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ORIGINAL ARTICLE

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, $CD34^+$ blastic 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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INTRODUCTION

Recent massive parallel sequencing has enabled the analysis of whole genomes and transcriptomes from clinical samples derived from acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). Currently it is possible to comprehensively identify genetic mutations, including single base changes, deletions, insertions and genomic rearrangements in early events for AML/MDS development.[1] As a hypothetical model, the molecular pathogenesis of AML requires cooperating mutations of several genes such as early mutation, class-I and class-II mutations.[1] Early mutations are present in clinically silent pre-leukemic cells, and involve genes for nucleolar ribonucleoprotein, nucleophosmin (NPM1) and the RNA splicing machinery.[2,3] Class-I and class-II mutations are mainly present in leukemic cells.[1] Class-I mutations constitutively activate genes in the kinase signaling pathways, including *FLT3, c-KIT, c-FMS* and *RAS*, which enhance the proliferation of leukemic cells, whereas class-II mutations inactivate hematopoietic transcription factors such as AML1, ASXL, TET2 and IDH, which are associated with epigenetic regulation of gene expression.[4] Although combined genetic abnormalities are likely essential for the development of AML/MDS, aberrant function and chromosomal abnormality of human bone marrow (BM) stromal cells could be involved MDS development and subsequent secondary AML.[5] Interestingly, impaired microRNA biogenesis in human BM stromal cells from MDS patients, with *Dicer1* and *Drosha* gene and protein downregulation, correlated with abnormal gene and microRNA expression.[6] Moreover, mice genetically deficient for Dicer1 in BM stromal cells, developed dysplastic changes in hematopoietic cells, subsequent genetic mutations and eventually leukemic transformation.[7,8] Based on these findings, the function of BM stromal cells in AML/MDS has gained increasing attention in hemato-oncology.

We and others have demonstrated that the hedgehog (Hh) signaling pathway is important in the regulation of stem/progenitor cell expansion and lymphocyte differentiation.[9–11] In particular, Indian Hh (Ihh), its receptor patched (Ptc) and a constitutively active signal transducer, smoothened (SMO), are expressed in cord blood (CB) $CD34^+$ cells and BM stromal cells. Furthermore, changes in the cytokine expression profile of human stromal cells treated with Ihh ligand derived from $CD34^+$ cells through Hh receptor complex signaling induced the proliferation of hematopoietic stem/progenitor cells.[11]

Thus, Hh acts on stromal cells to regulate hematopoietic stem/progenitor cells. However, conditional SMO overactivation has no significant effect on self-renewal and function of adult hematopoietic stem cells although expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hh pathway activation *in vivo*.[12–14] Therefore, it is controversial whether Hh signaling is self-limiting in hematopoietic/leukemic stem cell expansion in BM where there is continuous Hh stimulation.[15,16]

Aberrant Hh ligand expression has been described in human cancers[17–19] including leukemia.[14,20] We recently demonstrated that Hh ligand induced proliferation of AML cells in an autocrine manner.[21] Hh-interacting protein (HIP), a membrane-associated or soluble glycoprotein that binds Hh ligand with an affinity

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comparable to the Ptc receptor, functions as an endogenous Hh ligand inhibitor.[22,23]

Importantly, reduced expression of the human HIP (*HHIP*) gene was observed in several tumor cells[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 $CD34^+$ hematopoietic cells, acute leukemic 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). 5-Aza-2′-deoxycytidine (5-aza-dC) was obtained from Sigma (St Louis, MO, USA). Total RNA derived from mobilized peripheral blood (PB), BM or CB $CD34^+$ cells was purchased from AllCells, LLC (Toronto, Ontario, Canada). Human myeloid leukemic 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). $CD34^+$ 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 mM pyruvate, 0.1% penicillin (100 U/ml) and streptomycin (100 mg/ml). For long-term culture of TF-1, 10 U/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.[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.[26,27]

Separation of primary $CD34^+$ leukemic cells and $CD271^+CD45^-$ mesenchymal stromal cells

Primary $CD34^+$ cells or $CD271^+CD45^-$ mesenchymal 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 $CD34^+$ cells was by positive selection using MACS Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotech, Bergish-Gladbach, Germany). For purification of $CD271^+$ $CD45^-$ mesenchymal stromal cells, BM MNCs were labeled with phycoerythrin (PE)-conjugated CD45 antibody and $CD45^-$ cells were separated by negative selection using anti-PE microbeads as previously described.[28] Subsequently, the $CD271^+$ fraction was purified by CD271 Micro Bead Kit (Miltenyi Biotech, Bergish-Gladbach, Germany) (allophycocyanin (APC)).

