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DNA methyltransferase inhibition accelerates the immunomodulation and migration of human mesenchymal stem cells

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DNA methyltransferase (DNMT) inhibitors regulate target gene expression through epigenetic modifications, and these compounds have primarily been studied for cancer therapy or reprogramming. However, the effect of DNMT inhibitors on the immunomodulatory capacity of human mesenchymal stem cells (hMSCs) has not been investigated. In the present study, we treated hMSCs with 5-azacytidine (5-aza), a DNMT inhibitor, and confirmed that the inhibitory effects on mononuclear cell proliferation and cell migration toward activated T cells were increased. To identify the immunomodulatory factors stimulated through 5-aza treatment, we investigated the changes in promoter methylation patterns using methylation arrays and observed that the promoters of immunomodulatory factors, *COX2* and *PTGES*, and migration-related factors, *CXCR2* and *CXCR4*, were hypomethylated after 5-aza treatment. In addition, we observed that the COX2-PGE$_2$ pathway is one of the main pathways for the enhanced immunosuppressive activity of hMSCs through 5-aza treatment. We also determined that the migration of hMSCs toward ligands for CXCR2/CXCR4 was increased after 5-aza treatment. Moreover, using an experimental colitis model, we showed that 5-aza pre-treatment could enhance the therapeutic effect of MSCs against immune-related diseases.

M esenchymal stem cells (MSCs) regulate inflammatory responses through cell-to-cell contact with immune cells and the secretion of anti-inflammatory soluble factors. MSC-derived soluble factors, including prostaglandin E$_2$ (PGE$_2$), indoleamine 2,3-dioxygenase (IDO), hepatocyte growth factor (HGF) and transforming growth factor β1 (TGF-β1), regulate inflammation[1–4]. Moreover, MSCs exhibit chemotaxis and migration toward various cytokines secreted from inflamed or damaged tissues. The Stromal cell-derived factor 1 (SDF-1)/Chemokine (C-X-C) motif receptor 4 (CXCR4) axis is a critical mechanism of MSC migration[5]. Based on these findings, a number of studies have shown that MSCs are potential therapeutics for various inflammatory diseases[1,4].

MSCs can be isolated from various tissues, such as bone marrow, adipose, dermis, synovial fluid, periosteum and umbilical cord blood[1]. Among these, umbilical cord blood has several benefits, including its acquisition through non-invasive manipulation during tissue extraction processes and its most primitive nature among human tissues[6]. Umbilical cord blood-derived MSCs (UCB-MSCs) and other tissue-derived MSCs show variations in cellular characteristics and therapeutic effects depending on the primary cells. Therefore, a number of studies have found it difficult to identify representative selection markers for effective MSCs or to increase the therapeutic efficacy of MSCs in association with reproducible therapeutic effects[7,8]. Several studies have shown that pretreating MSCs with interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β) increases immune-modulation and the therapeutic effects against inflammatory diseases[9–12]. Since the migration ability of MSCs is another key factor for therapeutic effect, there are efforts to increase migration ability through the enforced expression of chemokine receptors[13–15].
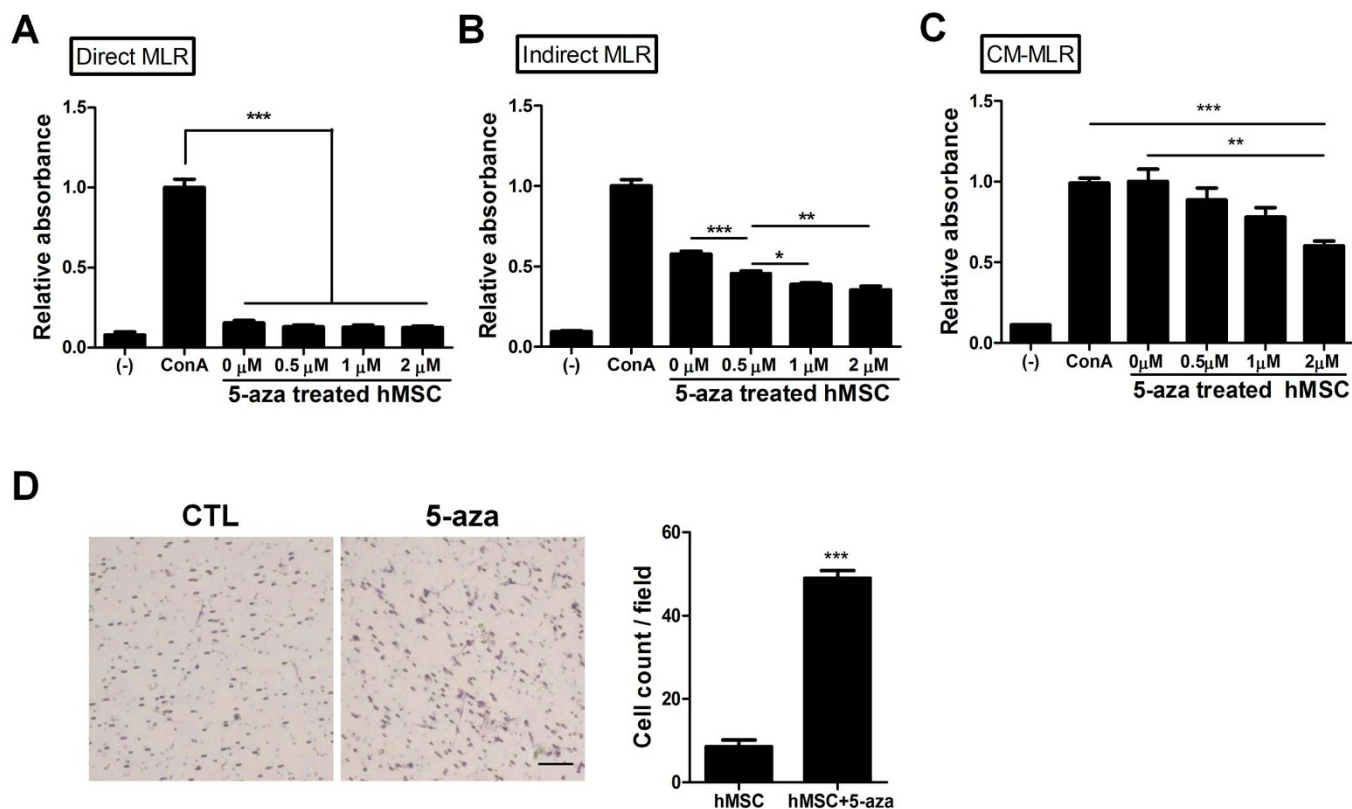
Figure 1 | **5-aza stimulates the immune-modulation and migration of hMSCs.** (A–C) After treating hMSCs with 5-aza for 24 hr, an MLR assay was performed. (A) Direct MLR; hMSCs were co-cultured with hMNCs and MNC proliferation was determined using the bromodeoxyuridine kit. (B) Indirect MLR; co-culture was performed using transwell plates, and MNC proliferation was detected. (C) CM-MLR; hMNCs were cultured with the CM from hMSC culture, and hUCB-MNC proliferation was measured. (D) After treating hMSCs with 5-aza for 24 hr, changes in the migration toward activated T cells were determined. The migrated cells were counted in at least five fields, and the average cell counts are graphically represented. Bar, 500 μm; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Results show one representative experiment out of at least three. Results are shown as mean ± SD.

DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) are representative epigenetic modulators that regulate DNA methylation and the histone acetylation status of genomic DNA, respectively, to regulate the expression of target genes. As different cell types possess different methylation and histone status profiles, different responses to DNMT or HDAC inhibitors are observed according to the individual cellular characteristics. Within identical cell types, the chemical concentration or treatment duration could result in different effects[16,17]. Generally, DNMT or HDAC inhibitors act as tumor suppressors that increase the expression of tumor suppressor genes in various cancer cell lines[18–22]. However, DNMT or HDAC inhibitors have been reported to increase the efficiency of induced pluripotent stem cell generation or reprogramming through epigenetic modifications[23–27]. DNMT inhibitors including 5-aza-2′-deoxycytidine (5-aza) and decitabine have also been reported to have immune suppressive effects. Choi et al. showed that a DNMT inhibitor mitigated graft-versus-host disease without sacrificing the graft-versus-leukemia effect through the conversion of effector T cells to regulatory T cells[28,29].

Although epigenetic modulators have various effects on cells and some modulators have demonstrated clinical effects, such as immune suppression, the effects of these molecules on the immune-modulatory properties of hMSCs have not been studied. Because both epigenetic modulators and hMSCs have common aspects in immune modulation, the effects of epigenetic modulators on immune modulation in hMSCs should be investigated to elucidate this potential cross-interaction. In the present study, we examined the effects of 5-aza on the immune-modulatory properties of MSCs. Moreover, we confirmed which immune modulation factors are regulated by 5-aza

treatment. Finally, we also investigated the therapeutic effect of 5-aza treated hMSCs in a DSS-induced mouse colitis model.

Results

The DNMT inhibitor augments the immunomodulatory function of hMSCs. To investigate the effects of DNMT inhibitors on the immunomodulatory properties of hMSCs, a mixed leukocyte reaction (MLR) was performed after treating hMSCs with 5-aza. A significant reduction in the mitogen-induced proliferation of mononuclear cells derived from cord blood was observed. However, when cell-to-cell contact was mediated during MLR, treatment with 5-aza did not influence the suppressive effect of hMSCs on mononuclear cell (MNC) proliferation, regardless of the treatment dose (Fig. 1A). We next examined whether 5-aza could alter the secretion of immunoregulatory factors from hMSCs. First, MNCs were cultured with naïve or 5-aza-treated MSCs using transwell plates. Interestingly, 5-aza treatment enhanced the inhibitory effect of hMSCs on MNC proliferation in a dose-dependent manner (Fig. 1B). Next, MNCs were cultured with conditioned media (CM) from naïve or 5-aza-treated hMSCs, and the results showed that 5-aza treatment could increase the suppressive effects of hMSC-derived CM on MNC proliferation in a dose-dependent manner (Fig. 1C). In addition, CM from 5-aza treated hMSCs significantly inhibited the proliferation of Jurkat cells, a human T-cell lymphoblast-like cell line (P = 0.007, Fig. S1). Because migration is another important function in the immunomodulation of hMSCs, we also confirmed the effects of 5-aza on the migration of hMSCs. We co-cultured hMSCs with T cell-activated MNCs using a CD3/CD28 antibody and subsequently
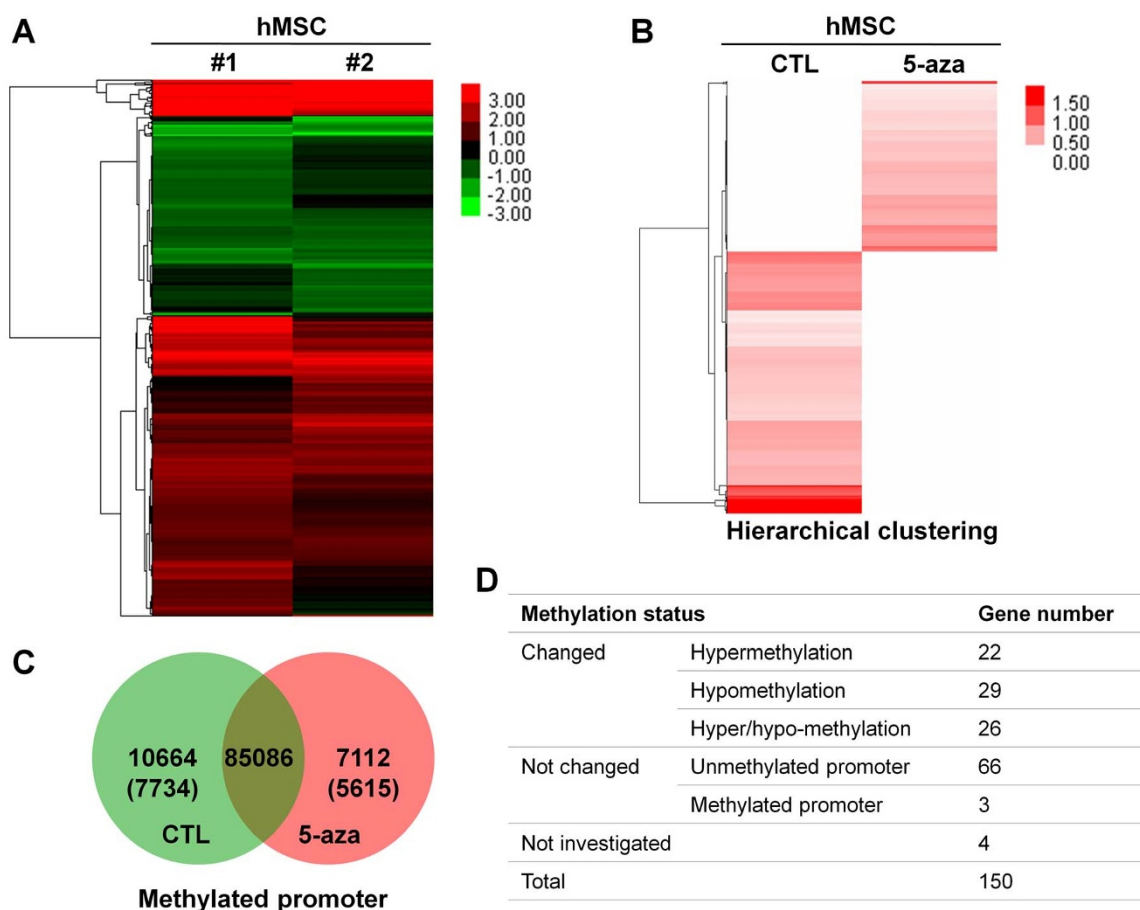
Figure 2 | **5-aza affects promoter methylation status, regulated through the inflammatory cytokines, IFN-γ and TNF-α.** (A) Two lots of hMSCs were treated with IFN-γ and TNF-α for 24 hr, and the up-regulated genes were investigated through microarray analysis. Changes in the gene expression were indicated through heat map imaging. (B) After treating hMSCs with 5-aza for 24 hr, changes in the methylation patterns in promoter regions were investigated through promoter array analysis. The changes in the methylation patterns were indicated through heat map imaging. (C) The changes of methylation status by treatment of 5-aza were indicated as a Venn diagram. Among the investigated promoters, the 10,664 promoters of 7,734 genes were hypomethylated, whereas 7,112 promoters of 5,615 genes were hypermethylated after 5-aza treatment. There was no change in methylation status of 85,086 promoters. (D) Changes in the methylation status of up-regulated genes through IFN-γ and TNF-α were investigated, and the results are presented in a table.

quantified hMSC migration. Interestingly, the number of migrated cells increased significantly after 5-aza treatment (P = 1.12E-09, Fig. 1D).

In a previous study, we showed that long-term treatment with 5-aza for more than 3 days increased the expression of senescence-related markers in hMSCs. Based on this finding, in the present study, we analyzed whether 5-aza treatment, which affects the immunomodulatory function of hMSCs, could influence the proliferation, senescence and apoptosis of hMSCs. Although 5-aza treatment for 24 hr slightly inhibited the proliferation of hMSCs, this treatment did not induce apoptosis (Fig. S2A–D). Furthermore, 5-aza treatment did not alter senescence-associated β-gal staining nor the expression of hMSC-specific markers and cellular senescence-related markers, such as p16^{INK4A} and p21$^{CIP1/WAF1}$ (Fig. S2E–G). Taken together, these findings suggest that 5-aza treatment for 24 hr enhances the secretory factor-mediated inhibitory effects of hMSCs on inflammatory cell proliferation and increases the migration of hMSCs toward activated T cells, without altering the expression of hMSC-specific or senescence-related markers.

