescence staining of MYH in C2C12 cells on day 5, which was cultured with DM or CMs from siCtl- and siBMP4-transfected HT29 cells. (D) Scale bar, 100 μm. (E) Differentiation and fusion indices were estimated and plotted on graph (n=10). (F) WB analysis of MyoD, myogenin, Myomaker and Myomixer in C2C12 cells on day 1 with DM or CMs from siCtl- and siBMP4-transfected HT29 cells. (G) RT-qPCR of Id1and Id3 in C2C12 cells on day 1 (n=3). (H) WB analysis of Id1, Id3 and Smad signaling proteins in C2C12 cells on day 1 with DM or CMs from siCtl- and siBMP4-HT29 cells. These experiments were independently repeated at least three times, and data are presented as mean ± SD. Statistical significance was determined by comparison between siBMP4 and siCtl. ***P<0.001. si, small interfering; WB, western blotting; RT-qPCR, reverse transcription-quantitative PCR; ctl, control; DM, differentiation medium; CM, conditioned medium; p, phosphorylation.

was inhibited by the addition of recombinant BMP4 protein. Conversely, blocking the BMP4-Smad1/5/8 signaling axis with the BMP antagonist Noggin or Dorsomorphin induced precocious differentiation (61). Furthermore, the study speculated that myogenic cells *per se* may secrete BMP4 and act on satellite cells *in vivo*, and concluded that during muscle regeneration, BMP4 signaling may be initially required to allow the expansion of the satellite cell pool by stimulating proliferation and preventing precocious differentiation (61). Given this previous study and the present observations, autocrine BMP4 secretion from satellite cells may be temporarily essential for myoblast proliferation during the early phase. However, secretion from cancer cells may continuously activate Smad1/5/8-Id signaling to prevent myoblasts from undergoing myogenic differentiation and may eventually induce muscle wasting.

The present study determined that KATOIII and BxPC3, consistent with HT29 and DLD1, could inhibit differentiation via BMP4-Smad1/5/8-Id signaling. These results suggest that the activation of BMP4-Smad1/5/8-Id signaling may be a central mechanism underlying myogenic differentiation