Coculture of $CD34^+$ leukemic cells or primary CB $CD34^+$ cells with human stromal cells

Two hundred thousand parental populations of primary human stromal cells, HTS or HTS clones were plated in 25 cm^2 plates in long-term culture medium.[26] Forty-eight hours after plating, cells were washed five times with phosphate-buffered saline (PBS) before addition of leukemic cell lines, CB $CD34^+$ cells (Takara, Tokyo, Japan) or primary $CD34^+$ leukemic cells. Twenty thousand primary $CD34^+$ leukemic cells or CB $CD34^+$ 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.[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.[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, 2×10^4 $CD34^+$ 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 2×10^4 cells per plate were replated at 7-day intervals.[30,31] The total numbers of colony-forming units in culture (CFU-C) and leukemia colony-forming cells were determined by microscopy.[11]

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 SuperScriptII (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′-TGCGG GCCGGGTCGGGTGGTG-3′ and 5′-GCCGCCCGTCTTGGCTGC-3′), Ptc (5′-CT GTTGGCATAGGAGTGGAGTTCACC-3′ and 5′-CTGCTGGGCCTCGTAGTGCCG AAGC-3′), SMO (5′-CAGAACATCAAGTTCAACAGTTCAGGC-3′ and 5′-ATA GGTGAGGACCACAAACCAAACCACACC-3′) or human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5′-ACATCAAGAAGGTGGTGAAGCA GG-3′ and 5′-CTCTCCCTCTTGTGCTCTTGCTGG-3′) as previously reported.[11] PCR amplification was performed with 30 cycles of 94 °C for 15 s, 58 °C for 30 s and 68 °C for 60 s. The PCR products were separated on a 2% agarose gel. For real-time RT-PCR analyses, Taqman Assays IDs of Ihh, HHIP, GAPDH, or 18 S (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 PRISM7700 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 utilized. 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 10 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.[32] 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.[10]

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).[11] The viral supernatants containing HHIP shRNA, TR20003 (control: shRNA empty pRS vector), TI316465, TI316466 or TI316468 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). Bisulphite modification of genomic DNA and MSP analysis were performed as described previously,[25] using primers corresponding to the HHIP promoter region sequences. HHIP methylation primer pair (5′-AAGATCTTTGAGGACTT GAGCTTC-3′ and 5′-CGCAGGCTCCTCTCTCCTCCCCGCTT-3′, product 299 size) and unmethylation primer pair (5′-AAGATTTTTGAGGATTTGAGTTTT-3′ and 5′-CACAAACTCCTCTCTCCTCCCCACTT-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.[33] Stromal cells were washed with 1 × 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 morphogenetic protein 2 (BMP-2), Wnt5A and HHIP 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.[27] However, expression of the soluble factors by stromal cells did not correlate with the supporting activity of CB-derived CFU-C (Figure 1a).[27] 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[11] and $CD34^+$ leukemic cell lines.[21] Recombinant mHIP is highly homologs with HHIP and mHIP can inhibit human Ihh activity.[10,22] Addition of recombinant mHIP into the coculture 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 Hh 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-dependant 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 replating 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

