MicroRNA-9 plays a role in interleukin-10-mediated expression of E-cadherin in acute myelogenous leukemia cells

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Interleukin-10 is a pleiotropic immunoregulatory cytokine that inhibits cytokine secretion and the effector function of T cells, monocytes, and macrophages. Interleukin-10 exerts its actions through a heterodimeric membrane receptor composed of a binding chain (IL-10R1) and a transducing chain (IL-10R2). Interaction between IL-10R1 and IL-10R2 activates a series of intracellular signaling molecules including the STATs.

Human BM contains a higher frequency of CD4⁺CD25⁺FoxP3⁺ regulatory T cells, which produce IL-10, than other secondary lymphoid organs, suggesting that the amount of IL-10 in the BM may be greater than that in other organs. The treatment of allogeneic HSPCs with an anti-IL-10 antibody led to a ~90% reduction in the number of surviving allogeneic HSPCs compared with control IgG antibody treatment. Interleukin-10 significantly increased the colony-forming ability of HSPCs in association with an increase in levels of anti-apoptotic Bcl-2 proteins. These observations suggest the important role of IL-10 in survival of HSPCs.

We previously showed that CD82 inhibits the activity of MMP9 and enhances the adhesion of AML cells to the BM microenvironment. We also found that CD82 stimulates production of IL-10 through STAT5 signal transduction pathways in AML cells.

CD82 acts as a tumor suppressor and inhibits tumor metastasis. Forced expression of CD82 inhibits cytokine-stimulated phosphorylation of β-catenin and stabilizes the E-cadherin/β-catenin complex, resulting in strengthened hemophilic intercellular cell adhesion in non-small-cell lung cancer cells. In addition, positive correlation between CD82 and E-cadherin expression was noted in non-small-cell lung cancer. Thus, it is reasonable to assume that CD82 may regulate the expression of E-cadherin in AML cells.

Mature miRNAs are endogenous, single-stranded, non-protein-coding small RNAs measuring 19–25 nt in length, which suppress the expression of target proteins by pairing with the 3'-UTR of target mRNAs. In carcinogenesis, miRNAs are classified as either oncogenic or tumor suppressor miRs. Recently, tumor suppressor miRNAs have been shown to be silenced by aberrant DNA hypermethylation in cancers. In humans, there are three independent miR-9 genes (miR-9-1 on chromosome 1, miR-9-2 on chromosome 5, and miR-9-3 on chromosome 15), with identical mature miR-9 sequences. In different cancers, miR-9 can function either as an oncomir or a tumor suppressor miRNA, depending on the type of cancer. For instance, overexpression of miR-9 can enhance metastasis or invasion in metastatic brain tumors or glioblastoma, indicating its oncogenic role. MicroRNA-9 interacts with the 3'-UTR of E-cadherin and downregulates its expression, which induces β-catenin nuclear translocation and subsequently upregulation of c-Myc and CD44.

MicroRNA-9 overexpression can induce...
epithelial–mesenchymal transition (EMT) and promote tumor metastasis through E-cadherin downregulation in esophageal squamous cell carcinoma.(25) Abnormal miR-9-1 and miR-9-3 methylation and their downregulation are frequently reported in many cancers.(20,26,27)

The present study examines the relationship between the CD82/STAT5/IL-10 axis and E-cadherin expression in AML cells. We also explore the biological function of IL-10/E-cadherin in AML cells.

Materials and Methods

**Cell sample collection.** Each study participant provided informed written consent, and the study was approved by the Kochi University ethics committee (Nankoku, Japan). Leukemia cells were isolated from patients with AML (n = 15; Table 1) who were classified, according to the WHO classification system, as having AML without maturation (cases 14 and 15), AML with maturation (cases 3, 5, and 8–12), acute myelomonocytic leukemia (cases 6 and 7), acute monocytic leukemia (cases 1, 2, and 13), or therapy-related AML (case 4). Normal BM MNCs were isolated from healthy volunteers (n = 5). The WHO classification system was categorized by genetic changes in leukemia cells and reverse transcribed according to the manufacturer’s instructions (PrimeScript RT reagent kit; Takara, Shiga, Japan). Real-time RT-PCR was carried out using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), a StepOnePlus Real-Time PCR System, and the following thermocycling conditions: 95°C for 10 min and 40 cycles at 95°C for 15 s, 60°C for 1 min. Expression of the 18S gene was used for normalization purposes. The sequences of the primers used are listed in Table 2.

