Stromal cells expressing hedgehog-interacting protein regulate the proliferation of myeloid neoplasms

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Aberrant reactivation of hedgehog (Hh) signaling has been described in a wide variety of human cancers including cancer stem cells. However, involvement of the Hh-signaling system in the bone marrow (BM) microenvironment during the development of myeloid neoplasms is unknown. In this study, we assessed the expression of Hh-related genes in primary human CD34+ cells, CD34+ blast cells and BM stromal cells. Both Indian Hh (Ihh) and its signal transducer, smoothened (SMO), were expressed in CD34+ acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS)-derived cells. However, Ihh expression was relatively low in BM stromal cells. Remarkably, expression of the intrinsic Hh-signaling inhibitor, human Hh-interacting protein (HHIP) in AML/MDS-derived stromal cells was markedly lower than in healthy donor-derived stromal cells. Moreover, HHIP expression levels in BM stromal cells highly correlated with their supporting activity for SMO+ leukemic cells. Knockdown of HHIP gene in stromal cells increased their supporting activity although control cells marginally supported SMO+ leukemic cell proliferation. The demethylating agent, 5-aza-2'-deoxycytidine rescued HHIP expression via demethylation of HHIP gene and reduced the leukemic cell-supporting activity of AML/MDS-derived stromal cells. This indicates that suppression of stromal HHIP could be associated with the proliferation of AML/MDS cells.

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comparable to the Ptc receptor, functions as an endogenous Hh ligand inhibitor.\textsuperscript{22,23}

Importantly, reduced expression of the human HIP (HHIP) gene was observed in several tumor cells.\textsuperscript{24,25} and may represent a mechanism for the induction of ectopic Hh signaling. However, whether aberrant expression of HHIP levels in BM can contribute to the development of myeloid malignancy is unknown.

Here, we examined the expression of Ihh, SMO and HHIP in primary CD34\textsuperscript{+} hematopoietic cells, acute leukemia cells and BM stromal cells, the major stromal growth factors required for the expansion of hematopoietic stem/progenitor cells, and the role of HHIP on stromal function in the regulation and inhibition of leukemic cell proliferation.

MATERIALS AND METHODS
Reagents and cell lines
Recombinant murine HIP (mHIP) was obtained from R&D Systems (Minneapolis, MN, USA). S-Aza-2′-deoxycytidine (S-aza-dC) was obtained from Sigma (St Louis, MO, USA). Total RNA derived from mobilized peripheral blood (PB), BM or CB CD34\textsuperscript{+} cells was purchased from AllCells, LLC (Toronto, Ontario, Canada). Human myeloid leukemia cell lines K562, HEL and KG-1 were cultured in RPMI1640 containing 10% heat-inactivated fetal calf serum (Gibco BRL, Rockville, MD, USA), 2 mM/L-glutamine, 0.1% penicillin (100 U/ml) and streptomycin (100 mg/ml). CD34\textsuperscript{+} leukemic cell lines, such as Kasumi-1, Kasumi-3 and TF-1 (American Type Culture Collection, Manassa, VA, USA), were cultured in RPMI1640 containing 20% heat-inactivated fetal calf serum, 2 mM/L-glutamine, 1 mu pyruvate, 0.1% penicillin (100 U/ml) and streptomycin (100 mg/ml). For long-term culture of TF-1, 1014/ml interleukin-3 (IL-3, R&D systems) was added to the complete medium. In some experiments, HEL, Kasumi-1, Kasumi-3 and TF-1 were cocultured with human stromal cells in StemPro-34 serum-free medium (Life Technologies, Tokyo, Japan).

Primary human stromal cells, hTERT-stromal cells (HTS) and clones Human BM was obtained by aspiration from the posterior iliac crest of healthy adult volunteers (age from 30 to 40), AML and MDS patients after informed consent. Informed consent from the Sapporo Medical University institutional review board was provided according to the Declaration of Helsinki. Human primary stromal cells were prepared as described previously.\textsuperscript{26} Only proliferative cells within 10 passages were used in this study and senescent stromal cells were excluded for analyses. The HTS and the HTS clones were established by retrovirus-mediated gene transfer of the human telomerase catalytic subunit (hTERT) gene as described previously.\textsuperscript{26,27}

Separation of primary CD34\textsuperscript{+} leukemic cells and CD271\textsuperscript{+}CD45\textsuperscript{−} mesenchymal stromal cells
Primary CD34\textsuperscript{+} cells or CD271\textsuperscript{+}CD45\textsuperscript{−} mesenchymal stromal cells were obtained from patients with AML, MDS or lymphoma (stage I or II) as a control sample by BM aspiration after obtaining informed consent. Low-density (< 1.077 g/ml) mononuclear cells (MNCs) were separated by Histopaque-1077 (Sigma-Aldrich, Tokyo, Japan). Purification of CD34\textsuperscript{+} cells was by positive selection using MACS Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotech, Bergish-Gladbach, Germany). For purification of CD271\textsuperscript{+}CD45\textsuperscript{−} mesenchymal stromal cells, BM MNCs were labeled with phycoerythrin (PE)-conjugated CD45 antibody and CD45\textsuperscript{−} cells were separated by negative selection using anti-PE microbeads as previously described.\textsuperscript{28} Subsequently, the CD271\textsuperscript{+} fraction was purified by CD271 Micro Bead Kit (Miltenyi Biotech, Bergish-Gladbach, Germany) (allophycocyanin (APC)).