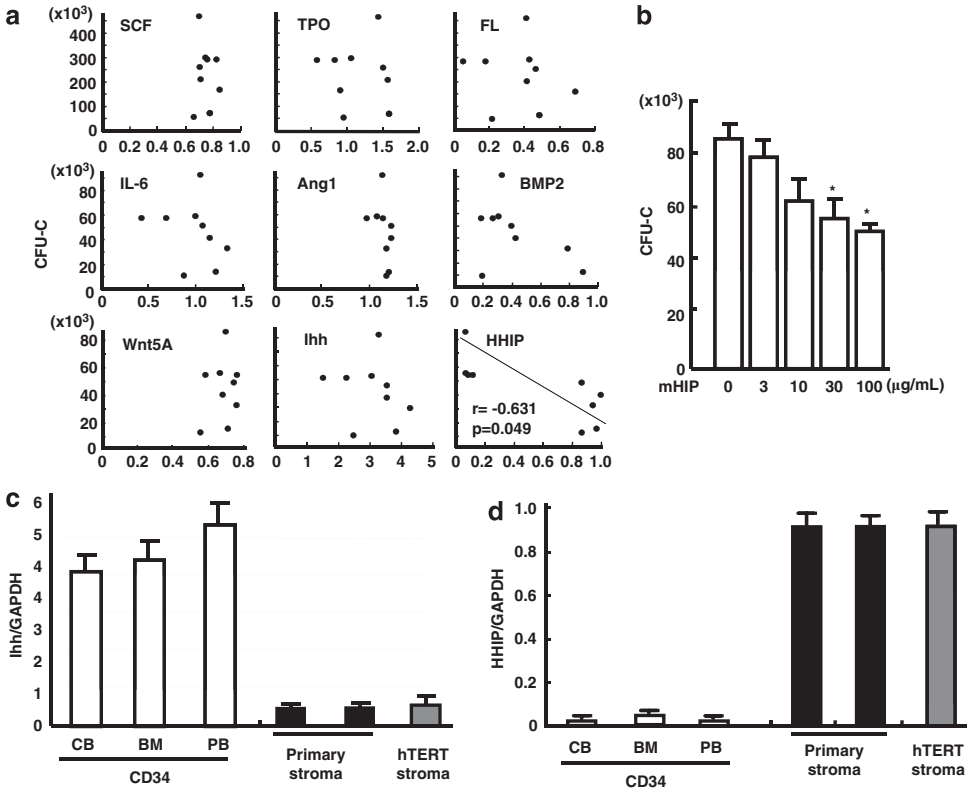

Figure 1. The expression of Ihh and HHIP in CD34$^+$ hematopoietic cells and primary and telomerized BM stromal cells. (**a**) Correlation between the number of CFU-C and various soluble factors expressed by HTS clones. A significant reverse correlation between the number of CFU-C and expression level 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 stromal (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$^-$ cell fraction.[34] Hence, we separated a primary uncultured stromal cell fraction using negative selection of CD45-PE and positive selection of CD271-APC (Figure 3e) when sufficient numbers of BM mononuclear cells were available. The collected cells were directly transcribed using TaqMan Gene Expression Cells-to-CT Kit. The BM MNSs from lymphoma patients were used as controls. The level of stromal HHIP expression in AML/MDS was significantly lower than in lymphoma patients without BM involvement (Figure 3e). Collectively, these results strongly indicated that HHIP expression was reduced in AML/MDS-derived stromal cells in the BM environment.

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 K562 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 K562 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

