Autonomous TGFβ signaling induces phenotypic variation in human acute myeloid leukemia

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Abstract

Heterogeneity of leukemia stem cells (LSCs) is involved in their collective chemoresistance. To eradicate LSCs, it is necessary to understand the mechanisms underlying their heterogeneity. Here, we aimed to identify signals responsible for heterogeneity and variation of LSCs in human acute myeloid leukemia (AML). Monitoring expression levels of endothelial cell-selective adhesion molecule (ESAM), a hematopoietic stem cell-related marker, was useful to detect the plasticity of AML cells. While healthy human hematopoietic stem/progenitor cells robustly expressed ESAM, AML cells exhibited heterogeneous ESAM expression. Interestingly, ESAM− and ESAM+ leukemia cells obtained from AML patients were mutually interconvertible in culture. KG1a and CMK, human AML clones, also represented the heterogeneity in terms of ESAM expression. Single cell culture with ESAM− or ESAM+ AML clones recapitulated the phenotypic interconversion. The phenotypic alteration was regulated at the gene expression level, and RNA sequencing revealed activated TGFβ signaling in these cells. AML cells secreted TGFβ1, which autonomously activated TGFβ pathway and induced their phenotypic variation. Surprisingly, TGFβ signaling blockade inhibited not only the variation but also the proliferation of AML cells. Therefore, autonomous activation of TGFβ signaling underlies the LSC heterogeneity, which may be a promising therapeutic target for AML.

KEYWORDS
acute myeloid leukemia, autonomous signaling, chemoresistance, endothelial cell-selective adhesion molecule, heterogeneity, leukemia stem cells, phenotypic variation, TGFβ

1 INTRODUCTION

Cancer stem cells (CSCs) has attracted increasing attention since it explains why several cancer patients experience relapse even after intensive treatment1,2. If a small subset of cancer cells inherently resistant to chemoradiotherapies remained and survived after therapeutic intervention, these would eventually lead to recurrence3,4. Although treatment-resistant cells are not
involved in initiating tumorigenesis, they are widely recognized as CSCs, which are critical in cancer treatment and clinical outcome. To eradicate CSCs, it is important to understand their features. Accumulating studies on CSCs, however, have shed light on their challenging complexities, including their heterogeneity.5-8

Transplantation of acute myeloid leukemia (AML) cells into severe combined immune-deficient (SCID) mice allowed the first identification of CSCs involved in human diseases.9 While the earliest studies determined the phenotype of stem cells in AML, namely leukemia stem cells (LSCs), as CD34+CD38−, subsequent studies with improved xenograft models proved that LSCs are phenotypically heterogeneous, and reside in other fractions such as CD34+CD38 or even in the CD34− fraction.10,11 In addition, Goardon et al. reported that two distinct but hierarchically related LSC populations coexist in most patients with AML.12,13 One LSC population shows immature phenotype with CD34+CD38−CD45RA+, which is identical to lymphoid-primed multipotent progenitors in normal hematopoietic differentiation, whereas the other expresses CD34, CD38, CD45RA, and CD123/IL-3Ra, which resembles granulocyte-macrophage progenitors. The former LSC population giving rise to the latter, but not vice versa, demonstrates the hierarchy in LSCs, which mirror normal hematopoietic process. However, other studies showed that LSC activity is detectable even in lineage marker (Lin)-expressing cells, which reconstitute the original AML profile (including the primitive Lin−CD34+CD38− fraction) after xenotransplantation to immunodeficient mice.14 Collectively, these results demonstrate that LSCs in AML are essentially heterogeneous and cannot be defined solely by the cell surface phenotype. While the diverse LSCs may originate in different stages and construct the hierarchy, LSCs may also undergo dedifferentiation or interconversion, which is observed in CSCs of other cancers, such as melanoma, skin squamous cell carcinoma, and pancreas cancer.5,15-17 The development of efficient strategies to eradicate LSCs would require understanding the precise molecular mechanisms that underlie LSC heterogeneity.

We have previously found that endothelial-cell selective adhesion molecule (ESAM) expression, the lial cell-selective adhesion molecule (ESAM) expression, the authors have shown the autonomous TGFβ signaling as one of the molecular mechanisms underlying heterogeneity and variability of leukemia stem cells associated with human acute myeloid leukemia (AML). By monitoring the endothelial cell-selective adhesion molecule (ESAM) expression, the authors found that human AML cells were phenotypically heterogeneous and variable. ESAM− and ESAM+ AML cells were mutually convertible in culture. The authors determined that autocrine TGFβ signaling was involved in AML cell heterogeneity and variability. Inhibiting the TGFβ pathway not only suppressed ESAM variability, but also induced AML cell apoptosis. Thus, mechanisms promoting the heterogeneity and variability of LSCs can be therapeutic targets against intractable AML.

