Received: 6 November 2017 | Revised: 26 January 2018 | Accepted: 12 February 2018
DOI: 10.1002/mc.22791

RESEARCH ARTICLE

WILEY Molecular Carcinogenesis

Abrus agglutinin stimulates BMP-2-dependent differentiation through autophagic degradation of β-catenin in colon cancer stem cells

Prashanta K. Panda[1] | Prajna P. Naik[1] | Prakash P. Praharaj[1] | Biswa R. Meher[2] | Piyush K. Gupta[3] | Rama S. Verma[3] | Tapas K. Maiti[4] | Muthu K. Shanmugam[5] | Arunachalam Chinnathambi[6] | Sulaiman A. Alharbi[6] | Gautam Sethi[5,7] | Rajesh Agarwal[8] | Sujit K. Bhutia[1]

[1] Department of Life Science, National Institute of Technology, Rourkela, India
[2] Department of Botany, Berhampur University, Berhampur, India
[3] Bhupat and Jyoti Mehta School of Biosciences, Department of Biotechnology, Indian Institute of Technology Madras, Chennai, India
[4] Department of Biotechnology, Indian Institute of Technology, Kharagpur, India
[5] Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore
[6] Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia
[7] School of Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth, Western Australia, Australia
[8] Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado Denver, Aurora, Colorado

Correspondence
Dr. Sujit Kumar Bhutia, Department of Life Science, National Institute of Technology Rourkela, Rourkela 769008, Odisha, India.
Emails: sujitb@nitrkl.ac.in, bhutiask@gmail.com

Funding information
Department of Biotechnology, Ministry of Science and Technology, Grant number: BT/PR7791/BRB/10/1187/2013; Board of Research in Nuclear Sciences, Grant number: 37(1)/14/38/2016-BRNS/37276; International Scientific Partnership Program ISPP at King Saud University, Grant number: ISPP# 0091; Science and Engineering Research Board, Grant number: EMR/2016/001246; National Cancer Institute, Grant number: CA195708

Eradicating cancer stem cells (CSCs) in colorectal cancer (CRC) through differentiation therapy is a promising approach for cancer treatment. Our retrospective tumor-specimen analysis elucidated alteration in the expression of bone morphogenetic protein 2 (BMP-2) and β-catenin during the colon cancer progression, indicating that their possible intervention through "forced differentiation" in colon cancer remission. We reveal that *Abrus* agglutinin (AGG) induces the colon CSCs differentiation, and enhances sensitivity to the anticancer therapeutics. The low dose AGG (max. dose = 100 ng/mL) decreased the expression of stemness-associated molecules such as CD44 and β-catenin in the HT-29 cell derived colonospheres. Further, AGG augmented colonosphere differentiation, as demonstrated by the enhanced CK20/CK7 expression ratio and induced alkaline phosphatase activity. Interestingly, the AGG-induced expression of BMP-2 and the AGG-induced differentiation were demonstrated to be critically dependent on BMP-2 in the colonospheres. Similarly, autophagy-induction by AGG was associated with colonosphere differentiation and the gene silencing of BMP-2 led to the reduced accumulation of LC3-II, suggesting that AGG-induced autophagy is dependent on BMP-2. Furthermore, hVps34 binds strongly to BMP-2, indicating a possible association of BMP-2 with the process of autophagy. Moreover, the reduction in the self-renewal capacity of the

Abbreviations: 3 MA, 3-methyl adenine; AGG, abrus agglutinin; ATRA, all-trans-retinoic acid; ALP, alkaline phosphatase; APC, adenomatous Polyposis Coli; BMP-2, bone morphogenetic protein 2; CSCs, cancer stem cells; CRC, colorectal cancer; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; IGFBP-2, endothelial growth factor binding protein 2; EGF, epidermal growth factor; ER, endoplasmic reticulum; FBS, fetal bovine serum; FGF, fibroblast growth factor; MD, molecular dynamics; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RIP II, ribosome inhibiting protein; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; shRNA, short hairpin RNA; TGF-β, Transforming growth factor-β.

colonospheres was associated with AGG-augmented autophagic degradation of β-catenin through an interaction with the autophagy adaptor protein p62. In the subcutaneous HT-29 xenograft model, AGG profoundly inhibited the growth of tumors through an increase in BMP-2 expression and LC3-II puncta, and a decrease in β-catenin expression, confirming the antitumor potential of AGG through induction of differentiation in colorectal cancer.

KEYWORDS

β-catenin, *Abrus* agglutinin, BMP-2, cancer stem cells, differentiation, hVps34

1 | INTRODUCTION

Abrus agglutinin (AGG), a plant lectin isolated from the seeds of *Abrus precatorius*, is (gal β [1–3] NAc gal) specific and belongs to type II ribosome-inactivating proteins (RIP-II), having a molecular weight of 134 kDa. AGG is composed of a toxic enzymatic subunit A of 30 kDa and the galactose-binding subunit B of 31 kDa, both linked covalently to each other by a single disulfide bond.[1,2] AGG exhibits a protein-synthesis inhibitory activity (IC_{50}) at 3.5 nM and a lethal dose (LD_{50}) of 5 mg/kg body weight. AGG inhibits the growth and proliferation of tumors originating from various tissues, even at sub-lethal doses, both in vitro and in vivo.[3–6] Our previous data demonstrated that AGG induces apoptosis through the activation of reactive oxygen species (ROS),[3] DNA damage response,[5] and endoplasmic reticulum (ER) stress[6] in different types of cancer cells. Further, the anti-angiogenic activity of AGG was demonstrated through the inhibition of insulin-like growth factor binding protein 2 (IGFBP-2) in human endothelial cells.[3] Recently, we also examined the autophagy-inducing potential of AGG through Akt dephosphorylation and activation of ER stress in cervical cancer.[5] Furthermore, we demonstrated that AGG inhibits the expression of β-catenin in an ROS-dependent manner in FaDu cell-derived orospheres.[7]

Colorectal cancer (CRC) is the third most-diagnosed cancer in men, and the second most commonly occurring cancer in women, at a global level.[8] Despite the availability of early-diagnosis techniques and modern treatment choices, including surgical resection and adjuvant chemo/radiotherapy (depending on the stage of malignancy), the efficacy of several first-line therapeutics is exhausting gradually, with 50% of the CRC patients developing recurrent disease, and the patients with advanced and metastatic CRC becoming susceptible to death.[9] A growing body of evidence indicates that a subset of tumor cells, referred to as cancer stem cells (CSCs), is responsible for such failure of the current treatment modules. The CSCs refer to a rare subpopulation of tumor cells that possess an exclusive ability of self-renewal, aggressive invasive potential, hierarchical differentiation, and tumorigenicity.[10] Most of the colon cancer cases have been associated with an abrupt activation of Wnt/β-catenin signaling. It has been demonstrated in certain previous reports that spheroids from colon CSCs express various proteins, including CD166, CD133, CD44, CD29, CD24, Lgr5, and β-catenin, in order to regulate the clonogenic and tumor-initiating potential.[11]

Autophagy is a ubiquitous, dynamic, and evolutionarily conserved catabolic process, which is essential for survival, differentiation, development, and cellular homeostasis.[12,13] Recent studies have demonstrated that the alteration in autophagy may determine cell fate during cellular development and differentiation. For instance, autophagy-related gene FIP200 plays a vital role in the maintenance and differentiation of postnatal neural stem cells,[14] and its deletion leads to accumulation of p62 in order to regulate the cell fate through activation of superoxide.[15] Another gene, Atg16, plays a critical role in gut homeostasis and its mutation causes an impaired Slit/Robo-dependent differentiation of the enteroendocrine cells to trigger inflammation, which may increase the risk of developing colorectal cancer.[16] The autophagy factor UVRAG, present in the Beclin1/Vps34 complex, is mutated in several cancers, causing an abnormal left-right axis formation, leading to heterotaxy.[17] Furthermore, depletion of Beclin1 has been reported to inhibit both autophagy and differentiation capabilities.[13] Although autophagy is known to be active in the normal colon cells,[18] its role in the differentiation of colon CSCs remains complex and unclear.

Colonic epithelium undertakes the continual rejuvenation maintained by colon stem cells located at the very base of the intestinal crypt. Bone morphogenetic proteins (BMPs)—the members of the transforming growth factor-β (TGF-β) superfamily of proteins—are important players in colon stem cell self-renewal and differentiation.[19–21] Moreover, BMP-2 has been reported to control a crucial early-commitment step in the differentiation of human embryonic stem cell.[20]Further, BMP-2 has been demonstrated to induce chondrogenic differentiation in vitro, through down-regulation of membrane-bound β-catenin.[19,21] Recently, it has been demonstrated that the expression of Atg7 and the Wnt signaling were elevated during BMP-2-mediated human osteoblast differentiation.[22] Reports have documented that BMP pathways are inactivated in the majority of the sporadic CRCs.[23,24] In the present study, our focus was to examine and establish the efficacy of AGG in colon CSCs elimination, inhibition of the self-renewal capacity of these cells, and differentiation in the context of anti-CRC efficacy. The present study was aimed at investigating the role of AGG in the differentiation of colon CSCs

through BMP-2-dependent autophagic degradation of β-catenin. Interestingly, we were also able to reveal a previously unknown function of AGG-induced BMP-2 to physically interact with hVps34; which might have a role in autophagosome biogenesis. Moreover, it is not very late to propose AGG as an antineoplastic agent, especially due to its therapeutic property of targeted abolition of cancer stem cells.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (D9542), dimethylsulfoxide (DMSO) (D8418), and 3-Methyladenine (3-MA) (M9281) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's minimal essential medium (DMEM), fetal bovine serum (FBS) (sterile-filtered, South American origin), fibroblast growth factor (FGF), epidermal growth factor (EGF), N_2 supplement, and the antibiotic-antimycotic (100×) solution were purchased from Invitrogen (Carlsbad, CA). Tissue array with 59 spots of various normal and cancerous colon tissue types from Imgenex (Bhubaneswar, India); RNeasy kit from Qiagen (Hilden, Germany); IHC kit from BioGenex (Fremont, CA); and primers from Integrated DNA Technologies (Coralville, IA) were purchased.