Identification of the DNMT inhibitor target using microarray analysis. To identify crucial factors and elucidate the underlying mechanisms responsible for the 5-aza-mediated regulation of hMSC immune functions, we performed microarray and promoter methylation array analysis. The gene expression in hMSCs was assessed after treatment with the prominent pro-inflammatory cytokines, IFNγ and TNFα, to mimic the inflammatory environment. The expression of 150 genes was elevated more than 2-fold after stimulation with IFNγ and TNFα (Fig. 2A–B, Table S1). In addition, the promoter methylation array revealed the demethylation of the promoters for 7,734 genes and the methylation of the promoters for 5,615 genes after 5-aza treatment (Fig. 2C). Furthermore, among the 150 genes showing elevated expression after treatment with IFNγ and TNFα, 29 genes were hypomethylated, and 26 genes were hyper- or hypomethylated after 5-aza treatment according to the promoter regions (Fig. 2D, Table S2).

The DNMT inhibitor regulates the expression of genes associated with MSC secretion of immunoregulatory factors and migration towards inflammatory sites. Among the 55 completely or partially hypomethylated genes, the immunomodulation-associated genes, cyclooxygenase 2 (COX2), prostaglandin E synthase (PTGES), leukemia inhibitory factor (LIF), and the cytokines IL-6 and IL-1β, were further examined. First, we determined the gene expression levels in hMSCs after treatment with inflammatory cytokines, IFNγ and TNFα, using quantitative real-time PCR. After treatment with IFNγ and TNFα, the expression of these genes was significantly increased in hMSCs isolated from different cord blood
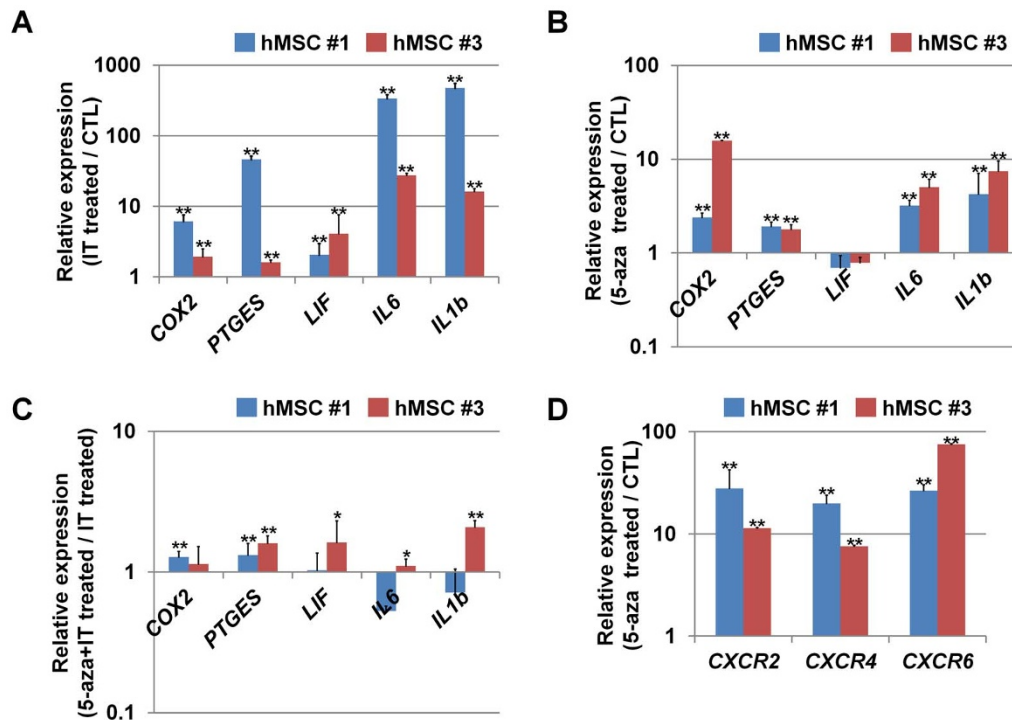
Figure 3 | 5-aza regulates the expression of genes associated with the hMSC secretion of immune-regulatory factors and migration into inflammatory sites. (A) After treating hMSCs with IFN-γ and TNF-α, changes in the expression of 5 representative genes, selected via microarray analysis, were investigated in 2 lots of hMSCs (Fig 2). The expression was confirmed through real-time qPCR, and the relative ratio to the control is graphically represented. (B) After treating hMSCs with 5-aza, the expression of indicated genes was detected and compared with that in control hMSCs (CTL). (C) The cells were pretreated with 5-aza (2 μM) for 24 hr and subsequently treated with IFN-γ/TNF-α for 24 hr (5-aza + IT treatment). The expression of indicated genes was determined, and the results were compared with those in hMSCs treated with IT only (IT-treated). (D) After treatment with 5-aza, the expression of *CXCR2*, *CXCR4* and *CXCR6* was measured and compared with that in control hMSCs (CTL). *, $p < 0.05$; **, $p < 0.01$. Results are shown as mean ± SD.

samples, designated #1 and #3 (P < 0.01, Fig. 3A). Next, we determined the gene expression levels after 5-aza treatment. The expression levels of *COX-2*, *PTGES*, *IL-6* and *IL-1β* were significantly increased after 5-aza treatment (P < 0.01, Fig. 3B). We also examined the gene expression levels in 3 different hUCB-MSCs, and the results showed that *COX2* and *IL-6* expression was increased after 5-aza treatment (Fig. S3A). To further determine whether pre-treatment with 5-aza affects the response of hMSCs against IFNγ and TNFα, these cells were treated with 5-aza for 24 hr, followed by treatment with IFNγ and TNFα for an additional 24 hr, and the expression of the related genes was subsequently assessed. Interestingly, 5-aza pre-treatment significantly increased the expression level of *PTGES* compared to the sole treatment of IFNγ/TNFα in both #1 and #3 hMSCs, whereas changes in the expression of other genes varied depending on the cord blood sources (Fig. 3C). In addition, 5 different hMSCs were treated with 5-aza for 24 hr, followed by treatment with IFNγ for an additional 24 hr, and subsequently COX2 expression was assessed. The pre-treatment with 5-aza increased *COX2* expression compared with IFNγ treatment alone (Fig. S3B). No migration-related genes were identified among the hypomethylated genes showing increased expression after IFNγ and TNFα treatment. However, the promoter array analysis showed that the promoters of *CXCR2*, *CXCR4*, *CXCR6*, metallopeptidase 2 (*MMP2*), *MMP11*, *MMP15* and *MMP16* were hypomethylated after 5-aza treatment (Table S3). We also examined whether the expression of *CXCR2*, *CXCR4* and *CXCR6* was increased after 5-aza treatment using real-time qPCR, and the results showed the increased expression of *CXCR2* and *CXCR4* in 5 different hUCB-MSCs (Fig. 3D, Fig. S3C). Moreover, the elevated expression of *MMP11*, *MMP15* and *MMP16* was observed after 5-aza treatment (Fig. S3D).