**Significance statement**

The authors have shown the autonomous TGFβ signaling as one of the molecular mechanisms underlying heterogeneity and variability of leukemia stem cells associated with human acute myeloid leukemia (AML). By monitoring the endothelial cell-selective adhesion molecule (ESAM) expression, the authors found that human AML cells were phenotypically heterogeneous and variable. ESAM− and ESAM+ AML cells were mutually convertible in culture. The authors determined that autocrine TGFβ signaling was involved in AML cell heterogeneity and variability. Inhibiting the TGFβ pathway not only suppressed ESAM variability, but also induced AML cell apoptosis. Thus, mechanisms promoting the heterogeneity and variability of LSCs can be therapeutic targets against intractable AML.

2 | MATERIALS AND METHODS

2.1 | Human samples

BM samples from patients with AML at diagnosis or relapse were obtained after receiving written informed consent in accordance with the Declaration of Helsinki. This study protocol was approved by the institutional review board of Osaka University Hospital (no. 13167).

2.2 | Cell lines

KG1a (CVCL_1824) was purchased from ATCC and used in this study. CMK (CVCL_0216) was provided from Otsuka Pharmaceutical Co., Ltd. Cell lines were cultured at 37°C with 5% CO2 and maintained in RPMI 1640 (Nacalai Tesque) supplemented with 10% FBS and 1% penicillin/streptomycin (P/S, Nacalai Tesque). In some experiments, recombinant human TGF-β1 (Biolegend) or was added to culture medium.

2.3 | Flow cytometry analysis and cell sorting

Antibodies used in this study are shown in Table S1. Human ESAM Antibody was biotinylated using EZ-Link Sulfo-NHS-Biotin and biotinylation kits (Thermo Fisher Scientific) according to the manufacturer’s instructions. APC Streptavidin was used for visualization of biotinylated antibody. Dead cells were excluded by staining with 7-aminoactinomycin D (7-AAD, Calbiochem). Cells were washed and then resuspended in PBS− containing 3% FBS (MP Biomedical). Cells were analyzed using FACS Aria IIu (BD Biosciences). In some cases, ESAM− or ESAM+ cells were sorted using FACS Aria IIu before use in subsequent experiments. FACs data was analyzed using FlowJo software (FlowJo, LLC).
2.4 | Primary leukemia cell culture

Primary leukemia cells were cultured at 37°C with 5% CO₂ in MEMα (Gibco) containing 10% FBS and 1% P/S, in the presence of recombinant human SCF (10 ng/mL, Biolegend), recombinant human Flt3-ligand (100 ng/mL, Biolegend), and recombinant human TPO (100 ng/mL, Biolegend).

2.5 | Real time RT-PCR

Total RNA was extracted with PureLink RNA Mini Kit (Invitrogen) according to the manufacturer's instructions. A High Capacity RNA-to-cDNA Kit (Applied Biosystems) was used for cDNA synthesis. Real time RT-PCR was performed on an ABI PRISM 7900 HT (Applied Biosystems, Inc). Expression levels were normalized to those of the internal reference β-actin. Primers used in this study are shown in Table S2.

2.6 | ELISA

KG1a were cultured in SF-03(Sekisui medical) medium for 3 days. Then, the supernatant was collected and TGFβ1 concentration was measured with LEGEND MAX Total TGF-β1 ELISA Kit (Biolegend) according to the manufacturer's instructions.

2.7 | Apoptosis assay

KG1a cells or primary AML cells were cultured with SB525334 (CAS356559-20-1, Calbiochem) and daunorubicin hydrochloride (CAS23541-50-6, Sigma-Aldrich), DMSO was added to control samples without SB525334. Subsequently, cells were resuspended in Annexin V Binding Buffer (BD Pharmingen) and stained with FITC-Annexin V and 7-AAD. Stained cells were analyzed using FACS Aria IIIu.

