


Oxidized high mobility group B-1 enhances metastability of colorectal cancer via modification of mesenchymal stem/stromal cells

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Funding information

This work was supported by MEXT KAKENHI Grant Numbers 19K16564 (RFT), 20K21659 (HK), 20K19349 (SK), 20K11260 (HO), 21K10143 (SM) and 20K19349 (IK)

Abstract

High mobility group box-1 (HMGB1) is known to be a chemotactic factor for mesenchymal stem/stromal cells (MSCs), but the effect of post-translational modification on its function is not clear. In this study, we hypothesized that differences in the oxidation state of HMGB1 would lead to differences in the function of MSCs in cancer. In human colorectal cancer, MSCs infiltrating into the stroma were correlated with liver metastasis and serum HMGB1. In animal models, oxidized HMGB1 mobilized three-fold fewer MSCs to subcutaneous tumors compared with reduced HMGB1. Reduced HMGB1 inhibited the proliferation of mouse bone marrow MSCs (BM-MSCs) and induced differentiation into osteoblasts and vascular pericytes, whereas oxidized HMGB1 promoted proliferation and increased stemness, and no differentiation was observed. When BM-MSCs pretreated with oxidized HMGB1 were co-cultured with syngeneic cancer cells, cell proliferation and stemness of cancer cells were increased, and tumorigenesis and drug resistance were promoted. In contrast, co-culture with reduced HMGB1-pretreated BM-MSCs did not enhance stemness. In an animal orthotopic transplantation colorectal cancer model, oxidized HMGB1, but not reduced HMGB1, promoted liver metastasis with intratumoral MSC chemotaxis. Therefore, oxidized HMGB1 reprograms MSCs and promotes cancer malignancy. The oxidized HMGB1-MSC axis may be an important target for cancer therapy.

KEYWORDS

cancer metastasis, colorectal cancer, HMGB1, mesenchymal stem cell, stemness

Shingo Kishi and Rina Fujiwara-Tani authors contributed equally to this work.

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1 | INTRODUCTION

Colorectal cancer (CRC) is the leading cause of cancer death worldwide, and its incidence has been increasing in recent years.¹ In Japan, it is the most common cancer, and the second leading cause of cancer death.² The average 5-year survival rate for CRC is 72.6%, but the 5-year survival rate is only 18.8% for stage 4 cases with distant metastasis.² One-fourth of advanced cases are associated with liver metastasis,³ which is a life-threatening event accounting for 30% of CRC deaths.^{4,5} We have studied the role of diabetes in the progression of liver metastasis.^{6,7} High mobility group box-1 (HMGB1) is overexpressed on a diabetic background, which promotes proliferation and invasion of CRC cells via its receptor, receptor for advanced glycation end-products (RAGE), suppresses host antitumor immunity by inducing cell death in monocytic cells, and subsequently promotes liver metastasis.⁸⁻¹²

Mesenchymal stem/stromal cells (MSCs) are self-renewing mesenchymal cells that differentiate into multiple lineages.¹³ In nontumor tissues, MSCs play a role in regeneration during inflammation and other disorders.¹⁴ MSCs have been isolated from many cancerous tissues, and influence the development and progression of cancer.¹⁵

HMGB1 is known as a chemotactic factor of MSCs.¹⁶ Other chemotactic factors of MSCs include platelet-derived growth factor, insulin growth factor, hepatocyte growth factor, fibroblast growth factor, transforming growth factor (TGF)- β , and chemokines, including stromal cell-derived factor-1.¹⁷⁻¹⁹ In contrast with these factors, HMGB1 is released from necrotic tissues and is thought to be involved in tissue regeneration in necroinflammatory reactions.²⁰ HMGB1 is also secreted by cancer cells and promotes cancer cell proliferation, invasion, and metastasis through RAGE expressed on cancer cells.¹⁰ The recruitment of MSCs via HMGB1 in cancer is thought to promote cancer progression, but the mechanism is unclear.

The action of HMGB1 is altered by post-translational modifications. HMGB1 is known to be post-translationally modified by acetylation, phosphorylation, and oxidation.²¹ Acetylation and phosphorylation alter DNA binding and bending properties of the HMGB1 protein and subsequently affect its secretion.^{22,23} Furthermore, HMGB1 contains three conserved redox-sensitive cysteines (C23, C45, and C106), and the modification of these cysteines determines the bioactivity of extracellular HMGB1.^{24,25} The disulfide bond between C23 and C45 in the HMGB1 molecule and the reduction of C106 (disulfide HMGB1; oxidized HMGB1) results in proinflammatory cytokine-stimulating activity, whereas the reduction of all cysteine residues (reduced HMGB1) results in chemotaxis mediator activity.^{24,26-28} When all cysteine residues are oxidized, HMGB1 is inactivated.²⁹ Oxidized HMGB1 is reduced by superoxide dismutase (SOD), catalase, and peroxidase, to its reduced form.²⁹

In this study, we investigated the effect of HMGB1 on bone marrow MSCs (BM-MSCs), in particular the role of HMGB1-pretreated BM-MSCs in cancer.

2 | MATERIALS AND METHODS

2.1 | Patients

We obtained frozen tissue samples from 16 patients with CRC with serosal invasion (pT3) and 1-3 regional lymph node metastases (pN1), who were diagnosed at the Department of Molecular Pathology, Nara Medical University, from 2014-2019 (Table S1). As written informed consent was not obtained from the patients for their participation in the present study, all identity-related information was removed from patient samples prior to their analysis to ensure strict privacy protection (unlinkable anonymization). All procedures were performed in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and with the approval of the Ethics Committee of Nara Medical University (approval number: 937, 2014/10/20).

2.2 | Immunohistochemistry

Sections (4- μ m thick) were immunostained with antibodies (0.5 μ g/ml) against OCT3 (Alexa 647-conjugated; Abcam, Cambridge, MA, USA) and CD73 (Alexa 488 or 647-conjugated, Abcam). Fluorescence images were observed using a BZ-X710 All-in-One fluorescence microscope (KEYENCE, Osaka, Japan).

Immunohistochemical staining for CD133 was performed using the immunoperoxidase technique⁶ with a CD133 antibody (0.5 μ g/ml, Abcam). The number of positive cells was counted, and the mean value was calculated from the microscopic observation of 30 high-power fields of view.