2.2 | *Abrus* agglutinin purification

AGG was isolated from *Abrus precatorius* seeds by 30-90% ammonium sulfate fractionation, followed by lactamyl sepharose affinity chromatography and bound proteins were eluted with 0.4 M lactose. After that, Sephadex G-100 gel-permeation chromatography was performed to isolate highly purified agglutinin from toxic abrin. The purity of the protein was subsequently confirmed by SDS-PAGE, native-PAGE and gel permeation by HPLC. Moreover, the lectin activity of the isolated AGG was confirmed by hemagglutination assay.[1,6]

2.3 | Antibodies

LC3 (NB100-2220) was purchased from Novus Biologicals (Littleton, CO); BMP-2 (ab14933-46), CK7 (ab9021), and CK20 (ab76126) from Abcam (Cambridge, UK); CD44 (21810441) from Immunotools (Friesoythe, Germany); β-actin (A2066) from Sigma; ATG5 (2630S), BECN1 (3738S), β-catenin (9562) from Cell Signaling Technology (Danvers, MA); and hVps34 (38–2100) was purchased from Invitrogen (Carlsbad, CA).

2.4 | Cell culture and sphere culture

HT-29, a human colorectal cancer cell line, was purchased from National Centre for Cell Science, Pune, India. The HT-29 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and containing 1× antibiotic-antimycotic, at 37°C in a humidified, 95% air and 5% CO_2 atmosphere.

In order to cause sphere formation, the HT-29 cells were seeded at 5000 cells per well in ultralow attachment plates in serum-free DMEM culture medium, in the presence of fibroblast growth factor (FGF; 20 ng/mL), epidermal growth factor (EGF; 20 ng/mL), 1% N_2 supplement and 1% penicillin-streptomycin. The plates were maintained at 37°C in a humidified 5% CO_2 incubator, and the culture media were replaced every three days. After 10-12 days of culture, the cells multiplied to form floating single-cell-cloned spheres, known as colonospheres, in the colon and/or CRC cells. The colonospheres were treated with AGG for three days and analyzed using various assays. In order to study differentiation, the AGG-treated colonospheres were cultured in the presence of serum (10% FBS) for three days more. At the end of the experiment, differentiated cells were subjected to differentiation assays.[7,25]

2.5 | Immunofluorescence staining of colonospheres and confocal imaging

The colonospheres grown were fixed on chamber slides in 10% formaldehyde, washed with PBS, permeabilized with 0.2% Triton X–100 for 20 min at RT, and incubated overnight with primary antibody CD44, β-catenin, CK7, or CK20. The cells were then washed with PBS and incubated with the secondary antibody for 6 h, followed by DAPI counterstaining. The expression of the marker proteins was analyzed by using a confocal laser microscope (Leica TCS SP8).[7]

2.6 | RNA extraction and semi-quantitative RT-PCR

Total RNA from the spheres of HT-29 cells was harvested using an RNeasy kit from Qiagen, following the manufacturer's instruction. cDNA was then synthesized from the harvested total RNA using reverse transcriptase enzyme, in accordance with the manufacturers' instruction. RT-PCR was used to study the expression of mRNAs for CD44, CK7, CK20, and GAPDH (internal control). The respective primers (from Sigma) and the PCR conditions were as follows: for CD44, forward 5′-AGATCAGTCACAGACCTGCC-3′ and reverse 5′-GCAAACTGCAAGCCAAGCC-3′ (annealing at 56.5°C, 35 cycles); for CK7, forward 5′-TGAATGATGAGATCAACTTCCTCAG-3′ and reverse 5′-TGTCGGAGATCTGGGACTGC-3′ (annealing at 54.5°C, 35 cycles); for CK20, forward 5′-CAGACACACGGTGAACTATGG-3′ and reverse 5′-GATCAGCTTCCACTGTTAGACG-3′ (annealing at 55.5°C, 35 cycles); for GAPDH, forward 5′- CAC AAT GCC GAA GTG GTC GT-3′ and reverse 5′-TCA CCA TCT TCC AGG AGC GA-3′ (annealing at 62°C, 35 cycles). The amplified products were separated by electrophoresis using 1.5% agarose gel, and visualized using the gel document system (BioRad) after ethidium bromide staining.[25]

2.7 | Western blot analysis

The spheres from the HT-29 cells were treated with various concentrations of AGG, followed by extraction of proteins. Cells were lysed with lysis buffer and an equal amount of the extracted proteins was resolved by SDS-PAGE and transferred to a nitrocellulose

membrane, which had been blocked beforehand with 5% BSA (in PBST) at room temperature for 1 h. Subsequently, the blots were incubated overnight at 4°C with Beclin1, ATG5, p62, β-catenin, BMP-2, hVps34, and LC3 antibodies, followed by incubation with the corresponding secondary antibodies for 1 h at room temperature. The expression of the protein of interest was analyzed by imaging the chemiluminescence using ImageQuant LAS 500 (GE HealthCare, Little Chalfont, UK).[7]

2.8 | Alkaline phosphatase assay

In order to detect the differentiation-inducing capacity of AGG, the HT-29 colonospheres were treated with 3-MA (5 mM) for 2 h prior to AGG treatment. The treated cells were lysed and equal amounts of protein were mixed with reaction buffer, followed by mixing with pNPP solution and equilibration at 37°C. The absorbance was measured at 450 nm, immediately.[22]

2.9 | Colocalization study by immunofluorescence analysis

HT-29 derived colonospheres were treated with various concentrations (max. = 100 ng/mL) of AGG for 72 h, followed by fixation with 10% formaldehyde. Cell permeabilization was performed using 0.1% Triton X–100, followed by blocking with 5% BSA. Following this, the cells were incubated with primary antibodies for BMP-2 (1:500), hVps34 (1:500), p62 (1:250), and β-catenin (1:250). After washing with PBST, the cells were incubated with secondary antibodies conjugated with Alexa Fluor. Imaging was done using a high-end fluorescence inverted microscope (Olympus IX71), using the cellSens Standard software. Colocalization was measured by applying JACoP plugin in single Z-stack sections of deconvoluted images.[22]

2.10 | Modeling BMP-2–hVps34 complex through docking and molecular dynamics simulation

The crystal structure of bone morphogenic protein BMP-2 was obtained from Protein Data Bank (PDB). The PDB entry was 1ES7.[26] Due to the unavailability of a complete and high-resolution crystal structure for the human protein hVPS34, the 3D structure for the same was predicted by using I-TASSER software. The hVps34 domain comprised 240 residues in the structure predicted using modeling through I-TASSER. The docking algorithm was then used to locate the optimal configuration of BMP-2 protein near to the binding site of hVps34. Initially, the BMP-2 domain was positioned near the binding site, and the docking algorithm was carried out using PATCHDOCK protein-protein docking server.[27] Using the protein-protein docking algorithms, the optimal orientation of the two proteins could be identified by scoring the energy of the protein-protein complex on the basis of van der Waals (VDW) interactions and the corresponding electrostatics. Therefore, the grid-based score was generated by calculating the nonbonded terms of the molecular mechanics force field, and the structure with the highest score was then considered for MD simulation.[22]

2.11 | Human colon cancer xenografts in athymic nude mice

In order to study the therapeutic potential of AGG in human colon carcinoma, athymic BALB/c nude mice were implanted with colon carcinoma HT-29 cells (2×10^6 cells), subcutaneously. The tumors were first propagated in vivo and subsequently implanted in the experimental animals. Using this method, 90-100% tumor induction rate is obtained. The actual therapeutic study was initiated when the tumors reached the size of 0.2 cm in diameter. The mice ($n = 5$) were administrated, intraperitoneally, either the vehicle control (1X PBS) or AGG (100 ng/kg body weight), and the positive control 5-fluorouracil (5-FU) (30 mg/kg body weight), five times a week for 4 wk. The tumor volume and body weight of the mice were measured at the end of every week for 4 wk. On completion of the treatment period, tumor diameter was measured. The tumor volume was calculated using the formula $(L \times W^2)/2$, where W and L are the width (short diameter) and length (long diameter) of the tumor. At the end of the treatment period, all mice were euthanized using carbon dioxide, followed by cervical dislocation to ensure complete immobilization of the mice prior to collecting the tumor tissues samples for biochemical and histological analysis.[4,5]

2.12 | Immunohistochemical staining and scoring

In order to perform the immunohistochemical analysis, formalin-fixed and paraffin-embedded specimens with 3-4 mm thickness were sectioned, and staining was performed with anti-β-catenin, anti-BMP-2, and anti-LC3, as described previously.[5,7] Semi-quantitative immunohistochemical score or Histoscore (H-score) was obtained using a modified scoring method as reported in our previous study.[5] The percentage of positive cells in each high power field (400× magnification) was calculated and assigned a relative staining-intensity score of 1 for low, 2 for intermediate, or 3 for high staining. The histoscore was finally calculated for each primary antibody as the sum of products of percent positive cells and staining intensity, using the formula (1 × % cells stained weakly positive) + (2 × % cells stained moderately positive) + (3 × % cells stained strongly positive). The H-scores ranged from 0 to 300 for each high power field.