**MicroRNA target prediction.** TargetScan (http://www.targetscan.org/) and miRBase (http://www.mirbase.org/) were used to identify putative miRNAs regulating expression of E-cadherin.

**MicroRNA expression.** Expression of miRNA was analyzed using a Mir-X miRNA quantitative RT-PCR SYBR Kit.
Fig. 1. Effect of interleukin-10 (IL-10) treatment on E-cadherin expression in AML cells. (a) MOLM13, MV4-11, and THP-1 cells were treated with IL-10 (100 ng/mL) for 48 h and analyzed for E-cadherin expression by real-time RT-PCR, flow cytometry, or Western blot. (b) UE6EFT-3 human bone marrow (BM)-derived mesenchymal stem cells were treated with IL-10 (100 ng/mL) for 48 h and analyzed for E-cadherin expression by real-time RT-PCR. (c) E-cadherin and IL-10 mRNA levels in AML cells isolated from patients (n = 15) and BM mononuclear cells isolated from healthy volunteers (n = 4). (d) AML cells isolated from patients (#1–#6, n = 6), BM mononuclear cells isolated from a healthy volunteer (n = 1), and MOLM13, THP-1, and MV4-11 cells were harvested and analyzed for IL-10, E-cadherin, and GAPDH expression by Western blot. *P < 0.05; **P < 0.01. –, untreated cells.
Fig. 2. (a) Sequence of microRNA-9 (miR-9) and its potential binding site in the E-cadherin 3′-UTR.

(b) Expression of miR-9 in leukemia cells treated with interleukin-10 (IL-10) was analyzed using a Mir-X miRNA qRT-PCR SYBR Kit. The results shown represent the mean ± SD of three experiments carried out in triplicate. (c) Expression of miR-9 in leukemia cells transfected with either a control mimic or an miR-9 mimic was analyzed using a Mir-X miRNA qRT-PCR SYBR Kit to measure the levels of the indicated gene. The results shown represent the mean ± SD of three experiments carried out in triplicate. (d, e) E-cadherin expression in leukemia cells transfected with either a control or miR-9 mimic was analyzed by real time RT-PCR and flow cytometry. (f) miR-9 levels in AML cells isolated from patients (n = 15) and bone marrow mononuclear cells isolated from healthy volunteers (n = 4). *P < 0.05; **P < 0.01.
MicroRNA levels were normalized to U6 mRNA expression, and relative miR quantities were determined by the 
\[ \Delta \Delta CT \] method.

Methylation analysis. DNA (300 ng) was isolated from leukemia cells and treated with bisulfite using an MethylEasy Xceed Rapid DNA Bisulphite Modification Kit (Takara), according to the supplier’s protocol. The recovered DNA was amplified by PCR on \( \text{miR-9-3} \) CpG islands. Amplification was carried out using the EpiScope MSP Kit (Takara) in a MyCycler thermal cycler (Bio-Rad Laboratories). The cycle conditions were as follows: 95°C initial activation for 30 s followed by denaturation for 45 cycles of 98°C for 5 s, annealing at 55°C for 30 s, and a final extension at 72°C for 1 min.

Overexpression of miR-9 precursor. A precursor miR-9 mimic (Sigma, Deisenhofen, Germany) was transfected into MOLM13, MV4-11, and THP-1 cells using the INTERFERin transfection reagent (Polyplus-transfection, New York, NY,

(Catalog #638314; Clontech Laboratories, Mountain View, CA, USA), following the manufacturer’s instructions. MicroRNA levels were normalized to U6 mRNA expression, and relative miR quantities were determined by the \( \Delta \Delta CT \) method. The sequence of the 5' primers used to detect miR-9-5p was as follows: 5' TTGGGAGGATGTAATGGAAT-3'. The 3' primer is the miRQ 3' Primer supplied with the kit.

