I 第2章 絶対に必要な医学の知識
第12節 がん関連の臨床研究

長村文孝

はじめに

がんは，わが国において死亡原因の約1/3を占める．また，固形がんにおいては，局所の浸潤，あるいは転移により手術で十分に切除できない時等の化学療法の効果は，ほとんどの場合，生存期間の延長もしくは生活の質（quality of life：QOL）の向上であり，治癒は期待できない．近年，分子標的療法が出現し，旧来の殺細胞性の抗がん剤よりも副作用が軽減される傾向にあるが，がん以外の疾患に用いる薬剤と比較すると，副作用は一般的に高度である．このような状況を背景として，より有効な，あるいはより副作用の軽い治療方法を開発することが，「がん」の治療開発として急務である．そのため，新たな治療法開発・検証を目指した「臨床研究」あるいは「臨床試験」が盛んに行われている．

1. 「臨床研究」，「臨床試験」とは

「臨床研究」とは，人を対象として，疾病の予防方法，診断方法および治療方法の改善，疾病原因および病態の理解並びに患者のQOLの向上を目的として実施される医学系研究である（厚生労働省「臨床研究に関する倫理指針」の「用語の定義」を改変）．これには，手術で摘出された検体，保存されている血清やDNAあるいは診療情報を用いた研究も含まれる．「臨床試験」は，「臨床研究」のうち，医薬品の投与あるいは医療機器を用いる等の被験者に対する介入行為を伴う研究であり，通常は研究計画を立て，実施計画書（プロトコール）の作成後に実施される（**図1**）．「治験」は，「臨床試験」のうち，医薬品

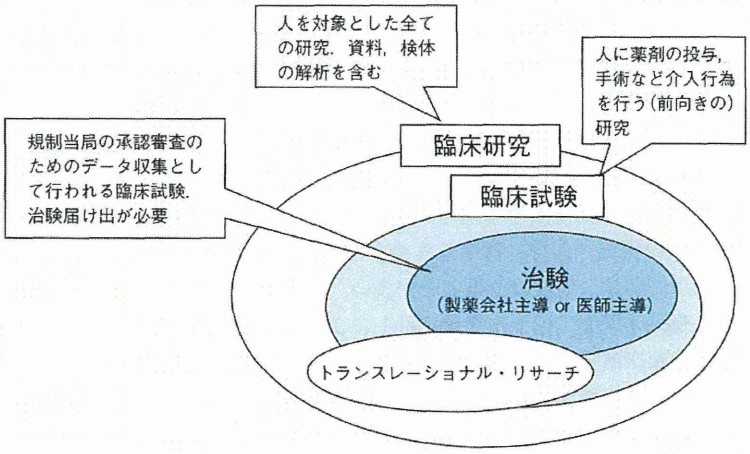

図1 臨床研究の分類

あるいは医療機器の厚生労働省等の規制当局からの製造・販売の認可を得るためのデータ収集を目的として行われる「臨床試験」を指し，試験開始に先立ち，わが国では医薬品医療機器総合機構（Pharmaceuticals and Medical Devices Agency：PMDA）を通じて厚生労働大臣宛に治験届けが提出され，PMDAで審査が行われる．「治験」は製薬企業等が主体のものと，医師が「自ら」実施する「医師主導治験」がある．後者は，対象患者数が少ないために製薬企業等が適応拡大等の治験を意図しない場合や，医師が研究者として自ら開発した治療法について承認申請を念頭に置き実施する場合に大別される．

「治験」ではない「臨床試験」は「自主臨床試験」あるいは「非治験臨床試験」等と呼ばれる．近年，なぜがんが発症するのか，あるいはがん細胞と正常細胞の違いは何か等の基礎研究が急速に発達し，これらの成果を基に臨床試験を行う「トランスレーショナルリサーチ（translational research：TR）」が盛んに行われるようになり，新たな治療法の開発として期待されている．TRは一般に早期の臨床試験に限定することが多く，「橋渡し研究」あるいは「探索型臨床研究」とも呼ばれる．抗がん剤の有効性，あるいは副作用の個人差等の臨床の結果を遺伝情報として研究することも多いが，このような臨床の結果から基礎に戻って行う研究もリーバースTRと呼称され，TRに含まれることがある．