2.3 | Cells and reagents

The human colon cancer cell lines KM12C and KM12SM, mouse colon cancer CT26 cell line, and IEC18RAS rat intestinal cancer cell line were provided by Professor Isaiah J. Fidler (MD Anderson Cancer Center, Texas University, Houston, TX, USA). The cells were cultured in DMEM (Wako Pure Chemical, Osaka, Japan) supplemented with 10% FBS (Sigma Chemical, St. Louis, MO, USA) at 37°C in a 5% CO₂ atmosphere. For oxidization of HMGB1, recombinant human HMGB1 (50 μ g; R&D Systems, Minneapolis, MN, USA) was incubated with 100 μ l of 50 μ M H₂O₂ (Wako) on ice for 1 h.³⁰ To prevent HMGB1 from being naturally oxidized, oxidized HMGB1 was newly prepared for each experiment and used while it was fresh. For assessment of drug resistance, cells (1×10^6) were treated with 5-fluorouracil (5-FU; Sigma) as shown in Figure 4.

2.4 | MSC preparation

BALB/c mice (5-week-old, male; SLC Japan, Shizuoka, Japan) or F344 rats (6-week-old, male, SLC) were euthanized, and bone marrow

cells were harvested by flushing the bone marrow from the femur with regular DMEM (WAKO). After centrifugation at 1500rpm for 5 min, the pellet was suspended in PBS (WAKO). After lysis of red blood cells, centrifugation was repeated at 1500rpm for 5 min, and the pellet was resuspended and cultured in MSC culture medium (MesenCult-ACF Plus; Veritas, Tokyo, Japan) for 3 days. Floating cells were carefully removed by PBS washing.

2.5 | Sphere formation assay

Cells were mixed with syngeneic BM-MSCs, at the indicated cell ratios, with a total cell number of 1000 cells, and were seeded onto uncoated bacteriological 35-mm dishes (Corning, Corning, NY, USA) in 3D Tumorsphere Medium XF (Sigma).³¹ After 7 days of culture, images of the spheres were acquired and analyzed on a computer, to count spheres using ImageJ software (version 1.52; NIH, Bethesda, MD, USA).

2.6 | Co-culture of CRC cells with BM-MSCs

For co-culture with cell-cell contact, cancer cells (1×10^6) were mixed with syngeneic BM-MSCs (1×10^4), respectively, or none, which were pretreated with reduced HMGB1 (10 $\mu\text{g}/\text{ml}$) or oxidized HMGB1 (10 $\mu\text{g}/\text{ml}$) for 24 h. The cell mixtures were cultured in regular medium.

For co-culture without cell-cell contact (non-contact), CT26 cells (1×10^6) and BM-MSCs (1×10^4) were seeded onto separate plates of NICO-1 (Ginrei Laboratory, Kanazawa, Japan) and cultured under conditions that allowed migration of extracellular vesicles.

2.7 | Animal model

BALB/c and BALB/c nude mice (4-week-old, male, SLC) were maintained in a pathogen-free animal facility at 23°C. The animal study was conducted in accordance with the institutional guidelines approved by the Committee for Animal Experimentation of Nara Medical University, Kashihara, Japan, following current regulations and standards of the Japanese Ministry of Health, Labor and Welfare (approval nos. 11528, 11569, 11596, 11725, 11716, 12777).

2.8 | Orthotopic tumor model

KM12C and KM12SM colon cancer cells (1×10^6) were inoculated into the cecal submucosa of nude mice. After euthanasia at 4 weeks, livers were excised and sectioned into 2-mm-thick slices, and metastatic foci were counted using a stereomicroscope (Nikon).³² For evaluation of serum HMGB1, cardiac blood was collected at euthanasia.

2.9 | Subcutaneous tumor model

CT26 cells were pretreated with siRNA for HMGB1 (10 nM) or siMix (10 nM). CT26 cells (1×10^7) were inoculated into the scapular subcutaneous tissues of six BALB/c mice; three were inoculated with siHMGB1-treated CT26 cells, and another three were inoculated with siC-treated cells. After euthanasia at 4 weeks for histological examination.

2.10 | Subcutaneous spongel model

Spongel® (5 mm \times 5 mm, LTL Pharma, Tokyo, Japan) was soaked with recombinant human HMGB1 (hrHMGB1, 10 $\mu\text{g}/\text{ml}$, R&D) or PBS (WAKO) and inserted into the subcutaneous tissue on the back of the BALB/c mice. Spongel was removed at 1 week after insertion and fixed in 10% formalin at 4°C for 24 h for histological examination.

2.11 | Bone marrow replacement model

Bone marrow cells were harvested from BALB/c mice and resuspended in DMEM and labeled with LuminiCell Tracker 540 (Sigma). BALB/c recipient mice underwent whole-body irradiation (10 Gy), and resuspended bone marrow cells (1×10^7 cells) were injected into the tail vein of four recipient mice. CT26 cells (1×10^7) were inoculated into the scapular subcutaneous tissues at 4 weeks after transplantation. Two of the mice were administered oxidized HMGB1 (R&D, 20 $\mu\text{g}/\text{mouse}$, ip, twice a week). The other two mice were administered reduced HMGB1 (R&D, 20 $\mu\text{g}/\text{mouse}$, ip, twice a week). After euthanasia at 2 weeks, tumors were excised for histological examination by frozen section.

2.12 | Mouse tumorigenesis model

CT26 cells were mixed with none, reduced HMGB1- or oxidized HMGB1-treated mouse BM-MSCs (1% of CT26 cells). Cell mixtures were inoculated into the subcutaneous of BALB/c mice (five mice each group). At 3 weeks after inoculation, tumor formation was assessed macroscopically.

2.13 | Depletion of extracellular vesicles (EV)

Cultured medium of mouse MSC cells (5 ml from 5×10^6 cells) was mixed with ExoQuick-TC (1 ml; System Bioscience, Palo Alto, CA, USA) at 4°C for 12 h. The mixture was centrifuged (1500g, 30 min) to precipitate EV as pellets. The supernatant was used for sphere formation assay as the EV-depleted culture medium. For control culture medium, the EV-depleted culture medium, in which EV pellets were redissolved, was used.

2.14 | Liver metastasis model

For establishment of liver metastasis, CRC cells were pretreated with siHMGB1 (10 nM). CRC cells (1×10^6) were mixed with syngeneic BM-MSCs (1×10^4) or none and were inoculated into the spleen of syngeneic rodents (BALB/c mice or Fisher F344 rats). Each group contained five rodents. After euthanasia at 4 weeks, livers were excised and sectioned into 2-mm-thick slices, to count metastatic foci using a stereomicroscope (Nikon).³²

2.15 | RT-PCR

To assess murine mRNA expression, RT-PCR was performed and PCR products images were measured using NIH ImageJ software (version 1.52; Bethesda, MD, USA).³⁰ The primer sets are listed in Table 1.