2.13 | Statistical analysis

All data were representative of a minimum of three independent experiments and were quantified and plotted as mean ± SD. Student's *t*-test was used for evaluating statistical differences between the experimental groups. Furthermore, a nonparametric test for statistical analysis among the groups was also performed using the alternative for one-way ANOVA, the Kruskal-Wallis test, along with Dunn's multiple comparisons tests as post hoc.

3 | RESULTS

3.1 | Analysis of expression of BMP-2 and β-catenin in colorectal cancer and non-cancer tissues

BMP signaling, which promotes the differentiation of normal and cancer stem cells, has been identified to be inactivated in the majority of the sporadic CRCs.[19,23] BMP-2 exhibits its tumor suppressive function by inhibiting the tumor-initiating ability of colon CSCs. In the present study, we analyzed the alteration in the expression pattern of BMP-2 and β-catenin in human normal, cancer, and metastatic colon tissue samples, through immunohistochemical analysis (Figure 1). The clinicopathological representation of the cohort is given in Table S1. We observed that there was a decrease in the expression of BMP-2 as the tumor progresses with the highest expression in the normal tissue, suggesting that BMP-2 is a tumor suppressor (Figure 1A). Interestingly, our clinical data in the present study reciprocated the earlier findings, which identified BMP-2 as a tumor-suppressive cytokine in colon cancer.[24] An aberrant expression of stemness and self-renewal protein β-catenin occurs due to a mutation in adenomatous polyposis coli (APC)/β-catenin signaling in colorectal cancer.[11] Our study demonstrated that β-catenin expression increased as the colon cancer progressed from normal to cancerous to metastatic stages (Figure 1B). It was evident from the H-score that the expression of BMP-2 and β-catenin decreased and increased, respectively, during the onset of cancer and its progression from stage I to metastasis (Figures 1C and 1D).

3.2 | AGG induces differentiation in colon cancer stem cells

It has been observed that AGG inhibits the growth of tumor spheres derived from various cancer cells (Supplementary Figure S1).[17] In order to investigate the role of AGG in inducing differentiation in colon cancer stem cells, we assessed the expression pattern of proteins associated with stemness and differentiation, using confocal microscopy. As expected, we observed a significant reduction in the expression of CD44, a CSC surface marker, and β-catenin, a self-renewal marker, in the HT-29 colonospheres, in a dose-dependent manner (Figure 2A). Moreover, we assessed the expression of differentiation markers CK7 and CK20 in AGG-treated colonospheres. Interestingly, the expression of CK7 and CK20 was decreased and increased, respectively, in a dose-dependent manner, with the AGG treatment in the HT-29 colonospheres (Figure 2B). The increase in the

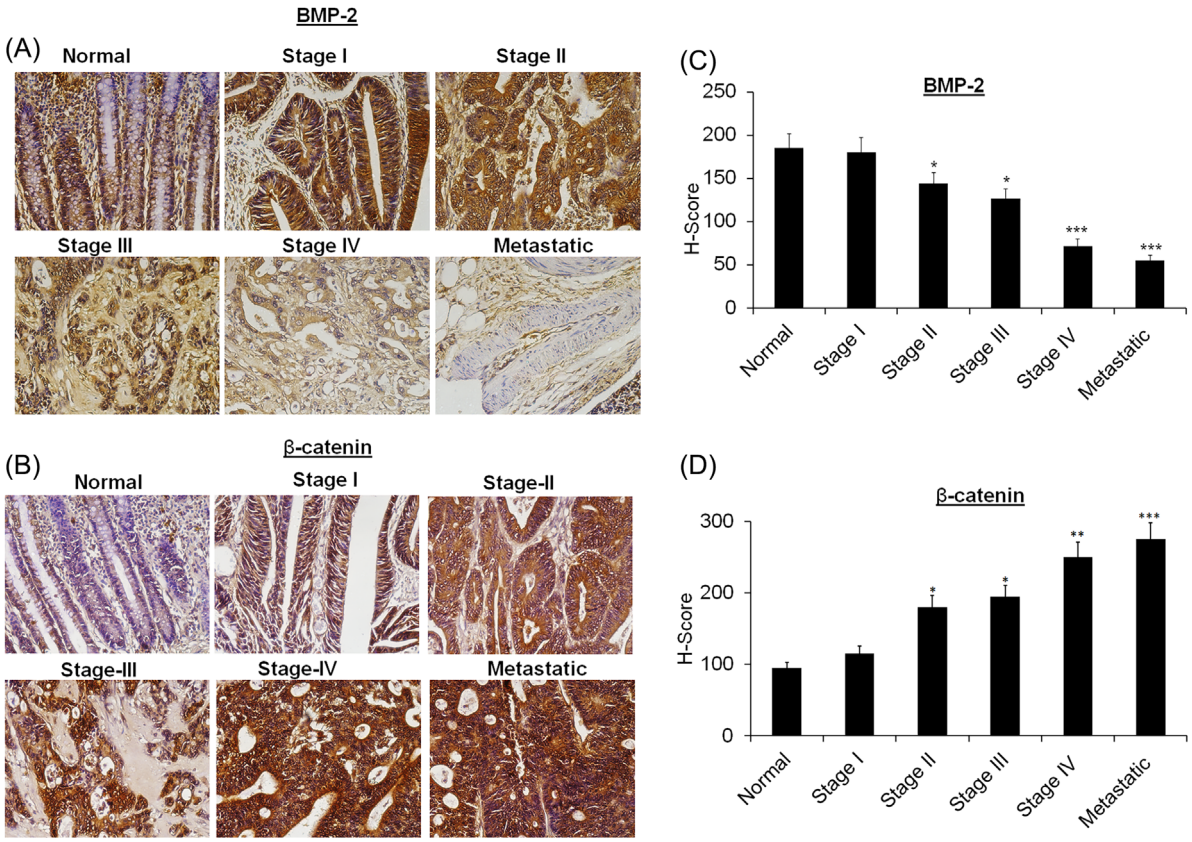

FIGURE 1 Immunohistochemistry analysis of expression of BMP-2 and β-catenin in colorectal cancer and non-cancer tissues. The slide shows the representative images of BMP-2 (A) and β-catenin (B) staining in normal colon tissue and different grades of colon cancer tissues. The semiquantitative immunoreactive analysis of BMP-2 and β-catenin was carried out by the histoscore method calculated from the percentage of positive cells and staining intensity of different tissue samples (C and D). **P*-value < 0.05; ***P*-value < 0.01; ****P*-value <0.001 were considered significant as compared with control

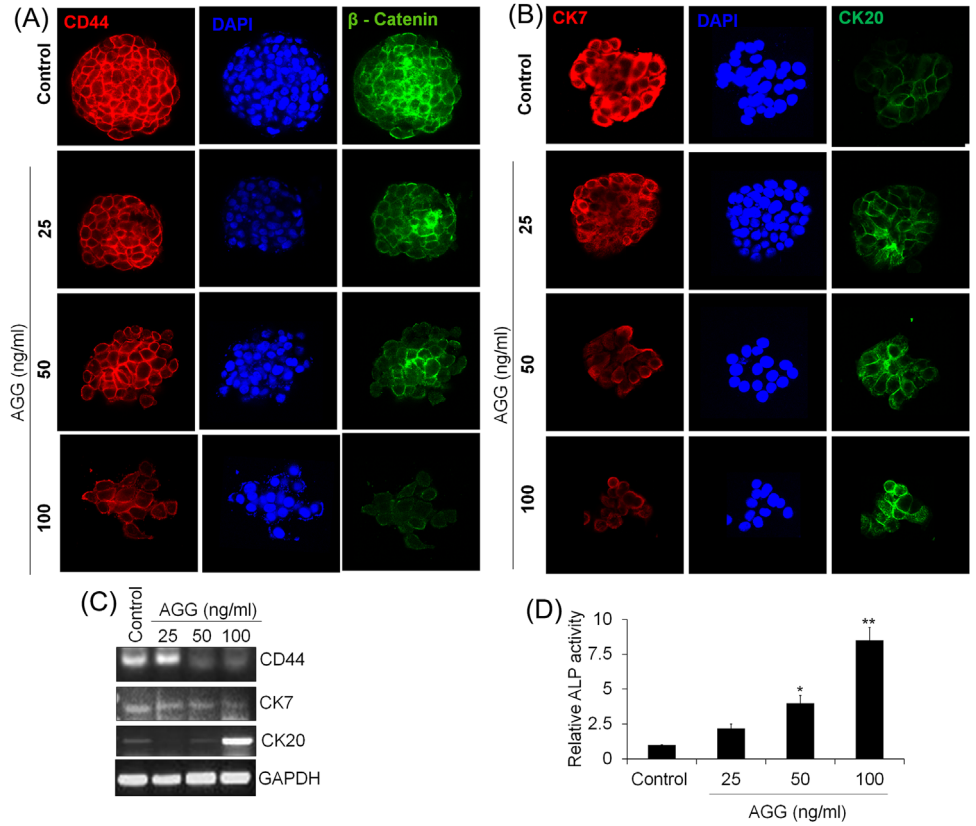

FIGURE 2 Role of AGG on stemness and differentiation potential in colonospheres. Colonospheres were treated with different concentration (25, 50, and 100 ng/mL) of AGG for 72 h and the expression of CD44/β-catenin (A) and CK7/CK20 (B) was analyzed by confocal microscopy. After treatment with AGG, the expression of CD44, CK7, and CK20 by RT-PCR (C) and alkaline phosphate activity (D) were quantified in colonospheres. The data reported as the mean ± SD of three independent experiments and compared to control. **P*-value < 0.05; ***P*-value < 0.01 were considered statistically significant as compared with the control

ratio of CK20/CK7 expression indicated increased differentiation in the AGG-treated groups. Similarly, our RT-PCR data demonstrated that AGG treatment could potentially reduce the relative mRNA expression of CD44 as well as CK7, and increase the mRNA level of CK20 in the colon CSCs (Figure 2C). Furthermore, we analyzed the alkaline phosphatase activity (ALP) as a measure of differentiation ability, and obtained an appreciable dose-wise increase in the ALP activity of the AGG-treated groups, compared to control (Figure 2D).