2．臨床研究とガイドライン等

臨床研究の倫理的規範として，世界医師会が策定した「ヘルシンキ宣言」が知られている．研究参加のためのインフォームド・コンセント（informed consent：IC）の手続き，実施計画書の作成，倫理審査委員会での承認等，実施するための原則等から構成されている．他の法令や指針，あるいはガイドラインの憲法的存在となっていて，これを規範として臨床研究あるいは臨床試験の種類に対応して法規，指針等が定められている．

臨床研究・臨床試験には，その実施を定める法令やガイドラインが存在する．「治験」は薬事法とその関連省令に拠って規定されている．「治験」を規定する省令は「医薬品の臨床試験の実施の基準に関する省令」であり，英名が“Good Clinical Practice”であるので略して「GCP」と呼ばれることが多い．ヒトゲノムや遺伝子解析の臨床研究は「ヒトゲノム・遺伝子研究に関する倫理指針」によって規定され，治験ではない遺伝子治療は「遺伝子治療臨床研究に関する指針」により，治験以外の再生医療臨床試験は「ヒト幹細胞を用いた臨床研究に関する指針」により，観察研究等の疫学研究は「疫学研究に関する倫理指針」により規定されている．前述以外の臨床研究・臨床試験は「臨床研究に関する倫理指針」により広くカバーされている．

3．がんにおける予防の臨床試験

がんの発症には遺伝，環境，食事，薬物，物理的刺激等多くの要素が関与する．がんの発症予防の研究は「疫学研究」として，がんの発症の危険度を高める（あるいは低下させる）要因が何であるかを多数の患者の資料を基に解析されることが多い．疫学研究から，受動喫煙を含めた喫煙が肺がんだけではなく，口腔・咽頭・食道・膵臓・腎臓等多くのがんの発症リスクであることが判明した．このようなものには，B型肝炎ウイルス・C型肝

炎ウイルス感染による肝がんの発症，肥満における結腸がん，膵がん，閉経後乳がんなどが示されている．このように蓄積したデータの解析から危険因子の発見がなされるが，この危険因子が本当にがんの発症に関わっているのかを実証することは現実には非常に大変である．過去にはコーヒーが発がんの危険因子とされていたが，実際にはコーヒーの摂取は発がんのリスクではなかったことが判明したこともある．ヘリコバクター・ピロリ菌の場合では，除菌群と非除菌群の比較を前向き試験（prospective study）として試験計画を立案し，被験者を募集して実施する必要があるが，非常に多数の被験者と長期間の観察が必要であること，多額の研究資金が必要であること，リスクを参加者が認識していた場合には，それを回避する可能性があることから，臨床試験として実施することは困難な場合も多い．

がんでは，治療による副作用の発症予防の臨床試験も存在する．たとえば，化学療法後の口内炎予防のケア（例：ブラッシングの教育の成果）など高度な医療を用いなくとも実施できるテーマは多く存在し，また，心理面接の導入や面談等の工夫により，罹病によるうつ状態の改善や，前向きな心理状態への改善等を研究することも考えられる．

4．がん診断の臨床試験

診断の場合には，血液や尿等の検体の解析を基にするものと，画像診断等の医療機器に関するものとに大別される．尿や便を使用する場合は体への侵襲はなく，採血の場合には侵襲性は低く，倫理的判断をあまり要しないことが多い．しかし，被曝等の副作用の可能性のある医療機器を使用する場合や，ラジオ・アイソトープを体内に注射し放射線の被曝を受ける場合等では，安全性・有益性の十分な検討と被験者へ適切なICが必須となる．