2.16 | Western blotting

Whole cell lysates were prepared according to our previous report.^{33,34} The Minute Cytoplasmic and Nuclear Extraction Kit (Invent; Biotechnologies, Inc., USA) was used to extract nuclear protein. Lysates were separated using 7.5% or 10.0% SDS-PAGE. Primary antibodies were specific to proliferating cell nuclear antigen (PCNA), phosphorylated ERK1/2 (pERK1/2) (Proteintech, Rosemont, IL, USA), phosphorylated p38 (pp38), and β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) by visualization using Fusion Solo (M&S Instruments, Osaka, Japan).

2.17 | ELISA

Levels of HMGB1 was measured using an ELISA kit (Shino-Test, Corp., Tokyo, Japan) according to the manufacturer's instructions.

2.18 | Small interfering RNA

Silence® siRNA targeting mouse *Hmgb1* (ID:158974 and 158,975) and rat *Hmgb1* (ID:197413 and 48,868) were purchased from Thermo Fisher. AllStars Negative Control siRNA was used as a control (Qiagen; Valencia, USA). The cells were transfected with 10 nM siRNA using Lipofectamine 3000 (Thermo Fisher) according to the manufacturer's recommendations.

2.19 | Statistical analysis

Statistical significance was calculated using a two-tailed Fisher's exact test and ordinary ANOVA using the InStat software (GraphPad, Los Angeles, CA, USA). A two-sided *p*-value of <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Effect of HMGB1 on human CRC liver metastasis

To investigate the effect of MSCs on metastasis of CRC in liver, we detected OCT3⁺/CD73⁺ MSCs in the primary tumor in 16 cases of serous invasion (pT3) and lymph node metastasis (pN1) (Figure 1A, B). MSCs were found in the stroma of the primary tumor, as shown in Figure 1B; the number of MSCs was five-fold higher in liver metastasis-positive cases in comparison with non-metastasized cases (Figure 1C). HMGB1 is known to be a chemotactic factor for MSCs.¹⁶ When the serum concentration of HMGB1 and the number of MSCs in the primary tumor were compared in CRC cases, a clear correlation was observed between the two (Figure 1D). This correlation was observed in both metastasis-positive and metastasis-negative cases, but both HMGB1 levels and number of MSCs were higher in positive cases,

TABLE 1 Primer sets

Gene symbol	Species	GenBank ID	Forward primer (5'-3')	Reverse primer (5'-3')
<i>Rage</i>	mouse	L33412.1	AATTGTGGATCCTGCCTCTG	AAGGTAGGATGGGTGGTTCC
<i>CD73</i>	mouse	L12059.1	CCTCTCAAATCCAGGGACAA	TTTGGAAAGTGGATTTCCTG
<i>CD44</i>	mouse	M27130.1	TGGATCCGAATTAGCTGGAC	AGCTTTTCTTCTGCCACA
<i>Gnl3</i>	mouse	BC037996.1	CAGGATGCTGACGATCAAGA	TTGATTGCTCAGGTGACAGC
<i>Gnl3</i>	rat	BC093602.1	CTGTCCCGGGTATAAGGAT	CAGGGGGATGGCAGTAGTAA
<i>Osx</i>	mouse	NM_130458.4	TCGGGGAAGAAGAAGCCAAT	CAATAGGAGAGAGCGAGGGG
<i>Acta2</i>	mouse	NM_007392.3	CTGACAGAGGCACCACTGAA	CATCTCCAGAGTCCAGCACA
<i>Alpi</i>	mouse	NM_001081082.2	CCTTCACAGGGACCCAAGTA	AGGACTGGGGACAAGAACCT
<i>Alpi</i>	rat	X17611.1	GTGCAAAGAAAGCAGGGAAG	TCCACCAAGGATCACATCAA
<i>Actb</i>	mouse	NM_007393.5	ACAATGAGCTGCGTGTGGCC	AGGGACAGCACAGCCTGGAT
<i>Actb</i>	rat	NM_031144.3	ACCGAGCGTGGCTACAGCTT	CGGAACCGCTCATTGCCGAT