2.8 | RNA-sequencing analysis

KG1a CD34⁺CD38⁻ ESAM-Neg or CD34⁺CD38⁻ ESAM-Hi cells were sorted using flow cytometry, and their total RNA was extracted with RNaseasy Mini Kit (Qiagen) according to the manufacturer's instructions. Library preparation was performed using a TruSeq stranded mRNA sample prep kit (Illumina) according to the manufacturer's instructions. Sequencing was performed on an Illumina HiSeq 2500 platform in a 75-base single-end mode. Illumina Casava1.8.2 software used for basecalling. Sequenced reads were mapped to the human reference genome sequences (hg19) using TopHat v2.0.13 in combination with Bowtie2 ver. 2.2.3 and SAMtools ver. 0.1.19. The number of fragments per kilobase of exon per million mapped fragments (FPKM) was calculated using Cuffnorm ver. 2.2.1. Access to raw data from this study was submitted under Gene Expression Omnibus (GEO) accession number GSE150081. The datasets were analyzed using Ingenuity Pathway Analysis (Qiagen).

2.9 | Immunoblotting assay

Total cell lysate was prepared in lysis buffer [10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% TritonX-100, 1 mM EDTA, 0.1% NP-40 and protease inhibitor cocktail (Nacalai Tesque)]. Cell lysates (5-10 μg total protein per lane) were separated using a 4-12% NuPAGE gel system (Invitrogen) under reducing conditions and immunoblotted using appropriate antibodies (Table S1). Immunoreactive proteins were visualized using an Odyssey infrared imaging system (LI-COR Biotechnology).

2.10 | Statistical analysis

Student's t-tests were used to compare data between two groups. Statistical analyses were conducted using error bars to represent SEM. Statistical analyses were performed using GraphPad Prism7(GraphPad). Results with P values <.05 were considered statistically significant.

3 | RESULTS

3.1 | Human AML cells are heterogeneous in terms of ESAM expression levels

Our previous study identified that a subset of human acute leukemia cell lines, particularly erythroid and megakaryocyte lines, express ESAM. Therefore, we initially evaluated ESAM expression levels in primary BM specimens from human acute leukemia patients (Figure S1A). Flow cytometry analyses showed that 12 out of 21 AML cases expressed ESAM on the leukemia cell-enriched fraction at a frequency higher than 20%, whereas none of the ALL cases expressed ESAM. Several reports demonstrated that, among leukemic cells, LSCs in human AML are enriched in the CD34⁺CD38⁻ fraction. Although LSC activity is also observed in the CD34⁺CD38⁺ and CD34⁻ fractions, the CD34⁺CD38⁻ population often exhibits higher LSC frequencies compared with the other two fractions. To determine whether ESAM can enrich LSCs, we evaluated the frequencies of ESAM-expressing leukemia cells in the three fractions of individual patients with AML. We found that the frequency of ESAM⁺ cells was significantly higher in the CD34⁺CD38⁻ fraction, but decreased with CD38 expression and CD34 disappearance (Figure 1A).

Notably, however, ESAM expression pattern remarkably differed among AML patients (Figures 1B and S1B). Indeed, ESAM expression
was negative or very low in the CD34^+CD38^− fraction of some patients with AML, which is aberrant because more than 80% of normal CD34^+CD38^− cells express ESAM. Furthermore, ESAM expression was apparently heterogeneous in most of AML cases (Figure S1B). These results suggest that ESAM expression might mark LSCs in a subset of AML cases, but there is intrinsic heterogeneity among patients and even within an individual case.

3.2 | AML LSCs present phenotypical variability of ESAM expression

While some studies showed a hierarchical relationship between AML LSCs in the CD34^+CD38^− and CD34^+CD38^+ fractions, reversible variability between LSCs with different phenotypes has also been reported. This issue is important to determine the
clonal origin of LSCs in individual patients with AML. To address this, we divided CD34+ cells of primary AML into ESAM− and ESAM+ fractions, and tested their interconversion in culture. In this experiment, we selected two AML cases whose CD34+ fractions consisted of ESAM− and ESAM+ cell populations (Figure 2). As shown in Figure 2A, CD34+ESAM+ cells from AML case #4 generated CD34+ESAM− cells along a hierarchical order, although a subtle but detectable reversibility was observed. Additionally, in AML case #21, both ESAM+ and ESAM− populations gave rise to the other robustly. These results were consistent with previous observations, in which a hierarchical relationship between LSCs existed in some AML cases, while phenotypical variability between LSCs was generally present, to a varying degree. In our previous study, we observed that normal CD34+ESAM− cells obtained from human cord blood were devoid of the HSC capability, that is, the long-term reconstituting potential. Additionally, those CD34+ESAM− cells generated no CD34+ESAM+ population in vitro or in vivo, suggesting that the phenotypic plasticity is likely to be more related to LSCs than normal HSCs.