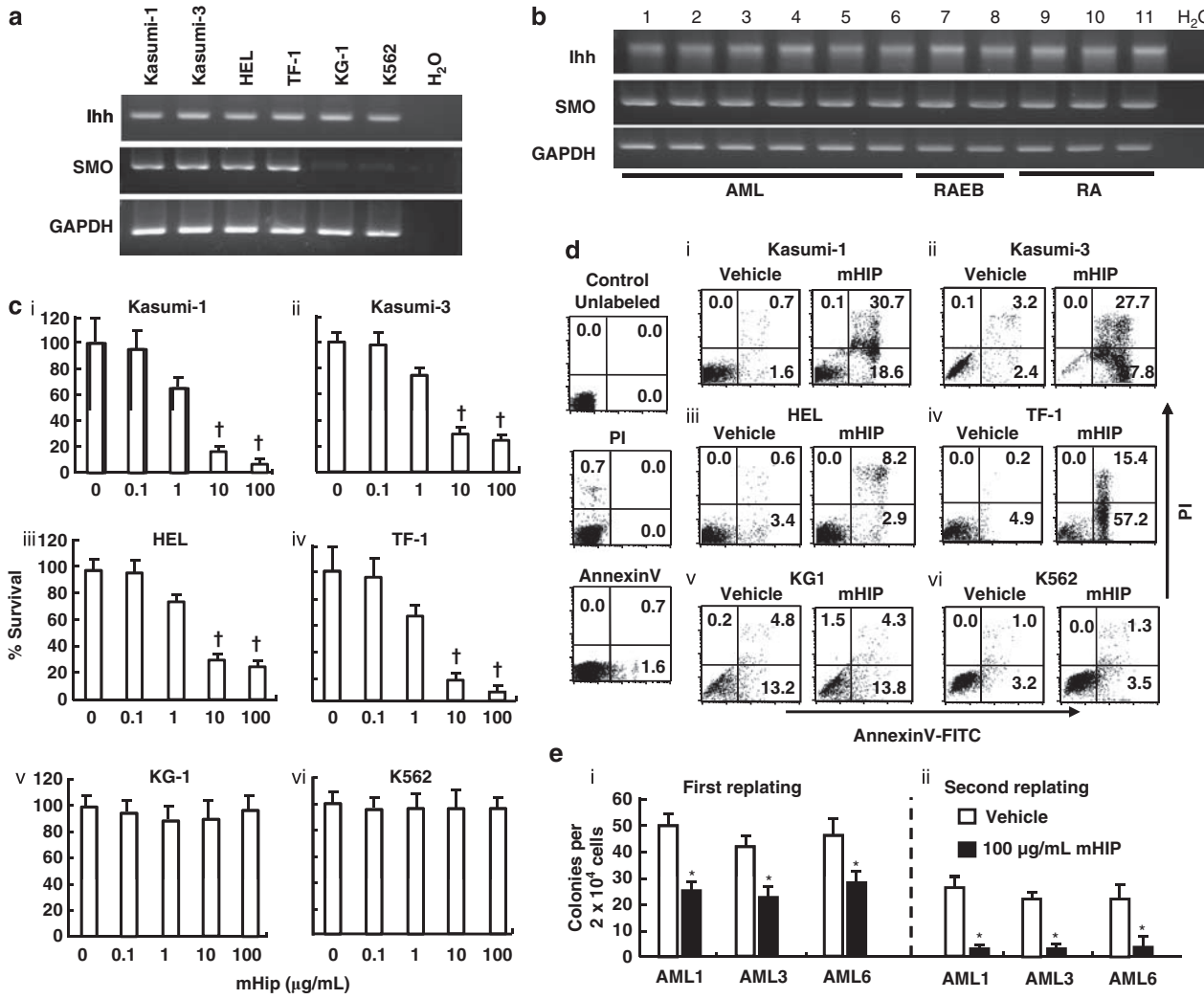

Figure 2. The effect of mHIP on leukemic cell lines in stroma-free conditions. (**a**) The expression of Ihh and SMO mRNA in six leukemic cell lines was analyzed by RT-PCR. The expression of GAPDH was used as an internal standard. (**b**) Ihh and SMO expression in the CD34$^+$ fraction of primary AML/MDS cells was analyzed by RT-PCR. Lane number 1–11 indicates the patient number suffering from AML or MDS (RA: refractory anemia; RAEB: refractory anemia with excess blast). H_2O was used as a negative control. (**c**) Cytotoxic assay of leukemic cell lines. *X* axis indicates the concentration of recombinant mHIP and *Y* axis indicates % survival of the cells. The number of surviving cells was assessed by WST-1 assay. (**d**) Double staining annexin V/PI assay of leukemic cell lines 48 h after exposure of mHIP. 'Control' indicates controls of unlabeled Kasumi-1 cells and single color controls of PI and Annexin V-FITC staining. Left panels indicate vehicle-treated control and right panels indicate mHIP-treated cells. *X* axis indicates Annexin V-FITC-positive cells and *Y* axis indicates PI-positive cells. (**e**) The replating capacity of the CD34$^+$ fraction of primary leukemic cells derived from patient ID no. 1, 3 and 6 was analyzed in the presence or absence of mHIP. Unlike control cultures (Vehicle), cultures with mHIP showed reduced replating capacity over three replatings (only two replatings are shown). Similar results were obtained in two independent experiments, each performed in triplicate. Results are expressed as means ± s.d.

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.[35,36] 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)(p10:q10)	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)(q11:q13)	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), − 7, add (7)(p22), + del (8)(q24), + 11, + 13, + 14, add(16)(q13), − 17, + mar; AML/MLD: AML with MLD.

