

pathway. Despite these findings, our preliminary study demonstrated that treatment of CD11b⁺ or CD11b⁻ IgA⁺ PCs with IL-10 alone did not induce their reciprocal differentiation into each other, and IL-10 KO mice with colitis possessed CD11b⁺ IgA⁺ PCs (J.K., unpublished data). Thus, IL-10 is redundant in some cases and additional factors are required for the maintenance of CD11b⁺ IgA⁺ PCs. Our current findings identified CD150 as a surface molecule that is highly expressed on CD11b⁺ IgA⁺ PCs. CD150 is a 70-kDa glycoprotein expressed on some B and T cells, thymocytes and macrophages²⁹. Homophilic interaction of CD150 induces proliferation of, and antibody synthesis by, B cells³⁹, and notably IL-10 synergistically enhances CD150-mediated B cell proliferation³⁹. Thus, it is likely that, at least partly, IL-10 and CD150 determine the unique features (for example, proliferation and high IgA production) of CD11b⁺ IgA⁺ PCs in the iLP. In addition, accumulating evidence has revealed an important immunological function of stromal cells as survival niches for PCs in the BM⁴⁰ and intestine^{41,42}. It is possible that complex immunological communications among commensal flora, epithelial and stromal cells, and the cells involved in innate and acquired immunity determine the differentiation and maintenance of IgA PCs in the intestine.

Taken together, our results provide new insights into the nature of IgA⁺ PCs in the murine intestine, and especially into the regulation of the early-phase IgA responses to intestinal antigens and requirement of microbe-dependent stimulation, IL-10, and the PP lymphoid structure. These findings add a new level of complexity to the intestinal IgA system of mice.

Methods

Mice. SPF and GF Balb/c mice were obtained from Japan CLEA (Tokyo, Japan). MyD88 KO mice, IL-10 mice (Balb/c background) and TCRβδ mice (C57/BL6 background) were maintained under SPF conditions at the Experimental Animal Facility, The Institute of Medical Science, The University of Tokyo, and WT littermates were used as controls. To deplete gut commensal bacteria, mice received broad-spectrum antibiotics, namely ampicillin (1 g l⁻¹; Sigma-Aldrich, St Louis, MO), vancomycin (500 mg l⁻¹; Shionogi, Osaka, Japan), neomycin sulphate (1 g l⁻¹; Sigma-Aldrich) and metronidazole (1 g l⁻¹; Sigma-Aldrich), in their drinking water for 4 weeks⁴³. To establish BM chimeric mice, we injected γ-irradiated (960 rad, Gammacell 40, Atomic Energy of Canada Limited, Ontario, Canada) recipient mice with 5 × 10⁶ BM cells through the tail vein and used them in experiments 8 weeks after injection. Under our experimental conditions, the reconstitution efficacy was about 90–95%. To obtain PP-null mice, pregnant BALB/c mice were injected intravenously and subcutaneously with 1 mg anti-IL-7Rα antibody (A7R34, BioLegend, San Diego, CA) at 14.5 days post coitus, as described previously²⁸. We confirmed the disruption of organized PPs and the existence of ILFs in the offspring, as described previously²⁸. To neutralize cytokines, mice were treated intraperitoneally with 250 μg of monoclonal antibodies specific for IL-5 (TRFK5), IL-6 receptor (D7715A7) or IL-10 (JES5.16E3) (BioLegend, San Diego, CA); control antibody (Rat IgG2b); or 100 μg of soluble TACI-Fc fusion protein (R&D Systems, Minneapolis, MN) every second day for 2 weeks^{44,45}. For assessing the role of SFB, mice purchased from the Jackson laboratory were orally inoculated with bacterial suspensions obtained by homogenizing faecal pellets from SFB-monoassociated mice in water. SFB colonization was confirmed by quantitative PCR³³ and CD11b⁺ IgA⁺ cells were analysed in the small intestine 2 weeks post gavage by flow cytometry. All experiments followed the guidelines of the Animal Care and Use Committee, The University of Tokyo and Columbia University.

Oral immunization. Mice were given sodium bicarbonate solution to neutralize stomach acid^{11,13}. Thirty minutes later, the mice were orally immunized with 1 mg OVA (Sigma-Aldrich) and 10 μg CT (List Biological Laboratories, Campbell, CA). This procedure was conducted on days 0, 7 and 14. In some groups, mice were intraperitoneally given CPM (35 mg kg⁻¹ each time, Sigma-Aldrich). One week after the final immunization, faecal samples and mononuclear cells from the iLP were collected for enumeration of OVA-specific antibody responses by enzyme-linked immunosorbent assay and ELISPOT, respectively¹³. *In vivo* CT challenge was performed by oral challenge of naive or immunized mice with 100 μg of CT as previously described⁴⁶.

Cell isolation. To isolate mononuclear cells from PPs, we stirred the tissues in RPMI-1640 medium containing 2% fetal calf serum plus 0.5 mg ml⁻¹ collagenase

(Wako, Osaka, Japan)^{11,13}. To isolate mononuclear cells from the iLP, PPs were carefully removed and the remaining intestines including ILFs were opened longitudinally, washed with RPMI-1640, cut into 2-cm pieces and stirred for 20 min at 37 °C into RPMI-1640 containing 0.5 mM EDTA and 2% fetal calf serum to remove epithelial cells and intraepithelial lymphocytes^{11,13}. The tissues were then stirred three times in 0.5 mg ml⁻¹ collagenase for 20 min before undergoing discontinuous Percoll gradient centrifugation (40 and 75%). Peritoneal cells were obtained by peritoneal flushing with 8 ml ice-cold phosphate-buffered saline (PBS)^{11,13}.