Coculture of CD34\textsuperscript{+} leukemic cells or primary CB CD34\textsuperscript{+} cells with human stromal cells
Two hundred thousand parental populations of primary human stromal cells, HTS or HTS clones were plated in 25 cm\textsuperscript{2} plates in long-term culture medium.\textsuperscript{29} Forty-eight hours after plating, cells were washed five times with phosphate-buffered saline (PBS) before addition of leukemic cell lines, CB CD34\textsuperscript{+} cells (Takara, Tokyo, Japan) or primary CD34\textsuperscript{+} leukemic cells. Twenty thousand primary CD34\textsuperscript{+} leukemic cells or CB CD34\textsuperscript{+} cells were seeded on a monolayer of human stromal cells pre-established in 10 ml of StemPro-34 serum-free medium (Life Technologies), supplemented with 10 ng/ml human stem cell factor (SCF, R&D Systems). The coculture was maintained by adding the same amount of SCF every week for 4 weeks. At the end of 4 weeks of coculture, hematopoietic cells that had expanded above the stromal cells were collected as the cobblestone area formation underneath the stromal layer was not observed in this culture.\textsuperscript{26} Adherent hematopoietic cells on the stromal layer were removed with PBS, and dissociated hematopoietic cells were mixed with nonadherent hematopoietic cells. Stromal layers were treated with two further cycles of pipetting. To avoid the presence of stromal cells in the resulting cell suspensions, a 30-min adhesion procedure was performed at 37 °C as described previously.\textsuperscript{26,29}

Clonogenic assay and replating assay of primary hematopoietic or leukemic cells
The clonogenic assay of primary hematopoietic cells was performed using MethoCult GF H4434V (Stem Cell Technologies, Vancouver, British Columbia, Canada). For the replating assay, 2 × 10\textsuperscript{4} CD34\textsuperscript{+} leukemic cells per plate were placed into methylcellulose (MethoCult GF H4434V) in the presence or absence of 100 μg/ml mHIP. For serial replating, cells were harvested from the methylcellulose, and 2 × 10\textsuperscript{4} cells per plate were replated at 7-day intervals.\textsuperscript{26} The total numbers of colony-forming units in culture (CFU-C) and leukemia colony-forming cells were determined by microscopy.

Analysis of Ihh and related gene mRNA expression
For reverse transcription (RT) reactions, total RNA was prepared from cells using the QIAGEN RNeasy kit (Chatsworth, CA, USA). Total RNA (1 μg) was reverse transcribed by SuperScript II (Invitrogen, Tokyo, Japan). For low numbers of separated primary cells, the RT reaction was directly conducted using TaqMan Gene Expression Cells-to-CT Kit (Applied Biosystems, Tokyo, Japan). PCR was performed using the Advantage GC 2 Polymerase Mix (Clontech, Tokyo, Japan) with primers specific for Ihh (5′-TGCAG GCCGGGTCGGGTGGTG-3′ and 5′-GCCGGGCTCTTGCCTGTC-3′), Ptc (5′-CTTTGGCAGATAGGTTGACC-3′ and 5′-CTGTCGGCTGTGATTGCGG-3′), SMO (5′-CAGAACATCAATGTCACAGGACCGG-3′ and 5′-ATA GTTGGAGACAAAAACACCCACCC-3′) or human glycolaldehyde 3-phosphate dehydrogenase (GAPDH) (5′-ACATCAAGAATGTGAATGGCAGGCGG-3′ and 5′-CTCTCCCTCTGGTCTCTGCTG-3′) as previously reported.\textsuperscript{11} PCR amplification was performed with 30 cycles of 94 °C for 15 s, 58 °C for 30 s and 72 °C for 1 min. The PCR products were separated on a 2% agarose gel. For real-time RT-PCR analyses, Taqman Assays IDs of Ihh, Hipp, GAPDH, or 18S (Applied Biosystems, Tokyo, Japan) were Hs00745531_s1, Hs00368450_m1, Hs9999905_m1 or Hs99999901_s1, respectively. The other Taqman Assays IDs are shown in supplementary Table 1. Real-time PCR was performed in triplicate using the Taqman PRISM 7700 Sequence Detection System (PE Applied Biosystems) in a 50-μl reaction volume. Relative gene expression was calculated as the signal ratio of target gene (FAM) to GAPDH cDNA.

Phenotypic characterization of human stromal cells
The phenotype of human primary stromal cells, HTS and HTS clones were determined by analyzing the expression of α-smooth muscle actin (α-SMA) and ALP (alkaline phosphatase). PE-conjugated CD105 (Ancell, Bayport, MN, USA) or CD166 (BD Bioscience, Tokyo, Japan), fluorescein isothiocyanate (FITC)-conjugated anti-α-SMA (Clone 1A4, Sigma), CD31 (BD Bioscience), CD14, CD45 (BD Bioscience) monoclonal antibodies (mAb) or isotype controls (Chemicon, Temecula, CA, USA) were used. For flow cytometric analysis of α-SMA, stromal cells were washed in PBS three times and fixed with 3.7% (v/v) formaldehyde in PBS at 4 °C for 60 min. Cells were permeabilized with Perm buffer I containing saponin (BD Bioscience) in PBS at 4 °C for 30 min with FITC-conjugate anti-α-SMA and anti-CD105 PE or isotype control (Chemicon). Labeled cells were analyzed by flow cytometry (FACSCalibur or FACSCanTo: Becton Dickinson, Mountain View, CA, USA) and dead cells were gated out by propidium iodide (PI) staining.