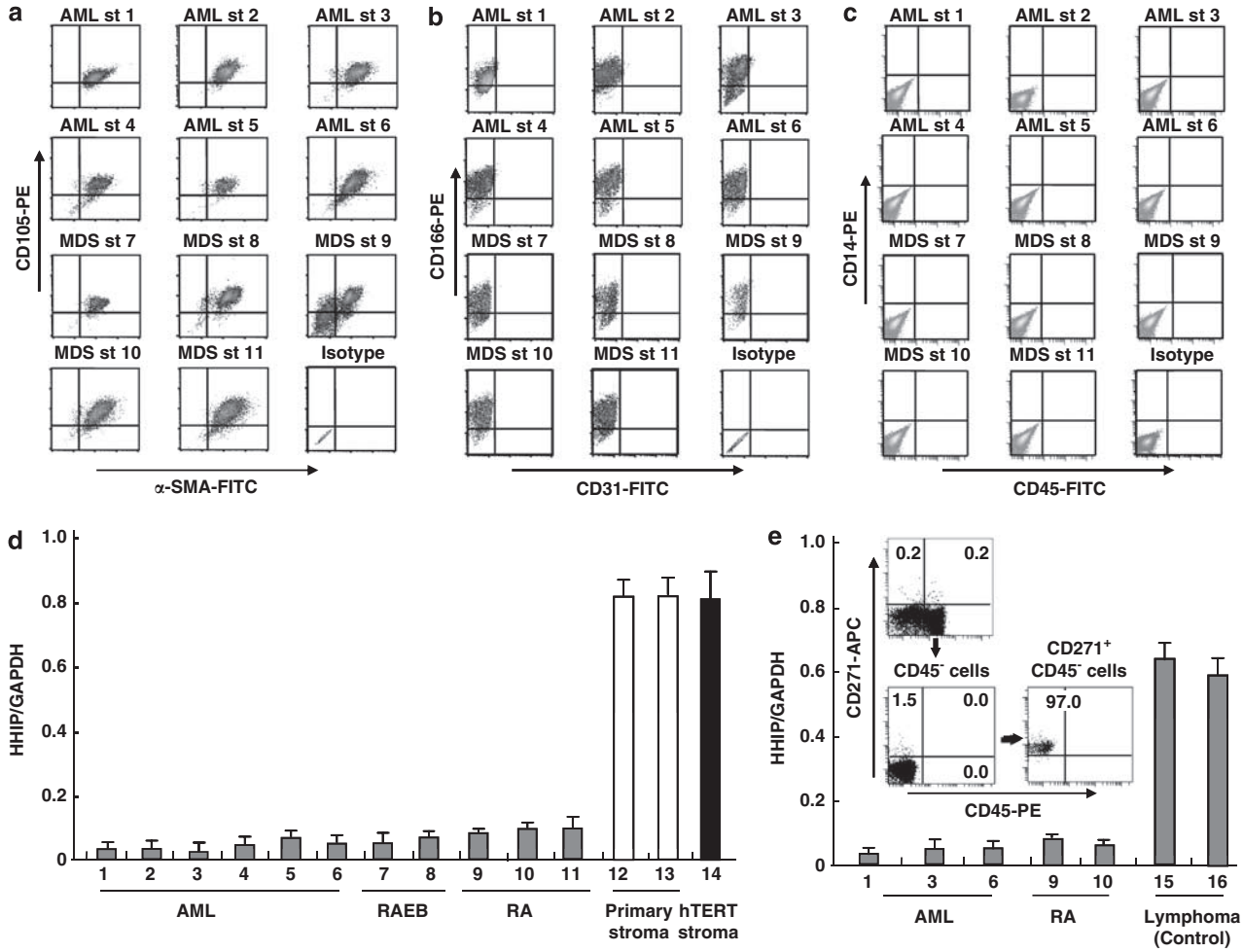

Figure 3. Immunophenotypical analysis and HHIP expression of primary stromal cells derived from AML/MDS patients. (**a**) Immunophenotypical analysis of stromal cells derived from AML/MDS patients. *X* axis indicates α-SMA-FITC and *Y* axis indicates CD105-PE. (**b**) Additional immunophenotypical analysis 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. The percentage of $CD271^+CD45^-$ 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).