5．がんの治療法の臨床試験

治療においても，過去のデータを基に推察する「臨床研究」と新たに介入行為を行う「臨床試験」に大別されるが，一般的には後者を指す．また，医薬品等の投与と手術等の手技においては「臨床試験」の概念がやや異なる．医薬品の臨床試験は第一相試験から第三相試験まで分類され，新薬開発では第一相試験から行われる．第一相試験は，動物実験等による非臨床試験での結果を基に，人への投与量，投与経路，投与スケジュール等を十分に検討した後に，人に初めて投与する段階である．抗がん剤以外の医薬品では，安全性，薬効の有無および薬理学的な情報の収集を目的として，健常人あるいは軽症の患者を対象として行われる．一方，抗がん剤の第一相試験は対象が異なり通常生存期間を延長する標準療法の無い段階の患者，すなわち標準療法の無くなった段階の患者を対象として行う．また，投与量を段階的に増加する用量漸増試験として行われ，人に投与できる最大耐用量（maximum tolerated dose：MTD）を決定する試験デザインであることが多い．これは，抗がん剤は，① 副作用が強い場合や，催奇性や2次発がんの危険性により健常人あるいは長期生存が見込まれる患者に投与することは倫理的に許容できない場合が多いこと，② 一般に抗がん剤は有効性と安全性を考慮した至適投与量の幅が狭く，有効性の低い低投与群あるいは毒性の強い高投与群の被験者が存在し，治療としての恩恵にあずかる

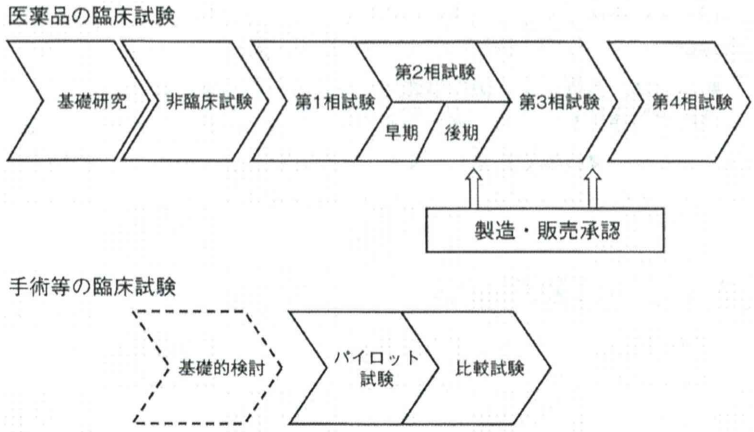

図2　臨床試験の流れ

ことのできる可能性のない被験者，あるいは毒性が強く生命の危機が生じる可能性のある被験者も存在することによる．これらを背景として，第一相試験は，被験者に有効性をもたらす可能性は低い．しかしながら，治癒の可能性を信じている患者も多いことに留意する必要がある．第二相試験は，第一相試験の結果を基に投与量・投与スケジュールを決定した後に，数十人程度の患者に投与し，有効性・安全性のある程度の情報を得ることを目的とする．1つの薬剤を単一投与量で最初の第二相試験を行うことが多いが，投与量の比較あるいは標準薬との比較試験として行われることある．また，「第二相試験」として行われる場合と，「早期第二相試験」と「後期第二相試験」とに分けて行われる場合がある．第三相試験は，標準療法との比較による有効性・安全性の検証を目的として数百人規模で実施される．迅速な第三相試験の実施と承認を目的として，最近は国際共同治験も増えている．第四相試験は市販後に情報を収集するために行われる．手術などの手技の臨床試験では，試験の分類が細分化されておらず，新たな手法を探索的に検討するパイロット試験と標準手技との比較試験に大別される（**図2**）．

臨床試験では，試験の目的を具体的数値で評価するためにエンドポイントを設定する．がんにおいては，最終的な目的は生存期間の延長あるいはQOLの向上であるが，これらを評価するためには多くの被験者と長い期間を必要とする．したがって，早期の試験では，これらと因果関係を有すると考えられる奏功率（腫瘍が縮小した被験者の割合）をサロゲート（代替）・エンドポイントとして用いることが多い．

サイコロジストへのメッセージ

臨床研究，特に臨床試験においては，被験者に対する十分な説明と適切な同意の取得が必須である．そのためには試験の目的，方法の理解が必要であり，更にその背景の理解として，対象とするがんの知識も必要となる．臨床試験が対象とするがんの種類，段階，予後，比較試験の場合には，その対象となる治療法等により患者の不安，選択への迷い，あるいは過度の期待が懸念されるので十分な知識をもって接することが重要である．