Drug cytotoxic assay
To assess the contribution of Hh signaling on cells, 0–100 μg/ml mHIP was added to each well and incubated for 48 h. The surviving cells were assessed by Annexin V-FITC Apoptosis Detection Kit (Medical and Biological Laboratories, Tokyo, Japan) and Premix WST-1 assay Cell Proliferation Assay System (Takara). The WST-1 assay is based on the
mitochondrial conversion of WST-1 to yellowish formazan, being indicative of the number of viable cells. The number of viable cells was evaluated by absorbance at OD450 nm (Abs) using a Model 680 microplate reader (Bio-Rad Laboratories, Tokyo, Japan).

Determination of cell cycle
Cell cycle analysis was performed by staining with equal volumes of 2 mg/ml RNase A in PBS and 0.6% NP40 containing 0.1 mg/ml PI (Calbiochem, La Jolla, CA, USA) in PBS at 4°C for 30 min. Thereafter, cell cycle distribution was analyzed by flow cytometry. Doublet particles were gated out by plotting FL2-W versus FL2-A in a dot plot as previously described.

Transduction of short hairpin RNA (shRNA) against HHIP in stromal cells Gene-specific shRNA vector of HuSh29mer shRNA construct against HHIP, catalog number TR304118 (tube ID, TI316456; TI316446; TI316468) was purchased from OriGene Inc. (Rockville, MD, USA) and TR20003 was utilized as a negative control. Retroviral supernatant was produced from phoenix-AMPHO cells (American Type Culture Collection) after transfection with purified plasmid DNA (Qiagen, Tokyo, Japan) in Lipofectamine 2000 transfection reagent (Life Technologies). The viral supernatants containing HHIP shRNA, TR20003 (control: shRNA empty pRS vector), TI1316465, TI131666 or TI1316468 were used to infect HTS-5, HTS-6, HTERT-stromal cell clones or primary stromal cells. After gene transduction of TR20003, TI316465, TI316466 or TI316468, transduced stromal cells (Cont-HTS-5, TI 65-HTS-5, TI 66-HTS-5 or TI 68-HTS-5; Cont-HTS-6, TI 65-HTS-6, TI 66-HTS-6 or TI 68-HTS-6) were selected with 3 μg/ml puromycin. The levels of mRNA of HHIP after transduction of shRNA were analyzed by real-time RT-PCR.

Bisulfite modification and methylation-specific PCR (MSP)
Bisulfite modification of genomic DNA (0.5 μg) was performed using Methylamp Universal Methylated DNA kit according to manufacturer’s instructions (Epigentek Group Inc., Brooklyn, NY, USA). Bisulfite modification of genomic DNA and MSP analysis were performed as described previously, using primers corresponding to the HHIP promoter region sequences. HHIP methylation primer pair (5’-AAGATCTTTGAGGACTT GAGCCTC-3’ and 5’-GGCGAGGGTTCTCTCCTGCCCCGTT-3’, product 299 size) and unmethylation primer pair (5’-AAGATTGGTGGAGATTGTGTTT-3’ and 5’-CACAAACTCCCTCTCCTGCCACTT-3’, product 299 size) were used. PCR amplification was performed using 30 cycles of 94°C for 30 s, 58°C for 30 s and 68°C for 40 s for methylated DNA and using 30 cycles of 94°C for 30 s, 53°C for 30 s and 68°C for 40 s for unmethylated DNA.

5-aza-dC treatment of human stromal cells
Human stromal cells were treated with 2 μM of 5-aza-dC (Sigma) on day – 6 and day – 3 before coculture with hematopoietic cells. Stromal cells were washed with 1 x PBS three times, and then used for coculture with leukemic cells.

Immunoblot analysis
Ten microliter of supernatant or 50 μg of lysate from stromal cells was subjected to electrophoresis on a 10% SDS-polyacrylamide gel and transferred to PVDF membranes using semidry transfer apparatus (Bio-Rad Laboratories). Cell lysate was prepared in a buffer containing 50 mM Tris-HCl (pH 7.4), 0.5% NP40, 150 mM NaCl and a protease inhibitor mixture (Roche Diagnostics, Tokyo, Japan). Anti-HHIP mAb (ab56406: Abcam Inc., Tokyo, Japan) and anti-β-actin mAb (Zymed Laboratories Inc, South San Francisco, CA, USA) were utilized as primary Abs. Proteins were visualized using the enhanced chemiluminescence method (GE Healthcare Life Sciences, Tokyo, Japan).

Statistical analysis
Each data set was first evaluated for normality of distribution by the Komolgorov-Smirnov test to decide whether a non-parametric rank-based analysis or a parametric analysis should be used. Two groups were compared by either the Wilcoxon signed-rank test or the Student’s t-test (two-tailed test). Results are expressed as the mean ± s.d.