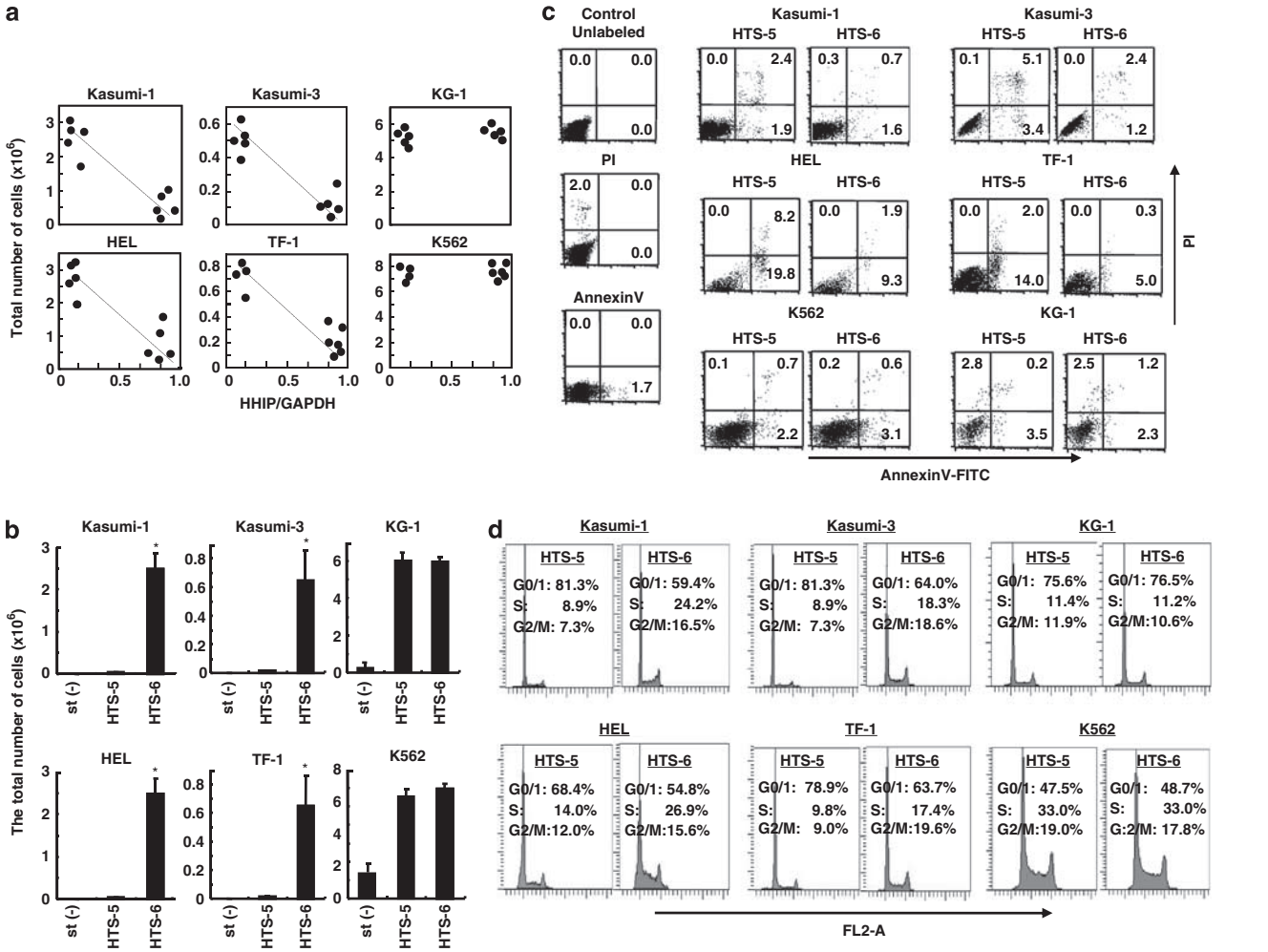

Figure 4. The supporting activity of BM-derived stromal clones on leukemic cells in aserum-free medium. (**a**) The correlation between the cell number of cocultured leukemic cell lines and the expression level of stromal HHIP in HTS clones. A significant reverse correlation was observed in four SMO$^+$ leukemic cell lines, but not in SMO$^-$ leukemic cells lines such as KG-1 and K562. (**b**) The growth of SMO$^+$ leukemia cell lines in coculture with stroma free (st (−)), HTS-5 (HHIP highest) or HTS-6 (HHIP lowest) stromal layer for 4 weeks. Twenty thousand leukemic cells were added to serum-free culture medium. For TF-1 culturing, 10 ng/ml of SCF was added every week. *Y* axis indicates the absolute number of cells from each culture system. *$P<0.05$, HTS-5 vs HTS-6. (**c**) The difference between the percentage of apoptotic cells on coculture with HTS-5 and HTS-6 by Annexin V/PI assay. *X* axis indicates Annexin V-FITC-positive cells and *Y* axis indicates PI-positive cells. 'Control' indicates controls of unlabeled Kasumi-1 cells and single color controls of PI and Annexin V-FITC. HTS-5: left panel; HTS-6: right panel. Data shown are from one representative experiment of three showing similar results. (**d**) Cell cycle analysis of leukemic cells by PI staining. Four SMO$^+$ leukemic cell lines and two SMO$^-$ leukemic cell lines (K562 and KG-1) were cocultured with HTS-5 or HTS-6 for 1 week and cell cycle entry of leukemic cells was analyzed. Data shown are from one representative experiment of three showing similar results.

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.[25] In addition, we investigated the effect of a demethylating regent, 5-aza-dC, on stromal supporting activity. For this experiment, HHIP-low HTS-6, α-SMA$^+$ primary AML stromal cell 1 (AML st-1) and α-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