Flow cytometry and cell sorting. Mononuclear cells were preincubated with 10 μg ml⁻¹ anti-CD16/32 antibody (BD Biosciences, San Diego, CA). They were then reacted with the following antibodies: Pacific blue-rat anti-mouse CD45R (B220) (RA3-6B2, 0.8 μg ml⁻¹), phycoerythrin (PE)-rat anti-mouse CD11b (M1/70, 0.1 μg ml⁻¹), PE-Cy7-hamster anti-mouse CD11c (HL3, 0.4 μg ml⁻¹), PE-rat anti-mouse CD18 (C71/16, 0.8 μg ml⁻¹), PE-rat anti-mouse CD19 (1D3, 0.8 μg ml⁻¹), PE-rat anti-mouse CD38 (90, 0.13 μg ml⁻¹), FITC-rat anti-mouse IgA (C10-3, 2 μg ml⁻¹), PE-Cy7-rat anti-mouse IgM (R6-60.2, 1 μg ml⁻¹), PE-anti-mouse I-A^d (AMS-32.1, 0.4 μg ml⁻¹), APC-Cy7-rat anti-mouse CD11b (M1/70, 1 μg ml⁻¹), APC-Cy7-anti-mouse β1-integrin (HMβ1-1, 4 μg ml⁻¹), APC-anti-mouse CD40 (3/23, 2 μg ml⁻¹), Pacific blue-anti-mouse CD11b (M1/70, 1 μg ml⁻¹), PE-Cy7-anti-mouse F4/80 (BM8, 0.4 μg ml⁻¹) and biotin mouse anti-CD138 (281-2, 10 μg ml⁻¹) (all antibodies from BD Biosciences) followed by incubation with streptavidin-APC (1 μg ml⁻¹, BD Biosciences), PE-anti-mouse CD150 (TC15-12F12.2, 0.1 μg ml⁻¹), Alexa Fluor 647-anti-mouse CD80 (16-10A1, 1 μg ml⁻¹) (BioLegend, San Diego, CA), anti-mouse CD267 (TACI) (8F10-3, 4 μg ml⁻¹) (eBioscience, San Diego, CA), PE-mouse CCR3 (83101, 0.5 μg ml⁻¹) (R&D Systems) or biotinylated anti-peanut agglutinin lectin (1 μg ml⁻¹, Vector Laboratories, Burlingame, CA), followed by staining with streptavidin PE (1 μg ml⁻¹, BD Biosciences). For staining for Blimp-1, cells were fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences) and stained with PE-conjugated anti-Blimp1 goat polyclonal IgG (0.4 μg ml⁻¹, Santa Cruz Biotechnology, Santa Cruz, CA). FSC-H and FSC-A discrimination was used to exclude doublet cells, and ViaProbe cell-viability solution (BD Biosciences) was used to discriminate between dead and living cells. To detect proliferating cells, mice received 1 mg BrdU intraperitoneally 24 h before analysis; the BrdU signal was detected with the manufacturer's protocol (BD Biosciences). Concentration-matched isotype antibodies were used as negative controls. Flow-cytometric analysis and cell sorting were performed with FACSCanto II and FACSAria (BD Biosciences), respectively. We confirmed that cell purity was about 95% (Fig. 2a).

Immunohistological analysis. Intestines were fixed in 4% paraformaldehyde for 15 h at 4 °C, washed with PBS and treated sequentially in 10 and 20% sucrose for 12 h at 4 °C¹³. The tissues were embedded in OCT compound (Sakura Fine-technical Co., Tokyo, Japan). Cryostat sections (7 μm) were pre-blocked with anti-CD16 and CD32 antibody for 15 min at room temperature and stained for 15 h at 4 °C with FITC-rat anti-mouse IgA (C10-3, 2 μg ml⁻¹) and biotin anti-mouse CD11b antibody (M1/70, 1 μg ml⁻¹). This was followed by incubation with horseradish peroxidase (HRP)-conjugated streptavidin (Pierce, Rockford, IL) for 30 min at 4 °C and amplification of the fluorescent signal with Cy3-tyramide (TSA-Direct kit; PerkinElmer, Waltham, MA)¹³. We confirmed that no signal was detected when the specimens were stained with the concentration-matched isotype antibodies. They were then counterstained with 4',6-diamidino-2-phenylindole (Sigma-Aldrich). Deconvoluted fluorescence images of specimens were obtained by fluorescence microscopy (BZ9000, Keyence, Osaka, Japan).

Detection of antibody responses by enzyme-linked immunosorbent assay and ELISPOT. To measure OVA- or CTB-specific IgA levels in faecal extracts, faeces were homogenized in PBS by vigorous vortexing^{11,13}. After centrifugation of the extracts (9,000g for 15 min) the supernatants were used as faecal extracts. Plates were coated with 1 mg ml⁻¹ OVA or 2 μg ml⁻¹ CTB in PBS; this was followed by blocking for 1 h at room temperature with 200 μl PBS containing 1% (w/v) bovine serum albumin. After extensive washing of the plates with PBS containing 0.05% Tween 20, serial sample dilutions were added for incubation overnight at 4 °C. Samples were then incubated for 1 h at room temperature with optimally diluted HRP-conjugated goat anti-mouse IgA (SouthernBiotech, Birmingham, AL). After sample washing, the colour reaction was developed at room temperature with 3,3',5,5'-tetramethylbenzidine (Moss, Pasadena, MD) and terminated by adding 0.5 M HCl. The colour reaction was measured as the optical density (wavelength 450 nm).

ELISPOT assay was used to enumerate IgA-producing AFCs in the iLP^{11,13}. Briefly, various concentrations of mononuclear cells were cultured at 37 °C for 4 h in 96-well nitrocellulose membrane plates (Millititer HA; Millipore, Bedford, MA) coated with 1 mg ml⁻¹ OVA and 5 μg ml⁻¹ bovine serum albumin-antigen-conjugated phosphorylcholine (Biosearch Technologies, Novato, CA). After vigorous washing of the plates with PBS and PBS containing 0.05% Tween 20, HRP-conjugated goat anti-mouse IgA was added; the plates were then incubated overnight at 4 °C. Spots of AFCs were developed with 2-amino-9-ethylcarbazole

(Polysciences, Warrington, PA). The size of each spot was measured with Zeiss KS ELISPOT software (Oberkochen, Germany).

In vitro culture. CD11b⁺ IgA⁺ or CD11b⁻ IgA⁺ PCs (10⁴ cells per well) were purified from the iLP and cultured with 100 ng ml⁻¹ phorbol 12-myristate 13-acetate plus 300 ng ml⁻¹ ionomycin, or 10 µg ml⁻¹ lipopolysaccharide (all from Sigma-Aldrich), for 24 h.

For the bacteria uptake assay, fluorescent *Staphylococcus aureus* was opsonized in accordance with the manufacturer's protocol (Molecular Probes). Mononuclear cells isolated from the iLP (2 × 10⁵ cells) were incubated with 1 × 10⁵ opsonized bacteria for 90 min. After being washed, the cells were stained with antibodies for PE-IgA (mA-6E1, 0.5 µg ml⁻¹, eBioscience) and Pacific Blue CD11b, and the bacterial uptake by each population was examined by flow cytometry.