RESULTS
The expression level of various factors in stromal cells and their supporting activity of CB clonogenic cells
We first analyzed the mRNA expression of various soluble growth factors, such as Ihh, SCF, thrombopoietin (TPO), FLT3-ligand (FL), interleukin-6 (IL-6), angiopoietin-1 (Ang-1), bone morphogenic protein 2 (BMP-2), WntsA and Hh in nine stromal clones (HTS-1 to HTS-9 cells) and the number of CFU-C derived from CB CD34+ cells after coculture with HTS stromal clones. However, expression of the soluble factors by stromal cells did not correlate with the supporting activity of CB-derived CFU-C (Figure 1a). Unexpectedly, endogenous HHIP expression in BM stromal cell clones negatively correlated with the number of CB CFU-C after coculture (Figure 1a). We previously demonstrated that Ihh and signal transducer SMO were highly expressed in CB CD34+ cells and CD34+ leukemic cell lines. Recombinant mHIP is highly homologs with HHIP and mHIP can inhibit human Ihh activity. Additio, Recombinant mHIP into the culture of CB CD34+ cells and human stromal cells, significantly reduced the number of clonogenic cells (Figure 1b). Thus, we compared the expression levels of Ihh and HHIP in hematopoietic cells and stromal cells to understand the inhibitory mechanism of Hh signaling in the BM microenvironment. As shown in Figure 1c, Ihh expression in PB, BM or CB CD34+ cells was markedly higher than in primary human stromal cells and HTS. In contrast, HHIP expression in the stromal cell population was dramatically higher than in PB, BM or CB CD34+ cells (Figure 1d), suggesting that HHIP expressed by stromal cells inhibited Ihh signaling in hematopoietic cells when the two cell types were present in the BM microenvironment.

Expression of Hh-related genes and the effect of recombinant mHIP on leukemic cells
We investigated whether recombinant mHIP could affect the proliferation of leukemic cells by examining the expression of Ihh and SMO in leukemic cell lines. Ihh was detected in all the leukemic cell lines examined (Figure 2a). However, the receptor component, SMO, was only detected in four out of six CD34+ leukemic cell lines or erythroleukemic cell lines (Kasumi-1, Kasumi-3, TF-1 and HEL). We next investigated the CD34+ fraction of BM cells derived from AML and MDS patients (Table 1). Ihh and SMO expression were detected in primary AML cells and MDS cells (Figure 2b). The significance of Ihh signaling in leukemic cells was examined by the addition of recombinant mHIP to the supernatant of leukemic cell lines in stroma-free conditions. As a result, the percentage survival of SMO+ leukemic cells was significantly decreased in a dose-dependent manner, although the percentage survival of SMO negative leukemic cells was not affected by mHIP (Figure 2c). Annexin V/PI assay revealed that mHIP-induced apoptosis in SMO+ leukemic cell lines (Figure 2d). We further assessed the effect of mHIP on primary leukemic cells. The replicating capacity of CD34+ fraction of leukemic cells derived from three AML patients (Table 1) was reduced by mHIP treatment and the effect of mHIP on leukemic colony numbers was pronounced in second replating (Figure 2e). This suggests that Hh signaling contributes to the proliferation and self-renewal capacity of leukemic cells in vitro.

The phenotype and expression of HHIP in stromal cells derived from AML/MDS
To investigate the HHIP expression in hematological disorders, we established stromal cells derived from AML or MDS patients. The stromal cell phenotype was determined by flow cytometric analysis. All stromal cells expressed the stromal antigen markers CD105 and CD166 (Figures 3a and b), but did not express CD31, CD14 or CD45, markers of either endothelial cells (Figure 3b) or stromal macrophages (Figure 3c). Most AML stromal (AML st) cells expressed cytoplasmic α-SMA (Figure 3a), a marker of

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Primary and telomerized BM stromal cells. (a) Correlation between the number of CFU-C and various soluble factors expressed by HTS clones. A significant reverse correlation between the number of CFU-C and expression level of HHIP in BM stromal cell clones was observed. X axis indicates the ratio of target mRNA to GAPDH mRNA in HTS clones by real-time PCR. (b) The effect of recombinant mHIP on the proliferation of clonogenic cells upon coculture with an hTERT-stromal clone 6 (HTS-6). The coculture was supplemented with 10 ng/ml SCF with or without mHIP. X axis indicates the concentration of mHIP and Y axis indicates the number of CFU-C. Data represent three independent experiments, performed in triplicate. Results are expressed as means ± s.d. (c) Endogenous Ihh expression levels in PB, BM or CB CD34+ cells, primary stromal cells or human hTERT-stromal cells were analyzed by real-time PCR. (d) Endogenous HHIP expression levels were analyzed by real-time PCR. The expression level of GAPDH was used as an internal standard.

Figure 1. The expression of Ihh and HHIP in CD34+ hematopoietic cells and primary and telomerized BM stromal cells. (a) Correlation between the number of CFU-C and various soluble factors expressed by HTS clones. A significant reverse correlation between the number of CFU-C and expression level of HHIP in BM stromal cell clones was observed. X axis indicates the ratio of target mRNA to GAPDH mRNA in HTS clones by real-time PCR. (b) The effect of recombinant mHIP on the proliferation of clonogenic cells upon coculture with an hTERT-stromal clone 6 (HTS-6). The coculture was supplemented with 10 ng/ml SCF with or without mHIP. X axis indicates the concentration of mHIP and Y axis indicates the number of CFU-C. Data represent three independent experiments, performed in triplicate. Results are expressed as means ± s.d. (c) Endogenous Ihh expression levels in PB, BM or CB CD34+ cells, primary stromal cells or human hTERT-stromal cells were analyzed by real-time PCR. (d) Endogenous HHIP expression levels were analyzed by real-time PCR. The expression level of GAPDH was used as an internal standard.