3.3 | Clonal AML LSCs represent the variability according to ESAM expression

Next, we assessed whether the phenotypical variability in LSCs occurred at the clonal level. To this end, we exploited human AML cell
lines as a clonal LSC model. In our previous study, KG1a cells maintained in our lab for long periods were found to be negative for ESAM expression. However, they became ESAM-positive and acquired more aggressive leukemic activity after propagated in vivo. Given the possibility that KG1a cells might have changed their phenotype during the repeated in vitro passage, we newly purchased original KG1a cells from ATCC and used in this study.

Among the tested cell lines, the original KG1a and CMK showed a broad distribution of ESAM levels (Figures 3A and S2A). KG1a cells showed exclusively immature CD34+CD38− phenotype, and displayed wide-ranging ESAM expression (Figure 3A). We sorted two subpopulations with the highest and lowest ESAM expression (henceforth denoted as ESAM-Hi and ESAM-Neg). Compared with ESAM-Hi cells, ESAM-Neg cells were characterized with abundant cytoplasm, conspicuous perinuclear halo, and fine chromatin structure (Figure 3B). When we cultured the two cell populations in standard growth medium, we observed marked differences in growth rate between them (Figure 3C). However, each population recreated a heterogeneous ESAM expression profile similar to that of the parental population (Figure 3D). The same result was obtained with CMK cells (Figure S2B). Restoration of the original parental profile was also observed using re-sorted ESAM-Hi and ESAM-Neg cells (Figure S2C). Collectively, these results indicate that AML LSCs are heterogeneous even in the genetically same clone.

3.4 Single LSCs reconstitute heterogeneity

Even within highly enriched populations, traces of contaminants may affect the experimental results. Moreover, a few outlier cells in a clonal population might have robust proliferating and fluctuating
characteristics, to which we could attribute the observed variability. To exclude these possibilities, we determined whether individual cells would reconstitute the parental profile of heterogeneous ESAM expression.

Cells of the ESAM-Neg or ESAM-Hi fraction were single-sorted into 96-well plates and cultured for 28 days under periodic microscopic observation. As shown in Figure 4 with four representative cells, single cells in both fractions unsurprisingly restored heterogeneous populations with a wide spectrum of ESAM levels. Indeed, even a cell with low initial ESAM expression level produced a robust ESAM+ population (Figure 4B upper). These results implicate that the variability in LSCs is not due to some aberrant clones but probably to the inherent nature of each individual LSC.

### 3.5 LSC heterogeneity is regulated at the gene expression level

To unveil what mechanism underlies the restoration of parental ESAM distribution from diverse LSCs, we next evaluated the amount of ESAM transcripts in ESAM-Neg and ESAM-Hi fractions sorted from parent KG1a cells. Real time RT-PCR examination revealed marked differences in ESAM mRNA levels between the two fractions (Figure 5A). Furthermore, recovered ESAM-Neg and ESAM-Hi fractions from single cells also presented clear differences in ESAM mRNA expression, in accordance with the levels of ESAM at the time of sorting on their surface (Figure 5B). These results suggest that the changes in ESAM expression occur at the transcriptional level.

To examine whether the phenotypic variation was caused by random fluctuation of the sole ESAM gene, we conducted RNA sequencing on ESAM-Hi and ESAM-Neg KG1a fractions immediately after sorting. This analysis revealed that the two fractions essentially differed in their transcriptomes, with 1186 genes showing significantly different expression (Figure 5C). Among the genes with the highest differences, IL1RL1, ITGA2B, and CHRM3 had over 60-fold higher expression in the ESAM-Hi fraction than in ESAM-Neg (Figure S3A). These genes encode cell surface receptors for IL33, fibrinogen/fibronectin/vitronectin, and muscarinic acetylcholine, respectively. Several genes encoding functional proteins were also upregulated in the ESAM-Neg fraction. Interestingly, these included KLRC1, RGS1, ITGB7, and BCL6, whose roles are intimately related to the lymphoid lineage. Real-time RT-PCR analysis confirmed the results of RNA sequencing (Figure S3B). Differences in ITGB7 expression levels between ESAM-Hi and ESAM-Neg showed reverse trend to that of ESAM expression even after serial sorting (Figure S3C). Transcriptome variability, along with changes in ESAM expression, was also observed in CMK, another AML cell line. However, the change pattern in CMK cells was found to essentially differ from that in KG1a cells (Figure S3D). That is, the fluctuation of ITGB7 and KLRC1 expression in CMK cells showed a similar pattern to that in KG1a cells, whereas that of ITGA2B, TIM3, and BCL6 showed an opposite pattern. These observations illustrate the robust transcriptome variability underlying the biological differences among different AML clones. Furthermore, functional diversity may exist even in a single AML clone, which can be reflected by the variation in ESAM expression levels.