Microarray analysis. Microarray analysis was performed as we previously reported⁴⁷. Briefly, CD11b⁺ IgA⁺ and CD11b⁻ IgA⁺ cells were isolated from the iLP, and total RNA was extracted from them with an RNeasy kit (Qiagen, Dusseldorf, Germany). cRNA was hybridized with DNA probes on a GeneChip Mouse Genome 430 2.0 array (Affymetrix), washed and fluorescence-labelled in accordance with the standard amplification protocol developed by Affymetrix. The fluorescence intensity of each probe was taken to represent the raw expression level and was quantified with GeneChip Operating software (Affymetrix). Data obtained from two independent experiments were analysed with GeneSpring 7.3.1 software (Silicon Genetics). All microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/) under the accession no. GSE37225.

Statistics. Results were compared by a non-parametric Mann-Whitney's *U*-test and unpaired *t*-test (two tailed) (GraphPad Software, San Diego, CA).

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Author contributions

J.K. planned the research and experiments, analysed data, wrote the paper and directed the research; M.G., E.H., I.I., M.H., Y.S., Y.G., C.P., I.I.I., R.S., L.A., T.W., S.S., Y.K. and S.S. conducted the immunological experiments; K.T. and S.A. provided key materials; and H.K. wrote the paper.

Additional information

Accession codes: Microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database under series accession code GSE37225.

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Mesenchymal Stem Cells Cancel Azoxymethane-induced Tumor

Initiation

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Key Words. mesenchymal stem cells • azoxymethane • tumor initiation • colorectal cancer • chemoprevention

ABSTRACT

The role of mesenchymal stem cells (MSCs) in tumorigenesis remains controversial. Therefore, our goal was to determine whether exogenous MSCs possess intrinsic antineoplastic or proneoplastic properties in azoxymethane (AOM)-induced carcinogenesis. Three *in vivo* models were studied: an AOM/dextran sulfate sodium colitis-associated carcinoma model, an aberrant crypt foci model, and a

model to assess the acute apoptotic response of a genotoxic carcinogen (AARGC). We also performed *in vitro* coculture experiments. As a result, we found that MSCs partially canceled AOM-induced tumor initiation but not tumor promotion. Moreover, MSCs inhibited the AARGC in colonic epithelial cells because of the removal of O⁶-methylguanine adducts through O⁶-methylguanine-DNA methyltransferase

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activation. Furthermore, MSCs broadly affected the cell-cycle machinery, potentially leading to G1 arrest *in vivo*. Coculture of IEC-6 rat intestinal cells with MSCs not only arrested the cell cycle at the G1 phase, but also induced apoptosis. The anti-carcinogenic properties of MSCs *in vitro* required transforming growth factor (TGF)- β signaling because such properties were completely abrogated by absorption of TGF- β under indirect coculture conditions. MSCs inhibited AOM-induced tumor initiation by preventing the initiating cells from sustaining DNA

insults and subsequently inducing G1 arrest in the initiated cells that escaped from the AARGC. Furthermore, tumor initiation perturbed by MSCs might potentially dysregulate WNT and TGF- β -Smad signaling pathways in subsequent tumorigenesis. Obtaining a better understanding of MSC functions in colon carcinogenesis is essential before commencing the broader clinical application of promising MSC-based therapies for cancer-prone patients with inflammatory bowel disease.

INTRODUCTION

Stem and progenitor cells are well-known direct cellular targets of genetic alterations in human carcinogenesis [1-3]. Previous studies have altered our perception of stromal cells from being innocent bystanders to active promoters in the neoplastic process [4-6]. Carcinoma formation accompanied by well-orchestrated desmoplastic reactions [7] closely resembles wound healing and scar formation, and entails the constant availability of growth factors, cytokines, and matrix-remodeling proteins that render the tumor site as a 'wound that never heals' [8]. Recent studies have shown that bone marrow-derived mesenchymal stem cells (MSCs) are recruited in large numbers to the stroma of developing tumors [9, 10].

However, the role of MSCs in tumorigenesis remains an intensely debated topic. Khakoo et al. demonstrated that intravenously injected human MSCs possess intrinsic antineoplastic properties in an *in vivo* model of Kaposi's sarcoma by inhibition of Akt activity in a

cell-cell contact-dependent manner [11]. In contrast, Karnoub et al. demonstrated that MSCs within the stroma of the tumor microenvironment facilitate metastatic spread via paracrine signals of C-C motif chemokine 5 that is secreted *de novo* by MSCs [12].

A meta-analysis of chemoprevention studies has suggested that azoxymethane (AOM)-based rodent models of carcinogenesis are valuable for prediction of chemopreventive efficacy in humans, which is better than that of other models [13, 14]. The prominent advantages of the AOM/dextran sulfate sodium (DSS) colitis-associated carcinogenesis model are that factors influencing tumor initiation [15, 16] should result in changes of the average tumor number per animal, whereas differences of the average tumor size typically provide evidence for factors involved in tumor progression [17, 18]. AOM is a genotoxic agent that initiates cancer by alkylation of DNA, in which O⁶-methylguanine (O⁶MeG) is a highly cytotoxic, apoptotic, mutagenic, recombinogenic, and clastogenic DNA adduct [19]. Conversely, DSS is not genotoxic, but

rather a proinflammatory tumor promoter [20]. Studies in rodents have revealed that AOM-induced tumors resemble human colorectal cancer at the molecular level, which displays dysregulation of the canonical WNT signaling pathway, similar target genes (21–24), and mutation of *K-ras* [25].

Our goal was to determine whether MSCs possess intrinsic antineoplastic or proneoplastic properties in an AOM-induced tumorigenesis model. Because MSCs are prime candidates for use in cell- and gene-based therapies [26, 27], this essential information must be obtained before implementing the broader clinical application of MSC therapies.

MATERIALS AND METHODS

For detailed Materials and Methods, please refer to Supplementary Data.

Experimental animals

Animal studies were performed under the supervision of the Committee for Animal Research of Sapporo Medical University in accordance with protocols approved by the Institutional Animal Care and Use Committee. All animals were maintained according to the guidelines of the Committee for Animal Research of Sapporo Medical University. Lewis rats were purchased from Charles River Laboratories Japan (Yokohama, Japan) and SD-TG (CAG-EGFP) rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan) [28]. All rats were aged 6 weeks and female unless indicated otherwise and were housed under pathogen-free conditions and received autoclaved food and water ad libitum.