myofibroblastic stromal cells although an α-SMA low-expressing population was also detected in MDS stroma (MDS st)-8 and MDS st-9 cells. We next examined the expression level of HHIP in patients BM stromal cells by real-time PCR. Interestingly, both AML and MDS-derived stromal cells expressed significantly low levels of HHIP compared with healthy volunteer-derived stromal cells or HTS as a human stromal cell line (Figure 3d). Recently, it was demonstrated that primary human BM mesenchymal stromal cells were highly enriched in the CD271+/CD45- population was also detected in MDS stromal (MDS st)-8 and MDS st-9 cells. We next examined the expression level of HHIP in nine HTS clones by real-time PCR. (Supplementary Figure 1b).27 HHIP expression in nine HTS clones was analyzed by real-time PCR. (Supplementary Figure 1a). Regarding the phenotype, HTS-5 is composed of fibroblastic osteoblastic stromal cells and HTS-6 is composed of fibroblastic stromal cells as previously reported (Supplementary Figure 1b).27 The expression of Ihh and HHIP in CD34+ hematopoietic cells and primary and telomerized BM stromal cells. (a) Correlation between the number of CFU-C and various soluble factors expressed by HTS clones. A significant reverse correlation between the number of CFU-C and expression level of HHIP in BM stromal cell clones was observed. X axis indicates the ratio of target mRNA to GAPDH mRNA in HTS clones by real-time PCR. (b) The effect of recombinant mHIP on the proliferation of clonogenic cells upon coculture with an hTERT-stromal clone 6 (HTS-6). The coculture was supplemented with 10 ng/ml SCF with or without mHIP. X axis indicates the concentration of mHIP and Y axis indicates the number of CFU-C. Data represent three independent experiments, performed in triplicate. Results are expressed as means ± s.d. (c) Endogenous Ihh expression levels in PB, BM or CB CD34+ cells, primary stromal cells or human hTERT-stromal cells were analyzed by real-time PCR. (d) Endogenous HHIP expression levels were analyzed by real-time PCR. The expression level of GAPDH was used as an internal standard.

Leukemic cell-supporting activity of BM-derived stromal clones

We next elucidated the relationship between expression levels of stromal HHIP and its leukemic cell-supporting activity. The stromal HHIP expression in nine HTS clones was analyzed by real-time PCR (Supplementary Figure 1a). HTS clones, SMO+ leukemic cell lines Kasumi-1, Kasumi-3, HEL and TF-1, and SMO+ leukemic cell lines KG-1 and KS62 were cocultured for 4 weeks in serum-free medium to exclude any effects of serum-derived factors. The number of proliferating leukemic cells was assessed. Stromal HHIP expression negatively correlated with the number of SMO+ leukemic cells after coculture although no correlation was observed in SMO- leukemic cell lines, suggesting stromal HHIP is a negative regulator of SMO+ leukemic cell proliferation (Figure 4a). Further detailed analysis was undertaken using the HTS-5 cell line, which expressed the highest level of HHIP and HTS-6, which expressed the lowest level of HHIP, among nine human stromal clones (Supplementary Figure 1a). Regarding the phenotype, HTS-5 is composed of osteoblastic stromal cells and HTS-6 is composed of fibroblastic stromal cells as previously reported (Supplementary Figure 1b).27 HTS-5 (HHIP-high) did not support proliferation of SMO+ leukemic cells (Figure 4b). In contrast, HTS-6 (HHIP-low) readily supported the proliferation of SMO+ leukemic cells (Figure 4b) although these SMO+ leukemic cells did not proliferate without stromal cells in serum-free medium (Figure 4b). Regarding SMO- leukemic cell lines, both KS62 and KG-1 readily proliferated on HTS-5 and HTS-6. We then analyzed the potential mechanism of leukemic cell-supporting activity of the two different stromal clones. Annexin V/PI analysis of leukemic and stromal cell cocultures revealed that HTS-5 cells induced higher apoptosis in SMO+ leukemic cell lines such as HEL and TF-1 than HTS-6, although no major differences were observed for Kasumi-1 and Kasumi-3, suggesting that stromal HHIP correlated with apoptosis
were reported to possess differentiation potential. The proliferation of SMO cocultured leukemic cells revealed high levels of cell cycle entry in some SMO+ leukemic cell lines (Figure 4c). Cell cycle analysis of cocultured leukemic cells revealed high levels of cell cycle entry in SMO+ leukemic cells, but not SMO− cells, in coculture with HTS-6 (HHIP-low) that was significantly higher than with HTS-5 (HHIP-high) (P < 0.05), suggesting that HHIP contributed to cell cycle entry suppression in SMO+ leukemic cells (Figure 4d). We further evaluated the possibility of whether stromal HHIP affected differentiation of SMO+ leukemic cells 4 weeks after coculture with HHIP-high stromal cells (HTS-5) as these SMO+ leukemic cells were reported to possess differentiation potential. The expression of lineage makers was not significantly different after coculture (Supplementary Figure 2), suggesting stromal HHIP did not affect differentiation of SMO− leukemic cell lines. Collectively, these results suggested that stromal HHIP could suppress the proliferation of SMO− leukemic cells.

shRNA-mediated inhibition of the HHIP gene on the leukemic cell-supporting activity of stromal cells