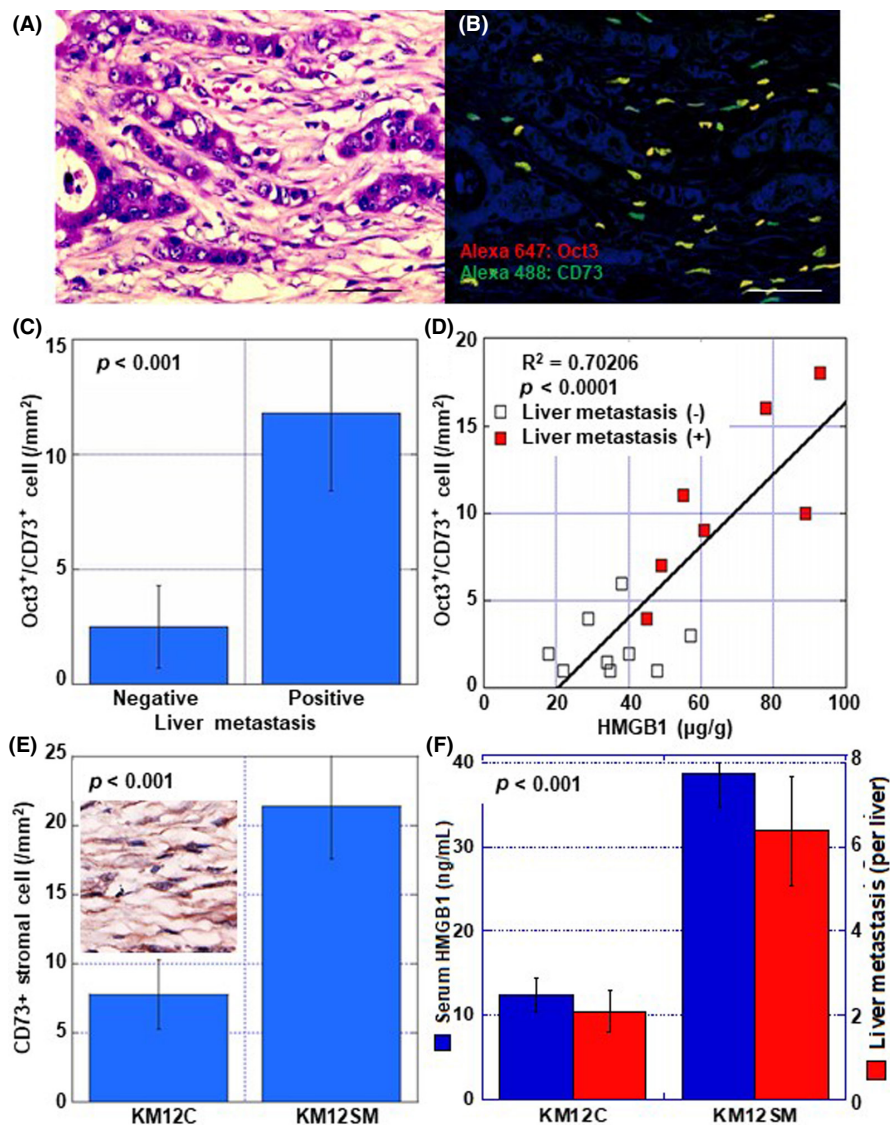


FIGURE 1 Effect of HMGB1 on CRC liver metastasis. (A) Invasive front of a CRC case with liver metastasis (H&E staining). (B) OCT3⁺/CD73⁺ MSCs were detected by fluorescence immunostaining and H&E. Scale bars, 100 μm. (C) Number of OCT3⁺/CD73⁺ MSCs in CRC stroma without liver metastasis (*n* = 9) and with liver metastasis (*n* = 7). (D) Comparison between stromal MSC number and serum HMGB1. (E, F) Mouse orthotopic colon cancer model. Low metastatic parental human CRC cells, KM12C (1×10^6) and high metastatic KM12SM cells were inoculated into the cecal wall of nude mice (*n* = 5). (E) Proportion of liver metastasis was counted 4 weeks after inoculation. (Inset) CD73⁺ MSCs by immunohistochemistry. Scale bar, 20 μm. (F) Serum HMGB1 concentration at euthanasia. Error bar, standard deviation

which suggests that HMGB1 might be responsible for recruitment of MSCs.

Next, we examined the relationship between CD73⁺ MSCs, serum HMGB1, and liver metastasis of the primary tumor using an orthotopic CRC model of the human CRC cell line KM12C and its high metastasis-inducing subclone KM12SM (Figure 1E, F). Liver metastasis was three-fold higher in the highly metastatic KM12SM model than in the parental KM12C model. The number of MSCs in the primary tumor was 2.5-fold higher in KM12SM than in KM12C. Serum HMGB1 was three-fold higher in KM12SM than in KM12C.

Therefore, number of MSCs and HMGB1 levels were shown to be strongly associated with liver metastasis of CRC.

3.2 | Differential effect of reduced HMGB1 and oxidized HMGB1 on translocation of BM-MSCs

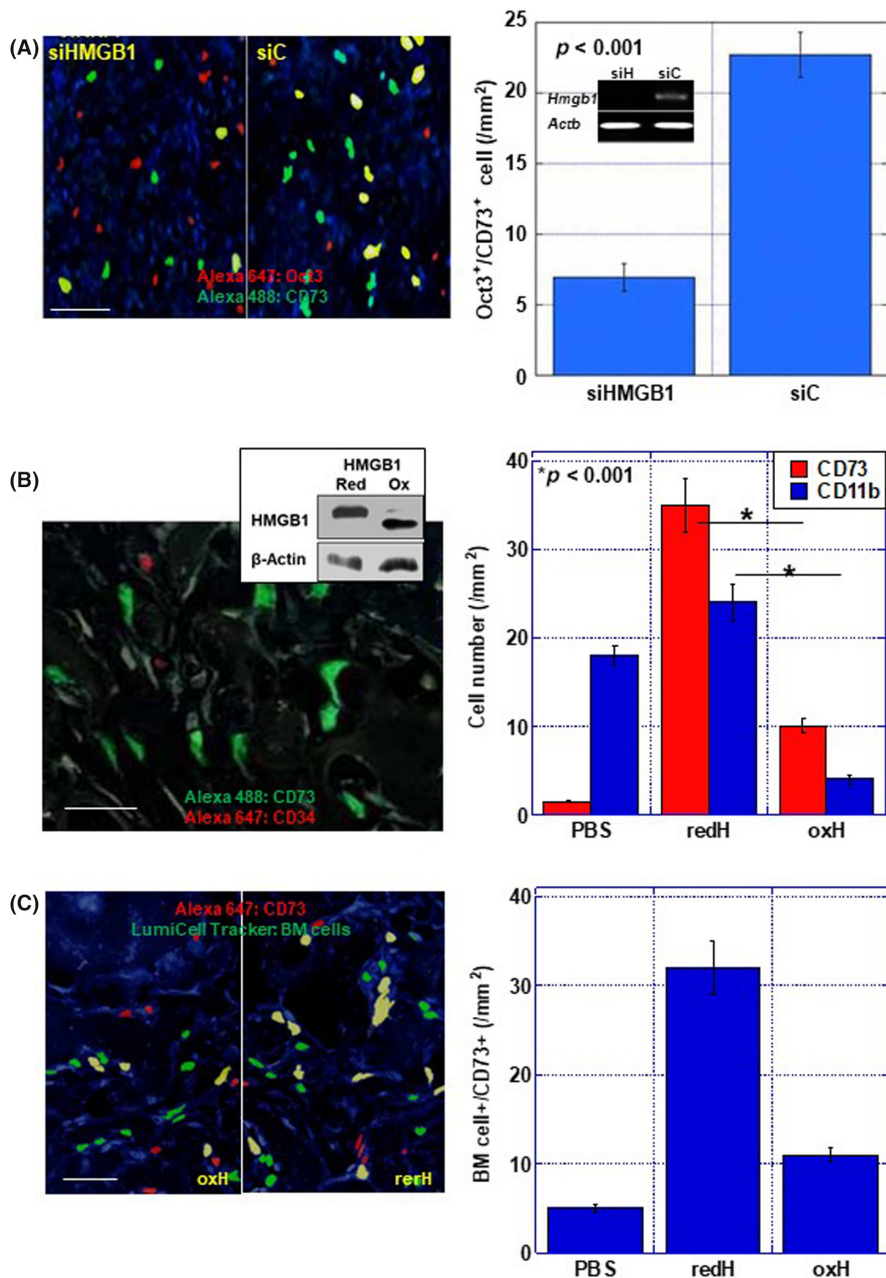
While HMGB1 is known to be a chemotactic factor for MSCs, its action is altered by oxidative modification; oxidized HMGB1

inhibits migration.³⁵ We compared the effects of reduced and oxidized HMGB1 on the migration of BM-MSCs into tumors in an animal model (Figure 2). Subcutaneous inoculation of CT26 mouse colon cancer cells knocked down for HMGB1 into syngeneic BALB/c mice showed that the number of CD73⁺/OCT3⁺ MSCs in the tumor was reduced by three-fold compared with that in HMGB1-expressing CT26 cells (Figure 2A).

Next, in a model of subcutaneous spongel implantation in BALB/c mice, reduced or oxidized HMGB1 was imbibed into the spongel (Figure 2B). Both CD73⁺ MSCs and CD11b⁺ myeloid cells were induced to migrate into the spongel by reduced HMGB1. In contrast, with oxidized HMGB1, the induction of migration of MSCs was reduced by three-fold, and the induction of myeloid cells migration was reduced by six-fold.