3.3 | AGG-induced BMP-2 prompted differentiation through autophagy activation in colon cancer stem cells

BMP-2, a member of TGF-β superfamily, plays important roles in apoptosis, autophagy, cell proliferation, and cell differentiation.[22,28] These properties of BMP-2 encouraged us to investigate its role in AGG-mediated CSCs differentiation, and surprisingly, we observed that there was a steep increase in the expression of BMP-2 with the exposure of AGG in the colonospheres, as visible in the confocal microscopy and western blot results (Figures 3A and 3B). Next, we investigated the potential role of BMP-2 in AGG-modulated differentiation in the BMP-2 knocked-down colonospheres of HT-29 cells (Figure 3C). Our data demonstrated that AGG was unable to decrease the expression of β-catenin in the BMP-2 knocked-down group, compared to the sicontrol group, in HT-29 derived stem cells (Figure 3D). Furthermore, we quantified the ALP activity and it revealed that BMP-2-deficient colonospheres of HT-29 cells exhibited a significant decrease in the ALP activity, compared to the sicontrol group, even in the presence of AGG (Figure 3E), indicating that the AGG-induced BMP-2 stimulates differentiation in the colon cancer stem cells.

Next, we intended to investigate whether autophagy induction in the HT-29 derived colonospheres regulates AGG-elicited differentiation. Colonospheres were treated with various concentrations of AGG and autophagy induction was analyzed by western blot (Figure 4A). We investigated changes in the expression of endogenous LC3 in the AGG-treated colon CSCs. It was observed that AGG could increase LC3-II accumulation in a dose-dependent manner, suggesting that AGG at low doses triggered autophagy induction. Furthermore, the autophagic molecules, including Beclin1, ATG5, and hVps34, were increased in the presence of AGG in the colonospheres. Similarly, we observed that the expression of p62, a selective adaptor for autophagic clearance, was decreased in a dose-dependent manner, confirming that AGG induces autophagy in colonospheres. Next, we

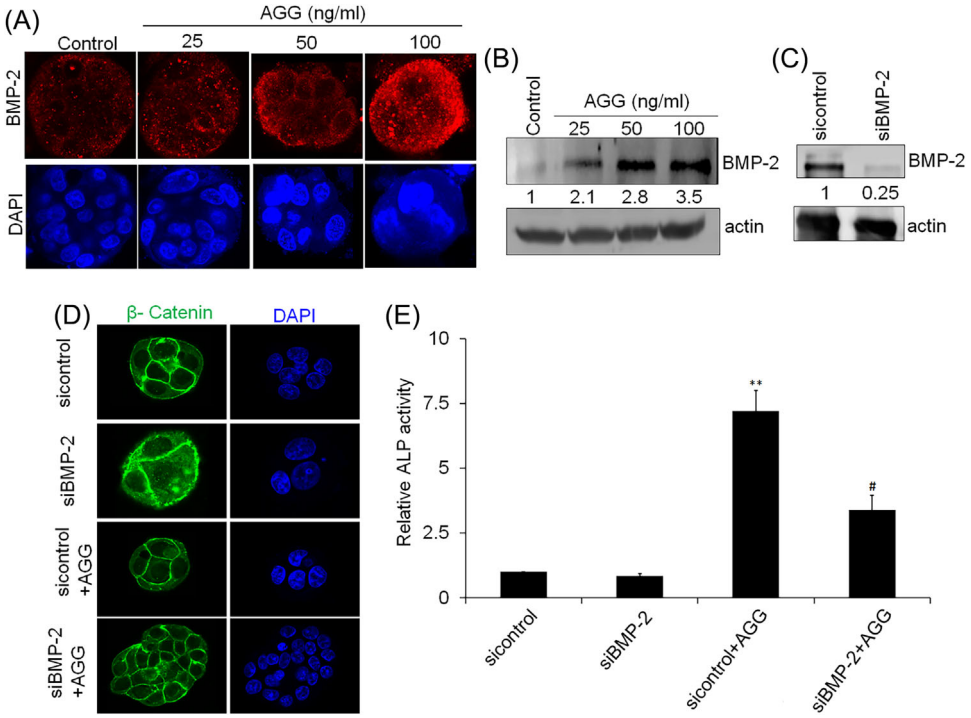

FIGURE 3 AGG-induced BMP-2 modulates stemness and differentiation in colonosphere. Colonospheres were treated with different concentrations of AGG for 72 h and the expression of BMP-2 was measured by confocal microscopy (A) and western blotting (B). Colonospheres cells were knocked down with siBMP-2 (C) and the expressions of β-catenin by confocal microscopy (D) and alkaline phosphate activity (E) was quantified in the AGG treated cells. The data reported as the mean ± SD of three independent experiments and compared with PBS control. ***P*-value < 0.01 was considered statistically significant. #*P*-value < 0.01 was considered statistically significant as compared to the AGG treated groups. Densitometry was performed on the original blots, considering the ratio of protein to actin in the control cells was 1

examined the role of autophagy in AGG-induced inhibition of self-renewal and differentiation potential in HT-29 derived colonospheres. It was observed that the self-renewal protein β-catenin is degraded in a manner independent of proteasomal degradation, via the lysosomal degradation pathway.[29] In this context, we examined the expression of β-catenin in the presence of 3-methyladenine (3-MA), an inhibitor of hVps34 activity. We observed that AGG was not able to inhibit the expression of β-catenin in presence of 3-MA (Figure 4B), suggesting that AGG-mediated inhibition of colonosphere is dependent on autophagic degradation of β-catenin that hinders the self-renewing ability of colonospheres. Furthermore, the differentiation activity of AGG was studied in the presence of 3-MA in HT-29 stem cells. The decrease in the ALP activity was observed in the colonospheres treated with both 3-MA and AGG, compared to the colonospheres treated only with AGG (Figure 4C), indicated that autophagy is essential for AGG-induced differentiation in colonospheres. Moreover, siBMP-2 transfected cells exhibited a decrease in LC3-II accumulation, compared to that in the AGG-exposed HT-29 stem cells (Figure 4D), concluding that BMP-2-mediated autophagy regulates differentiation in AGG-treated colonospheres.

A previous study had demonstrated that BMP-2 is localized to the late endosomal vesicles through a specific receptor[30] that might be involved in autophagosome biogenesis. Besides, it is documented that hVps34 is essential for the formation of internal vesicles within multivesicular bodies.[31,32] In order to investigate a possible involvement of BMP-2 in the induction of autophagy through the activation of hVps34, we studied the physical association of BMP-2 with hVps34. Analyzing the spatial distribution of BMP-2 and hVps34 by performing double immunofluorescence analysis using confocal microscopy, we observed that the concerned proteins visibly overlapped to display a dense yellow signal in the merged image (Figure 5A), depicting a total overlap coefficient and a Pearson's coefficient of 0.87 and 0.83, respectively, in the cytofluorogram scatter plot. Furthermore, the co-immunoprecipitation analysis was performed to pull out BMP-2 and hVps34 from the AGG-treated HT-29 colonospheres lysates, using anti-hVps34 or anti-BMP-2 antibodies for immunoprecipitation, followed by western blotting with anti-BMP-2 or anti-hVps34 (Figure 5B); which confirmed that BMP-2 interacted strongly with hVps34 to induce autophagy. In order to identify the residues of the individual domains involved in the interactions between BMP-2 and hVps34, a 5000 ps long molecular dynamics (MD) simulation of the docked hVps34-BMP-2 complex was performed; the complex appeared quite stable (Figure 5C), with C_α atom's root-mean-square deviation (RMSD) value in the range of 2-4 Å, as depicted in the Supplementary Figure S2. MM-GBSA based average binding free energy and the detailed contributions from various energy