Cell lines and culture conditions

Bone marrow cells were harvested by inserting a needle into the shaft of the femur or tibia and flushing it with 30 mL α -modified Eagle's medium (α MEM) containing 20% fetal bovine serum (FBS). To harvest rat MSCs [29], the cell suspensions were passed through a 70- μ m nylon filter (Becton Dickinson, Franklin Lakes, NJ, USA) and plated in 75-cm² flasks. Cells were grown in α MEM containing 20% FBS at 37°C and 5% CO₂. After 3 days, the medium was replaced with fresh α MEM containing 10% FBS, and the adherent cells were grown to 80% confluence to obtain passage 0. In accordance with the International Society for Cellular Therapy criteria [30], cells between passages 3 and 5 were used for subsequent experiments [26]. To harvest rat hematopoietic stem cells (HSCs) [31], CD90.1 (Thy1.1)⁺ cells were magnetically labeled with CD90.1 MicroBeads (Miltenyi Biotec GmbH, Gladbach, Germany) for 15 min. Then, the cell suspension was loaded onto a MACS[®] column that was placed in the magnetic field of a MACS[®] separator. The magnetically labeled CD90.1⁺ cells were retained and then eluted as the positively selected cell fraction. Detailed protocols and data sheets are available at www.miltenyibiotec.com. To prepare conditioned medium from rat MSCs (MSC-CM), MSCs (4×10^5 cells/150-mm culture dish) were seeded and cultured to confluency. Then, the medium was changed to serum-free Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA), and the rat MSCs were cultured for a further 48 h. The conditioned medium was collected, centrifuged at 300 \times g for 5 min, filtered using a

0.22- μm syringe filter, and then stored at -80°C until use.

IEC-6 cells obtained from the American Type Culture Collection (Manassas, VA, USA) and 3Y1 rat fibroblasts (3Y1-B Clone 1-6) [32] obtained from JCRB Cell Bank were maintained as recommended by the depositors.

AOM/DSS colitis-associated carcinoma model and evaluation of tumor growth

We adopted the two-stage colon tumor model that mimics colitis-driven tumor development as described by Tanaka *et al.* [33]. Thirty-nine female Lew rats were divided into three groups ($n = 13$ each group): two treatment groups and one control group (Fig. 1). The control group designated as ‘MSC (-)’ was administered a single intraperitoneal injection of AOM (15 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) and was not treated with MSCs. Starting at 1 week after injection, the animals received 2.5% DSS (MW 9,000–20,000; Sigma-Aldrich) in drinking water for 7 days, and then received no further treatment for 18 weeks. The group designated as ‘MSC Day0’ was intravenously administered 2×10^4 MSCs/g body weight on Day 0 when AOM was injected, and the group designated as ‘MSC Day9’ was administered MSCs on Day 9 following the AOM administration corresponding to Day 2 after receiving DSS in drinking water. Evaluation of tumor growth in the AOM/DSS colitis-associated carcinoma model is described in Supplementary Data.

Analysis of aberrant crypts (ACs)

Fifteen female Lew rats were divided into three groups ($n = 5$ each group): two treatment groups

and one control group (Fig. 3). The control group designated as ‘MSC (-)’ was administered two separate intraperitoneal injections of AOM (15 mg/kg body weight) at 7 days apart (Days 0 and 7) and was not treated with MSCs. No further treatment was performed for 3 weeks. The group designated as ‘MSC Day-1’ was intravenously administered 2×10^4 MSCs/g body weight on Day 1, which was 1 day before AOM was first injected, and the group designated as ‘MSC Day8’ was administered MSCs on Day 8 (1 day after the second AOM administration). The rats were sacrificed at the end of the study (Week 4) by transcardial perfusion with 4% paraformaldehyde in PBS. The colon was removed and cut open from the anus to the cecum along the longitudinal axis. We defined the rectum as the segment at 2 cm proximal to the anus, and divided the entire colon into three segments each measuring approximately 7 cm in length: the distal colon including the rectum and the middle and proximal colon. The colon was spread flat between sheets of filter paper and fixed in 10% buffered formalin. Then, the colon tissues were stained with 0.2% methylene blue in saline according to the procedure described by Bird [34] to observe ACs. Based on the McLellan and Bird [35] definition, ACs were defined as those that (i) were larger than normal crypts, (ii) had an increased pericryptal space that separated them from normal crypts, (iii) had a thicker layer of epithelial cells that often stained darkly, and (iv) generally had oval rather than circular openings. The number of aberrant crypt foci (ACF) per colon, the number of ACs in each focus, and the location of each focus were determined by stereomicroscopy (Olympus, Tokyo, Japan) at $\times 40$ magnification.

The mucosa of the distal segments was scraped off and subjected to western blot analysis.

Effects of MSCs on the acute apoptotic response to a genotoxic carcinogen (AARGC)

The five experimental groups included the MSC-untreated control group administered PBS, the group designated as 'MSC' administered MSCs, groups designated as 'HSC' and '3Y1' administered HSCs or 3Y1 rat fibroblasts, respectively, and the group designated as 'MSC-CM' treated with MSC-CM at 24 h prior to AOM administration. Each group consisted of five rats and received a single subcutaneous injection of AOM (15 mg/kg body weight) at 09:00 h. The rats were then sacrificed by CO₂-induced narcosis at the indicated intervals from 8 to 48 h. The entire colon was removed immediately, cut open, and flushed with ice-cold saline. Segments measuring 2 cm were taken from the rectal end of the distal portion. These segments were immediately fixed in 10% paraformaldehyde overnight at room temperature and then embedded in paraffin. The mucosa on the remaining segments was scraped off and subjected to subsequent analyses.

β-catenin nucleotide sequence

Sequencing was performed by the classical Sanger method [36].

WNT signaling pathway PCR array analysis

A Rat WNT Signaling Pathway RT² Profiler™ PCR Array (SuperArray Bioscience, Frederick, MD, USA) was performed according to the manufacturer's instructions.

Analysis of the cell cycle and apoptosis

The cell cycle was assessed by flow cytometry and Ki67 immunohistochemistry. The apoptotic cell fraction was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) reactions.