Regulatory network analysis using Ingenuity Pathway Analysis (IPA) software revealed that “maturation of blood cells” is the
network most affected by the difference in ESAM expression levels (Figure 5D). A detailed network analysis also uncovered that the genes related to cell maturation were mostly inhibited in the ESAM-Hi fraction (Figure S4). These results point to a hierarchical order among AML LSCs from ESAM-Hi to ESAM-Neg, which reflects normal hematopoiesis. Collectively, while the ESAM-Neg fraction exhibited a differentiation-prone transcriptome, this feature is not irreversibly fixed, while some ESAM-Neg cells could reverse to immature stages. This finding supports the observation made in primary AML cells (Figure 2) and the notion that the differentiation hierarchy and the variability in AML LSCs are not mutually exclusive.

3.6 Autonomous TGFβ1 signaling promotes phenotypic variability of LSC

To elucidate the mechanisms underlying the transcriptome variability regulation in LSCs, we performed upstream analysis of RNA sequencing data. This approach helped us identify several cytokine-signaling pathways that could be responsible for the transcriptome differences between ESAM-Neg and ESAM-Hi. Among the identified pathways, most inflammatory cytokine signals such as TNFα and IFNα had negative z-value, meaning that they were active in ESAM-Neg. On the other hand, only the TGFβ signaling showed positive z-value, suggesting its activation in ESAM-Hi (Figure 5E).

Higher phosphorylation levels of Smad2/3 were detected in ESAM-Hi, which indicates the activation of TGFβ signaling (Figure S5). Additionally, the amount of TGFβ1 transcripts was substantive in KG1a cells whereas no transcripts for TNFA or IFNA were detected (as shown by the RNA-sequence results). This suggests that TGFβ may be more directly involved in the transcriptome signature of ESAM-Hi cells.

As the standard growth medium for KG1a cells with 10% FBS contains natural TGFβ1 at approximately 1 ng/mL, we analyzed how the expression of ESAM would change in the absence of FBS. When cultured in serum-free condition, the entire cell population homogeneously converged to an ESAM-intermediate stage (Figure 6A middle).

**FIGURE 5** LSC heterogeneity is regulated at the gene expression level. A, ESAM mRNA levels analyzed using real time RT-PCR (n = 5). B, ESAM mRNA levels after single-cell culture analyzed using real time RT-PCR (n = 3). Single cell with ESAM-Neg or ESAM-Hi of CD34+CD38− fraction of KG1a was sorted and cultured for 28 days. ESAM-Neg and ESAM-Hi fraction cells were sorted again from the clones after culturing. C, Gene expression profiles of CD34+CD38− cells between ESAM-Neg and ESAM-Hi were compared through RNA sequencing analysis. Scatterplots comparing transcript levels (in fragments per kilobase of exon per million fragments) in ESAM-Neg (x-axis) and -Hi (y-axis). D, Regulator network analyses. The significantly altered networks are shown. E, Upstream regulator analyses. The significantly altered upstream regulators are shown. Pathways with P-values below 10−12 were extracted and arranged in descending order of z-value. Data are shown as means ± SEMs. Statistically significant differences are represented by asterisks (**P < .001)
However, adding 1 ng/mL TGFβ1 to the serum-free medium restored the heterogeneous ESAM expression (Figure 6A right). These results support the findings from the upstream analysis, suggesting that TGFβ1 is involved in the regulation of ESAM expression. Next, we analyzed how TGFβ1 influences ESAM expression of ESAM-Neg cells. When TGFβ1 was added to the medium, the mean expression intensity of ESAM significantly increased in sorted ESAM-Neg cells, suggesting that TGFβ1 signaling upregulated ESAM levels in these cells (Figure 6B).