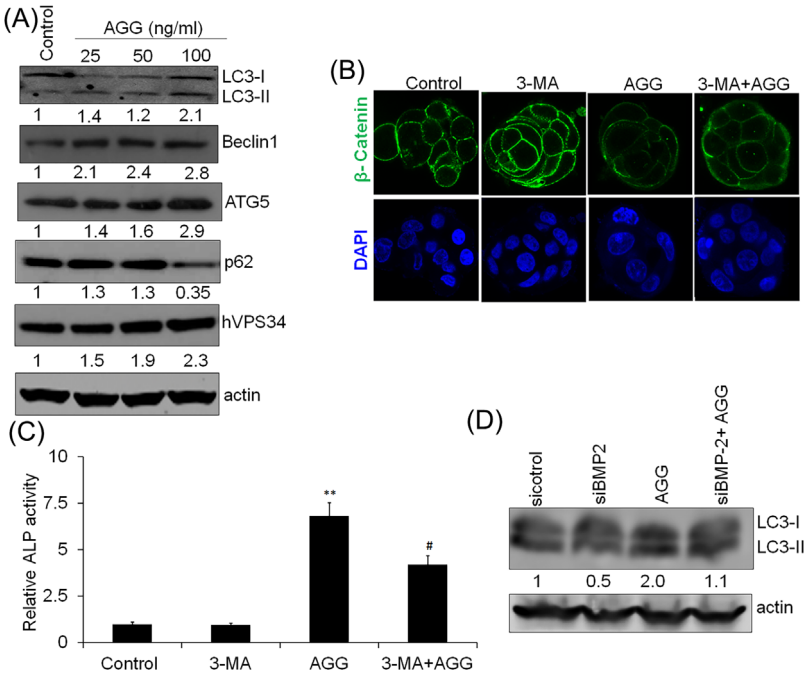

FIGURE 4 AGG induces BMP-2-dependent autophagy in HT-29 colonospheres. Colonospheres were treated with different concentrations of AGG for 72 h and the expression of autophagic proteins was analyzed by western blot (A). Colonospheres were treated with AGG (100 ng/mL) in the presence of 3-MA (5 mM; 2 h) for 72 h and the expression of β-catenin through confocal microscopy (B) and alkaline phosphatase activity (C) was quantified in colonospheres. After AGG (100 ng/mL) for 72 h, the accumulation of LC3-II was analyzed by western blot in BMP-2 knocked down colonospheres (E). The data reported as the mean ± SD of three independent experiments and compared with the PBS control. ***P*-value < 0.01 was considered significant. #*P*-value < 0.01 was considered significant as compared with the AGG treated groups. Densitometry was performed on the original blots, considering the ratio of protein to actin in control cells was 1

components were calculated for the hVps34-BMP-2 complexes, by taking 500 snapshots from the 5 ns of the MD trajectory, as depicted in Table S2. It was observed that the calculated binding free energy for the complex, according to the MM-GBSA method, was −37.838 kcal/mol. The favorable contribution from direct electrostatic interactions between hVps34 and BMP-2 was recompensed by the electrostatic desolvation free energy upon binding, which progressed to an unfavorable contribution as a whole, consistent with the other MM-GBSA and MM-PBSA studies. In contrast, the nonpolar interactions, $\Delta G_{nonpolar}$ (including van der Waals interactions and nonpolar solvation), contributed −130.657 kcal/mol, which is very favorable to the binding process and consistent with the large hydrophobic binding surface between hVps34 and BMP-2. Decomposing the binding free energy into contributions from each residue helped to recognize the binding hotspots between hVps34 and BMP-2 (Figure 5E). It revealed several residues of hVps34 that favorably contributed >−2.0 kcal/mol of the free energy – Glu52, Tyr71, Tyr135, Ala140, Pro142, Val143, Gly144, Pro168, Arg231, Asn234, and Met236. In addition, some other residues – Leu60, Pro63, Gly145, Val166, Trp167, and Asn169 – contributed >−1.0 kcal/mol of the free energy. It can be seen that these key residues of hVps34 domain, which interacted with BMP-2, are basically from the longer loop and the α-helix present between the anti-parallel beta sheet-1 and beta sheet-2 structures (Figure 5E). Residue energy decomposition results for BMP-2 were as follows: His54, Thr58, Asn59, and Ile62 in BMP-2 contributed >−2.0 kcal/mol of the free energy; while Ala52, His60, Glu109, and Cys111 contributed >−1.0 kcal mol^{-1} of the free energy. It was observed that these key residues on the BMP-2 surfaces are basically from the beginning portion of the single α-helix present in the BMP-2 structure and from the last anti-parallel β-sheet existing near the C-terminal region. The chief contributor for the binding, ie, His54, resides in the loop region just before the α-helix (Figure 5F). Our results revealed that BMP-2 interacts with hVps34 to augment the efficiency of Hvps34 for autophagosome formation to induce autophagy for differentiation in AGG-treated cells; although a detailed investigation is further required.

3.4 | AGG induces β-catenin degradation through autophagy to promote differentiation in colon cancer stem cells

We examined the molecular mechanism of β-catenin degradation in AGG-treated HT-29 colonospheres. It was observed that cytosolic β-catenin becomes phosphorylated through tumor-suppressing APC destruction complex and undergoes ubiquitination and subsequent proteasomal degradation.[33] The present study analyzed whether AGG induces the ubiquitination of β-catenin through autophagic degradation in HT-29 CSCs. The confocal microscopy data in our study

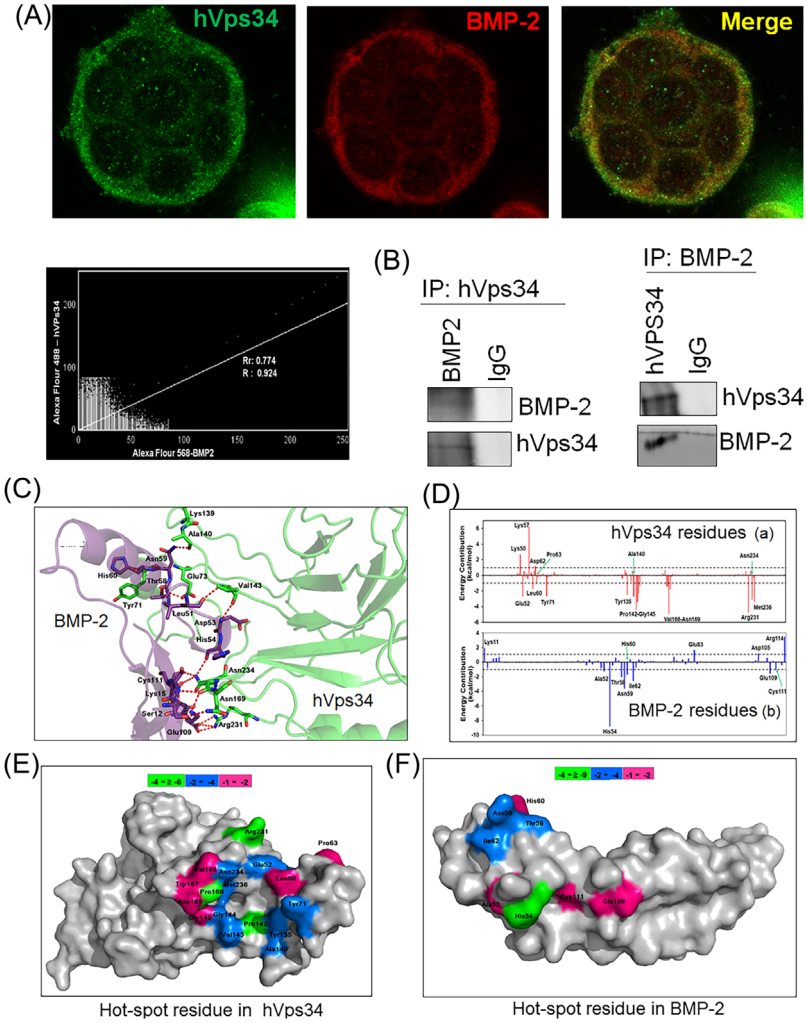

FIGURE 5 Interaction of BMP-2 and hVps34. HT-29 colonospheres were treated with AGG (100 ng/mL) for 72 h and the interaction of BMP-2 (Red) and hVps34 (Green) was analyzed by confocal microscopy (A). The colocalization parameters were analyzed by using JaCoP plugin in ImageJ software. HT-29 colonospheres were treated with AGG (ng/mL) for 72 h and immunoprecipitated with anti-BMP-2 and anti-hVps34 followed by immunoblotting with anti-Vps34 or anti-BMP-2 antibodies (B). A schematic ribbon representation of the BMP-2-hVps34 complex structure is shown in different colors. The hVps34 domain is shown in green color while the BMP-2 structure is shown in violet color. The residues showing the interactions are shown in sticks along the domain interface for both BMP-2 and hVps34 (C). The decomposition of ΔG on per-residue basis or the pair interaction energy between BMP-2 and hVps34: (a) the contribution of each residue in hVps34 to BMP-2 domain binding; (b) the contribution of each residue in the BMP-2 domain to hVps34 binding (D). The distributions of the identified hotspot residues on the hVps34 (E) and BMP-2 (F) domain cartoon representation. Colored bars show the range of contributions by the residues in the unit kcal/mol

revealed that AGG treatment promoted strong colocalization between ubiquitin and β-catenin, with the appearance of an intense yellow color in the cells. In contrast, much-diffused colocalization was observed in the control colonospheres (Figures 6A and 6B). The ubiquitination level was further examined by immunoprecipitation studies, which demonstrated that the expression of ubiquitinated β-catenin in the AGG-treated group was significantly higher than that in the control HT-29 colonospheres (Figure 6C). Interestingly, the immunoprecipitation data revealed that a decline in the interaction of ubiquitin with β-catenin was observed in 3-MA-treated AGG-exposed colonosphere, compared to the group treated only with AGG (Figure 6D). The adaptor protein p62 interacts selectively with ubiquitinated proteins for the clearance of autophagic cargo.[34] Our study is a pioneer in demonstrating, through co-immunoprecipitation and colocalization studies, that β-catenin physically interacts with p62. The co-immunoprecipitation study was employed to examine p62 and β-catenin obtained from AGG-treated HT-29 colonosphere lysates, using anti-β-catenin or anti-p62 antibodies for immunoprecipitation, followed by western blotting with anti-p62 or anti-β-catenin (Figure 6E). Similarly, the confocal analysis demonstrated colocalization of p62 with β-catenin in AGG-treated cells, in comparison to control HT-29 colonospheres (Figure 6F). Taken together, the above findings suggest that β-catenin undergoes ubiquitination in the AGG-treated cells for its autophagic degradation.