**Fig. 3.** Effect of interleukin-10 (IL-10) on DNA methyltransferase 3A (DNMT3A) expression in leukemia cells. (a) DNMT3A expression in leukemia cells treated with IL-10 was analyzed by real-time RT-PCR. (b) Western blot analysis. Leukemia cells treated with IL-10 were harvested, and subjected to Western blot analysis to monitor the levels of the indicated proteins. (c) Methylation-specific PCR. Leukemia cells were treated with IL-10, and DNA was extracted from the cells. DNA with methylated CpG was processed using a MethylEasy Xceed Rapid DNA Bisulphite Modification Kit. The recovered DNA was amplified by PCR on \( \text{miR-9-3} \) CpG islands. The experiments were repeated three times. M, methylation of the gene promoter; U, unmethylated gene promoter. (d-f) DNMT3A expression in MOLM13 (d), MV4-11 (e), and THP-1 (f) cells transfected with either control siRNA or DNMT3A siRNAs was analyzed by real-time RT-PCR. E-cadherin expression in leukemia cells transfected with either control or DNMT3A siRNAs was analyzed by real-time RT-PCR and flow cytometry. (g) Expression of miR-9 in leukemia cells transfected with either control or DNMT3A siRNAs was analyzed using an Mir-X miRNA qRT-PCR SYBR Kit to measure the levels of the indicated gene. The results shown represent the mean ± SD of three experiments carried out in triplicate. * \( P < 0.05; \) ** \( P < 0.01. \)
USA), according to the manufacturers’ instructions. A non-targeting oligonucleotide mimic was used as a negative control.

Small interfering RNAs and transfections. A control siRNA and two siRNAs against DNMT3A were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Sigma, respectively. THP-1 cells were transfected with either the control or DNMT3A siRNAs (300 nM) using an Amaxa Electroporator Nucleofector II (Wako Pure Chemical Industries, Osaka, Japan) and the Nucleofector Kit V (program V-001), as previously described.(31)

Fluorescent microscopy. THP-1 cells were transfected with a GFP vector (0.5 μg) using an Amaxa Electroporator Nucleofector II (Wako Pure Chemical Industries) and the Nucleofector Kit V (program V-001), as previously described.(31) Subsequently, GFP-positive THP-1 cells were transfected with either control or E-cadherin siRNAs (300 nM) using the same procedure. After 24 h, the cells were cocultured with UE6E7T-3 BM-MSCs and treated with AraC and/or IL-10. After 48 h, floating cells were removed and the remaining adherent cells were washed twice with PBS. The GFP-positive cells were analyzed by fluorescent microscopy (BZ-9000; KEYENCE, Osaka, Japan). Cell counts were undertaken using BZ-II Dynamic Cell Count software (KEYENCE).

Bone marrow transplantation. NOD.Cg-Rag1tm1Mom Il2rgtm1Wjl/+SzJ mice (stock number 007799) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and bred in a pathogen-free environment in accordance with the guidelines of the Kochi University School of Medicine; 6-week-old female and male animals were used for the experiments. Human MOLM13 cells (5 x 10^6/mouse) transfected with a control shRNA or IL-10-specific shRNA were injected into the tail vein. At 1 week post-AML cell transplantation, the mice were treated with AraC (20 mg/kg; 3 days/week for 2 weeks); control mice received PBS (100 μL/mouse, 3 days/week for 2 weeks). Cell engraftment was analyzed by flow cytometry after staining PBMCs with a phycoerythrin-conjugated anti-human CD82 mAb (BioLegend). On days 18 and 28 post-transplantation, mice were killed and the BM was removed. Bone marrow cells were flushed from the femurs using 25-gauge needles (Becton Dickinson Biosciences, New Jersey, USA) and collected to extract mRNA. Human cell engraftment was analyzed by

Fig. 3. Continued.
flow cytometry after staining BM cells with a phycoerythrin-conjugated anti-human CD82 mAb (BioLegend). Plasma was separated by centrifugation, and mRNA was isolated from plasma using the NucleoSpin miRNA Plasma Kit (Takara). The levels of hsa-miR-9 in plasma were assessed using a Mir-X miRNA qRT-PCR SYBR Kit (Clontech Laboratories), following the manufacturer’s instructions.