We generated a mouse model in which bone marrow cells were replaced with fluorescently labeled cells. BALB/c mice were subcutaneously inoculated with HMGB1 knockdown CT26 cells, and the infiltration of MSCs into the tumor was examined (Figure 2C). BM-MSC infiltration into the tumor was low in mice treated with PBS,

FIGURE 2 Differential effect of reduced HMGB1 and oxidized HMGB1 on infiltration of BM-MSCs. (A) Effect of HMGB1 knockdown on recruitment of MSCs into CT26 subcutaneous tumor. (Left) Fluorescence image of the tumors. OCT3⁺/CD73⁺ MSCs were detected in CT26 tumors. (Right) Quantification of intratumoral MSCs. (Inset) Protein expression of Hmgb1 in siH1-, siH2-, or siH1+2-treated CT26 cells. β -Actin was used as the internal control. (B) Effect of oxHMGB1 and redHMGB1 on recruitment of MSCs into subcutaneous spongel. CD73⁺ MSCs or CD34⁺ myelocytic cells were detected. (Right) Quantification of intratumoral MSCs and myelocytic cells. (Inset) Western blotting of redHMGB1 and oxHMGB1. (C) Effect of oxHMGB1 and redHMGB1 on recruitment of BM-MSCs into CT26 subcutaneous tumor. (Left) Fluorescence image of the tumors. CD73⁺ MSCs recruited from LumiCell Tracker-labeled replaced BM were detected. (Right) Quantification of intratumoral BM-MSCs. Scale bars, 100 μ m; error bar, standard deviation calculated from five animals



vehicle. High BM-MSC infiltration was observed in mice treated with reduced HMGB1. In contrast, in mice treated with oxidized HMGB1, BM-MSCs infiltration was reduced to one-third of that with reduced HMGB1.

Therefore, oxidized HMGB1 showed less induction of BM-MSC chemotaxis and much less induction of myeloid cells chemotaxis compared with reduced HMGB1.

3.3 | Differential effect of reduced HMGB1 and oxidized HMGB1 on stemness and differentiation of MSCs

Next, we compared the effects of reduced and oxidized HMGB1 on stemness and differentiation of BM-MSCs (Figure 3). As shown in

Figure 3A, BM-MSCs from BALB/c mice were treated with reduced or oxidized HMGB1. With reduced HMGB1 treatment, CD73 expression was maintained, but expression of stem cell markers CD44 and *Gn3* decreased. In contrast, the expression of the osteoblast differentiation marker osterix (*Osx*) and the vascular pericyte marker *Acta2* was induced. With oxidized HMGB1 treatment, CD73 expression was maintained, but CD44 and *Gn3* expression increased. Reduced HMGB1 inhibited BM-MSC proliferation compared with vehicle (PBS) (Figure 3B). In contrast, oxidized HMGB1 enhanced BM-MSC proliferation three-fold. Examination of the proliferative signals showed that expression of PCNA, a marker of proliferation, was decreased, and pERK1/2 and p38 phosphorylation was increased by reduced HMGB1. In contrast, with oxidized HMGB1, levels of PCNA and phosphorylated ERK1/2 increased, whereas that of phosphorylated p38 remained unchanged (Figure 3C). Therefore,

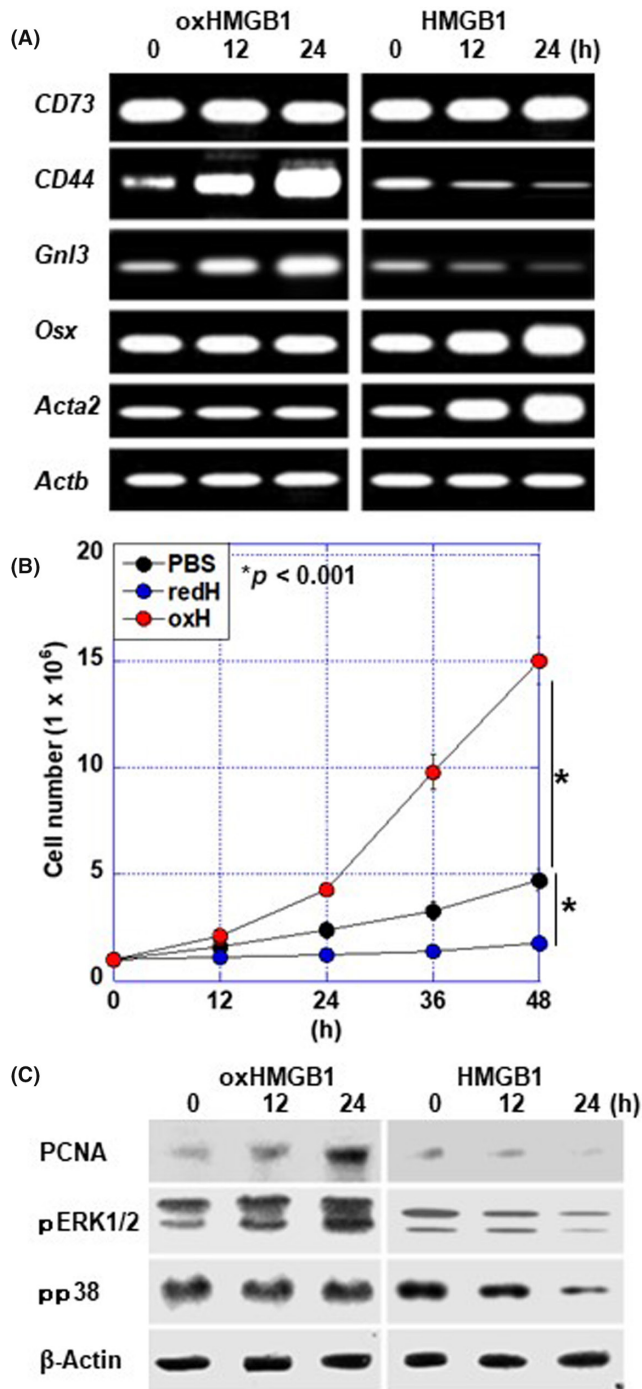


FIGURE 3 Differential effect of reduced HMGB1 and oxidized HMGB1 on stemness and differentiation of MSCs. (A) Expression of genes associated with stemness (*CD73*, *CD44*, *Gln3*), and differentiation (*Osx*, *Acta2*) of MSCs in mouse bone marrow MSCs treated with oxHMGB1 (10 µg/ml) or redHMGB1 (10 µg/ml) for 24 h. *Actb* was used as a loading control. (B) Effect of oxHMGB1 and redHMGB1 on proliferation of bone marrow MSCs. Error bar, standard deviation from three independent trials. (C) Protein levels of proliferation-associated signal factors. β-Actin was used as a loading control

oxidized HMGB1 exhibited proliferation-promoting, stemness-promoting, and differentiation-suppressing effects on BM-MSCs, in contrast with reduced HMGB1.