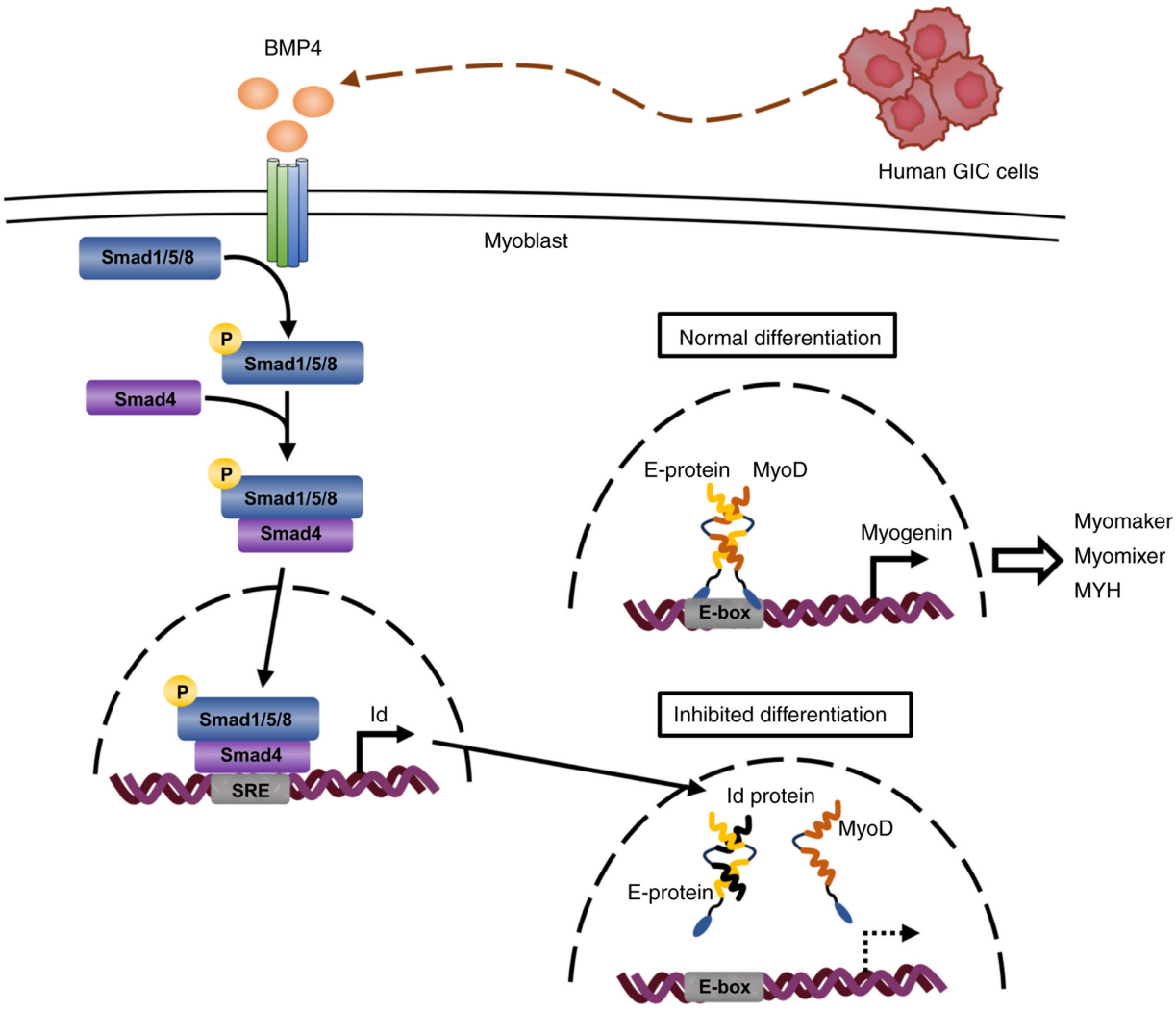

Figure 7. A possible mechanism of inhibitory effect of human GIC-derived BMP4 on myoblast differentiation through Smad-Id signaling. During the normal process of myogenic differentiation, a heterodimer complex MyoD/E-protein is formed through each HLH domain and activates the transcription of myogenin gene via binding to E-box DNA element within its promoter. Subsequently, myogenin protein transactivates the expression of the downstream genes, and eventually completes the terminal differentiation to myotube. By contrast, human GIC cells-derived BMP4 binds to BMP receptors, which in turn phosphorylates Smad1/5/8. The p-Smad1/5/8 forms a complex with Smad4 and translocates to the nucleus. The p-Smad1/5/8-Smad4 complex binds to the Smad responsive DNA element (SRE) in the Id gene promoter to upregulate mRNA expression. As Id protein contains an HLH domain, but it lacks a basic DNA binding region, Id prevents MyoD activity by forming antagonistic dimers with E-protein through each HLH. GIC, gastrointestinal cancer; p, phosphorylated; HLH, helix-loop-helix; BMP, bone morphogenetic protein.

inhibition in human GIC cells, because four (including HT29, DLD1, KATOIII and BxPC3) of the five GIC cell types inhibited C2C12 differentiation via this signaling pathway. Additionally, the present study investigated the cross-species interaction between human GIC cells and mouse myoblasts. Future validation using human primary myoblasts would improve the contextualization of the findings for human pathophysiology. However, the present study suggests that BMP4 derived from human GIC cells inhibited myogenic differentiation in murine C2C12 cells using pharmacological inhibition and genetic knockdown. Recombinant human BMP4 is well established to be biologically active in C2C12 cells, where it induces Smad1/5/8 phosphorylation and inhibits myogenic differentiation, indicating effective activation of murine BMP receptors (62). Furthermore, BMP signaling and receptor activation mechanisms are highly conserved across species (63-65). Notably, the amino acid sequence identity of the BMP receptor BMPR-II between humans and mice is ~96.6%, indicating structural conservation (66). Taken together, these findings suggest that potential species-specific differences in receptor affinity are unlikely to affect downstream signaling or confound the interpretation of the results.

Meanwhile, previous studies have reported that cancer-secreted BMPs promote proliferation, invasion and epithelial-mesenchymal transition in an autocrine manner (46-50). Notably, autocrine BMP4-Smad1/5/8-Id signaling was activated in HT29 and DLD1 cells *per se*, and contributed to accelerating tumor growth of the HT29 xenograft in nude mice (49). Therefore, BMP4-expressing GIC cancer, such as HT29 and DLD1, may carry out dual roles in promoting tumor growth and cancer cachexia. Furthermore, an *in vitro* study demonstrated that muscle differentiation in human skeletal muscle myoblasts was inhibited by sera from cancer-cachectic patients, including patients with colorectal cancer (67). Moreover, previous studies have reported that circulating BMP4 has been detected at ~230 pg/ml by ELISA in human serum, and its levels are

associated with disease status in patients with cancer, suggesting that BMP4 may function as a systemic factor in cancer progression (68,69). In the future, analyzing the association between the serum BMP4 levels and the cachexia grade in patients with GIC may be important to verify these *in vitro* findings.

In conclusion, the present study identified cancer-derived BMP4 as an essential factor that inhibits the myogenic differentiation of C2C12 in human GIC cells. As the present study was limited to *in vitro* experiments, further *in vivo* studies using mouse xenografts or clinical samples from patients are needed to clarify whether cancer-derived BMP4 inhibits skeletal muscle differentiation and eventually causes cachexia. However, this novel insight may provide clues for the elucidation of the complicated mechanisms underlying cancer-induced cachexia in humans.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

KH designed and performed the experiments, analyzed and interpreted the data, and drafted the manuscript. YK designed and supervised the study, analyzed and interpreted the data, and drafted and reviewed the manuscript. NK, SI and SM performed the experiments. TT interpreted the data and reviewed the manuscript. HN contributed to the conception and design of the study, provided critical interpretation of the data, and supervised the overall research direction. KH and YK confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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