Immunofluorescence of the DNA adduct of O⁶MeG

The level of DNA alkylation was analyzed by immunofluorescence of distal colon sections using an antibody specific for the DNA adduct of O⁶MeG. Frozen sections (4 μm) were prepared, rehydrated, and incubated with 3% H₂O₂ in 50% ethanol for 15 min at room temperature. Antigen retrieval was carried out with a Retrieval Solution (DAKO, Carpinteria, CA, USA) for 10 min at 105°C in an autoclave. RNase treatment (20 μL RNase A at 10 mg/mL and 5 μL RNase T at 10 U/mL in 1,000 μL PBS, pH 7.5) was carried out for 1 h at 37°C, and then stopped by treatment with a 140 mmol/L NaCl solution for 5 min at 4°C. DNA unwinding was achieved by alkali treatment (1,500 μL of 70 mmol/L NaOH/140 mmol/L NaCl and 1,000 μL of absolute methanol) before applying Protein Block (DAKO) for 10 min at room temperature. The sections were then incubated at room temperature overnight with an anti-O⁶MeG monoclonal antibody (clone EM 2-3; Squarix Biotechnology, Marl, Germany) diluted at 1:1,000 in PBS. The next day, the sections were washed in PBS three times for 5 min each before applying an Alexa Fluor[®] 594-labeled secondary anti-mouse IgG. Sections were counterstained with DAPI, dehydrated, and cover slipped for observation under a LSM 510 META. The primary antibody was omitted for the negative control.

MTT assay

Cell proliferation was measured by a MTT dye reduction assay [37].

RNA isolation and quantitative real-time PCR (qPCR) analysis

qPCR was performed using TaqMan[®] Universal PCR Master Mix (Applied Biosystems) for 40 cycles at 95°C for 15 s and 60°C for 1 min by standard methods.

Western blot analysis

Western blot analysis was performed according to standard methods.

MSC and IEC-6 cell coculture experiments

Green fluorescent protein (GFP)-labeled rat MSCs were cocultured with the rat small intestinal cell line IEC-6. MSCs and IEC-6 cells cultured separately were included as controls. The cells were cocultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C with 5% CO₂. A total of 1×10^6 MSCs were seeded per 100-mm dish for the MSC control and harvesting MSC-CM, and 1×10^6 IEC-6 cells per dish for the cell line control. For direct coculture, 1×10^6 MSCs per dish were preseeded for 2–3 h. Then, 1×10^6 IEC-6 cells per dish were added and cultured for up to 72 h with/without AOM treatment. For indirect coculture, 1,250 IEC-6 cells per well were preseeded in the lower chamber of a Transwell[®] (0.4-µm pores, 48 wells; Corning, Lowell, MA, USA) for 2–3 h. Then, the same number of MSCs per well were added to the upper chamber of the Transwell[®] and cultured for up to 72 h with/without AOM, methylazoxymethanol (MAM; Wako Pure

Chemical Industries, Tokyo, Japan), or O⁶-benzylguanine (O⁶BG; Sigma-Aldrich), which binds irreversibly to and inhibits the DNA repair enzyme O⁶-methylguanine-DNA methyltransferase (Mgmt). Because AOM is metabolized into the active metabolite MAM by Cyp2e1, we confirmed whether *Cyp2e1* was expressed in IEC-6 cells [38]. For absorption of transforming growth factor (TGF)-β, 1.0 µg/mL anti-TGF-β neutralizing antibody (Clone # 9016; R&D Systems, Minneapolis, MN, USA) was added to the direct coculture condition.

Statistical analysis

To compare means between two groups, parametric and nonparametric analyses were performed using the unpaired Student's *t*-test and the Mann-Whitney U-test, respectively. Categorical variables were compared using the chi-square test, exact *P* value based on Pearson's statistic, or the Monte Carlo method. For multiple comparisons, we applied analysis of variance (ANOVA), especially in serial assessments, and two-way repeated measures (mixed between-within subjects) ANOVA followed by the Bonferroni test [39]. A difference was considered significant at *P* < 0.05 in all two-tailed tests. The SPSS Statistics 17.0 software package (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

RESULTS**MSCs reduce the tumor number but not the tumor size in AOM/DSS colitis-associated tumorigenesis**

We explored whether MSCs affected tumor initiation or promotion in the AOM/DSS model

and the associated mechanism (Fig. 1A). The average tumor number per rat was significantly decreased by up to half of the expected level when MSCs were simultaneously injected with AOM (MSC Day0 group) ($P = 0.008$ compared with the untreated control and $P = 0.023$ compared with the MSC Day9 group; upper panel in Fig. 1B and Supplementary Fig. S1A). In contrast, the average tumor diameter was not significantly different among the groups as shown in the lower panel of Figure 1B. In this model, factors that influence tumor initiation should result in changes of the average tumor number per animal, whereas differences in average tumor sizes typically provide evidence of factors involved in tumor progression [18]. Therefore, these results suggest that MSC partially cancel AOM/DSS-induced tumor initiation.

MSCs profoundly affect the mutational spectra during the tumor initiation phase

As shown in Figure 1C, the tumor β -catenin expression analyzed by western blotting was not significantly different among MSC (-), MSC Day0, and MSC Day9 groups. However, β -catenin was more frequently phosphorylated (56%, 14/25 tumors) in the MSC Day0 group than that in the MSC (-) group (13.8%, 4/29 tumors) and the MSC Day9 group (12.5%, 2/16, $P = 0.0006$). Furthermore, the mutation spectrum of β -catenin was quite different between MSC (-) and MSC Day0 groups. The codon 34 missense mutation (GGA–GAA) was the most frequent (11/25, 44%) in the MSC (-) group. In addition to the above mutation (5/15, 33.3%), the codon 32 missense mutation (GAT–AAT) was also frequently mutated (5/15, 33.3%) in the MSC Day0 group. Four

(66.7%) of the six mutated regions detected in the MSC Day0 group and 12 (85.7%) of the 14 regions detected in the MSC (-) group appeared to be unique (Supplementary Table S3). Seventy-nine (88.8%) of the 89 genes of the WNT signal pathway examined in the WNT PCR array were downregulated in MSC Day0 tumors compared with that in MSC (-) tumors (Fig. 1D).