STEM CELLS

TISSUE-SPECIFIC STEM CELLS

Mesenchymal Stem Cells Cancel Azoxymethane-Induced Tumor Initiation

MASANAO NASUNO,[a] YOSHIAKI ARIMURA,[a] KANNA NAGAISHI,[b] HIROYUKI ISSHIKI,[a] KEI ONODERA,[a] SUGURU NAKAGAKI,[a] SHUHEI WATANABE,[a] MASASHI IDOGAWA,[c] KENTARO YAMASHITA,[a] YASUYOSHI NAISHIRO,[d] YASUSHI ADACHI,[a] HIROMU SUZUKI,[e] MINEKO FUJIMIYA,[c] KOHZOH IMAI,[f] YASUHISA SHINOMURA[a]

Key Words. Mesenchymal stem cells • Azoxymethane • Tumor initiation • Colorectal cancer • Chemoprevention

[a]Department of Gastroenterology, Rheumatology, and Clinical Immunology, [b]Department of Anatomy, [c]Department of Medical Genome Sciences, Research Institute for Frontier Medicine, [d]Department of Educational Development, and [e]Department of Molecular Biology, Sapporo Medical University, Chuo-ku, Sapporo, Japan; [f]Center for Antibody and Vaccine Therapy, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Correspondence: Yoshiaki Arimura, M.D., Ph.D., Department of Gastroenterology, Rheumatology, and Clinical Immunology, Sapporo Medical University, S-1, W-16, Chuo-ku, Sapporo 060-8543, Japan. Telephone: +81-11-611-2111, Ext. 3211, 3213; Fax: +81-11-611-2282; e-mail: arimura@sapmed.ac.jp

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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 O^6-methylguanine (O^6MeG) adducts through O^6MeG-DNA methyltransferase 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-carcinogenetic 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. STEM CELLS *2014;32:913–925*

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 (O^6MeG) is a highly cytotoxic, apoptotic, mutagenic, recombinogenic, and clastogenic DNA adduct [19]. Conversely, dextran sulfate sodium (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, refer to Supporting Information.

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; http://www.crj.co.jp), and SD-TG (CAG-EGFP) rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan; http://www.jslc.co.jp/) [28]. All rats were aged 6 weeks and were 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 (αMEM) containing 20% fetal bovine serum (FBS). To harvest rat MSCs [29], the cell suspensions were passed through a 70-μm nylon filter (Becton Dickinson, Franklin Lakes, NJ; http://www.bd.com/us/) and plated in 75-cm^2 flasks. Cells were grown in alpha-modified Eagle's medium (MEM) containing 20% FBS at 37°C and 5% CO_2. After 3 days, the medium was replaced with fresh αMEM 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; https://www.miltenyibiotec.com/en/) for 15 minutes. 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 http://www.miltenyibiotec.com. To prepare conditioned medium from rat MSCs (MSC-CM), MSCs (4×10^5 cells per 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; lifetechnologies.com), and the rat MSCs were cultured for a further 48 hours. The conditioned medium was collected, centrifuged at 300*g* for 5 minutes, 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; http://www.atcc.org/) and 3Y1 rat fibroblasts (3Y1-B Clone 1–6) [32] obtained from JCRB Cell Bank (Saito, Japan; http://cellbank.nibio.go.jp/) 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]. A total of 39 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; http://www.sigmaaldrich.com/united-states.html) and was not treated with MSCs. Starting at 1 week after injection, the animals received 2.5% DSS (molecular weight 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 Supporting Information.

Analysis of Aberrant Crypts

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 Day1" 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 killed at the end of the study (week 4) by transcardial perfusion with 4% paraformaldehyde in phosphate buffered saline (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 aberrant crypts (ACs).