The DNMT inhibitor augments PGE₂ production in hMSCs through the up-regulation of synthesis enzymes. PGE_2 is a well-known immune modulator that plays a role in the MSC-mediated regulation of immune cell activation[2,30,31]. To determine whether the COX2-PGE_2 pathway is involved in the 5-aza-mediated enhancement of hMSC immune function, we examined the expression of COX2 and PTGES, crucial enzymes for PGE_2 synthesis, after treatment with different doses of 5-aza. After treating hMSCs with 5-aza for 24 hr, the expression of COX2 and PTGES was increased on both mRNA and protein levels (Fig. 4A–B). The PGE_2 concentration in the CM was also elevated after 5-aza treatment (Fig. 4C). Furthermore, COX2 inhibition through siRNA significantly restored the robust inhibitory effect of 5-aza-treated hMSCs on MNC proliferation (Fig. 4D). To determine whether the increase in COX2 and PTGES expression through 5-aza is associated with demethylation of the gene promoter, changes in the methylation pattern following 5-aza treatment were analyzed using methyl-specific PCR (Fig. 4E). The methylation of the promoters of both *COX2* and *PTGES* was reduced after 5-aza treatment (Fig. 4F).

The DNMT inhibitor increases CXCR2 and CXCR4 expression and hMSC chemotaxis. MSCs express CXCR2 and CXCR4 and show chemotactic activity towards their ligands, SDF-1/CXCL12 and IL-8/CXCL8[32]. Moreover, the SDF-1/CXCR4 axis is important for the migration of MSCs towards target tissues with inflammatory reactivity[14,33]. We investigated whether the increased CXCR2 and CXCR4 expression after 5-aza treatment affects the hMSC migration. After the treatment of 5-aza on hMSCs for 24 hr, the expression of CXCR2 and CXCR4 was increased on mRNA and protein levels (Fig. 5A, B). Next, we confirmed whether the migration of hMSCs toward CXCR2 and CXCR4 ligands is altered through 5-aza treat-
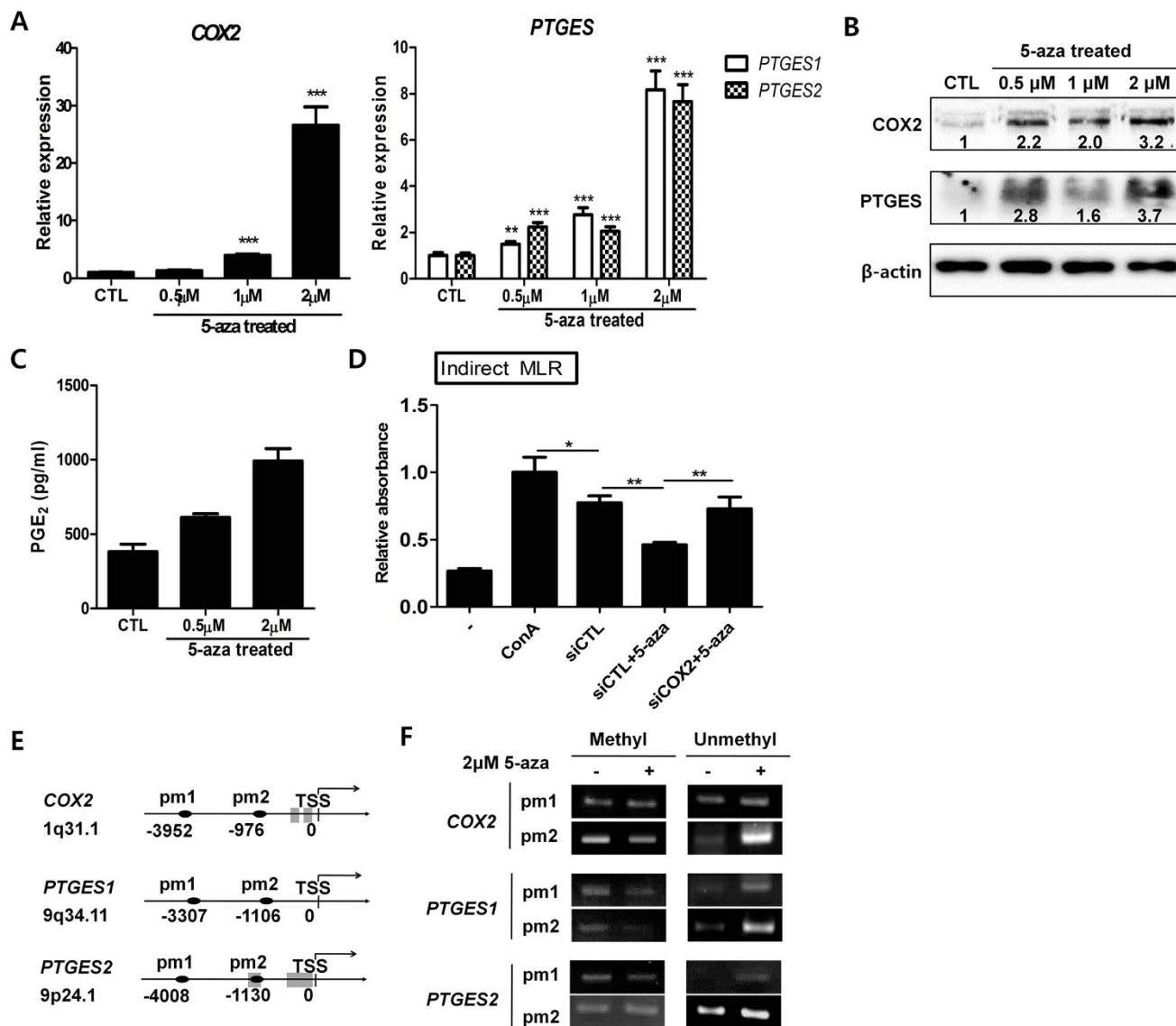
Figure 4 | 5-aza increases the production of PGE$_2$ from hMSCs through the up-regulation of synthesis enzymes. (A-B) After treating hMSCs with 5-aza for 24 hr, COX2 and PTGES expression was detected through (A) real-time qPCR and (B) western blot analysis (C) After treating hMSCs with 5-aza for 24 hr, the changes in PGE$_2$ expression levels were measured using ELISA. PGE$_2$ secretion from hMSCs was increased by 5-aza treatment. (D) After treatment of 5-aza with or without the inhibition of COX2 (siCOX2), indirect-MLR was performed. The suppression of MLR by hMSCs was increased by treating with 5-aza and the effect was rebounded by inhibition of COX2. (E) Schematic diagrams indicating the locations of the promoter primers. (F) After treating hMSCs with 5-aza, bisulfide conversion and methyl-specific PCR were performed. In 5-aza treated hMSCs, the promoters of *COX2*, *PTGES1* and *PTGES2* were hypomethylated compared with control MSCs. pm, promoter locus; TSS, transcription start site; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Results show one representative experiment out of at least three. Results are shown as mean $\pm$ SD.

ment. The number of migrated cells toward IL-8, CXCL1 and SDF-1 was significantly increased after 5-aza treatment ($P = 0.0028, 0.0001, 0.0023$, respectively, Fig. 5C). To determine whether the increase in CXCR2 and CXCR4 expression through 5-aza is associated with demethylation of the gene promoter, changes in the methylation pattern following 5-aza treatment were analyzed using methyl-specific PCR (Fig. 5D). The promoters of both *CXCR2* and *CXCR4* were hypomethylated after 5-aza treatment (Fig. 5E).