To confirm that stromal HHIP is critical for the leukemic cell-supporting activity of BM stromal cells, we analyzed the effect of shRNA-mediated inhibition of stromal HHIP before coculture. HHIP shRNA expression vector or control vector (shRNA empty pRS vector) were transduced into HTS-5 and the following HHIP shRNA-transfected or control vector-transfected cell lines were established: TI 65-HTS-5, TI 66-HTS-5, TI 68-HTS-5 and Cont-HTS-5. Although HHIP expression levels were significantly decreased in all three HHIP shRNA-transduced stromal cell lines, the strongest reduction was observed in TI 66-HTS-5 (Figure 5a). Subsequently, we conducted the coculture experiment in serum-free medium over 4 weeks using SMO+ leukemic cell lines, Kasumi-1, Kasumi-3, HEL and TF-1 and evaluated the number of proliferative cells.
Table 1. Karyotype of BM cells and clinical outcome in AML and MDS patients

| Case | Age | Sex | WHO/FAB classification | Karyotype of neoplasm | Karyotype of stromal cells |
|------|-----|-----|------------------------|-----------------------|---------------------------|
| 1    | 43  | M   | AML (M1)               | 46, XY                | 46, XY                    |
| 2    | 44  | F   | AML (M2)               | 46, XX                | 46, XX                    |
| 3    | 77  | M   | AML (M5)               | 46, XY, del(11)(q14q23) | 46, XY                    |
| 4    | 70  | M   | AML/MLD                | 46, XY                | 46, XY                    |
| 5    | 79  | F   | AML/MLD                | 46, XX                | 46, XX                    |
| 6    | 80  | M   | AML (M4)               | 46, XY                | 46, XY                    |
| 7    | 63  | M   | RAEB-2                 | 45, XY, der(7;22)(p10q10) | 46, XY                    |
| 8    | 64  | M   | RAEB-2                 | 50, XY, complex       | 46, XY                    |
| 9    | 76  | F   | RA low                 | 46, XX                | 46, XX                    |
| 10   | 72  | F   | RA int-1               | 46, XX                | 46, XX                    |
| 11   | 75  | F   | RA int-2               | 46, XX, del(20)(q11q13) | 46, XX                    |

Abbreviations: AML, acute myeloid leukemia; BM, bone marrow; F, female; FAB, French-American-British; M, male; MDS, myelodysplastic syndrome; MLD, multilineage dysplasia; RA, refractory anemia; RAEB-2, refractory anemia with excess blast-2; WHO, world health organization; 50, XY, complex; 50, XY, + Y, der (1) add (1)(p11) add (1)(q32), del (5)(q15q33), add(6)(p23), /C0 7, add (7)(p22), + del (8)(q24), + del (10)(q24), + 11, + 13, + 14, add(16)(q13), − 17, + mar; AML/MLD: AML with MLD.

Figure 3. Immunophenotypical analysis and HHIP expression of primary stromal cells derived from AML/MDS patients. (a) Immunophenotypical analysis of stromal cells derived from AML/MDS patients. X axis indicates α-SMA-FITC and Y axis indicates CD105-PE. (b) Additional immunophenotypical analysis of CD31-FITC and CD166-PE (ALCAM) antibodies. Data shown are representative of one experiment of three showing similar results. (c) Immunophenotypical analysis of CD45-FITC and CD14-PE antibodies. (d) HHIP expression in BM stromal cells was analyzed by real-time PCR. Lane number 1–11 indicates each patient number. Lane 12 and 13 indicate HHIP expression in primary BM stromal cells derived from different healthy volunteers. Lane 14 indicates hTERT-stromal cells (around 30 population doubling after hTERT gene transfer). Y axis indicates the ratio of HHIP to GAPDH mRNA. (e) HHIP expression in primary uncultured CD271+ CD45− cells before selection was analyzed by CD271-APC/CD45-PE (upper panel). Cell debris and dead cells were excluded from the analysis based on scatter signals and PI fluorescence. BM mononuclear cells were negatively selected by CD45-PE/anti-PE microbeads (lower left panel) and positively selected by CD271-APC/anti-APC microbeads (lower right panel) when sufficient numbers of BM MNCs were obtained (number 1, 3, 6, 9 and 10). Purified CD271+ CD45− cells were directly transcribed and HHIP expression was analyzed by real-time PCR. BM MNCs obtained from lymphoma patients without BM involvement were used as a control for BM cells (number 15 and 16).
above the stromal layer. SMO- leukemic cells did not proliferate in serum-free medium without stromal cells present, and only modestly in the presence of control vector-transfected Cont-HTS-5 cells (Figure 5b). In contrast, SMO+ leukemic cells proliferated significantly when cocultured with HHIP shRNA-transduced stromal cells. This indicated that stromal HHIP is an important suppressor of leukemic cell proliferation and that reduced levels of HHIP in human stromal cells may allow the uncontrolled proliferation of SMO+ leukemic cells.

