Aberrant fucosylation of extracellular vesicles remodels the vascular microenvironment and promotes chemoresistance in myelodysplastic syndromes and acute myeloid leukemia

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Abstract

Primary resistance to hypomethylating agents (HMA) remains a major obstacle in the treatment of elderly patients with myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). An altered integrity of the vascular wall is suspected to contribute to this resistance, yet the underlying molecular mechanisms remain unclear. Here, we show that small extracellular vesicles (sEV) derived from leukemic cells resistant to decitabine (DAC-R), a commonly used HMA, promote vascular permeability by down-regulating tight junction proteins, including ZO-1, occludin and claudin5, in endothelial cell monolayers. This disruption of vascular integrity may facilitate vascular leakage and leukemic cell dissemination. Mechanistically, DAC-R cells exhibit increased expression of the fucosyltransferase *FUT4*, driven by the transcription factor TWIST1, leading to enhanced biosynthesis of non-sialylated Lewis x (Le^X) structures. FUT4-mediated Le^X modification stabilizes intercellular adhesion molecule 3 (ICAM3) on sEV, and the delivery of Le^X-modified ICAM3 to endothelial cells suppresses NF-κB signaling, impairing endothelial barrier function. Functionally, vascular remodeling driven by fucosylated sEV promotes leukemic dissemination, suggesting that disruption of vascular homeostasis represents an additional layer of therapeutic resistance. These findings define a TWIST1–*FUT4*–Le^X–ICAM3 axis and highlight glycosylation as a critical mediator of vascular microenvironment remodeling in MDS/AML.

Introduction

Primary resistance to hypomethylating agents (HMA) such as 5-aza-2'-deoxycytidine (decitabine, DAC) represents a major obstacle to the treatment of elderly patients with myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML).[1-3] Leukemic cells that are refractory to the epigenetic and cytotoxic effects of the drug rapidly expand, leading to death. These refractory cells modulate the bone marrow (BM) niche, establishing a protective microenvironment that further promotes their survival and expansion under therapeutic pressure.[4] One of their targets is the vascular endothelium in which alteration of endothelial cell functions increases vessel permeability.[5] Small extracellular vesicles (sEV) have emerged as key mediators in this dialogue between leukemic cells and their microenvironment as they facilitate the transfer of diverse molecules from one cell type to another.[6,7]

One of the features of sEV that contribute to their biological effects is their glycosylation profile. This crucial post-translational modification affects the folding, trafficking, stability, and activity of multiple glycoproteins. Accordingly, cancer initiation and progression, angiogenesis, invasion, metastasis, and chemoresistance were all associated with abnormal expression of glycosyltransferase enzymes and characteristic glycosylation profiles. For example, therapeutic

resistance of chronic myeloid leukemia cells was related to the downregulation of *ST3GAL4*, which encodes an enzyme of the β-galactoside-α2,3-sialyltransferase family,[8] while the resistance of AML cells to cytotoxic drugs was associated with overexpression of the glycosyltransferase ALG9 in leukemic cells.[9] Importantly, the glycosylation profile of sEV reflects that of donor cells and significantly influences their biological effects. For example, sEV decorated with sialyl-Lewis x (sLe^X) facilitate bladder cancer metastasis by increasing vascular permeability,[10] whereas elevated bisecting GlcNAc modifications on sEV derived from breast cancer cells mitigate their pro-metastatic potential.[11]

Our previous studies involved the interaction of the transcription factor TWIST1 with DNA methyltransferases 3 (DNMT3)[12] and the cellular transfer of miR-4755-5p in leukemic cell resistance to DAC.[13] The contribution of the glycosylation profile of leukemic cells and their sEV to this resistance remained poorly explored. Here, we show that DAC-resistant MDS/AML cells demonstrate TWIST1-driven overexpression of the fucosyltransferase encoding the *FUT4* gene, which promotes protein modifications with Lewis x (Le^X) structures. In turn, sEV expressing Le^X-modified proteins such as ICAM-3 compromise endothelial integrity, increase vascular permeability, and favor leukemic cell homing. These findings provide new insights into interactions between leukemic cells that are resistant to HMA and surrounding vessels.

Methods

Patient samples

Plasma samples from healthy individuals (HD) and MDS/AML patients who are sensitive to DAC treatment (DAC-S group) or are refractory to DAC treatment (DAC-R-group) were collected from the People's Hospital of Shaanxi Province (*Online Supplementary Table S1*). Blood samples were collected into precoated EDTA tubes and immediately centrifuged at room temperature for 15 minutes (min) at 2,000 g, and plasma samples were collected and frozen at –80°C until further use. Written informed consent was obtained from all patients in accordance with the principles of the Declaration of Helsinki. The study was approved by the Medical Ethics Committee of Northwest University (approval number: 230306006, 6 March 2023).