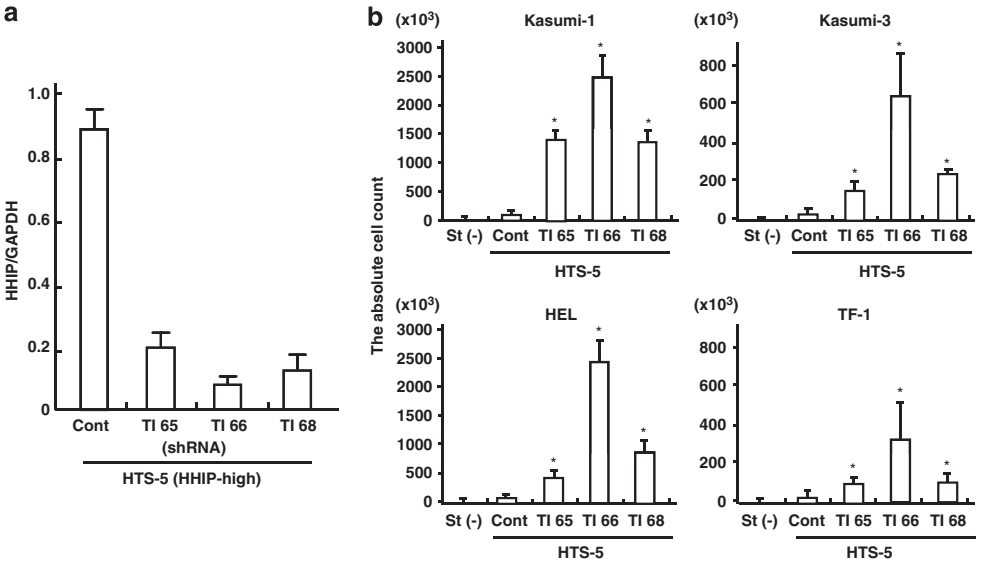

Figure 5. The effect of HHIP knockdown on the leukemia-supporting activity of stromal cells. (**a**) HHIP shRNA expression vectors or control vector (shRNA empty pRS vector) were transduced into HTS-5. The expression of HHIP in Cont-HTS-5 (Control), TI 65-HTS-5 (TI 65), TI 66-HTS-5 (TI 66) or TI 68-HTS-5 (TI 68) was analyzed by real-time PCR. (**b**) The growth of SMO^+ leukemic cell lines, Kasumi-1, Kasumi-3, HEL and TF-1 in coculture with control, TI 65, TI 66 or TI 68 stromal cells in a serum-free medium. The absolute number of cells was counted after 4 weeks coculture. The initial number of cells was 2×10^4. Data shown are from one representative experiment of three showing similar results. $^*P < 0.01$, control vs TI 65, TI 66, or TI 68.

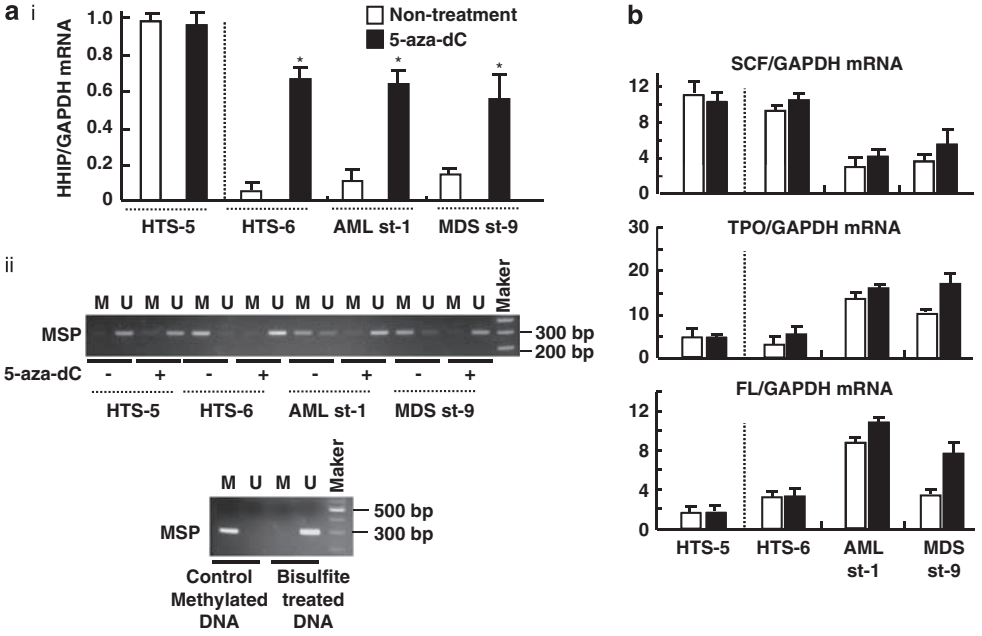

Figure 6. Methylation of HHIP promoter of HHIP-reduced stromal cells. (**a**) Reactivation of HHIP expression by 5-aza-dC treatment in HTS-6, primary AML st-1 or MDS st-9. HTS-5 was used as a negative control. (i) The restoration of HHIP expression was analyzed by real-time RT-PCR. Open bars represent HHIP mRNA expression in non-treated stromal cells and closed bars represent 5-aza-dC-treated stromal cells. $^*P < 0.05$, HHIP/GAPDH mRNA levels in non-treated cells vs that in 5-aza-dC-treated cells. (ii) Upper panel indicates methylation in the promoter region of the *HHIP* gene in stromal cell lines with or without 5-aza-dC treatment by MSP analysis. Lower panel indicates MSP analysis of HHIP promoter lesion in control methylated DNA and bisulfite treated DNA provided by Methylamp Universal Methylated DNA kit. Maker indicates 100 bp DNA ladder. Data shown are from one representative experiment of two showing similar results. 'M' represents methylated cytosine and 'U' represents non-methylated cytosine that was converted to uracil after bisulfite treatment. (**b**) The changes in expression levels of SCF, TPO and FL after 5-aza-dC treatment were examined by real-time PCR. The expression levels of GAPDH mRNA were used as a standard. Open bars represent mRNA expression in non-treated stromal cells and closed bars represent that in 5-aza-dC-treated stromal cells.