Promoter methylation of HHIP-low expressing stromal cells and the effect of demethylating regent
In the present study, a reduction in stromal HHIP expression was associated with the acquisition of leukemic cell-supporting activity in stromal cells. However, the mechanism of HHIP reduction is not clear. We investigated the methylation status of the HHIP promoter region, as a CpG island was previously described in the HHIP promoter. In addition, we investigated the effect of a demethylating regent, 5-aza-dC, on stromal supporting activity. For this experiment, HHIP-low HTS-6, +SMA+ primary AML stromal cell 1 (AML st-1) and +SMA+/ALP+ MDS st-9 were used (Figure 6a), because these two primary AML/MDS-derived stromal cells could be readily propagated. First, we assessed the expression of HHIP mRNA in BM stromal cells by real-time RT-PCR before or after 5-aza-dC treatment. An increase in HHIP mRNA expression in all stromal cells was observed following 5-aza-dC treatment (Figures 6a and i). Methylation of the promoter region of the HHIP gene in these three stromal cell lines was detected by the bisulphite modification of genomic DNA and MSP analysis before 5-aza-dC treatment. HHIP gene expression was restored following 5-aza-dC treatment (Figures 6a and ii). Methylation of the HHIP promoter was observed in other HHIP-reduced stromal cells, including telomerized stromal clones and other...
AML/MDS-derived stromal cells (Supplementary Figure 3). Quantitative analysis of HHIP and major hematopoietic growth factors by real-time PCR demonstrated no significant changes in expression levels of SCF, TPO and FL (Figure 6b), consistent with previous reports showing that promoter methylation of CpG islands were not observed in mesenchymal stromal cells.37 In addition, the
expression of other cytokines including IL-6, Angiopoietin-1, Ihh and Wnt5A were unchanged in stromal cells after 5-aza-dC treatment (data not shown). These results suggested that the promoter methylation of HHIP partly contributed to the HHIP reduction observed in BM stromal cells without a change in the expression of cytokines involved in stem/progenitor cell support.

Reduction of leukemia cell-supporting activity of primary leukemia-associated stromal cells after HHIP reactivation

We examined whether leukemia cell-supporting activity could be reduced by reactivation of HHIP in HHIP-low expressing stromal cells. HHIP-low-expressing stromal cells, HTS-6, primary AML st-1 and MDS st-9 were used. These stromal cells were treated twice with 2 μM 5-aza-dC on day −3 and −6 before coculture with leukemia cells. We also conducted shRNA-mediated inhibition of HHIP mRNA levels in pre-5-aza-dC-treated HTS-6 cells. Real-time PCR and immunoblot analysis revealed that HHIP expression was increased in HTS-6 after pretreatment with 5-aza-dC, whereas the elevation of HHIP mRNA was reduced in HHIP shRNA-transduced HTS-6 (Figure 7a). Subsequently, we examined the proliferation of leukemic cells after 4 weeks coculture with 5-aza-dC pretreated HTS-6 and 5-aza-dC pretreated HHIP shRNA-transduced HTS-6 in serum-free medium. The growth of SMO⁺ leukemia cell lines, Kasumi-1, Kasumi-3, HEL and TF-1 were cocultured with HTS-6 or 5-aza-dC-pretreated HTS-6 and HHIP knockdown Ti66-HTS-6 in serum-free medium for 4 weeks. *P < 0.05, HTS-6 vs 5-aza-dC-pretreated HTS-6. *P < 0.05, 5-aza-dC-pretreated HTS-6 vs 5-aza-dC-pretreated Ti66-HTS-6. ‘Pre-5-aza-dC’ indicates coculture with 5-aza-dC-pretreated stromal cells, ‘HHIP shRNA (−)’ indicates coculture with Ti66-HTS-6 and ‘HHIP shRNA (−)’ indicates coculture with mock-transfected Cont-HTS-6. (c) The replating capacity of CD34⁺ fraction of primary leukemia cells cocultured with AML/MDS-derived stromal cells was analyzed. The primary CD34⁺ leukemic cells derived from patient ID no. 1, 3 and 6 was cocultured with (i) primary AML st-1 and 5-aza-dC pretreated AML st-1 cells or (ii) primary MDS st-9 and 5-aza-dC-pretreated MDS st-9 cells with transduction of control or HHIP shRNA expression vector. Four weeks after coculture, 2 × 10⁴ leukemic cells cocultured on a stromal layer were placed into methylcellulose. Y axis indicates the number of colonies per 2 × 10⁴ cells. *P < 0.05, primary AML/MDS-derived stromal cells vs 5-aza-dC-pretreated stromal cells (Student’s t-test, two-tailed). *P < 0.05, 5-aza-dC-pretreated AML/MDS-derived stromal cells vs 5-aza-dC-pretreated AML/MDS-derived stromal cells with HHIP shRNA transduction. Data shown are from three independent experiments, each done in triplicate. Results are expressed as means ± s.d.

Figure 7. The leukemia-supporting activity in HHIP-low stromal clones (HTS-6) and primary AML/MDS-derived stromal cells after modulation of stromal HHIP expression. (a) HHIP shRNA expression retrovirus vector was transduced into HTS-6, establishing Ti66-HTS-6. HTS-6 and Ti66-HTS-6 were exposed to 5-aza-dC and washed with PBS before use. The expression of HHIP in HTS-6, 5-aza-dC-pretreated HTS-6 and Ti66-HTS-6 was confirmed by (i) real-time PCR and (ii) immunoblot analysis. (b) SMO⁺ leukemic cell lines, Kasumi-1, Kasumi-3, HEL and TF-1 were cocultured with HTS-6 or 5-aza-dC-pretreated HTS-6 and HHIP knockdown Ti66-HTS-6 in serum-free medium for 4 weeks. *P < 0.05, HTS-6 vs 5-aza-dC-pretreated HTS-6. ‘Pre-5-aza-dC’ indicates coculture with 5-aza-dC-pretreated stromal cells, ‘HHIP shRNA (−)’ indicates coculture with Ti66-HTS-6 and ‘HHIP shRNA (−)’ indicates coculture with mock-transfected Cont-HTS-6. (c) The replating capacity of CD34⁺ fraction of primary leukemia cells cocultured with AML/MDS-derived stromal cells was analyzed. The primary CD34⁺ leukemic cells derived from patient ID no. 1, 3 and 6 was cocultured with (i) primary AML st-1 and 5-aza-dC pretreated AML st-1 cells or (ii) primary MDS st-9 and 5-aza-dC-pretreated MDS st-9 cells with transduction of control or HHIP shRNA expression vector. Four weeks after coculture, 2 × 10⁴ leukemic cells cocultured on a stromal layer were placed into methylcellulose. Y axis indicates the number of colonies per 2 × 10⁴ cells. *P < 0.05, primary AML/MDS-derived stromal cells vs 5-aza-dC-pretreated stromal cells (Student’s t-test, two-tailed). *P < 0.05, 5-aza-dC-pretreated AML/MDS-derived stromal cells vs 5-aza-dC-pretreated AML/MDS-derived stromal cells with HHIP shRNA transduction. Data shown are from three independent experiments, each done in triplicate. Results are expressed as means ± s.d.