The DNMT inhibitor augments the immunomodulatory and migratory properties of hMSCs in a murine inflammatory bowel disease (IBD) model. According to previous studies, PGE$_2$ secretion from hMSCs is important for the regulation of immune reactions and regulatory T cell (Treg) populations, preventing the development of disease in a mouse IBD model[2,6,14]. In addition, CXCR4 up-regulated

MSCs showed an increased therapeutic efficacy of transplantation in DSS-induced colitis[11]. Based on these results and the results of *in vitro* analyses, we confirmed whether 5-aza treatment increases the immunomodulation and migration of hMSCs in a DSS-induced colitis mouse model. hMSCs with or without 5-aza treatment were injected intraperitoneally at 24 hr after colitis induction with DSS. Body weight changes were monitored for 12 days, and the results showed that 5-aza-treated hMSCs exerted protective effects on the reduction of body weight and lethality in mice with DSS-induced colitis (Fig. 6A). The shortening of the colon length was protected by hMSC injection, and further improved by 5-aza-treated hMSC infusion (Fig. 6B). In addition, we detected and quantified the cell migration toward the inflamed colon and observed that 5-aza-treated hMSCs were significantly more infiltrated in the colon tissues ($P = 0.0137$, Fig. 6C, 6D, S4).
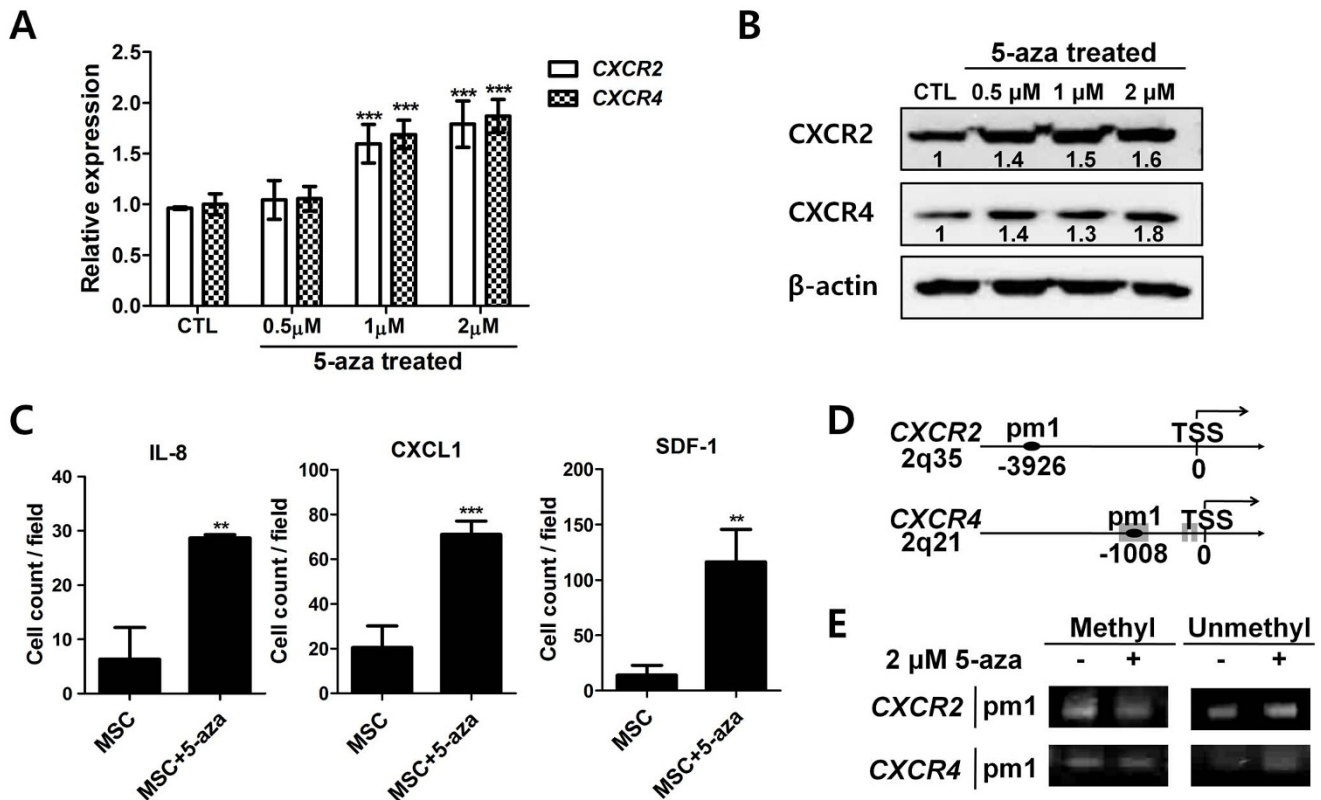
Figure 5 | 5-aza stimulates the migration ability of hMSCs through the increase of CXCR2 and CXCR4. (A-B) After treating hMSCs with 5-aza, CXCR2 and CXCR4 expression was confirmed through (A) real-time qPCR and (B) western blot analysis. (C) After 5-aza treatment, the migration of hMSCs was determined after treatment with the CXCR2 or CXCR4 ligands. (D) Schematic diagrams indicating the locations of the promoter primers. (E) After treating hMSCs with 5-aza, bisulfide conversion and methyl-specific PCR were performed. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Results show one representative experiment out of at least three. Results are shown as mean $\pm$ SD.

The DNMT inhibitor-mediated COX-2 activation in hMSCs is critical for the amelioration of colitis. Given that DNMT inhibitor treatment enhanced the expression of COX2 in hMSCs, we explored the physiological role of COX2 activation in the immunoregulatory capability of 5-aza-treated hMSCs. As expected, COX2 inhibition by specific siRNA abrogated the ability of 5-aza-treated hMSCs to protect mice from body weight loss and lethality (Fig. 7A). Although the administration of non-treated (naïve) hMSCs exerted a limited protective effect in body weight loss, naïve hMSCs significantly decreased the disease activity index, and 5-aza pretreatment further improved this effect (Fig. 7B). In contrast, COX2 inhibition in 5-aza-treated hMSCs resulted in the loss of this protective effect (Fig. 7B). On histological examination, destruction of colonic epithelium and infiltration of inflammatory cells were observed in DSS-induced colitic mice. In hMSC-injected mice, mucosal destruction and lymphocyte infiltration were partially reduced. Importantly, the administration of 5-aza-treated hMSCs greatly suppressed the histological damage and the treatment of siRNA for COX2 on 5-aza-treated hMSCs neither prevented the epithelial destruction nor the lymphocyte infiltration (Fig. 7C and D).

Discussion

In the present study, we investigated the effect of the DNMT inhibitor, 5-aza, on the immune-modulatory and migratory properties of hMSCs. We observed that 5-aza treatment augmented immune-modulation and migration through the up-regulation of immune modulators and chemotaxis-related factors in hMSCs. In addition, 5-aza treatment increased the immunosuppressive effects of hMSCs in both MLR, using transwell plates or CM. These findings suggest that 5-aza increases the secretion of immunomodulatory soluble

factors from hMSCs, regardless of the interaction between hMSCs and lymphocytes. However, when cell-to-cell contact between hMSCs and lymphocytes was facilitated, naïve hMSCs suppressed lymphocyte proliferation to a similar extent as unactivated lymphocytes, and the additive effect of 5-aza treatment could not be determined, suggesting that additional factors, such as adhesion molecules, are involved in the direct MLR.