KG1a cells restore their phenotypic heterogeneity even in serum-free conditions in the long term (data not shown). From this observation, we inferred that autocrine/paracrine TGFβ1 might be involved in the phenotypic variability of LSCs. In fact, after culturing KG1a in serum-free medium, TGFβ1 concentration in the culture supernatant increased in a cell-number dependent manner (Figure 6C). Furthermore, real-time RT-PCR showed that ESAM-Hi expressed higher levels of TGFβ1 than ESAM-Neg (Figure 6D left). Interestingly, the expression of TGFBR2, of which levels are known to correlate with the activation of TGFβ signaling pathway,22 showed a reciprocal change (Figure 6D right).

3.7 | Blocking TGFβ signaling induces cell death of leukemia cells

Our ultimate goal is to identify efficient therapeutic strategies to eradicate AML LSCs. Based on the results described above, AML LSCs are fluctuating in terms of transcriptomes as well as surface phenotypes, due at least in part to autocrine/paracrine TGFβ1, which we presumed may be involved in treatment resistance. Therefore, we examined whether inhibiting TGFβ signaling would block the fluctuations and
enhance the chemosensitivity of AML cells. To this aim, we added SB252334 (TGFβRI Kinase Inhibitor VIII), a selective TGFβRI inhibitor that blocks TGFβ1-induced Smad2/3 activation,23 to the KG1a culture. In ESAM-Neg KG1a, the addition of a small amount of SB252334 inhibited the upregulation of ESAM expression (Figure S6A). Surprisingly, when cultured with SB252334 for 3 days, the growth of KG1a cells was markedly inhibited (Figure 7A left); the variability of ESAM expression was also suppressed (Figure S6B). Moreover, Annexin V staining revealed that blocking TGFβ signaling by SB252334 induced cell death of KG1a (Figure 7A right). These results indicate that blocking the TGFβ signaling not only inhibited the phenotypic variability but also induced apoptosis in KG1a cells. While long-term inhibition of TGFβ signaling induced significant cell death, the effect of short-term SB252334, for 24 hours, was subtle (Figure 7B). However, together with antitumor drug daunorubicin, short-term SB252334 treatment significantly enhanced the antitumor effect (Figure 7B), suggesting that inhibition of TGFβ signaling in combination with other drugs would have a stronger antitumor activity.

Finally, we analyzed the effects of TGFβ signaling blockade on primary leukemia cells obtained from patients with AML. In ESAM− fractions of primary CD34+ AML cells, TGFβ signaling blockade by SB252334 inhibited the upregulation of ESAM as observed in KG1a (Figure S6C–D). In addition, SB252334 suppressed the proliferation of primary CD34+ AML cells (Figures 7C left and S6F) and induced apoptosis (Figures 7C right and S6F) in AML case #4 and #17. In AML case #8, SB252334 alone did not suppress cell proliferation or induce apoptosis (Figure S6E) but enhanced the antitumor effect of daunorubicin (Figure 7D). From these results, we conclude that interfering with autocrine/paracrine TGFβ signaling mechanisms might be a promising new strategy to treat unresponsive AML.

4 | DISCUSSION

The principal aim of this study was to further investigate the features of LSCs in human AML. We hypothesized that ESAM expression could mark LSCs in human AML; hence, we examined its levels in various AML samples. Although our finding of AML LSCs with variable expression of ESAM did not agree with our hypothesis, our approach provided insight into their inherent features, namely heterogeneity and variability. By analyzing the molecular mechanisms involved in the heterogeneity and variability of LSCs, we identified autocrine TGFβ secretion in AML cells, which provides an important clue for developing new strategies to treat AML.

While treatment results for many blood cancers have greatly improved in the past decades, AML remains a poor-prognosis disease due to its high recurrence rate after treatment. The high recurrence rate is considered at least partly due to residual chemoresistant LSCs. Therefore, developing novel treatments targeting LSCs is essential. Previous studies have searched for specific antigens that are expressed specifically on LSCs, but not on normal HSCs, as therapeutic targets. Several molecules have been reported as LSC-related antigens in human AML, such as CD44,24 CD47,25,26 CD96,27 CLL-1/CD371,28 and TIM-3.29 Although therapies targeting these antigens are promising, it is uncertain if they are constantly expressed on LSCs during the treatment course. Indeed, AML cells exhibit heterogeneity in gene as well as surface antigen expression,30 and the phenotype of LSCs varies significantly among patients.10,11 Moreover, even within the same patient, LSCs show plasticity. It is therefore possible that a small subset of LSC clones without LSC-specific antigens may remain and expand during antigen-specific treatments. Furthermore, non-LSCs may acquire additional mutations and transition to new LSC clones. For example, melanoma CSCs heterogeneously express numerous antigens, and the heterogeneous CSCs are reconstituted over time even when specific antigen-positive cells are removed.15 Thus, understanding the mechanisms underlying LSC heterogeneity is indispensable to realize curative remedies for AML.