**Statistical analysis.** Student’s t-test was used when comparing results between two groups. All statistical analyses were carried out using PRISM statistical analysis software (GraphPad Software, San Diego, CA, USA) and the results were considered significant in cases where the P-value was <0.05, or highly significant when the P-value was <0.01.

**Results**

**Effect of IL-10 on E-cadherin expression in leukemia cells.** The exposure of leukemia cells (MOLM13, MV4-11, and THP-1 cells) to IL-10 (100 ng/mL, 48 h) increased E-cadherin expression at both the mRNA and protein levels (Figs 1a,S1). E-cadherin expression was also upregulated in U6E7T-3 BM-MSCs following treatment with IL-10 (Fig. 1b). The levels of both IL-10 and E-cadherin in BM-MNCs isolated from AML patients (n = 15) were higher than those in BM-MNCs isolated from healthy volunteers (n = 4) at both the mRNA and protein levels (Fig. 1c). Western blot analyses also found that BM-MNCs isolated from AML patients (n = 6) and AML cell lines (MOLM13, MV4-11, and THP-1) express detectable levels of IL-10 and E-cadherin proteins. However, these proteins were not detectable in BM-MNCs isolated from healthy volunteers (Fig. 1d).

**Effect of IL-10 on miR-9 expression in leukemia cells.** To verify the mechanisms by which IL-10 increases levels of E-cadherin in AML patients, we focused on miRNA. We identified homology between miR-9 and the 3′-UTR of the human E-cadherin gene by searching mRNA databases (Fig. 2a). These observations suggested that miR-9 might bind to 3′-UTR of E-cadherin and suppress its expression. We first examined whether IL-10 modulates expression of miR-9 in AML cells. Exposure of AML cells to IL-10 downregulated miR-9 expression by nearly half in these cells (Fig. 2b). We next examined whether miR-9 regulates expression of E-cadherin. As expected, forced expression of miR-9 in AML cells significantly downregulated levels of E-cadherin at both mRNA and protein levels (Fig. 2c–e).
These observations suggested that IL-10 decreases levels of miR-9, leading to upregulation of E-cadherin. Notably, the levels of miR-9 in BM-MNCs isolated from AML patients (n = 15) were significantly lower than those in BM cells isolated from healthy volunteers (n = 4, Fig. 2f).

**Effect of IL-10 on the methylation status of** miR-9 **CpG islands in leukemia cells.** We next examined whether the expression of miR-9 was regulated by epigenetic DNA methylation changes in the miR-9 gene. To test this possibility, we examined the methylation status of miR-9 CpG islands in leukemia cells by methylation-specific PCR. Interestingly, exposing leukemia cells to IL-10 increased the levels of DNTM3A in both mRNA and protein (Fig. 3a,b). Concomitantly, the hypermethylation of miR-9-3 CpG islands was noted in these leukemia cells (Fig. 3c). These observations suggested that IL-10 downregulates miR-9 expression through DNMT3A activity, resulting in E-cadherin induction. As expected, the downregulation of DNMT3A by siRNAs decreased E-cadherin expression in association with an increase in miR-9 expression in leukemia cells (Fig. 3d–g).