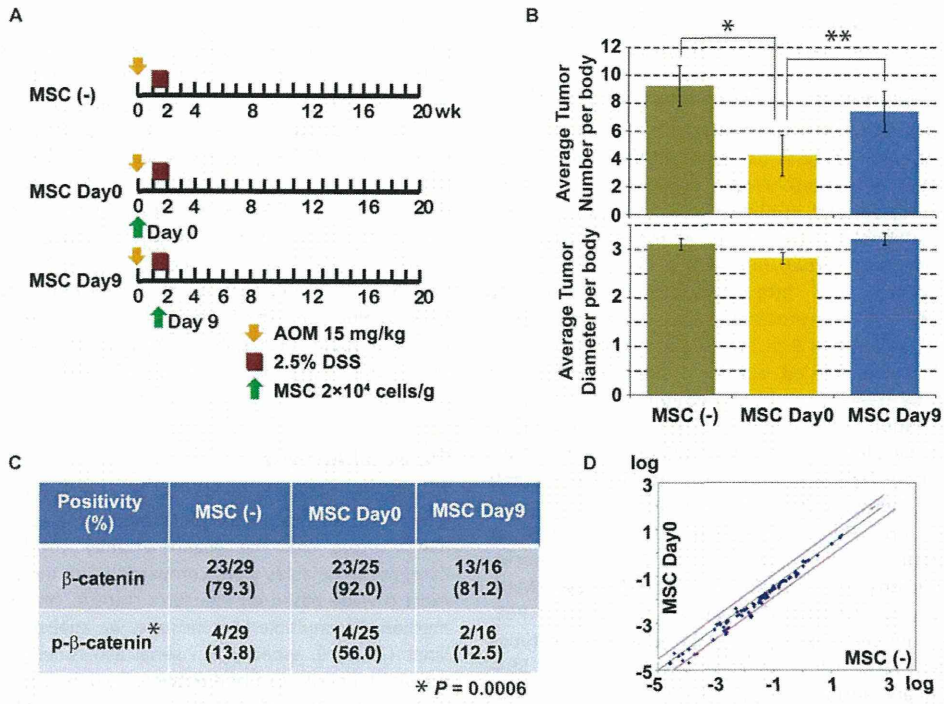

Figure 1. Effects of mesenchymal stem cells (MSCs) in the azoxymethane (AOM)/dextran sulfate sodium (DSS) colitis-associated carcinoma model. AOM/DSS model rats ($n = 39$) were classified into three groups ($n = 13$ each group) according to the timing of MSC administration in carcinogenetic phases: MSC-untreated 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. *, $p = .023$; **, $p = .008$. 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. In all, 23 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 (*, $p = .0006$). The WNT signal pathway polymerase chain reaction (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. Abbreviations: AOM, azoxymethane; DSS, dextran sulfate sodium; MSC, mesenchymal stem cell.

Based on the McLellan and Bird [35] definition, aberrant crypts (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; http://www.olympus.co.jp/jp/) at 40× magnification. The mucosa of the distal segments was scraped off and subjected to Western blot analysis.

Effects of MSCs on the AARGC

The five experimental groups included the MSC-untreated control group administered PBS, the group designated as "MSC" administered MSCs, groups designated as "hematopoietic stem cells (HSCs)" and "3Y1" administered HSCs or 3Y1 rat fibroblasts, respectively, and the group designated as "MSC-conditioned medium (MSC-CM)" treated with MSC-CM at 24 hours before AOM administration. Each group consisted of five rats and received a single subcutaneous injection of AOM (15 mg/kg body weight) at 09:00 hours. The rats were then killed by CO_2-induced narcosis at the indicated intervals from 8 to 48 hours. 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 RT^2 profiler polymerase chain reaction (PCR) array (SuperArray Bioscience, Frederick, MD; http://www.sabiosciences.com/) 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 O^6MeG

The level of DNA alkylation was analyzed by immunofluorescence of distal colon sections using an antibody specific for the DNA adduct of O^6MeG. Frozen sections (4 μm) were prepared, rehydrated, and incubated with 3% hydrogen peroxide in 50% ethanol for 15 minutes at room temperature. Antigen retrieval was carried out with a Retrieval Solution (DAKO, Carpinteria, CA; http://www.dako.com/) for 10 minutes 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 hour at 37°C, and then stopped by treatment with a 140-mmol/l sodium chloride (NaCl) solution for 5 minutes 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 minutes at room temperature. The sections were then incubated at room temperature overnight with an anti-O^6MeG monoclonal antibody (clone EM 2–3; Squarix Biotechnology, Marl, Germany; http://www.squarix.de/) diluted at 1:1,000 in PBS. The next day, the sections were washed in PBS three times for 5 minutes 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 [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] dye reduction assay [37].