Receptor-regulated Smad representing canonical TGF- β -Smad signaling was confined to the cell membrane of the lamina propria stromal cells, and β -catenin was expressed only on the cell membranes of crypt epithelial cells in the normal colon (Fig. 2A). Phospho-Smad2 expression representing activated TGF- β signaling was not significantly different between MSC (-) and MSC Day0 groups in western blot analyses (data not shown). The total Smad2 protein level was upregulated locally in the cytoplasm and partially in the nuclei (white arrowheads in Fig. 2B). β -catenin was slightly upregulated in membranous and cytoplasmic staining of the colon carcinomas in MSC (-) group rats (Fig. 2B). In contrast, both Smad2 and β -catenin were localized only on the membrane of colon carcinoma cells in MSC Day0 group rats (Fig. 2C). MSC engraftment was observed in tumors established at 20 weeks after AOM administration (data not shown). Therefore, these results suggest that MSCs profoundly affect the mutational spectra during the tumor initiation phase, leading to distinct WNT and canonical TGF- β -Smad signaling in subsequent tumorigenesis and even in the established tumors.

MSCs reduce the formation of ACF

Next, we determined whether MSCs affect ACF formation and the timing of MSC administration during tumor initiation induced by AOM for the most efficacious chemoprevention (Fig. 3A). The average ACF density was significantly lower in both pre-AOM (MSC Day-1; $P = 4.7E-4$) and post-AOM (MSC Day8; $P = 0.001$) treatment groups than that in the MSC (-) control group (Fig. 3B and Supplementary Fig. S1B). As depicted in Figure 3C, ACF were formed more frequently in the distal colon than in the proximal colon as reported previously [34]. ACF formation was suppressed in both the distal and middle colons of both treatment groups (MSC Day-1 and MSC Day8) with no significant differences between the two treatment groups. The multiplicity of ACs per focus, as shown in Figure 3D1 (one AC) to D4 (>four ACs), was reciprocally related to the frequency of the ACs. Among these ACs, one AC/focus was significantly reduced by MSC treatment, although there were no significant difference between the treatment groups (Fig. 3E). Canonical TGF- β -Smad signaling represented by phospho-Smad2 was activated in all colonic epithelia (5/5) of the MSC Day8 group and not in the colonic epithelia of MSC (-) or MSC Day-1 groups (Fig. 3F). Surprisingly, these results suggest that MSCs elicit a chemopreventive effect on formation of the prototype ACF (one AC/focus), both as a preventive measure in the pre-initiation phase (MSC Day-1) and a treatment measure in the post-initiation phase (MSC Day8). However, it is unknown why the canonical TGF- β -Smad signals were distinctly activated by the two measures.

MSCs suppress the AARGC

To obtain a further mechanistic insight into the antineoplastic properties of MSCs in AOM-induced carcinogenesis, we examined whether MSCs affect the AARGC *in vivo* (Fig. 4A) [40]. The AARGC peaked at 8 h after AOM administration, which was significantly suppressed only in the MSC-treated group compared with that in the MSC-untreated control, HSC, 3Y1, and MSC-CM groups (Fig. 4B and 4C). The Ki-67 labeling index of the colonic epithelia was significantly decreased at 24 and 48 h only in the MSC group compared with that in the other groups (Fig. 4D and 4E). Western blot analyses revealed suppression of Akt in the AARGC (8 h) observed in control groups was significantly activated in MSC groups, whereas activation of p38 in the AARGC observed in control groups was slightly suppressed in MSC groups (Fig. 4F). Consequently, these results suggest that AARGC suppression is a specific property of MSCs, which does not involve other cell types or humoral factors produced by MSCs. Because the AARGC is accepted as one of the *in vivo* mechanisms that suppress tumorigenicity, further experiments are necessary to explain why MSCs possess chemopreventive and AARGC suppression effects.

MSCs reduces the amount of DNA adducts of O⁶MeG

After administration, AOM is metabolized into MAM by Cyp2e1, which causes a DNA adduct of genotoxic O⁶MeG. The methyl moiety in O⁶MeG is then enzymatically removed by Mgmt. Therefore, we examined whether MSCs affect the level of O⁶MeG using the above model to assess the AARGC *in vivo*. At 8 h after

AOM treatment, massive amounts of O⁶MeG adducts were found in the colonic epithelia of MSC (-) control rats by O⁶MeG immunofluorescence analysis (Fig. 5A). Furthermore, the amount of O⁶MeG adducts was significantly reduced in the MSC-treated group (Fig. 5B). There were almost no positive signals for DNA adducts in healthy rats without AOM treatment at a basal physiological level (Fig. 5C). Moreover, at 8 h after AOM exposure, the amount of *Mgmt* transcripts was more abundant in MSC-treated groups than that in MSC-untreated control groups (Fig. 5D). These results suggest that MSCs indirectly remove O⁶MeG adducts, likely through *Mgmt* activation.

MSCs profoundly affect the cell-cycle machinery

In western blot analyses of the cell-cycle machinery (Fig. 5E), phospho-Smad2 was upregulated at 24 h in the MSC-treated group. Expression of both I κ B α and p21 was gradually upregulated in the treated group, whereas it was gradually downregulated in the control group. Accordingly, *p21* expression was upregulated at 4–8 h in the MSC group as revealed by qPCR (data not shown). IKK phosphorylates NF κ B bound to I κ B α , leading to degradation of I κ B α and allowing NF- κ B signal activation. p21 Waf1/Cip1 are members of the Cip/Kip family of cyclin-dependent kinase (Cdk) inhibitors, which form heterotrimeric complexes with cyclins and Cdks, inhibiting kinase activity and blocking progression through G1/S phase. Expression of Bax, a pro-apoptotic protein belonging to the Bcl-2 family, was slightly reduced in the treated group. Cyclin Ds induced by mitogenic stimuli form a complex with

Cdks. Cyclin D-dependent kinase 4 (Cdk4) complexes, which sequentially phosphorylate the retinoblastoma protein (Rb), facilitate G1–S transition. Cdk4 and phospho-Rb appeared to be downregulated in the treated group. Taken together, MSCs might induce G1 arrest in colon epithelia, even after the AARGC, partially through the canonical TGF- β -Smad signaling pathway.