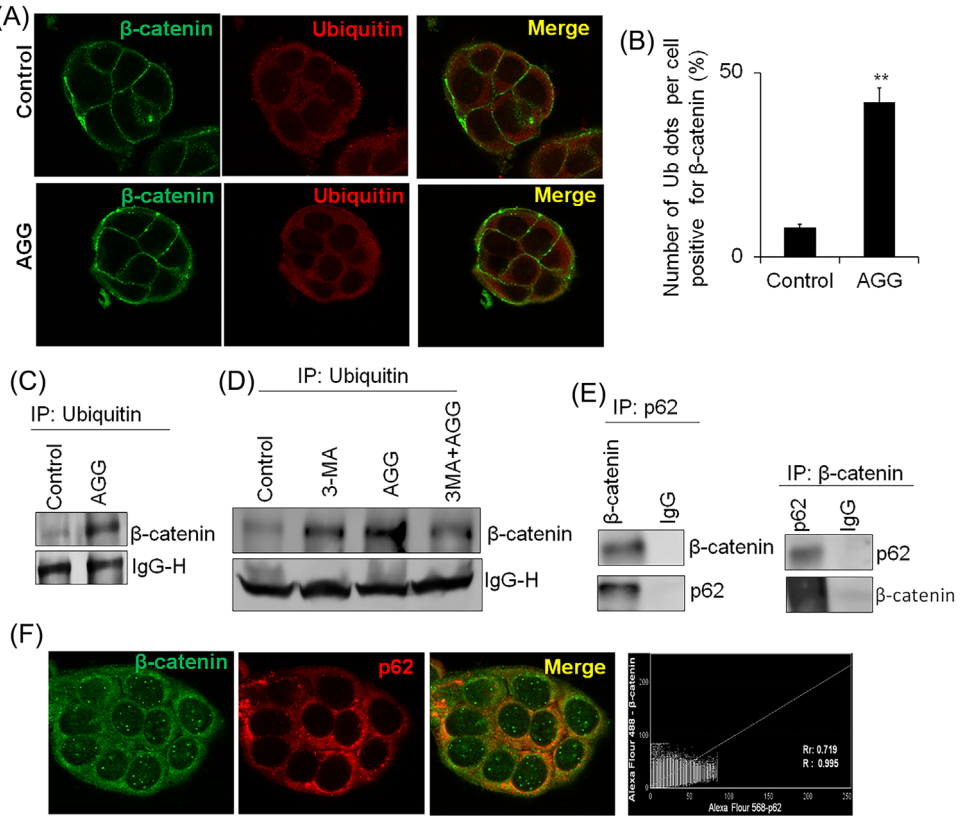

FIGURE 6 AGG induces β-catenin degradation through autophagy to promote differentiation in colonospheres. After AGG treatment HT-29 derived colonospheres were stained with ubiquitin and β-catenin and the colocalization was studied through confocal microscopy (A and B). Colonospheres were treated with AGG (100 ng/mL) in the absence and presence of 3-MA (5 mM, 2 h) and β-catenin/Ub interaction was studied by immunoprecipitation analysis (C and D). HT-29 colonospheres were treated with AGG for 72 h and immunoprecipitated with anti-p62 and anti- β-catenin followed by immunoblotting with anti-β-catenin or anti-p62 antibodies (E). After AGG treatment, HT-29 colonospheres were stained for p62 and β-catenin and colocalization was studied through confocal microscopy (F). The data were reported as mean ± SD of three independent sets of the experiment and compared with the PBS control. $^{**}P$-value < 0.01 was considered statistically significant

3.5 | Effect of AGG on tumor growth and expression of β-catenin, BMP-2, and LC3 in HT-29 xenograft model

After examining the in vitro efficacy of AGG on differentiation in colon CSCs, we evaluated the efficacy of AGG in inhibiting the growth of HT-29 cell xenografts in nude mice. The athymic BALB/c nude mice were implanted with colon carcinoma HT-29 cells subcutaneously. The mice were injected with either PBS as a vehicle (n = 5) or AGG (100 ng/kg body weight) (n = 5), and a positive control (5-fluorouracil, 30 mg/kg body weight) (n = 5), intraperitoneally, five times a week for 4 wk. The in vivo study demonstrated that AGG significantly suppressed the tumor growth of HT-29 cells following the 4 wk of treatment (Figure 7A-C). It should also be noted that there was no significant loss in the body weight of the AGG (100 ng/kg body weight) treated mice, compared to control mice (Figure 7B). In addition, AGG at the dose level of 100 ng/kg body weight was observed to be well tolerated by the mice; whereas in the 5-fluorouracil (5-FU) treated group, three mice died in the fourth week of treatment. Although there was no significant difference between the total body weights, there was a significant reduction in the tumor volume, from 201 ±31.5 mm^3 to 50.4 ± 23.7 mm^3, at the end of the fourth week (Figure 7C). The tumor tissues were subjected to immunohistochemistry for the analysis of expression of stemness-, differentiation-, and autophagy-related molecules (Figure 7D). In the xenograft, the expression of β-catenin decreased significantly in the AGG-exposed group, compared to control mice (Figure 7D). Moreover, the AGG-treated group exhibited a significant increase in the expression of BMP-2 and LC3 staining, compared to control (Figure 7D). The H-score significantly distinguished the expression of β-catenin as high and low; and BMP-2 as low and high, in the control and AGG-treated groups, respectively (Figure 7E). Interestingly, it was observed that the AGG-treated group exhibited significantly increased LC3 puncta, compared to control (Figure 7E). These results clearly indicate that AGG decreases stemness and activates differentiation through autophagy, resulting in a potent anticancer effect in HT-29 cells (Figure 8).

4 | DISCUSSION

In colon cancer, a small population of cells termed as cancer stem cells is responsible for tumor initiation, metastasis, therapy resistance, and

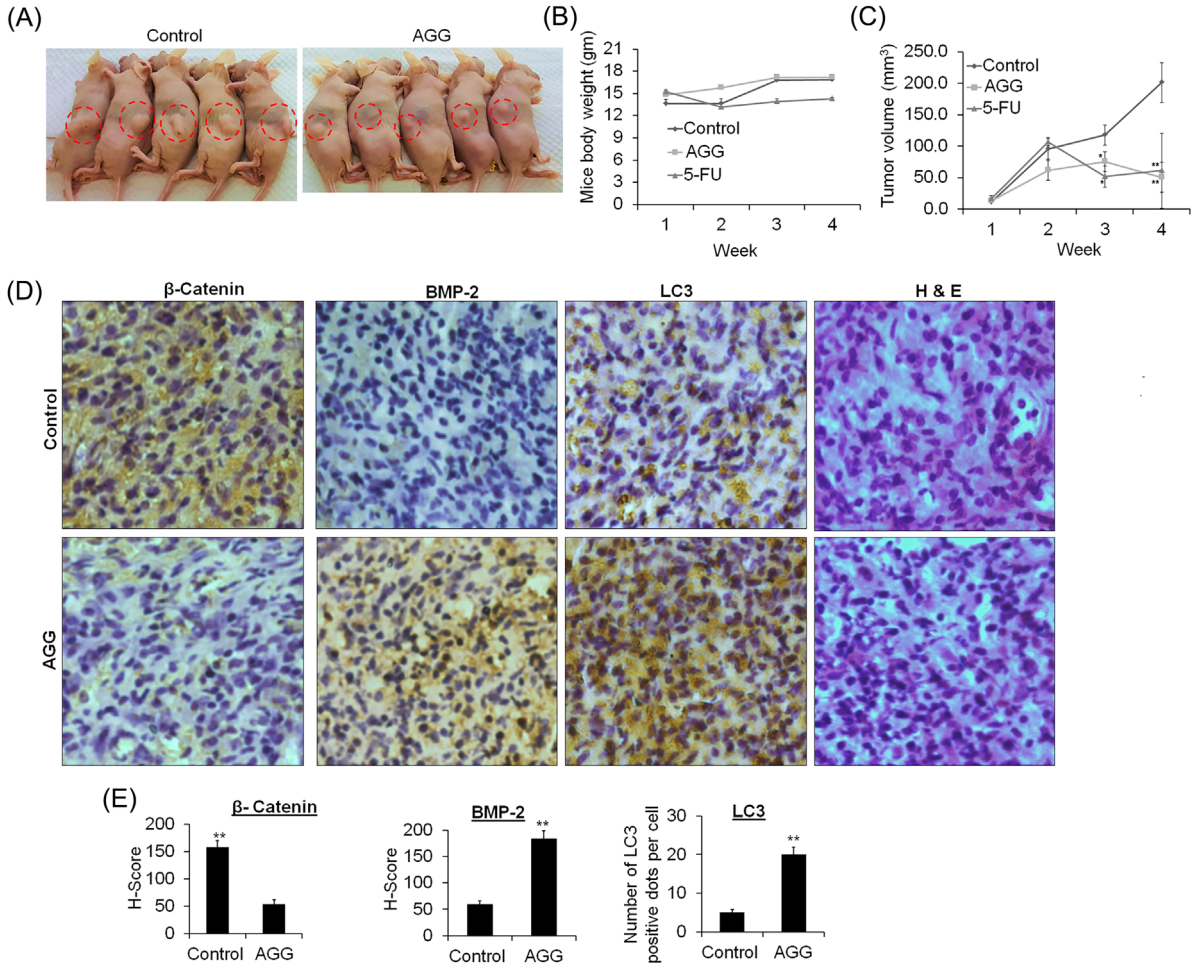

FIGURE 7 AGG inhibits tumor growth in HT-29 xenograft model. HT-29 cells were subcutaneously injected (2×10^6 cells/mouse) to each mice ($n = 5$ for each group). The mice in control group were treated with normal saline (0.9%) and in the treatment group with intraperitoneal AGG at a dose of 100 ng/kg body weight. Tumor weight and tumor volume are plotted in the form of histogram and graph (A-C). Tumor tissues were harvested followed by fixation with formalin and paraffin-embedded sections were immunostained for BMP-2, β-catenin, and LC3 in the control and treated groups (D). The semiquantitative immunoreactive analysis for β-catenin, BMP-2 and LC3 was performed by the histoscore method (E). The data are reported as the mean ± SD of three independent experiments and compared with the PBS control. $^{**}P$-value < 0.01 was considered statistically significant as compared to the control