RNA Isolation and qPCR Analysis

Quantitative Real-Time PCR (qPCR) was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA; lifetechnologies.com) for 40 cycles at 95°C for 15 seconds and 60°C for 1 minutes 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 1,640 (Sigma-Aldrich) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C with 5% CO_2. 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 hours. Then, 1×10^6 IEC-6 cells per dish were added and cultured for up to 72 hours 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, Tewksbury, MA; http://www.corning.com) for 2–3 hours. Then, the same number of MSCs per well were added to the upper chamber of the Transwell and cultured for up to 72 hours with/without AOM, methylazoxymethanol (MAM; Wako Pure Chemical Industries, Tokyo, Japan; http://www.wako-chem. co.jp/), or O^6-benzylguanine (O^6BG; Sigma-Aldrich), which binds irreversibly to and inhibits the DNA repair enzyme O^6MeG-DNA methyltransferase (Mgmt). Because AOM is metabolized into the active metabolite methylazoxymethanol (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-transforming growth factor (TGF)-β neutralizing antibody (Clone # 9016; R&D Systems, Minneapolis, MN; http://www.rndsystems.com/) 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) analysis of variance (ANOVA) followed by the Bonferroni test [39]. A difference was considered significant at $p < .05$ in all two-tailed tests. The SPSS Statistics 17.0 software package (SPSS Inc., Chicago, IL; http://www.spss.com/) 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; $p = .008$ compared with the untreated control and $p = .023$ compared with the MSC Day9 group; upper panel in Fig. 1B and Supporting Information 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 of 25 tumors) in the MSC Day0 group than that in the MSC (−)