The up-regulated expression and demethylation of the *COX2* and *PTGES* promoters and increased PGE$_2$ secretion from hMSCs were also observed after 5-aza treatment. In the present study, we observed that the increased PGE$_2$ secretion after 5-aza treatment augmented the suppressive effect of hMSCs on lymphocyte proliferation. PGE$_2$ is a powerful immunomodulator secreted by hMSCs. Previous studies have reported that hMSCs inhibit Th1/Th17 differentiation and induce regulatory phenotypes through COX2-dependent PGE$_2$ secretion[34–36]. Moreover, PGE$_2$ secretion from MSCs in response to inflammatory cytokines or ligands is critical for the immunomodulatory function of MSCs in various immune disease models, including arthritis and colitis[2,37]. The secretion of PGE$_2$ from MSCs has been reported to regulate dendritic cells, CD4$^+$ helper T cells, B cells, NK cells, monocytes and macrophages, exerting anti-inflammatory effects[1]. Interestingly, the regulation of COX2 expression through promoter methylation has been reported in other cell types. Fang et al. reported that the enhancement of COX2 expression in antiviral defense is regulated through DNA hypomethylation. These authors showed that COX2 expression is regulated through DNMT3a and DNMT3b in both A549 cells and peripheral blood mononuclear cells infected with influenza A virus[38]. Akhtar et al. reported that *Helicobacter pylori* (*H. pylori*)-stimulated COX2 expression in gastric epithelial cells was dependent on the methylation status of the individual *COX2* promoter. Based on these findings, the methylation
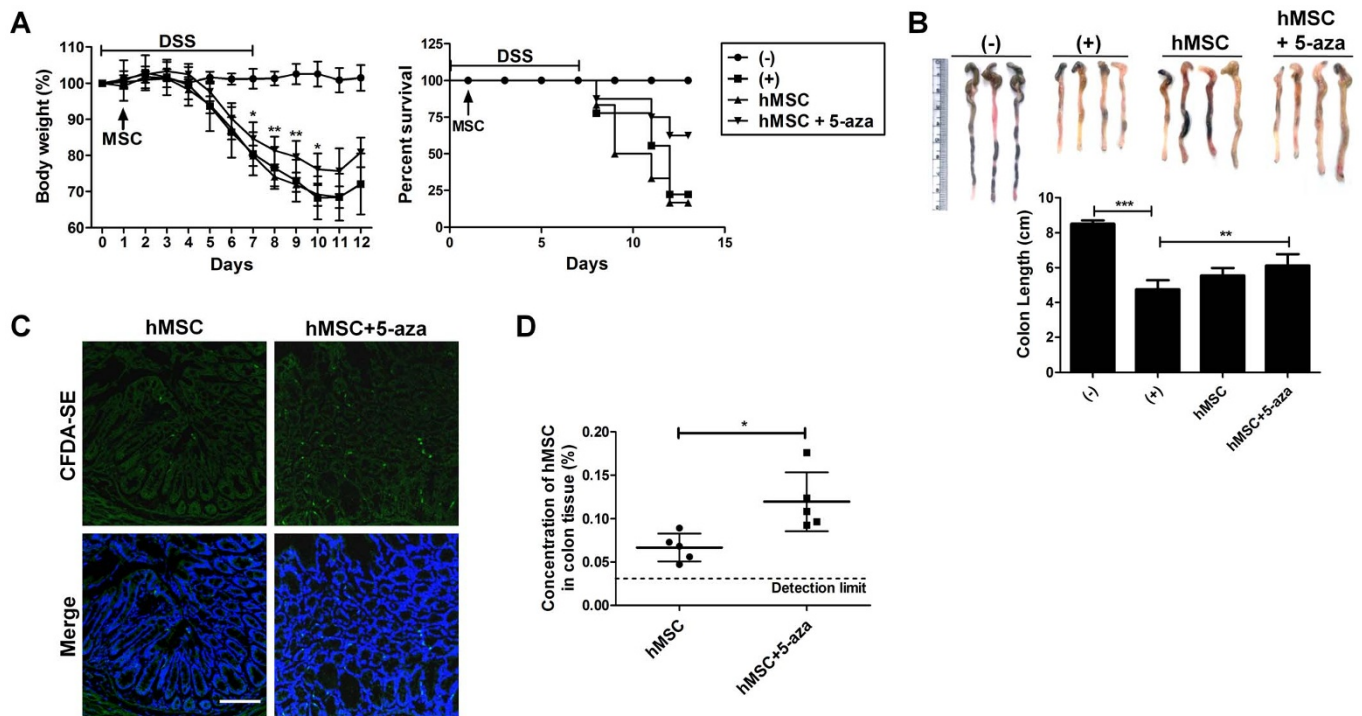
Figure 6 | 5-aza enhances the immune-modulation and migration ability of hMSCs in DSS-induced colitis mice. (A) hMSCs with or without 5-aza treatment were injected into mice with DSS-induced colitis. Body weight loss and survival rate were monitored. Number of mice for each group: (-) = 7, (+) = 9, hMSC = 10, 5-aza treated hMSC =8. (B) The colon length was measured and shown in a graph. Number of mice for each group: (-) = 7, (+) = 9, hMSC = 10, 5-aza treated hMSC = 8. (C) CFDA-SE-labeled hMSCs were detected through confocal image analysis. Bar, 100 μm. (D) The hMSC distribution in colon tissues was analyzed using real-time qPCR of the human-specific ALU gene. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Results are shown as mean ± SD.
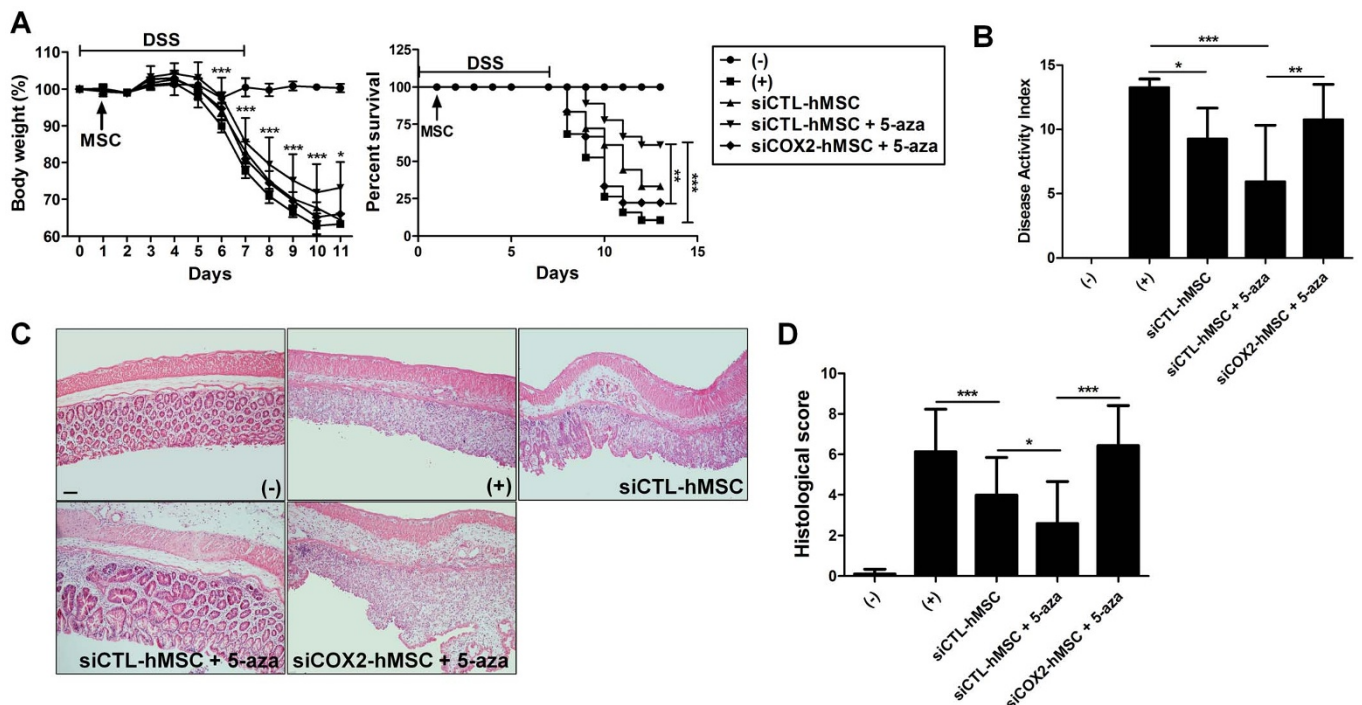

Figure 7 | 5-aza-mediated immunomodulatory effect of hMSCs is COX-2 dependent. siRNA for COX-2 was transfected into hMSCs. siRNA-transfected hMSCs with or without 5-aza treatment intraperitoneally injected into DSS-induced colitic mice. (A) Body weight loss and survival rate were monitored. Number of mice for each group: (-) = 12, (+) = 19, siCTL-hMSC = 18, siCTL-hMSC + 5-aza = 18, siCOX2-hMSC + 5-aza = 18. (B) Disease activity index for colitis severity was evaluated. (C-D) Histopathological analysis of colon, Bar, 200 μm, Five to seven mice/group were used. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Results are shown as mean ± SD.

status of the *COX2* promoter is different among different donor-derived gastric epithelial cells, suggesting that the increased COX2 expression in response to *H. pylori* was dependent on the methylation status of *COX2* promoters. Moreover, after treatment with 5-aza, gastric epithelial cells containing methylated COX2 promoters showed a significant enhancement of COX2 expression and PGE_2 secretion in response to *H. pylori*[39].