re-occurrence.[9,11,25] The colon CSCs are more resistant to conventional chemotherapy; however, "forced differentiation therapy" is now emerging as a novel approach to render the differentiated cancer stem cells more sensitive to traditional chemotherapy.[35] Several drugs including sodium butyrate,[36] all-trans-retinoic acid (ATRA),[37] and Arsenic trioxide[38] have been demonstrated as differentiation-inducing agents in several cancers including the cancer of the colon. More importantly, several natural compounds such as resveratrol,[39] curcumin,[40] Tetrandrine,[41] inhibit the self-renewing potential of the stem cells and stimulate differentiation in these cells. In the present study, we uncovered a previously unknown role of AGG, ie, the induction of differentiation in the colon CSCs through autophagic degradation of β-catenin in the colorectal carcinoma, indicating that AGG could serve as a promising differentiation-inducing agent in cancer therapeutics.

Differentiation therapy is focused on restoring the endogenous differentiation programs leading to terminal maturation of CSCs, rendering them available for being targeted using the routine therapeutic approaches.[42] The present investigation focused on the differentiation-inducing ability of AGG at a nontoxic dose (max. dose = 100 ng/mL) to establish its promising candidature as a suitable anticancer agent against CSCs. The modulation of APC,[43] BMP,[17] caveolin,[44] and R-spondin[45] genes, which are mutated in cancer, induce differentiation in colon cancer and sensitize the colon CSCs to conventional chemotherapy. In the present study, it has been demonstrated that AGG-induced BMP-2 activation and autophagy-mediated β-catenin degradation is responsible for the differentiation of colonospheres.

According to previous reports, while the typically activated Wnt/β-catenin pathway exerts a significant influence on the initiation of

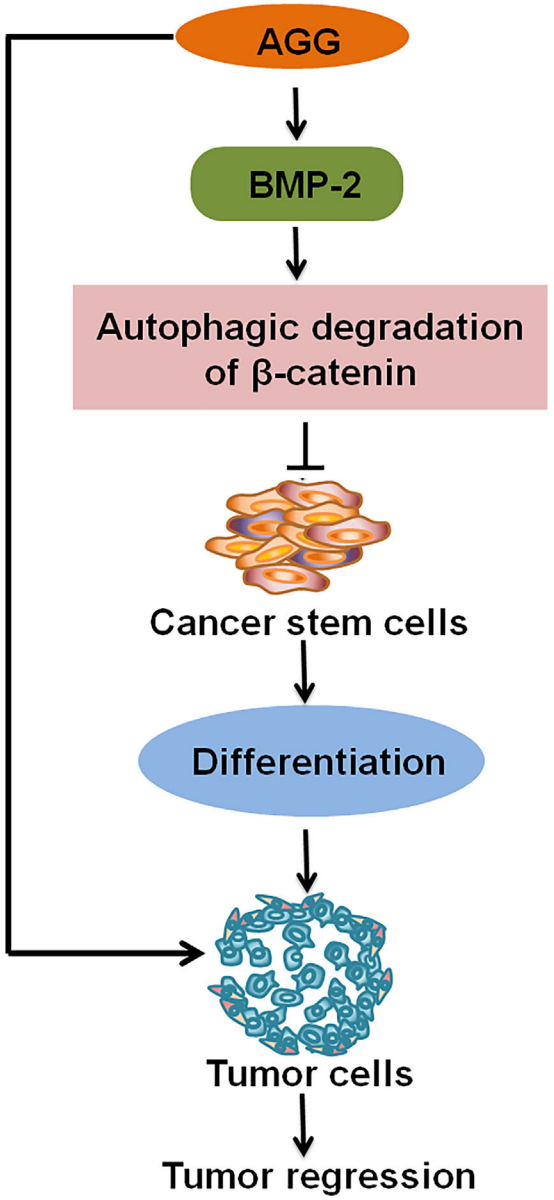

FIGURE 8 Schematic representation of BMP-2 activation by AGG induced differentiation through autophagic degradation of β-catenin in colon cancer stem cells

CRC development,[19] aberrant activation of the BMP pathway has been demonstrated to contribute to juvenile polyposis syndrome, suggesting the potential role of BMP signaling in CRC progression.[23] Intriguingly, our tissue microarray data established that the expression of BMP-2 was down-regulated; on the contrary, the expression of β-catenin was increased during tumor progression. Using the CSC-enriched population of CRC cells, we demonstrated that AGG exhibited an efficient inhibitory effect on both the number and the size of colonospheres, confirming AGG as a promising agent in various cancer settings, for targeting the self-renewal of CSCs as well as bulk tumor cells.[5,7] Consistent with the work of Kumar et al.,[25] it was demonstrated that AGG promotes the decrease of self-renewal properties through downregulation of CD44 and β-catenin and through an increase in the CK20/CK7 ratio, indicating increased differentiation. Interestingly, a recent report describes that β-catenin is selectively degraded via autophagy, through the formation of a β-catenin-LC3 complex, attenuating the adaptation during metabolic stress.[29] In a similar vein, an AGG-induced decrease in β-catenin content was an autophagy-dependent degradation, and 3-MA treatment was associated with a reduction in the decrease of expression of β-catenin and thus, a reduction in the differentiation potential in HT-29 colonospheres. Previously, a fucose-binding lectin LecB has been identified as an effective differentiation-inducing agent through autophagic degradation of β-catenin in acute myeloid leukemia.[46]

BMP signaling plays a significant role in controlling various cellular processes including autophagy and differentiation.[21,22] Recently, it has been demonstrated that BMP-2-induced differentiation in human stem cell-derived osteoblast cells involves autophagy protein Atg7.[22] Our data revealed that AGG upregulates BMP-2 expression to promote differentiation in CSCs, providing a potent antitumor activity in colon cancer. Previously, statin, a DNA methyltransferase inhibitor, was demonstrated to induce differentiation and reduce stemness in CRC cells in vitro and in vivo, by activating the BMP-2-dependent pathway and sensitizing the cells to 5-FU chemotherapy.[47] Activation of autophagy has been described as an essential step for differentiation. For instance, cannabidiol stimulates autophagy to induce differentiation in glioma-initiating cells.[48] Although the detailed mechanism is required to be investigated, autophagy-regulatory molecule hVps34 has been identified to interact with AGG-elicited BMP-2 to activate autophagy for differentiation in colon CSCs. What is the mechanism of BMP-2 stimulation in AGG-treated CSCs in colon cancer? Whether AGG regulates the promoter methylation of BMP-2 or inhibits the degradation of BMP-2 association in late endosomal vesicle to involve in different cellular processes including autophagy induction. It would also be interesting to examine how BMP-2 interacts with hVps34 present in the internal vesicles within the multivesicular bodies to regulate phagophore formation, establishing a crossroad between endocytosis and autophagy. In conclusion, AGG-induced BMP-2 regulates differentiation in colon cancer stem cells through autophagic degradation of β-catenin in colorectal carcinoma (Figure 8), promising the candidature of AGG as a suitable differentiation-inducing agent in cancer therapeutics.

ACKNOWLEDGMENTS

Research support was partly provided by Department of Biotechnology (Grant Number: BT/PR7791/BRB/10/1187/2013; Science and Technology Department, Government of Odisha; the Board of Research in Nuclear Sciences (BRNS) (Number: 37 [1]/14/38/2016-BRNS/37276), Department of Atomic Energy (DAE); Science and Engineering Research Board (SERB) (Number: EMR/2016/001246), Department of Science and Technology. Research infrastructure was partly provided by Fund for Improvement of S&T infrastructure in universities & higher educational institutions (FIST) (Number: SR/FST/LSI-025/2014), Department of Science and Technology, Government of India. PKP is obliged to DBT (BT/PR1/5090/GBD/27/309/2011) and MHRD (Government of India) for providing fellowship. ICMR International Fellowship for Indian Bio-medical Scientists (INDO/FRC/

452(Y-64)/2015-16-IHD) to SKB is acknowledged. Authors sincerely thank Sushanta Pradhan for assisting in the confocal facility for this research work.