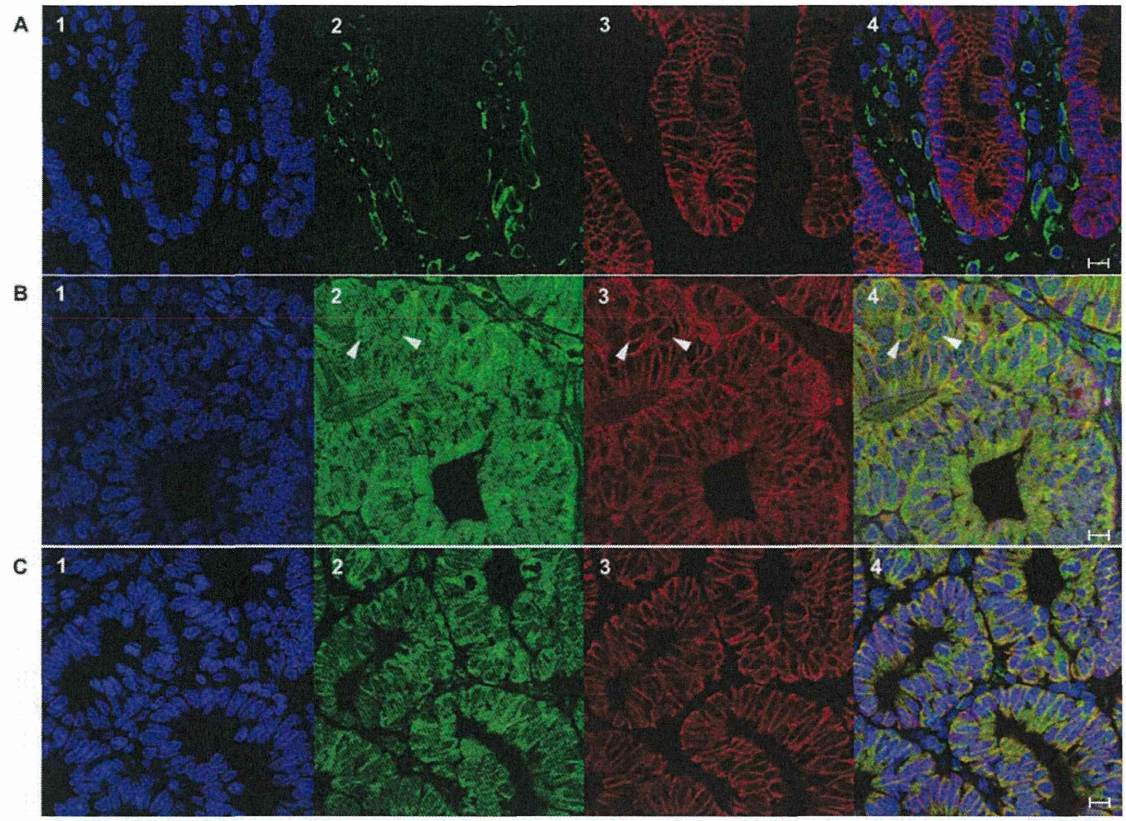

Figure 2. Double immunofluorescence staining of Smad2 and β-catenin. From the left to right, panels show nuclear counterstaining with DAPI (1), visualization of Smad2 (2) and β-catenin (3) using Alexa Fluor 488- and 594-labeled secondary antibodies, respectively, and merged images (4). Panel A shows normal colonic tissues of azoxymethane-untreated healthy rats, panel B shows tumor tissues from the mesenchymal stem cell (MSC) (−) group, and panel C shows tumor tissues from the MSC Day0 group. White arrowheads in panel B indicate nuclear staining of Smad2. Bar scales at the right lower corner in each panel indicated 10 μm.

group (13.8%, 4 of 29 tumors) and the MSC Day9 group (12.5%, 2 of 16, $p = .0006$). 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 of 25, 44%) in the MSC (−) group. In addition to the above mutation (5 of 15, 33.3%), the codon 32 missense mutation (GAT–AAT) was also frequently mutated (5 of 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 (Supporting Information Table S3). Of the 89 genes, 79 (88.8%) 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 laminar 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 aberrant crypt foci (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; $p = 4.7E\text{-}4$) and post-AOM (MSC Day8; $p = .001$) treatment groups than that in the MSC (−) control group (Fig. 3B; Supporting Information 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 Day1 and MSC Day8) with no significant differences between the two treatment groups. The multiplicity of ACs