Herein, we found that human AML cells display phenotypic heterogeneity in terms of ESAM expression. We have previously shown that normal HSC activity correlates positively with ESAM expression levels in the CD34+CD38− fraction.17 The CD34+CD38− phenotype, which reflects immaturity of hematopoietic cells in healthy human BM, is also known to enrich LSC activity in AML.9 Hence, we expected that ESAM would be useful as one of the LSC-related antigens in human AML. However, even in the same AML clones, ESAM expression level and pattern differed substantially. In addition, primary AML cells obtained from patients not only exhibited heterogeneous ESAM expression pattern but also phenotypic variability regarding ESAM levels. Although there appears to be a downward hierarchical relation from ESAM+ to ESAM− AML cells, we also observed mutual interconversion between ESAM− and ESAM+ cells in most AML cases. While the rate of recovery of ESAM expression varied from case to case, in some cases both ESAM+ and ESAM− populations gave rise to the other with a similar activity. These results support previous
reports suggesting the inherent plasticity of AML LSCs,14,31,32 and imply that we could resolve the fundamental mechanisms underlying LSC plasticity by analyzing the molecular pathways regulating ESAM expression.

A similar phenotypic fluctuation in EML cells, a murine HSC clone, has been previously studied.33,34 EML cells are heterogeneous regarding Sca1 expression, a traditional mouse HSC-related marker. When sorted according to Sca1 expression levels and cultured, both Sca1hi and Sca1lo EML cells reconstitute the original heterogeneous population, whereas the two fractions differ in their affiliation to the erythroid lineage. This functional difference results from their transcriptomes, which vary even at the single-cell level. In this study, we observed a similar phenomenon with respect to ESAM expression on human AML cells. The fluctuation of ESAM expression levels was regulated by gene transcription in concert with other genes involved in HSC function and differentiation (Figures 5 and S3). RNA sequencing results revealed the substantial differences between ESAM⁺ and ESAM⁻ KG1a cells, particularly in the expression levels of cytokine receptors and adhesion molecules such as integrins. These observations suggest that ESAM expression distinguishes functionally different LSCs in one AML clone, whose preferable locations to grow, the so-called “LSC niche,” probably also differ in the BM. Furthermore, this notion again emphasizes the importance of determining the molecular mechanisms that modulate the functional as well as phenotypic versatility of AML LSCs in order to eradicate them from the BM.

ESAM mediates cell-cell interactions through homophilic binding and plays physiological roles in the tight junction of endothelial cells.35-37 We previously found that ESAM has functional significance in normal HSCs. ESAM deficiency disrupts erythropoietic potential in both fetal and adult HSCs.38,39 Since the migration of neutrophils to inflammatory sites is inhibited in ESAM-deficient mice, ESAM is also thought to play a role in the motility of hematopoietic cells.40 Notably, crosslinking of ESAM on normal HSCs with an anti-ESAM antibody activates Rho GTPase, which is known to induce cancer cell motility by actin polymerization.40 Although direct evidences are lacking at this stage, ESAM expression may also contribute to the motility of AML LSCs.

We identified TGFβ signaling as an upstream regulator of ESAM expression; the deprivation of natural TGFβ from culture medium reduced the variety of ESAM expression pattern in KG1a cells, leading to a homogeneous ESAM-intermediate population (Figure 6). This phenomenon suggests that the sensitivity and/or response to TGFβ signaling differ between ESAM⁺ and ESAM⁻ cells even in a clonal population. This differential sensitivity might, at least in part, reflect the reciprocal pattern of TGFβRI expression (Figure 6D). ESAM⁻ cells with high TGFβRI expression may activate the TGFβ signaling pathway, even under conditions of very low TGFβ1 concentrations, resulting in the upregulation of ESAM expression. Thus, there may be a possible feedback loop between TGFβ1-producing ESAM⁺ cells and TGFβRI-expressing ESAM⁻ populations, which might contribute to the reconstitution of heterogeneity in clonal AML cells. A similar observation was reported with discrete normal HSC subtypes. In fact, at low concentrations, TGFβ stimulates the growth of myeloid-biased HSCs but inhibits lymphoid-biased HSCs.41,42 Notably, the ESAM-Hi fraction highly expressed CD33, a myeloid-related marker, whereas the ESAM-Neg fraction presented a lymphoid-related transcriptome signature (Figure S3). The action of TGFβ thus seems to be complex and context-dependent, and this cytokine is likely to confer different phenotypes and functions on individual hematopoietic cells.