**Role of E-cadherin in adhesion of leukemia cells.** We next examined the biological function of E-cadherin in leukemia cells. The GFP-expressing THP-1 cells were transfected with either control or E-cadherin siRNAs (Fig. 4a), which were then incubated with AraC and/or IL-10 in plates coated with UE6E7T-3 BM-MSCs. After 48 h, the number of GFP-positive adherent cells was analyzed by fluorescent microscopy. The results showed that treatment with IL-10 increased the number of adherent cells in control siRNA transfectants. The exposure of these cells to AraC decreased the number of adherent cells; however, the number of adherent cells increased in the presence of IL-10 (Fig. 4b,c). In contrast, IL-10 was not able to enhance the adhesion of THP-1 cells to UE6E7T-3 BM-MSCs when E-cadherin was depleted by siRNA. Nor did IL-10 counteract the ability of AraC to detach leukemia cells from MCS cells in E-cadherin-depleted THP-1 cells (Fig. 4c). Importantly, the number of adherent E-cadherin siRNA-transfected THP-1 cells was less than that of control siRNA transfectants (Fig. 4c).

**Effect of IL-10 on leukemia cell engraftment in vivo.** Interleukin-10 downregulation by an shRNA decreased the levels of E-cadherin in MOLM13 cells to approximately 25% (Fig. S2). We next explored the effect of IL-10/E-cadherin on AML cell growth in severely immunocompromised mice. MOLM13 cells (5 × 10⁶) transduced with control shRNA or IL-10-specific shRNA were transplanted into NOD.Cg-Rag1<sup>tm1Mom</sup> Il2rg<sup>tm1Wjl</sup> Sj1 mice (control shRNA mice or IL-10 shRNA mice) by tail vein injections (n = 10 mice/group). The treatment was initiated after 1 week of transplantation. The mice received either cytarabine (AraC) (n = 10; 20 mg/kg, 3 days/week for 2 weeks) or PBS (n = 10; 100 μL, 3 days/week for 2 weeks). (a) Kaplan–Meier analysis of mouse survival (n = 10). At day 18 (b) and 28 (c) of transplantation, peripheral blood (PB) and bone marrow (BM) cells were collected and incubated with anti-CD82 antibody. Positive cells were analyzed by flow cytometry (n = 10). BM cells were collected on day 18 (d) and 28 (f) post-transplantation and analyzed for IL-10, DNA methyltransferase 3A (DNMT3A), and E-cadherin mRNA expression by real-time RT-PCR (n = 10). Plasma was collected on day 18 (e) and 28 (g) post-transplantation and analyzed for microRNA-9 (miR-9) expression by real-time RT-PCR (n = 10). *P < 0.05; **P < 0.01.

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initiated on day 8 of transplantation. Kaplan–Meier survival curves indicated that the mice transplanted with IL-10-depleted MOLM13 cells (hereafter referred to as IL-10 shRNA mice) survived longer than the mice transplanted with control shRNA transduced MOLM13 cells (hereafter referred to as control shRNA mice, Fig. 5a). The treatment with AraC only slightly prolonged the survival of control shRNA mice (Fig. 5a). Of note, treatment of IL-10 shRNA mice with AraC significantly prolonged their survival compared with untreated IL-10 shRNA mice (Fig. 5a, \( P < 0.01 \)). We also examined the MOLM13 leukemic cell burden in the transplanted mice. On day 18 of transplantation, engraftment of MOLM13 cells was severely impaired in IL-10 shRNA mice compared with control shRNA mice, as judged by quantification of human CD82 expression by FACS (PBMCs: control shRNA, 33%; IL-10 shRNA, 15%) (BM: control shRNA, 71%; IL-10 shRNA, 29%) (Fig. 5b). Moreover, the treatment of either control shRNA or IL-10 shRNA mice with AraC decreased leukemia cell numbers in both peripheral circulation and BM (PBMCs: control shRNA, 2%; IL-10 shRNA, 3%) (BM: control shRNA, 14%; IL-10 shRNA, 6%) (Fig. 5b). All of control shRNA and IL-10 shRNA mice died because of leukemia by day 28 of transplantation. Fewer MOLM13 leukemia cells were noted in IL-10 shRNA mice treated with AraC than control shRNA mice treated with AraC in both peripheral circulation and BM (PBMCs: control shRNA, 17%; IL-10 shRNA, 7%) (BM: control shRNA, 46%; IL-10 shRNA, 11%) (Fig. 5c). Simultaneously, we measured the levels of IL-10, DNMT3A, and E-cadherin in MOLM13 cells isolated from BM of control shRNA and IL-10 shRNA mice on day 18 of transplantation to examine the effect of depletion of IL-10 by an shRNA on expression of these genes in vivo. Real-time RT-PCR found that IL-10 was effectively downregulated in MOLM13 cells removed from IL-10 shRNA mice in parallel with a decrease in levels of DNMT3A and E-cadherin (Fig. 5d). Real-time RT-PCR using murine plasma found that expression of miR-9 was significantly elevated in plasma isolated from IL-10 shRNA mice compared with that in plasma removed from control shRNA mice (Fig. 5e).