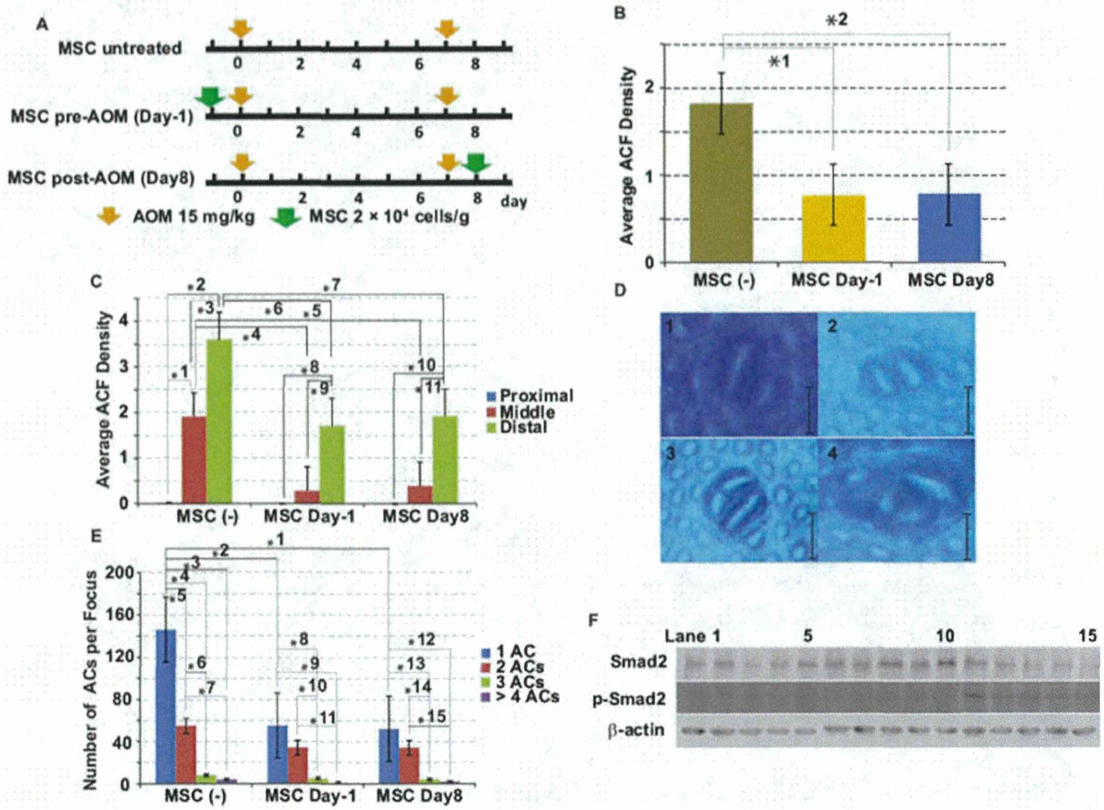

Figure 3. Analysis of the aberrant crypt foci (ACF) model. ACF model rats ($n = 15$) were classified into three groups ($n = 5$ each group) according to the timing of mesenchymal stem cell (MSC) administration either before or after administration of two separate of azoxymethane (AOM) on Days 0 and 7: MSC (−), MSC pre-AOM (Day–1), and MSC post-AOM (day 8) groups (A). A total of 400 ACF developed: 213, 95, and 92 in MSC (−), MSC Day–1, and MSC Day8 groups, respectively. The average ACF density is shown in panel B. *1, $p = 4.7E\text{-}4$; *2, $p = .001$. The average ACF density in the proximal, middle, and distal colon is shown in panel C. *1, $p = .02$; *2, $p = 1.6E\text{-}4$; *3, $p = .037$; *4, $p = .017$; *5, $p = .022$; *6, $p = .002$; *7, $p = .004$; *8, $p = 4.0E\text{-}5$; *9, $p = 2.2E\text{-}4$; *10, $p = 2.1E\text{-}7$; *11, $p = 1.9E\text{-}6$. Representative ACs, one to more than four ACs per focus, are shown in panels D1–4, respectively. Scale bars: 50 μm. The average density of ACs per focus, one to more than four ACs, is shown in panel E. *1, $p = .009$; *2, $p = .011$; *3, $p = 5.2E\text{-}5$; *4, $p = 7.3E\text{-}5$; *5, $p = .005$; *6, $p = 7.3E\text{-}5$; *7, $p = 5.3E\text{-}5$; *8, $p = 1.7E\text{-}4$; *9, $p = 7.9E\text{-}5$; *10, $p = .021$; *11, $p = .005$; *12, $p = 4.0E\text{-}6$; *13, $p = 6.4E\text{-}6$; *14, $p = .001$; *15, $p = .001$. Panel F shows the analysis of transforming growth factor-β signaling by Western blotting of Smad2 and phospho-Smad2. Lanes 1–5, 6–10, and 11–15 show data for MSC (−) control MSC Day–1, and MSC Day8 groups, respectively. Abbreviations: AC, aberrant crypt; ACF, aberrant crypt foci; AOM, azoxymethane; MSC, mesenchymal stem cell.

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 was 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 of 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 preinitiation phase (MSC Day1) 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 acute apoptotic response of a genotoxic carcinogen (AARGC) in vivo (Fig. 4A) [40]. The acute apoptotic response of a genotoxic carcinogen (AARGC) peaked at 8 hours 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, 4C). The Ki-67 labeling index of the colonic epithelia was significantly decreased at 24 and 48 hours only in the MSC group compared with that in the other groups (Fig. 4D, 4E). Western blot analyses revealed suppression of Akt in the AARGC (8 hour) 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 necessity to explain why MSCs possess chemopreventive and AARGC suppression effects.