Accumulating evidence has shown that aberrant TGFβ signaling is involved in the pathogenesis of human leukemia.43,44 However, the activity of TGFβ on AML cells remains controversial. For example, mutations and deletions in SMAD4 or TGFβRII, which permit AML-initiating cells to evade inhibition by TGFβ signaling pathway, are involved in some AML cases.45,46 suggesting that TGFβ has suppressive effects on AML LSCs. Meanwhile, TGFβ produced by activated BM microenvironment enhances the aggressiveness of human MLL-AF9 oncogene-induced AML in mouse transplantation models.47 These contradictory results may be explained by the different sensitivity to TGFβ among AML LSCs or by the different magnitude of TGFβ induction in each experimental model. Alternatively, TGFβ may inhibit the emergence of AML LSCs at the initiation of disease, but support their proliferation afterwards in most cases.

Our findings demonstrate that AML cells produce TGFβ at low concentration, which in turn supports their diversity and variability. This is critical since TGFβ is normally secreted in a latent form and its activation requires multiple steps including the accumulation in the extracellular matrix and the interaction with cell surface integrins.48 Our data postulate that the activation of TGFβ signaling in AML cells occurs in a cell-autonomous manner. In addition, previous studies have primarily focused on TGFβ1 production from leukemia-supporting microenvironment, the “LSC niche,” which formed a conceptual therapeutic target. As observed with CSCs in glioma,49,50 it is likely that AML LSCs produce TGFβ1 by autocrine secretion and thus form a favorable environment by themselves. In this context, it is remarkable that inhibition of TGFβ signaling by SB525334, a TGFβRI-selective inhibitor, exhibited growth inhibitory and cell death-inducing effects in primary AML cells as well as KG1a.

Given the differences in susceptibility to SB525334 among primary AML cells, each AML case must have a genetic status which corresponds to different dependence on TGFβ signaling. Nevertheless, inhibition of TGFβ signaling significantly enhanced the effects of daunorubicin even in cases where single agents were ineffective. Notably, the 5 μM concentration of SB525334 used during in vitro experimentation would be feasible with 10 mg/50 kg body according to our provisional calculations. Inhibition of TGFβ signaling has also been reported to enhance the efficacy of tyrosine kinase inhibitors in chronic myelogenous leukemia51,52 and of cytarabine in AML cells.53 Our results support those findings, and further emphasize that small compounds inhibiting TGFβ signaling could be promising therapeutic strategy against human AML.

Although our data propose a promising new therapeutic strategy, this study has some limitations. First, the phenotypic variability in
LSCs has only been demonstrated in vitro. We have shown that primary AML cells present phenotypic heterogeneity and variability in culture, but it remains uncertain whether they are variable in vivo. Second, although our results are in accordance with recent findings using large data sets of cancer genomics, the expression levels of only a single marker is not sufficient to determine the features of LSCs or associate them with patient survival. Thus, studies on other multiple LSC-associated markers in addition to ESAM are warranted. Finally, whether inhibition of TGFβ signaling would enhance the therapeutic effects of antitumor drugs for AML in humans should be carefully tested. In future, it is necessary to extend our research to in vivo models to apply our findings to the clinical field.

5 | CONCLUSION

We demonstrated that human AML cells show heterogeneous ESAM expression, and that ESAM+ and ESAM− AML cells are mutually convertible. ESAM variability is regulated by TGFβ1, involving autocrine cytokine secretion. Blockade of TGFβ signaling not only inhibits ESAM variability, but also induces leukemic cell growth inhibition and apoptosis. Although efficacy and safety of TGFβ signaling inhibitors in vivo warrants confirmational studies, we believe that the molecular mechanisms underlying the regulation of leukemic cells variability can potentially be therapeutic targets, and the combination of known drugs with new strategies may lead to complete eradication of AML LSCs.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Y.S.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; T.Y.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; D.O., N.H.: collection and assembly of data, manuscript writing; T.S., T.I., Y.D., T.U., R.N., A.T., M.I., H.S., Y.K.: collection and assembly of data.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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