ORCID

Sujit K. Bhutia http://orcid.org/0000-0001-9719-9594

REFERENCES

1. Hegde R, Maiti TK, Podder SK. Purification and characterization of three toxins and two agglutinins from Abrus precatorius seed by using lactamyl-Sepharose affinity chromatography. *Anal Biochem*. 1991;194:101–109.
2. Bagaria A, Surendranath K, Ramagopal UA, Ramakumar S, Karande AA. Structure-function analysis and insights into the reduced toxicity of Abrus precatorius agglutinin I in relation to abrin. *J Biol Chem*. 2006;281:34465–34474.
3. Bhutia SK, Behera B, Nandini Das D, et al. Abrus agglutinin is a potent anti-proliferative and anti-angiogenic agent in human breast cancer. *Int J Cancer*. 2016;139:457–466.
4. Mukhopadhyay S, Panda PK, Das DN, et al. Abrus agglutinin suppresses human hepatocellular carcinoma in vitro and in vivo by inducing caspase-mediated cell death. *Acta Pharmacol Sin*. 2014;35:814–824.
5. Sinha N, Panda PK, Naik PP, et al. Abrus agglutinin promotes irreparable DNA damage by triggering ROS generation followed by ATM-p73 mediated apoptosis in oral squamous cell carcinoma. *Mol Carcinog*. 2017;56:2400–2413.
6. Panda PK, Behera B, Meher BR, et al. Abrus agglutinin, a type II ribosome inactivating protein inhibits Akt/PH domain to induce endoplasmic reticulum stress mediated autophagy-dependent cell death. *Mol Carcinog*. 2017;56:389–401.
7. Sinha N, Panda PK, Naik PP, Maiti TK, Bhutia SK. Abrus agglutinin targets cancer stem-like cells by eliminating self-renewal capacity accompanied with apoptosis in oral squamous cell carcinoma. *Tumour Biol*. 2017;39:1010428317701634.
8. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics. *CA Cancer J Clin*. 2012;2015:87–108.
9. Kumar R, Price TJ, Beeke C, et al. Colorectal cancer survival: an analysis of patients with metastatic disease synchronous and metachronous with the primary tumor. *Clin Colorectal Cancer*. 2014;13:87–93.
10. Naik PP, Das DN, Panda PK, et al. Implications of cancer stem cells in developing therapeutic resistance in oral cancer. *Oral Oncol*. 2016;62:122–135.
11. Vermeulen L, Todaro M, de Sousa Mello F, et al. Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. *Proc Natl Acad Sci USA*. 2008;105:13427–13432.
12. Panda PK, Mukhopadhyay S, Das DN, Sinha N, Naik PP, Bhutia SK. Mechanism of autophagic regulation in carcinogenesis and cancer therapeutics. *Semin Cell Dev Biol*. 2015;39:43–55.
13. Wang J. Beclin 1 bridges autophagy, apoptosis and differentiation. *Autophagy*. 2008;4:947–948.
14. Wang C, Liang CC, Bian ZC, Zhu Y, Guan JL. FIP200 is required for maintenance and differentiation of postnatal neural stem cells. *Nat Neurosci*. 2013;16:532–542.
15. Wang C, Chen S, Yeo S, et al. Elevated p62/SQSTM1 determines the fate of autophagy-deficient neural stem cells by increasing superoxide. *J Cell Biol*. 2016;212:545–560.
16. Nagy P, Szatmári Z, Sándor GO, Lippai M, Hegedűs K, Juhász G. Drosophila Atg16 promotes enteroendocrine cell differentiation via regulation of intestinal Slit/Robo signaling. *Development*. 2017;144:3990–4001.
17. Lee G, Liang C, Park G, Jang C, Jung JU, Chung J. UVRAG is required for organ rotation by regulating Notch endocytosis in Drosophila. *Dev Biol*. 2011;356:588–597.
18. Groulx JF, Khalfaoui T, Benoit YD, et al. Autophagy is active in normal colon mucosa. *Autophagy*. 2012;8:893–902.
19. He XC, Zhang J, Tong WG, et al. BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. *Nat Genet*. 2004;36:1117–1121.
20. Pera MF, Andrade J, Houssami S, et al. Regulation of human embryonic stem cell differentiation by BMP-2 and its antagonist noggin. *J Cell Sci*. 2004;117:1269–1280.
21. Zhang WV, Stott NS. BMP-2-modulated chondrogenic differentiation in vitro involves down-regulation of membrane-bound beta-catenin. *Cell Commun Adhes*. 2004;11:89–102.
22. Ozeki N, Mogi M, Hase N, et al. Bone morphogenetic protein-induced cell differentiation involves Atg7 and Wnt16 sequentially in human stem cell-derived osteoblastic cells. *Exp Cell Res*. 2016;347:24–41.
23. Kodach LL, Wiercinska E, de Miranda NF, et al. The bone morphogenetic protein pathway is inactivated in the majority of spo- radic colorectal cancers. *Gastroenterology*. 2008;134:1332–1341.
24. Lombardo Y, Scopelliti A, Cammareri P, et al. Bone morphogenetic protein 4 induces differentiation of colorectal cancer stem cells and increases their response to chemotherapy in mice. *Gastroenterology*. 2011;140:297–309.
25. Kumar S, Raina K, Agarwal C, Agarwal R. Silibinin strongly inhibits the growth kinetics of colon cancer stem cell-enriched spheroids by modulating interleukin 4/6-mediated survival signals. *Oncotarget*. 2014;5:4972–4989.
26. Kirsch T, Sebald W, Dreyer MK. Crystal structure of the BMP-2-BRIA ectodomain complex. *Nat Struct Biol*. 2000;7:492–496.
27. Schneidman-Duhovny D, Inbar Y, Nussinov R, Wolfson HJ. PatchDock and SymmDock: servers for rigid and symmetric docking. *Nucleic Acids Res*. 2005;33:W363–W367.
28. Zhang Y, Chen X, Qiao M, et al. Bone morphogenetic protein 2 inhibits the proliferation and growth of human colorectal cancer cells. *Oncol Rep*. 2014;32:1013–1020.
29. Petherick KJ, Williams AC, Lane JD, et al. Autolysosomal β-catenin degradation regulates wnt-autophagy-p62 crosstalk. *EMBO J*. 2013;32:1903–1916.
30. Alborzinia H, Schmidt-Glenewinkel H, Ilkavets I, et al. Quantitative kinetics analysis of BMP2 uptake into cells and its modulation by BMP antagonists. *J Cell Sci*. 2013;126:117–127.
31. Futter CE, Collinson LM, Backer JM, Hopkins CR. Human VPS34 is required for internal vesicle formation within multivesicular endosomes. *J Cell Biol*. 2001;155:1251–1264.
32. Obara K, Noda T, Niimi K, Ohsumi Y. Transport of phosphatidylinositol 3-phosphate into the vacuole via autophagic membranes in Saccharomyces cerevisiae. *Genes Cells*. 2008;13:537–547.
33. Li VS, Ng SS, Boersema PJ, et al. Wnt signaling through inhibition of β-catenin degradation in an intact Axin1 complex. *Cell*. 2012;149:1245–1256.
34. Johansen T, Lamark T. Selective autophagy mediated by autophagic adapter proteins. *Autophagy*. 2011;7:279–296.
35. Wielenga MCB, Colak S, Heijmans J, et al. ER-Stress-Induced differentiation sensitizes colon cancer stem cells to chemotherapy. *Cell Rep*. 2015;13:489–494.
36. Bai Z, Zhang Z, Ye Y, Wang S. Sodium butyrate induces differentiation of gastric cancer cells to intestinal cells via the PTEN/phosphoinositide 3-kinase pathway. *Cell Biol Int*. 2010;34:1141–1150.
37. Degos L, Dombret H, Chomienne C, et al. All-trans-retinoic acid as a differentiating agent in the treatment of acute promyelocytic leukemia. *Blood*. 1995;85:2643–2653.

38. Masciarelli S, Capuano E, Ottone T, et al. Retinoic acid and arsenic trioxide sensitize acute promyelocytic leukemia cells to ER stress. *Leukemia*. 2018;32:285–294.
39. Guo L, Wang L, Wang L, et al. Resveratrol induces differentiation of human umbilical cord mesenchymal stem cells into neuron-Like cells. *Stem Cells Int*. 2017;2017:1651325.
40. Zhuang W, Long L, Zheng B, et al. Curcumin promotes differentiation of glioma-initiating cells by inducing autophagy. *Cancer Sci*. 2012; 103: 684–690.
41. Liu T, Men Q, Wu G, et al. Tetrandrine induces autophagy and differentiation by activating ROS and Notch1 signaling in leukemia cells. *Oncotarget*. 2015;6:7992–8006.
42. Sell S. Stem cell origin of cancer and differentiation therapy. *Crit Rev Oncol Hematol*. 2004;51:1–28.
43. Dow LE, O'Rourke KP, Simon J, et al. Apc restoration promotes cellular differentiation and reestablishes crypt homeostasis in colorectal cancer. *Cell*. 2015;161:1539–1552.
44. Dasgupta N, Kumar Thakur B, Ta A, Das S. Caveolin−1 is transcribed from a hypermethylated promoter to mediate colonocyte differentiation and apoptosis. *Exp Cell Res*. 2015;334:323–336.
45. Storm EE, Durinck S, de Sousa e Melo F, et al. Targeting PTPRK-RSPO3 colon tumours promotes differentiation and loss of stem-cell function. *Nature*. 2016;529:97–100.
46. Kühn K, Cott C, Bohler S, et al. The interplay of autophagy and β-Catenin signaling regulates differentiation in acute myeloid leukemia. *Cell Death Discov*. 2015;1:15031.
47. Kodach LL, Jacobs RJ, Voorneveld PW, et al. Statins augment the chemosensitivity of colorectal cancer cells inducing epigenetic reprogramming and reducing colorectal cancer cell 'stemness' via the bone morphogenetic protein pathway. *Gut*. 2011;60:1544–1553.
48. Nabissi M, Morelli MB, Amantini C, et al. Cannabidiol stimulates AmL^{-1}a-dependent glial differentiation and inhibits glioma stem-like cells proliferation by inducing autophagy in a TRPV2-dependent manner. *Int J Cancer*. 2015;137:1855–1869.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Panda PK, Naik PP, Praharaj PP, et al. *Abrus* agglutinin stimulates BMP-2-dependent differentiation through autophagic degradation of β-catenin in colon cancer stem cells. *Molecular Carcinogenesis*. 2018;1–14. https://doi.org/10.1002/mc.22791