In the present study, we also observed that 5-aza treatment increased hMSC migration through the CXCR2/IL-8, CXCR2/CXCL1 and CXCR4/SDF-1 axes. IL-8 and CXCL1 are proinflammatory cytokines that play a role in the recruitment of neutrophils in inflamed tissues, and the expression of these cytokines is elevated in inflammatory diseases, including IBD[40–45]. Xu et al. reported that the over-expression of CXCR1/CXCR2 in mesenchymal stromal cells could increase recruitment to the sites of degenerated myocardium[46]. On the other hand, SDF-1 plays a role in the recruitment of lymphocytes, hematopoietic stem cells (HSCs) and MSCs[5,47,48]. It has been reported that the SDF-1/CXCR4 axis regulates the migration of transplanted bone marrow MSCs towards the inflamed tissues in an acute pancreatitis rat model[33]. A number of studies have reported that the SDF-1/CXCR4 axis is important for the migration of MSCs, and there have been several attempts to improve the migration of MSCs through the up-regulation of CXCR4[5,49–51]. In the present study, we observed that the expression of CXCR2 and CXCR4 was up-regulated and that their associated promoters were demethylated in hMSCs after 5-aza treatment. The epigenetic regulation of CXCR2 or CXCR4 expression has been addressed in several studies. CXCR2 has been reported to be epigenetically regulated through histone H3 acetylation[52,53]. Considering the association between DNA methylation and histone acetylation, these reports propose the regulation of CXCR2 through DNA methylation[54]. Sato et al. reported that CXCR4 is regulated through DNA methylation in pancreatic cancer. These authors observed a significant inverse correlation between methylation and the mRNA expression of CXCR4 in a large panel of pancreatic cancer cell lines[55].

In the DSS-induced colitis mouse model, we observed that 5-aza-treated hMSCs showed augmented therapeutic effects and migration capacities compared with naïve hMSCs. In the present study, naïve hMSCs showed a limited protective effect on body weight loss and lethality in colitic mice and slightly reduced the shortening of colon length. Because hMSCs are derived from different donor tissues, there might be variations in DNA methylation among the hMSCs, and these variations could reflect the inconsistent therapeutic efficacy of MSCs against IBD[56,57]. Moreover, the difference in the promoter methylation status could account for the different responses to DNA demethylating agents, including 5-aza. The hMSCs with hypomethylated immunomodulatory gene promoters would have more significant effects than naïve cells and show weak responses to DNA demethylating agents. In contrast, hMSCs carrying hypermethylated gene promoters would only exhibit slight immunomodulatory effects compared with naïve cells and have a strong response to DNA demethylating agents. Further studies regarding the relationships between variations in DNA methylation and the therapeutic effects of hMSCs are needed for the clinical application of hMSCs. However, regardless of the variations among hMSCs derived from different donor tissues, DNMT inhibitor treatment consistently augmented the physiological effect of hMSCs through the activation of COX-2 signaling.

Taken together, treatment with a demethylating agent (5-aza) increased the immunomodulation and migration of hMSCs through the demethylation of target gene promoters. These findings provide new insight to enhance the immune-modulatory effects of hMSCs via epigenetic modifications. Moreover, the different responses to 5-aza of hMSCs derived from different donors suggest that variations in DNA methylation influence immune-modulatory effects of hMSCs. Further studies concerning the epigenetic regulation of the immune-modulatory gene expression in hMSCs would be beneficial for the controlled use of effective hMSCs as therapeutics and the development of novel methodologies to increase the therapeutic effects of hMSCs in human diseases.

Methods

Isolation and Culture of hUCB-Derived MNCs and MSCs. hUCB-MSCs and MNCs were isolated and maintained as described in the Supplementary Methods section.

Mixed Leukocyte Reaction (MLR). hMSCs were plated onto 6-well plates at 3×10^5 cells/well. After 24 hr, the cells were treated with 2 μM 5-aza for 24 hr. For direct co-culture, hMSCs were treated with 25 mg/ml mitomycin C for 1 hr, and the cells were plated at 1×10^4 cells/well onto 96-well plates. MNCs were activated using Concanavalin A (ConA) and seeded onto plates at 5×10^4 cells/well. For indirect co-culture, 0.4-μm-poresize transwells (24-well plate, Corning, Corning, NY) were used. MNCs were activated with ConA and plated at 1×10^6 cells/well in the lower chamber, and 5-aza-treated hMSCs were seeded at 1×10^5 cells/well in the upper chamber. After 3 days, the proliferation rate was analyzed using a BrdU assay (Bromodeoxyuridine kit, Roche, Upper Bavaria, Germany).

For the MLR assay using CM, MSCs were seeded at 3×10^5 cells/well onto 6-well plates and treated with 5-aza for 24 hr. After washing 3 times with PBS, the media were changed to RPMI 1640 (Gibco BRL) without FBS and enriched for 4~5 days. The CM was collected and used for MNC culture. MNCs were plated at 1×10^5 cells/well onto 96-well plates and treated with CM containing 10% FBS with or without ConA. After 3 days, the BrdU assay was performed.

RNA Interference. The cells were transfected with siRNA at 60% confluency. The siRNAs for PTGS2 (siCOX-2, L-004557-00) and the nontargeting control (siControl 1, D-001810-01) were purchased from Dharmacon (Chicago, IL). The experiments were conducted using DharmaFECT1 (Dharmacon) as a transfection agent and siRNA at a concentration of 50 nmol/L. After 48 hr, the medium was changed, and the cells were used for the following studies.

Animal study and Ethical Statement. C57BL/6J mice (male; age, 8–10 wk) were obtained from Jackson Laboratory (Bar Harbor, ME). The mice were housed in groups under conventional conditions in the animal facility of Seoul National University. Acute colitis was induced in mice after the addition of 3% (w/v) dextran sulfate sodium (DSS, MP Biochemicals, Solon, OH) in drinking water for 7 days. hMSCs were treated with siRNA or 2 μM 5-aza before administration and washed with PBS to remove residual 5-aza. The body weight and morbidity were monitored over 11 days. At day 7, colitis severity was measured by evaluating the disease activity index through the scoring of weight loss (0~4), stool consistency (0~4), bleeding (0~4) and mouse activity (0~2). At the peak of disease (day 10), the mice were sacrificed, and colon length was measured. Mice were monitored until they had reached criteria for humane endpoints. Mice losing above 40% of their original body weight or showing signs of distress (unphysiological bodily posture, shaggy fur, and breathlessness/panting etc.) were sacrificed using CO_2 asphyxiation. At the designated time points for tissue harvesting, mice were sacrificed by CO_2-asphyxiation. Every effort was made to minimize suffering. All experimental protocols were approved by the Seoul National University Institutional Animal Care and Use Committee (Approval #; SNU-100125-8, SNU-111223-1, and SNU-130130-2) and carried out in accordance with the approved guidelines.

Histopathological Evaluation. Colon samples were collected, fixed in 10% formalin, subjected to consecutive steps of alcohol–xylene changes, and embedded in paraffin. Sections that were 5-μm thick were prepared and stained with H&E. Leukocyte infiltration (0~4) and intestinal damage (0~4) were graded blindly.