MSCs induce G1 arrest and/or apoptosis in IEC-6 cells mediated by TGF- β *in vitro*

Intriguingly, MSC suppressed ACF tumorigenesis even in the post-initiation phase (Fig. 3). Furthermore, in the model assessed on the AARGC, MSCs inhibited the cell-cycle machinery of cells evading the AARGC (Fig. 5). These observations strongly suggest that there are additional chemopreventive mechanisms of MSC other than a reduction of mutation load with O⁶MeG. To investigate further, we conducted the following coculture experiments under AOM exposure. O⁶BG irreversibly inhibits *Mgmt* that demethylates O⁶MeG caused by MAM. Thus, before AOM treatment, we confirmed that *Cyp2e1* was expressed in IEC-6 cells (data not shown) as previously reported [38]. AOM exposure maintained proliferation and suppressed apoptosis of IEC-6 cells without coculture (the first blue bar in Fig. 6A and 6B, and the red line in Fig. 6F). In contrast, IEC-6 cells cocultured with MSCs always showed inhibition of cell proliferation and acceleration of apoptosis depending on the received mutation load. The mutation load corresponded to the level of O⁶MeG adduct depending on both the concentration and exposure time of the active metabolite, MAM (Fig. 6A and 6B). AOM

treatment represented a minimal mutation load, whereas MAM plus O⁶MeG treatment represented the maximal mutation load in this setting. IEC-6 cells cocultured with MSCs under AOM exposure for 72 h exhibited a significant reduction of proliferative activity (the first blue and second red bar in Fig. 6A, 6C, and 6D). Cell-cycle analysis by flow cytometry revealed that IEC-6 cells cocultured under AOM exposure developed G1 arrest after 72 h (Fig. 6E). To obtain a mechanistic insight into these phenomena, MTT assays under AOM exposure were performed using IEC-6 cells, IEC-6 cells treated with MSC-CM, IEC-6 cells directly or indirectly cocultured with MSCs, and IEC-6 cells directly cocultured with MSCs under anti-TGF- β antibody exposure (Fig. 6F). Because the cells in both direct and indirect coculture were able to retain antiproliferative properties, the humoral factors present under a direct heterotypic cell-cell interaction appeared to be important. This property of MSCs was completely abrogated by absorption of TGF- β by neutralizing antibodies.

DISCUSSION

MSCs exhibit chemopreventive properties by primarily canceling AOM-induced tumor initiation

MSC administration led to a dramatic decrease of tumor incidence by up to half of the expected level without affecting the tumor size in the AOM/DSS colitis-associated carcinoma model (Fig. 1B). Consequently, our findings strongly suggest that MSCs exhibit chemopreventive properties by canceling AOM/DSS-induced tumor initiation. ACF, which are putative precursor lesions of colon tumors, represent

alternative early end-points in AOM-induced colon carcinogenesis [34, 41, 42]. Although the early form of ACF, namely a single AC per focus, was most frequently observed in our analysis, it was significantly decreased by MSC treatment independent of the timing of AOM administration (Fig. 3). This finding strongly suggests that MSCs do not reduce dysplastic ACF, but rather block ACF formation itself. This result motivated us to analyze the chemopreventive mechanisms exerted by MSCs as early as the AARGC in AOM-induced tumorigenesis.

The AARGC is apparently suppressed by MSC administration

Because evasion of apoptosis is one of the hallmarks of cancer [16], induction of apoptosis during carcinogenesis is a critical step in chemoprevention. It is an accepted notion that the AARGC might regulate the mutational load in the colon or eliminate DNA-damaged cells that might otherwise progress to malignancy [43]. Therefore, most chemopreventive agents enhance the AARGC. Counterintuitively, in this study, the AARGC was seemingly suppressed by up to one-third following MSC treatment in the *in vivo* model compared with that in the MSC-untreated control (Fig. 4C). In contrast to our *in vivo* observations, *in vitro* experiments revealed that MSCs not only inhibited the proliferation of IEC-6 cells in coculture, but also accelerated apoptosis depending on the mutation load of the O⁶MeG adduct level (Fig. 6). This discrepancy between *in vitro* and *in vivo* observations concerning apoptosis arises from investigations of the different phases of tumorigenesis between the two observations. MSCs can apparently prevent the AARGC *in*

in vivo because of pruning the DNA insults of O⁶MeG adducts in the initiating cells, likely through Mgmt activation [44] or because of reducing production of the DNA adduct through Cyp2e1 inhibition [45]. These assumptions are directly supported by the immunofluorescence analysis showing that O⁶MeG adducts were significantly decreased by MSC treatment. However, because MSCs upregulated *Mgmt* expression in the colonic mucosa of the AARGC model (Fig. 5A–C), the former pruning mechanism is more likely. In contrast to *in vivo* observations, increasing the mutation load of O⁶MeG adducts using a combination of AOM or MAM with O⁶BG linearly induced an increase of IEC-6 cell apoptosis *in vitro* (Fig. 6B). Cocultured MSCs directly augmented apoptosis caused by the mutation load of O⁶MeG adducts *in vitro*, causing a discrepancy in the results regarding apoptosis between *in vitro* and *in vivo* observations. Our *in vitro* experiments examined the effects of MSCs on the survival of initiated cells beyond the AARGC, which corresponds more to the later phase of tumorigenesis than that in *in vivo* experiments. The detailed underlying mechanisms warrant further analyses.

MSCs induce O⁶MeG-triggered G1 arrest and/or apoptosis in the initiated cells evading the AARGC

We determined whether cells that escape from the AARGC subsequently undergo G1 arrest or apoptosis *in vivo*. A reduction of ACF formation was observed by post-AOM treatment of MSCs representing initiated cells entering the post-initiation stage and pre-AOM treatment of MSCs representing cells that remained at the pre-initiation stage (Fig. 3A and

3B). Therefore, additional chemopreventive mechanisms appear to be exerted by MSCs other than a reduction of DNA insults. MSC-IEC-6 coculture experiments revealed that MSCs induced not only G1 arrest in IEC-6 cells, but also enhanced apoptosis depending on the mutational load received by AOM (Fig. 6A and 6B). These antineoplastic and proapoptotic properties of MSCs observed *in vitro* required TGF- β signaling because they were completely abrogated by absorption of TGF- β in cocultures (Fig. 6F). However, in contrast to the *in vitro* study, MSC treatment broadly affected the cell-cycle machinery to facilitate G1 arrest in colon epithelial cells *in vivo* (Fig. 5E). Moreover, it is unlikely that massive apoptosis occurs beyond the AARGC because the apoptotic index was distributed unimodally by steadily increasing and reaching a peak at the AARGC and then steadily decreasing for up to 48–72 h (Fig. 4C). Apoptosis is caused by a series of complex responses involved in mismatch repair machineries and DNA double-strand breaks in subsequent consecutive and replicative cell cycles [46]. Consequently, a longer and closer observation is required to fully determine whether MSCs can induce O⁶MeG-triggered apoptosis after evasion of the AARGC *in vivo*. In summary, MSCs reduce the number of initiating cells by pruning O⁶MeG and/or inducing G1 arrest through TGF- β signaling in early initiated cells (Fig. 7).