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

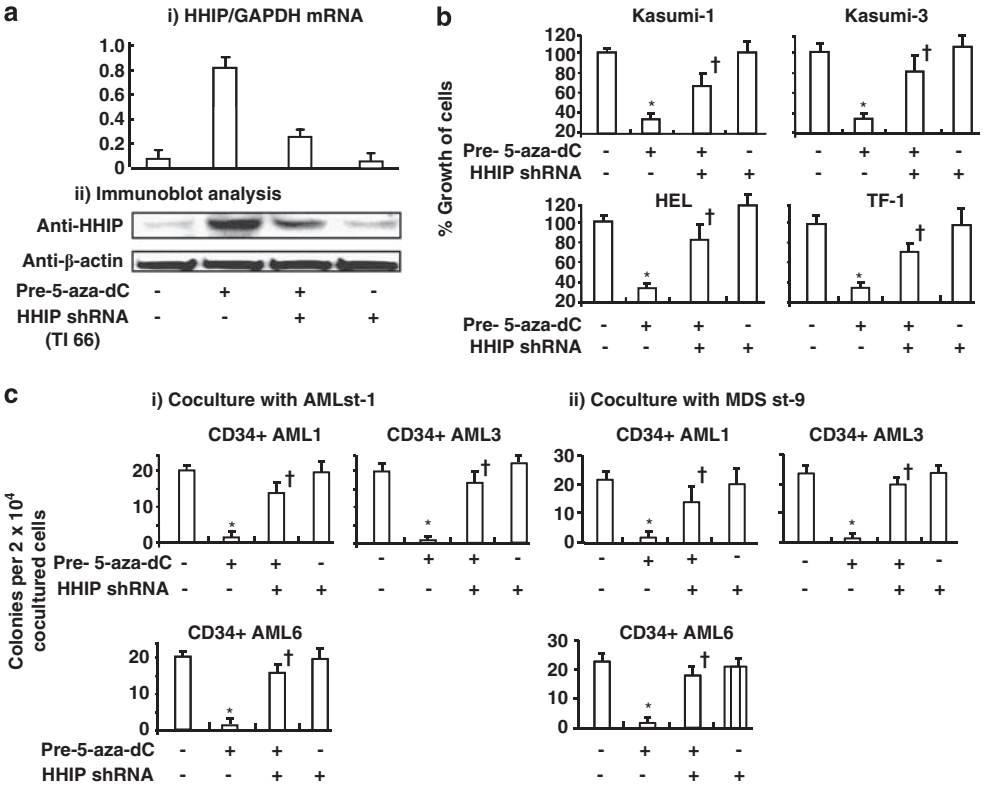

Figure 7. The leukemia-supporting activity in HHIP-low stromal clones (HTS-6) and primary AML/MDS-derived stromal cells after modulation of stromal HHIP expression. (**a**) HHIP shRNA expression retrovirus vector was transduced into HTS-6, establishing TI66-HTS-6. HTS-6 and TI66-HTS-6 were exposed to 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. $^{\dagger}P<0.05$, 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 TI 66-HTS-6 and 'HHIP shRNA (−)' indicates coculture with mock-transfected Cont-HTS-6. (**c**) The replating capacity of $CD34^+$ fraction of primary leukemic 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, 2×10^4 leukemic cells cocultured on a stromal layer were placed into methylcellulose. *Y* axis indicates the number of colonies per 2×10^4 cells. $^*P<0.05$, primary AML/MDS-derived stromal cells vs 5-aza-dC pretreated stromal cells (Student's *t*-test, two-tailed). $^{\dagger}P<0.05$, 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.

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

We examined whether leukemic 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 leukemic 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^+ leukemic cell lines was significantly reduced when cocultured with 5-aza-dC pretreated HTS-6, and this effect was negated by HHIP shRNA transfer into HTS-6 (Figure 7b).

We next examined whether these effects could be observed using coculture between primary AML/MDS-derived stromal cells and primary $CD34^+$ leukemic cells. The replating capacity of the primary $CD34^+$ leukemic cells was significantly reduced following coculture with 5-aza-dC-pretreated AML st-1 or MDS st-9, compared with non-treated stromal cells. Remarkably, this reduction of replating capacity by 5-aza-dC pretreatment was partially but significantly restored following transduction with an HHIP shRNA expression vector (Figure 7c). These results indicated that 5-aza-dC pretreatment restored HHIP expression and concomitantly reduced leukemic cell-supporting activity in primary AML/MDS-derived stromal cells. Moreover, this effect was significantly negated by HHIP shRNA transfer. Thus, HHIP expression in primary stromal cells could be highly associated with leukemic cell-supporting activity.

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.[38] 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 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.[15,16] 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 5′ CpG island existed in the HHIP promoter lesion, and that 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, transcriptomes 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; YS and RT helped to analyze the data. JK extensively edited the paper.

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