3.4 | Differential effect of reduced HMGB1 and oxidized HMGB1 on stemness and differentiation of CRC cells

Next, we compared the effects of BM-MSCs exposed to reduced or oxidized HMGB1 on syngeneic CRC cells in a co-culture system (1% of CRC cell population) (Figure 4). The sphere-forming ability of CRC cells was not significantly changed on co-culture with BM-MSCs pretreated with reduced HMGB1, whereas the sphere-forming ability of both CRC cells increased on co-culture with BM-MSCs pretreated with oxidized HMGB1 (Figure 4A). As the sphere-forming ability of BM-MSCs was quite low, sphere formation by BM-MSCs was negligible compared with sphere formation by CRC cells. Next, we examined changes in the expression of the cancer stem cell marker *Gln3* and the colonic differentiation marker small intestinal alkaline phosphatase *Alpi* in CRC cells co-cultured with MSCs (Figure 4B). In both CRC cells, *Gln3* expression was clearly increased by co-culture with oxidized HMGB1-pretreated BM-MSCs, but not by those treated with reduced HMGB1. In contrast, *Alpi* expression was mildly decreased by co-culture with reduced HMGB1-pretreated BM-MSCs and markedly decreased by co-culture with oxidized HMGB1-pretreated BM-MSCs. Furthermore, we examined the effect of BM-MSCs on tumorigenicity (Figure 4C). Approximately 1 × 10⁵ CT26 cells without BM-MSCs and with BM-MSCs pretreated with reduced HMGB1 were required for inducing tumorigenesis. In contrast, tumor formation was observed with 1 × 10³ CT26 cells when oxidized HMGB1-pretreated BM-MSCs were added. As cancer stem cells are known to confer anticancer drug resistance,³⁶ we examined the effect of BM-MSCs on anticancer drug resistance in CRC cells (Figure 4D). In CRC cells without BM-MSCs or with BM-MSCs pretreated with reduced HMGB1, there was no difference in 5-FU sensitivity. In contrast, the IC50 was seven- and six-fold higher in CT26 and IEC18SRC cells, respectively, with BM-MSCs pretreated with oxidized HMGB1. Therefore, MSCs treated with oxidized HMGB1 increased the stemness of cancer cells and induced tumorigenesis and anticancer drug resistance.

3.5 | Effect of extracellular vesicles from MSCs

As shown in Figure 4E, to evaluate the effect of the EV from MSCs on cancer stemness, we compared sphere-forming ability of CT26 cells among CT26 cells alone, contact co-culture with oxHMGB1-treated MSCs and CT26 cells, non-contact co-culture with oxHMGB1-treated MSCs and CT26 cells. Only the contact co-culture of both cells showed an increased sphere-forming ability. Moreover, when we compared sphere-forming ability of CT26 cells between treatment with MSC cultured medium and treatment with EV-depleted MSC cultured medium, no difference was observed between these two conditions. These findings suggest that effects of MSCs might be depend on cell-cell contact.

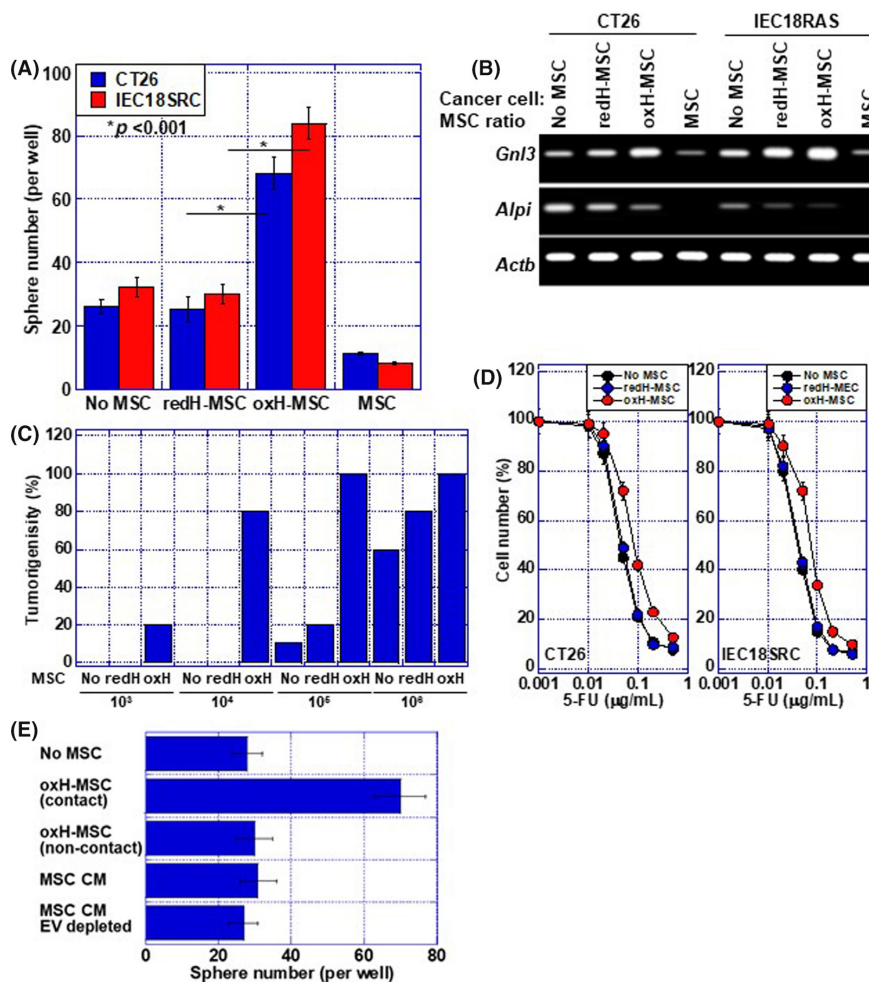


FIGURE 4 Differential effect of reduced HMGB1 and oxidized HMGB1 on stemness and differentiation of CRC cells. (A) Sphere formation of CT26 and IEC18SRC cells co-cultured with 1% MSCs. MSCs were pretreated with oxidized HMGB1 (10 μg/ml) or reduced HMGB1 (10 μg/ml) for 24 h. (B) Expression of genes associated with stemness (*Gnl3*) and colonic differentiation (*Alpi*) in CRC cells co-cultured with 1% MSCs. (C) Tumorigenicity of various cell numbers of CT26 cells inoculated into the subcutaneous space of syngeneic BALB/c mice (five mice each group). CT26 cells were mixed with 1% MSCs pretreated with oxidized (oxH) or reduced (redH) HMGB1. (D) Sensitivity of CRC cells co-cultured with 1% MSCs pretreated with oxidized or reduced HMGB1 for 24 h to 5-FU. (E) Sphere-forming ability was compared among CT26 cells alone (no MSC), contact co-culture with oxHMGB1-treated MSCs, non-contact co-culture with oxHMGB1-treated MSCs, CT26 cells treated with control MSC cultured medium (MSC CM, 50% v/v), and CT26 cells treated with the extracellular vesicle-depleted MSC cultured medium (MSC CM EV depleted, 50% v/v). Error bar, standard deviation from three independent trials