Cell Tracking. To track the injected cells, hUCB-MSCs were labeled with 10 μmol/L CFSE (Molecular Probes) according to the manufacturer's protocol. CFSE-labeled cells were injected intraperitoneally. At 3 days after injection, 10-μm frozen colon sections were cut and examined for green fluorescence with a confocal microscope. To analyze hMSC concentration using real-time qPCR with Alu primers, DNAs were isolated from colon segments. Standard samples of mouse colon DNA which containing 16%, 4%, 1%, 0.25%, 0.063%, 0.031%, 0.015%, 0.006% and 0%(NC) of hMSCs DNA were prepared, respectively and obtained standard curve. We fixed the detection limit as 0.031% hMSCs of which Cp value has no difference with that of negative control. Based on standard curve, we obtained the concentration of hMSCs in each sample. The sequences of Alu primers which were used for real-time qPCR are as follows;

GTCAGGAGATCGAGACCATCCC (forward) and TCCTGCCTCAGCCT-CCCAAG (reverse)[58].

Microarray analysis. Total RNA was extracted from human cells using the TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. Following homogenization, 1 ml of solution was transferred to a 1.5-ml Eppendorf tube and centrifuged at 12,000 g for 10 min at 4°C to remove insoluble material. The supernatant containing RNA was collected, mixed with 0.2 ml of chloroform, and centrifuged at 12,000 g for 15 min at 4°C.

Subsequently, the RNA in the aqueous phase was transferred to a new tube, precipitated with 0.5 ml of isopropyl alcohol and recovered through centrifugation at 12,000 g for 10 min at 4°C. The RNA pellet was briefly washed in 1 ml of 75% ethanol and centrifuged at 7,500 g for 5 min at 4°C. The RNA pellet was dissolved in nuclease-free water, and the quality and quantity of the RNA was assessed through Agilent Bioanalyzer2100 analysis. Gene expression was analyzed using a GeneChip® Affymetrix Primeview array (Affymetrix, Santa Clara, CA), comprising more than 530,000 probes representing approximately 20,000 well-characterized human genes. For each gene, eleven pairs of oligonucleotide probes were synthesized *in situ* on the arrays.

Biotinylated cRNA was prepared according to the standard Affymetrix protocol from 250 ng of total RNA (Expression Analysis Technical Manual, 2001, Affymetrix). Following fragmentation, 12 μg of RNA was hybridized for 16 hr at 45°C onto the GeneChip Human Genome Array. The GeneChips were washed, stained in the Affymetrix Fluidics Station 450 and scanned using the Affymetrix Gene Chip Scanner 3000 7G. The data were analyzed using Microarray Suite version 5.0 (MAS 5.0) with Affymetrix default analysis settings and global scaling as a normalization method. The trimmed mean target intensity of each array was arbitrarily set to 100. The normalized and log-transformed intensity values were analyzed using GeneSpring GX 12.5 (Agilent Technologies, Santa Clara, CA).The fold-change filters required that the genes be present in at least 200% of controls for up-regulated genes and less than 50% of controls for down-regulated genes. Hierarchical clustering data were clustered into groups that behave similarly across experiments using GeneSpring GX 12.5 (Agilent Technologies). The Euclidean distance, with average linkage, was used as the clustering algorithm.

Promoter array. After treatment with 2μM 5-aza for 24 hr, the samples for promoter array analysis were prepared using the MethylMiner™ Methylated DNA Enrichment Kit (Applied Biosystems) according to the manufacturer's instructions. We used arrays comprising 0.4 million 50-mer to 60-mer oligonucleotides covering chromosome 21 at a median probe density of 1 per 60 bp (Agilent Technologies). Microarray protocols, including labeling, hybridization and post-hybridization washing procedures, were conducted according to the manufacturer's instructions and are available at http://www.agilent.com.

The MeDIP (cyanine 5-dUTP) and reference DNA (cyanine 3-dUTP) were labeled (Genomic DNA Enzymatic Labeling Kit, Agilent Technologies), cleaned (Amicon filters, Millipore) and quantified. Competitive hybridization onto the microarray was subsequently performed (Methylation Hybridization Kit, Agilent Technologies) according to the manufacturer's instructions in a rotating SureHyb chamber at 67°C for 40 hr. The washed slides were scanned (High-Resolution C Scanner, Agilent Technologies), and fluorescence was measured using Feature Extraction software.

These two signal values were normalized using background subtraction, and the signal ratio (MeDIP/input), signal log ratio [log2 (MeDIP/input)], P[X], and P were obtained using Agilent Genomic Workbench software (Agilent Technologies). The log2 value is the Cy5 : Cy3 fluorescence ratio (methylated DNA recovered by IP: total input DNA) for each probe, converted to a log2 scale, representing a relative measure of the amount of methylated DNA at each locus. We applied Median and the Lowess normalization to the raw data and filtered outlier probes to remove low-quality data points. The P[X] and P values, used in the Methylation IP (MeDIP) array analysis to obtain a binding call, were defined as the probability of how the X ( ) value deviates from the Gaussian distribution of X ( ) values of the entire genome of a sample. Here, the X value for a probe was obtained as the difference between the MeDIP and the input signals after adjusting the symmetry of its distribution. The value for a probe was calculated as an average X, considering the signals for neighboring probes (within 1 kb of the probe).

We defined hypermethylation as a normalized log2 ratio > 1.0 (equivalent to ~2.0-fold MeDIP/input signal intensity) based on previous validation experiments.

Methylation-specific PCR. For methylation-specific PCR, genomic DNA was extracted from cells using Accuprep® (Bioneer) according to the manufacturer's instructions. The bisulfite conversion of genomic DNA was performed using the MethyCode™ (Invitrogen) according to the manufacturer's instructions. The sodium bisulfite-modified DNA was amplified using the Accupower PCR premix (Bioneer). The primers used for each promoter were designed through an online web site (www.urogene.org/methprimer/), and the primer sequences are listed in Table S4.

Migration assay. The migration assay was performed based on the previous report[59]. Briefly, the migration of hMSCs was assessed using transwell chambers with polycarbonate membrane filters of 6.5 mm diameter and 8 μm pore size. The cells (4 × 10⁵) in 400 μl of media (RPMI1640, 1% BSA) were added to the upper chamber, and the lower chamber was filled with 800 μl of media (RPMI1640, 1% BSA) containing CD3/CD28 activated MNCs (8 × 10⁶) with 100ng/ml IL-8, 100ng/ml CXCL1 or 100ng/ml SDF-1. T cell activation was performed using Dynabeads Human T-Activator CD3/CD28 (Invitrogen) according to the manufacturer's instructions. The transwell chambers were incubated for 24 hr at 37°C and 5% CO₂ to facilitate cell migration. The cells on the top surface of the filters were wiped off with cotton swabs, and the cells that had migrated into the lower chamber and attached to the lower surface of the filter were stained with crystal violet. To count the migrated hMSCs, at least three fields per sample were randomly chosen and photographed at × 40 magnification. The measurements were performed at least three times using different samples.

Statistical analysis. All experiments were conducted at least three times (n = 3), and the results are expressed as the mean ± SD. The statistical analysis was conducted via Student's t-test or one-way ANOVA. A value of p < 0.05 was considered significant.

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Acknowledgments

Author contributions

S.L. designed the study, collected and analyzed the data, and wrote the manuscript; H.-S.K. designed the study, collected and analyzed the data, and wrote the manuscript; K.-H.R. designed the study, collected and analyzed the data, and wrote the manuscript; B.-C.L. collected and analyzed the data; T.-H.S. collected and analyzed the data; J.-M.Y. collected and analyzed the data; Y.-L.K. collected and analyzed the data; K.-R.Y. collected and analyzed the data; K.-S.K. and K.-W.S. designed, supervised the study, and critically contributed to analyzing the data

Additional information

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