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DISCUSSION

In the present study, we demonstrated that stromal HHIP expression could suppress leukemic cell proliferation. HHIP expression in AML/MDS-derived BM stromal cells was significantly reduced, and these low HHIP-expressing stromal cells readily supported the proliferation of leukemic cells. Moreover, 5-aza-dC pretreatment partially restored HHIP expression and leukemic cell proliferation on coculture was concomitantly reduced, indicating that aberrant reduction of HHIP expression is associated with the leukemic cell-supporting activity of BM stromal cells.

It was previously suggested that genetic abnormalities are necessary for the development of MDS and AML, and genetic aberrations, such as single base changes, deletions, insertions and genomic rearrangements, could be detected in early events during AML/MDS development. However, it remains unclear how genetic aberrations could be induced in BM. Mice with conditional knockout of Dicer1 or Sbds in BM stromal cells developed dysplastic changes in hematopoietic cells, subsequent genetic mutations, and eventually leukemic transformation.7,8 This suggests that stromal dysfunction may be an early event in the development of AML/MDS. Consistent with these findings, the reduction of stromal HHIP expression was observed in AML samples and MDS samples. Thus, reduction may be an early event in AML/MDS patients.

The precise molecular mechanism of how stromal HHIP reduction could contribute to the proliferation of leukemic cells remains unclear. In this regard, we and others have demonstrated that HHIP binds to the Hedgehog (Hh) ligands, including Ihh and sonic Hh, are highly expressed in CD34+ stem/progenitor cells.9,11 Moreover, Hh stimulates the cell cycle of hematopoietic stem/progenitor cells and erythroid progenitor cells in an autocrine or paracrine manner.21–39,41 However, it is unclear how Hh signaling is self-limiting in BM microenvironment.35,36 In the present study, we found that HHIP is highly expressed in primary stromal cells, but not in primary PB, BM or CB CD34+ hematopoietic cells (Figure 1d), suggesting that stromal HHIP is part of an inhibitory system of Hh signaling (Figures 1a and b). Hh signaling has no significant effects on adult hematopoietic stem cell function in vivo.12,42 Whether the Hh pathway has an important role in malignant hematopoiesis is controversial.43 The expansion of Bcr-Abl-positive leukemic stem cells was dependent on Hh pathway activation,13,14 although Hh signaling is not required for the development of MLL-AF9-mediated AML.44 Collectively, the high level of stromal HHIP expression in BM could suppress the proliferation of Hh signaling-dependent leukemic cells.

Understanding the mechanisms of reduced stromal HHIP in leukemia is a critical step towards future therapies. In this regard, we focused on the marked reduction of HHIP expression in several human stromal cell clones and AML/MDS-associated stromal cells (Figure 1a, Supplementary Figure 1a and Figure 3c). Analysis of the methylation status of the HHIP promoter region determined that the HHIP promoter was methylated in HHIP-low stromal cells (Figure 6a and Supplementary Figure 3). These results were consistent with previous reports demonstrating that a CpG island methylated in the HHIP promoter lesion and methylation of the HHIP promoter is detected in some types of tumors.25,45 Although the demethylating agent 5-aza-dC has begun to be used in the treatment of AML/MDS and genes involved in the regulation of DNA methylation (DNMT3a, IDH1/IDH2 and TET2) are found to be mutated in MDS,46 the precise molecular mechanism how 5-aza-dC mediates its effects is still unclear especially regarding BM microenvironment.47,48 Hence, we tested the effects of 5-aza-dC on the leukemic cell-supporting activity of primary AML/MDS-derived cells. Remarkably, HHIP expression in stromal cells was restored following 5-aza-dC treatment and their leukemic cell-supporting activity was concomitantly reduced and associated with demethylation of the HHIP promoter (Figure 6a and Figure 7c). Although several transcription factors and adhesion molecules could be associated with the efficacy of 5-aza-dC in the treatment of AML/MDS,49,50 stromal HHIP may be an important target for 5-aza-dC. Genome-wide analysis, transcriptome analysis or non-coding RNA analysis of AML/MDS-derived stromal cells are required to fully elucidate the mechanisms in future studies.

In conclusion, stromal HHIP expression was reduced in AML/MDS-derived stromal cells. The reduction of stromal HHIP expression may correlate with the proliferation of leukemic cells. Thus, aberrant stromal HHIP reduction could contribute to the progression of AML/MDS.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

MK designed the experiments and performed flow cytometry and drafted paper; SI, SK, HH, TS performed the experiments, analyzed the data; KM, YK, KT, KO, YK and HT followed patients and obtain informed consent; KM helped statistic analysis of the data; VS and RT helped to analyze the data. JK extensively edited the paper.

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