**Discussion**

The present study showed that AML cells aberrantly express immunosuppressive cytokine IL-10 and an adhesion molecule E-cadherin. Real-time RT-PCR analyses revealed that AML cells also express receptors for IL-10 (data not shown).
Because of the paucity of the sample number (n = 15), we were not able to identify the specific leukemia subtype that highly expresses these molecules. Also, close correlation between IL-10 and E-cadherin expression in AML cells was not noted (data not shown); nevertheless, exposing AML cells to IL-10 significantly increased levels of E-cadherin (Fig. 1a), suggesting the critical roles of IL-10 in regulation of E-cadherin expression. We previously showed that IL-10 activates STAT5 that binds to the promoter region of the IL-10 gene, resulting in IL-10 production in AML cells. Interleukin-10 produced by AML cells may support their survival by an autocrine mechanism. We also showed that STAT5A bound to the promoter region of DNMT3A gene and modulated the levels of DNMT3A in AML cells. As we found that IL-10 elevated the expression of DNMT3A in AML cells, we strongly postulated that IL-10-induced hypermethylation and downregulation of miR-9 in AML was most likely due to DNMT3A activity. Downregulation of DNMT3A by siRNAs resulted in IL-10 production in AML cells. Interleukin-10 may be a promising molecular target for eradicating AML cells.

The frequency of aberrant DNA methylation among AML patients was 13–69% for E-cadherin. This pattern was markedly different between each patient. As the treatment of IL-10 increased the levels of DNMT3A in leukemia cells, upregulation of DNMT3A could induce the methylation of the E-cadherin promoter region, resulting in suppression of E-cadherin expression. Contrary to our expectation, downregulation of DNMT3A decreased the levels of E-cadherin in leukemia cells. Interleukin-10 may also cause downregulation of miR-9 in AML cells. Another therapeutic strategy targeting the CD82/STAT5/IL10 axis: the use of an antibody against CD82 mobilized the AML cells into peripheral circulation and potentiated the cytotoxic effects of AraC and significantly prolonged the survival of human AML bearing mice.

Previous findings showed that ectopic overexpression of miR-9 inhibited the proliferative, migratory, and invasive capacities of nasopharyngeal carcinoma cells in vitro and in vivo. Overexpression of miR-9 reduced the levels of CXCR4 by binding to the 3′-UTR of CXCR4 in nasopharyngeal carcinoma cells. Interaction between HSCs and stromal cells is regulated by CXCR4/SDF-1. The CXCR4 antagonist AMD3100 mobilizes leukemia cells into the peripheral circulation, which were then sensitized to the in vivo effects of cytotoxic chemotherapy. Thus, miR-9 could induce the migration of leukemia cells through downregulation of CXCR4.

CD82 inhibits p38 MAPK and increases expression of EZH2, the polycomb group member in AML cells. The elevated expression of EZH2 was associated with histone H3 lysine 27 trimethylation in the promoter region of tumor suppressor genes such as PTEN and their downregulation. Activation of EZH2 may also cause downregulation of miR-9 in AML cells.

Delodigier et al. (2017) reported that the frequency of aberrant DNA methylation among AML patients was 13–69% for E-cadherin.
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**Supporting Information**

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

**Fig. S1.** Fluorescence-activated cell sorting of leukemia cells.

**Fig. S2.** Interleukin-10 (IL-10) and E-cadherin mRNA expression by real-time RT-PCR. **P < 0.01.**