3.6 | Differential effect of reduced HMGB1 and oxidized HMGB1 on liver metastasis of CRC cells

When we examined the effects of reduced and oxidized HMGB1 on cell proliferation (Figure 5A, B), no difference in cell proliferation was observed between CRC cells without BM-MSCs and CRC cells with reduced HMGB1-pretreated BM-MSCs. In contrast, addition of oxidized HMGB1-pretreated BM-MSCs promoted proliferation in both CRC cell lines. Finally, we compared the effects of reduced and oxidized HMGB1 on liver metastasis using a liver metastasis model in which CRC cells knocked down for HMGB1 were mixed with BM-MSCs (1% of CRC cells), which had been pretreated with reduced or oxidized HMGB1, and inoculated into the spleens of syngeneic rodents (Figure 5C, D). The proportion of liver metastases was reduced by three-fold in siHMGB1-treated CRC cells compared with the control siRNA-treated CRC cells (Figure 5C). siHMGB1-treated

CRC cells plus reduced HMGB1-pretreated BM-MSCs had the same proportion of metastases as the control siRNA-treated CRC cells. In contrast, when siHMGB1-treated CRC cells were treated with oxidized HMGB1-pretreated BM-MSCs, the proportion of liver metastases increased two-fold and 1.7-fold compared with control siRNA (siC)-treated CRC cells and siHMGB1-treated CRC cells in co-culture with reduced HMGB1-pretreated BM-MSCs, respectively. Therefore, oxidized HMGB1 induced BM-MSC migration into the tumor, enhanced the metastatic potential of CRC cells, and promoted liver metastasis.

4 | DISCUSSION

In this study, we showed that the effects of HMGB1 on MSCs were different between the reduced and oxidized forms of HMGB1.

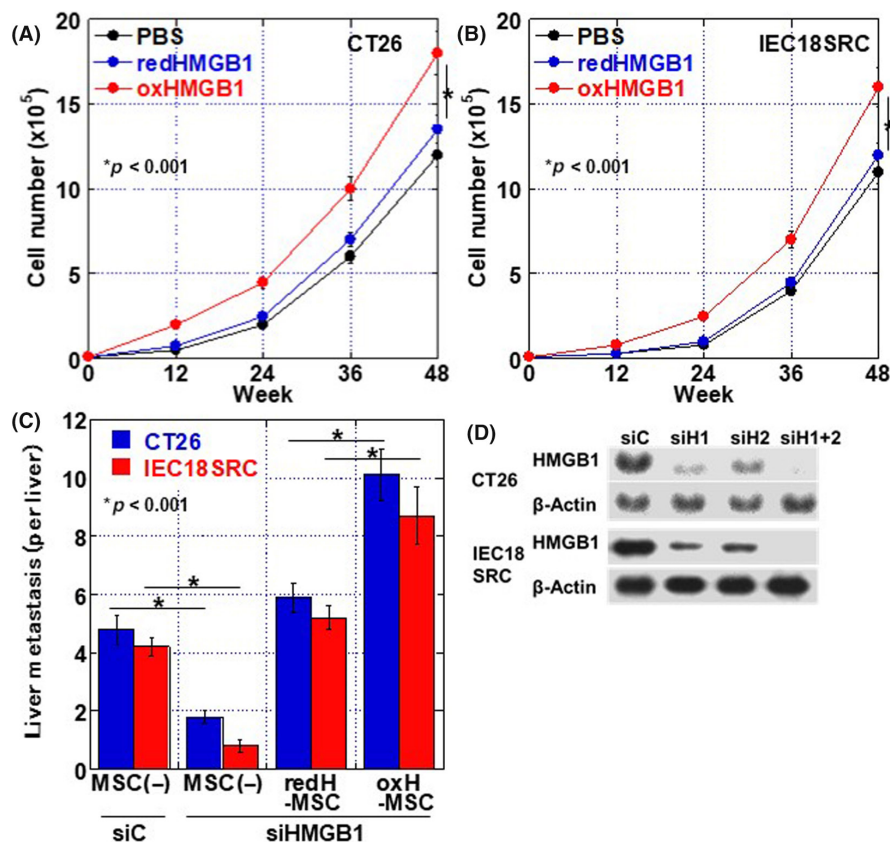


FIGURE 5 Differential effect of reduced HMGB1 and oxidized HMGB1 on liver metastasis of CRC cells. (A, B) Effect of co-culture of 1% MSCs pretreated with oxidized or reduced HMGB1 on cell proliferation of CT26 and IEC18SRC cells. (C) Number of liver metastatic foci. CRC cells were mixed with 1% MSCs pretreated with oxidized or reduced HMGB1 and inoculated into the spleen. (D) Effect of knockdown of HMGB1 in CT26 and IEC18SRC cells. For the animal model, siH1 + H2 was used. Error bar, standard deviation from five mice

Reduced HMGB1 promoted chemotaxis of MSCs, decreased their stemness, and promoted their differentiation. In contrast, oxidized HMGB1 increased stemness and promoted proliferation of MSCs, and chemotaxis was decreased. Furthermore, MSCs exposed to oxidized HMGB1 increased the stemness of cancer cells and promoted metastatic potential compared with those exposed to reduced HMGB1.