Future challenges for better understanding chemopreventive property of MSC

The mechanism of the first chemopreventive property of MSC, which was attributed to activation of *Mgmt* expression levels by MSCs, remains to be elucidated. MGMT expression is

lost by epigenetic silencing in a variety of human cancers including nearly half of sporadic colorectal cancers [44]. On the other hands, similar to human colorectal cancer, AOM-induced tumors display global DNA hypomethylation. In addition, *Mgmt* is methylated in both AOM tumors and normal colon mucosa [49]. Collectively, these data suggest that epigenetic silencing of *Mgmt* by AOM treatment can be abrogated by MSCs via unknown mechanisms. Thus far, at least three different layers have been elucidated for epigenetic regulation influencing the expression of MGMT: promoter methylation, histone modifications [50], and alternative polyadenylation with consecutive miRNA targeting [51]. Further studies are needed to confirm how MSCs cancel epigenetic silencing of *Mgmt* and which of the above regulatory mechanisms contribute to this process.

The level of cytosolic β -catenin is controlled by the so-called “ β -catenin destruction complex” that facilitates casein kinase 1 alpha (CKI α)- and GSK3 (glycogen synthase kinase)-mediated serial phosphorylation and subsequent polyubiquitination/degradation of β -catenin. However, missense β -catenin mutations at phosphorylation sites are known to prevent β -catenin degradation. In our study, we showed that MSCs facilitated β -catenin phosphorylation in the established tumors of the AOM/DSS model because MSCs induced a unique mutation spectrum of the β -catenin gene during the tumor initiation phase (Figure 1C and Supplementary Table S3). Degradation of Phospho β -catenin is more likely to occur, which can lead to a reduction of Wnt signal activity. In support of this notion, the expression

of most WNT signaling pathway molecules appeared to be suppressed in the tumors from MSC Day0 groups as shown by the WNT PCR array analysis (Fig. 1D). However, it largely remains to be clarified how alteration of the mutational spectra leads to a distinct sequela of tumor promotion and progression. The best-defined location of TGF- β /Wnt cross-talk is in the nucleus, where the Smad/ β -catenin/Lymphoid enhancer-bind (Lef) protein complex regulates numerous shared target genes, often in a synergistic manner. The implication of cooperative TGF- β and Wnt signaling in tumor progression has recently been examined by Labbé et al. [52]. We demonstrated activation of both canonical pathways of TGF- β and Wnt, whereas both pathways were suppressed by MSCs even in established tumors in which we could not detect any engrafted MSCs. Collectively, these findings suggest that MSCs appear to exert broader and more long-lasting so-called third mechanisms of chemoprevention, which extend beyond the tumor initiation phase in the AOM-induced tumorigenesis via regulation of the cross-talk between canonical Tgf- β and Wnt signaling pathways than discussed heretofore.

Loss of canonical TGF- β -SMAD signaling is considered an essential step in carcinogenesis [53]. In fact, the activity of the Tgf- β pathway is decreased by AOM treatment [54-56]. Tgf- β expression has been found to be reduced in ACF [57, 58] but enhanced in colonic adenomas [57] and adenocarcinomas [59]. These observations suggest dual roles of Tgf- β as an initial tumor suppressor and later as a tumor promoter in AOM-induced carcinogenesis as reported previously [47, 48]. Therefore, MSC activation

of canonical Tgf- β -Smad signaling as a tumor suppressor only in the early initiated cells of the post-AOM MSC treatment group on day 8 in the ACF model (MSC Day8; Fig. 3F) suggests “the second” chemopreventive mechanism of MSCs as discussed in detail earlier. Although canonical Tgf- β -Smad signaling was found to be activated in the established AOM/DSS tumors, Tgf- β signal as a tumor promoter was inhibited in the tumor tissues from the MSC Day0 group (Fig. 2). This alteration of the signal probably reflects the whole integrated signals of a wide array of mutational events and likely does not reflect the effects of MSCs injected in the prior 20 weeks. However, it should be clarified whether this alteration of the Tgf- β signal is related to the above “third” mechanisms of chemoprevention via the cross-talk between the canonical Tgf- β and Wnt signaling pathway.

CONCLUSION

Exogenous MSCs possess intrinsic antineoplastic properties against AOM-induced

carcinogenesis. Although a complete mechanistic insight into the properties of MSCs has yet to be achieved, MSCs act as early as tumor initiation events either to reduce the number of initiating cells and/or to induce G1 arrest in early initiated cells (Fig. 7). Obtaining this information is essential before commencing the broader clinical application of promising MSC-based therapies for cancer patients, particularly cancer-prone patients with inflammatory bowel disease.

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Disclosure of potential conflicts of interests

The authors indicate no potential conflicts of interest.

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Figure 1. Effects of MSCs in the AOM/DSS colitis-associated carcinoma model

AOM/DSS model rats (n = 39) were classified into three groups (n = 13 each group) according to the timing of MSC administration in carcinogenetic phases: MSC untreated control [MSC (-)], tumor initiation (day 0; ‘MSC Day0’), and tumor promotion phases (day 9; ‘MSC Day9’), as shown in panel A. Brown, orange, and blue bars represent data obtained from MSC (-), MSC Day0, and MSC Day9 groups, respectively. The upper panel of B shows the average tumor number per rat (a total of 271 tumors developed), and the lower panel indicates the average tumor size. *, *P* = 0.023; **, *P* = 0.008. The upper row of the panel C shows that β-catenin protein expression (n = 70 tumor tissues) was regarded as positive when the band intensity was stronger than that in the normal colon in western blot analyses. Twenty-three of 29 (79.3%), 23 of 25 (92.0%), and 13 of 16 (81.2%) tumors examined were positive for β-catenin expression in MSC (-), MSC Day0, and MSC Day9 groups, respectively. The lower row indicates positivity of phospho-β-catenin at Ser33/37/Thr41 in western blot analyses in the three groups. The contingency table analysis reached statistical significance (**P* = 0.0006). The WNT signal pathway PCR array of representative samples pairs obtained from MSC (-) and MSC Day0 groups was depicted in panel D. PCR and western blot analyses were performed in triplicate unless specified otherwise.