HMGB1 is affected by oxidation. The redox cycle of HMGB1 is involved in the role of HMGB1 as a damage-associated molecular pattern (DAMP). Reduced HMGB1 mobilizes BM-MSCs into necro-inflammatory lesions, and oxidized HMGB1 activates macrophages and neutrophils, which are abrogated when all cysteine residues are sulfonated.^{25,37} Reduced HMGB1 also promotes differentiation into vascular pericytes and osteoblasts of MSCs and promotes tissue regeneration and repair.^{16,38,39}

The role of MSCs in cancer has received much attention. Our data suggested that MSCs infiltrating into tumors have metastasis-promoting effects. The tumor-promoting effects of MSCs include suppression of antitumor immunity, induction of epithelial-mesenchymal transition (EMT) by cross-talk with cancer cells, induction of tumor-promoting properties of cancer-associated fibroblasts (CAFs), and promotion of tumor angiogenesis.⁴⁰⁻⁴³ A meta-analysis of preclinical studies revealed that MSCs promoted a 100-fold relative risk of cancer metastasis.⁴⁴ However, it has also been reported that tumor-infiltrating MSCs inhibit nuclear factor (NF)- κ B and suppress tumors.⁴⁵

These conflicting results may involve the education of MSCs by tumor cells⁴⁶; whereas naïve MSCs are antitumor, reprogramming by education changes MSCs to be tumor promoting. Our data showed that reduced HMGB1 and oxidized HMGB1 pretreatment of MSCs have different effects on cancer cells. MSCs treated with oxidized HMGB1 increased the stemness of cancer cells and promoted metastasis. This suggests that oxidized HMGB1 may be a factor that reprograms MSCs to be tumor promoting.

According to our data, oxidized HMGB1 induced stemness in colon cancer cells. MSCs are known to promote cancer cell proliferation, motility, and invasion in the cancer microenvironment.⁴⁷ In the cancer stem cell niche, together with immune cells, endothelial cells, and CAFs, MSCs are involved in the maintenance of cancer stem cells through humoral factors.⁴⁸ The mechanisms include induction of EMT by TGF β secreted by MSCs⁴¹ and induction of CAFs,⁴⁹ but this requires further investigation.

It has been reported that HMGB1 stimulates MSCs to increase chemotaxis, inhibit proliferation, secrete cytokines, and promote osteoblast differentiation.^{38,50,51} Our results showed that reduced HMGB1 had a similar effect on BM-MSCs. In contrast, reports on the effects of oxidized HMGB1 are scarce. It has been reported that oxidized DAMP abrogates the effect of reduced DAMP on MSCs.³⁵ Our data showed that oxidized HMGB1 has different activities from the reduced form, namely, decrease of chemotaxis and differentiation and promotion of proliferation. Oxidation of HMGB1 by H₂O₂, which is frequently used to generate oxidized HMGB1 in

experimental studies, can lead to the loss of HMGB1 activity if the conditions are strong enough to oxidize all cysteine residues, eliminating disulfide HMGB1. We have confirmed the formation of disulfide linkage in HMGB1 by western blotting (Figure 2B inset).

How is oxidized HMGB1 produced in cancer is an interesting question that needs to be addressed. HMGB1 has no signal peptide and is secreted into the extracellular space by a variety of non-canonical secretory mechanisms.⁵² HMGB1 that forms disulfide bonds binds to the nuclear exportin chromosomal region maintenance 1, which preferentially transports it out of the nucleus, resulting in its secretion.⁵³ Previously, we have reported that nuclear oxidation by deoxycholic acid decreases nuclear HMGB1 levels.⁵⁴ Peroxiredoxins I and II induce the formation of intramolecular disulfide bonds of HMGB1 in the nucleus.⁵⁵ As a result, nuclear export and even extracellular secretion of HMGB1 is induced.⁵³ In cancer, the expression of extracellularly localized SOD3 is epigenetically suppressed,⁵⁶ increasing oxidative stress in the cancer microenvironment. In such an oxidative environment, HMGB1 may be modified to an oxidized form in the cancer microenvironment, and its modification may be maintained. In our study, extracellularly secreted HMGB1 was mainly in the oxidized form.³⁰

We have previously shown that the co-expression of HMGB1 and RAGE in cancer cells correlates with malignancy potential of various cancers, including CRC.¹⁰ The effect of reduced HMGB1 on MSCs in cancer is due to the expression of RAGE as a receptor.³⁵ In our previous study, phosphorylation of RAGE and AKT and nuclear translocation of NF- κ B p65 are at low levels by reduced HMGB1 and high levels by oxidized HMGB1.³⁰ Therefore, oxidized HMGB1 was considered to be a highly functional ligand for RAGE in cancer. In contrast, in MSCs, RAGE is induced and activated by HMGB1 to promote the expression of CXCR4,⁵⁷ TGF β , and proinflammatory cytokines.^{58,59} However, reports analyzing the modification of HMGB1 and its function in MSCs are scarce. The differential action of the oxidized and reduced forms in this study may be important for future targeting of MSCs. Recently, it has been noticed that exosomes secreted by MSCs in tumors play a major role in the alteration of the cancer cell phenotype.⁶⁰ As we compared co-culture with cell-cell contact and non-contact condition, the contact was essential for the pro-stemness effect of MSCs. Moreover, the culture medium of MSCs did not affect the sphere-forming ability of CT26 cells with or without EVs. However, it is still important to examine the differential effect of oxidized or reduced HMGB1 on MSC-derived exosomes in further studies.

This study suggested that oxidized HMGB1 recruits BM-MSCs into tumors and reprograms them, thereby increasing cancer stemness and promoting liver metastasis. Targeting of the post-translational modifications of HMGB1 is expected to inhibit tumor-specific MSCs and suppress cancer stemness,⁶¹ and could be an important therapeutic option for CRC in the future.

AUTHOR CONTRIBUTIONS

Study concept and design: HK. Acquisition of data: SK, RFT, RS. Analysis and interpretation of data: SK, RFT, SM, HO, YM. Drafting

and editing of the manuscript: SK, RFT. Critical revision of the manuscript: TS, IK. All authors gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

ACKNOWLEDGMENTS

The authors thank Ms. Tomomi Masutani for expert assistance with the preparation of this manuscript.

DISCLOSURE

The authors disclose no potential conflicts of interest.

ETHICAL APPROVAL

As written informed consent was not obtained from the patients for their participation in the present study, all identity-related information was removed from patient samples prior to their analysis to ensure strict privacy protection (unlinkable anonymization). All procedures were performed in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and with the approval of the Ethics Committee of Nara Medical University (approval number: 937, 2014/10/20). The animal study was conducted in accordance with the institutional guidelines approved by the Committee for Animal Experimentation of Nara Medical University, Kashihara, Japan, following current regulations and standards of the Japanese Ministry of Health, Labor and Welfare (approval nos. 11528, 11569, 11596, 11725, 11716, 12777). Registry and the Registration No. of the study/trial: N/A.

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SUPPORTING INFORMATION

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How to cite this article: Kishi S, Fujiwara-Tani R, Honoki K, et al. Oxidized high mobility group B-1 enhances metastability of colorectal cancer via modification of mesenchymal stem/stromal cells. *Cancer Sci*. 2022;113:2904-2915. doi: [10.1111/cas.15400](https://doi.org/10.1111/cas.15400)