In vivo mouse model

All mouse experiments were approved by the Animal Care and Use Committee of Northwest University (approval number: NWU-AWC-20231202M, 2 December 2023).

Briefly, 6-8-week-old B-NSG mice (NOD-Prkdcscid*IL2rg*tm1/Bcgen, NSG; Biocytogen Pharmaceuticals, Beijing, China) were irradiated with 3 Gy, followed by tail vein injection of 10^7 KG1a or KG1a-DAC cells. After 16 hours (hr), mononuclear cells in blood, BM, and spleen stained with antibody against human CD45 (#561865, BD Biosciences; Franklin Lakes, NJ, USA) and the percentages of human $CD45^+$ cells were analyzed by FACS.

For sEV pre-conditioned mice, sEV (50 μg in 100 μL PBS) from HD, KG1a, KG1a-FUT4, KG1a-DAC, KG1a-DAC-shFUT4 were intravenously (i.v.) injected into NSG mice three times per week for two weeks, and then irradiated with 3 Gy, followed by i.v. injection of 10^7 KG1a cells. The percentages of human $CD45^+$ cells in blood, BM, and spleen were analyzed by FACS.

Statistical analysis

Each experiment was performed at least three times. Prism 8.0 Statistical Software program (GraphPad Software; La Jolla, CA, USA) was used for statistical analysis. Intergroup means were compared using Student *t* test, and multiple group comparisons were evaluated by one-way ANOVA with Bonferroni post hoc test. Data are presented as mean ± standard error of mean (SEM). $P<0.05$ was considered statistically significant.

Results

Decitabine-resistant leukemic cells increase endothelial permeability

We first performed a xenotransplantation experiment in which KG1a parental cells and DAC-resistant (KG1a-DAC) cells were injected i.v. to irradiated B-NSG mice. Sixteen hours after injection, the fraction of KG1a-DAC cells was lower in the peripheral blood but higher in the BM and the spleen when compared to KG1a parental cells (Figure 1A). One of the features associated with chemoresistance of MDS/AML is an increased vascular permeability that may promote leukemic cell homing.[14,15] Accordingly, in co-culture experiments performed in a transwell device, KG1a-DAC cell increased the permeability of human umbilical vein endothelial cell (HUVEC) monolayers (*Online Supplementary Figure S1A*). In accordance with the role of cell-cell junctions in endothelium permeability,[16] immunoblot analyses detected a decreased expression of ZO-1, occludin and claudin5 in HUVEC co-cultured with KG1a-DAC compared to KG1a cells (*Online Supplementary Figure S1B*).

Interestingly, a similar decrease in the expression of ZO-1, occludin and claudin5 was detected in HUVEC co-cultured for 48 hr with plasma collected from MDS/AML patients refractory to DAC (DAC-R), as compared to plasma collected from patients sensitive to the drug (DAC-S) and healthy donor (HD) plasma (Figure 1B). Plasma collected from MDS/AML patients who responded to DAC was observed to increase the trans-endothelial migration of GFP-labeled KG1a cells (Figure 1C), the permeability of HUVEC monolayers to rhodamine (Figure 1D), and the formation of tubes (Figure 1E, *Online Supplementary Figure S1C*). All these effects were amplified when plasma was collected from MDS/AML patients who did not respond to DAC (Figure 1C-E, *Online Supplementary Figure S1C*). Similar results were observed

when HUVEC monolayers were treated with conditioned media (CM) collected after 48 hr of culture of KG1a-DAC, compared to KG1a-CM collected in the same conditions (*Online Supplementary Figure S1D-G*).

Small extracellular vesicles released by myelodysplastic syndromes and acute myeloid leukemia cells impair the endothelial barrier

Recognizing the pivotal role of sEV in facilitating metastasis by compromising vascular integrity,[16,17] we performed ultracentrifugation to sort sEV from KG1a and KG1a-DAC CM. These sEV exhibited a typical spherical shape (*Online Supplementary Figure S2A, B*), a consistent size distribution peaking at approximately 100 nm (*Online Supplementary Figure S2C, D*), and a strong expression of canonical sEV markers such as CD63, Alix, and Tsg101 (*Online Supplementary Figure S2E, F*). Treatment of HUVEC monolayers with sEV derived from KG1a-DAC-CM resulted in a significant decrease in ZO-1, occludin, and claudin5 expression (Figure 2A), increased permeability (Figure 2B), and enhanced tube formation (Figure 2C) compared to sEV collected from KG1a-CM. Consistent with these observations, HUVEC monolayers exposed to plasma sEV collected from DAC-R patients exhibited a significant reduction in ZO-1, occludin and claudin5 (Figure 2D), increased permeability (Figure 2E), and enhanced tube formation (Figure 2F, *Online Supplementary Figure S2G*) when compared to those treated with plasma sEV collected from HD and DAC-S patients.

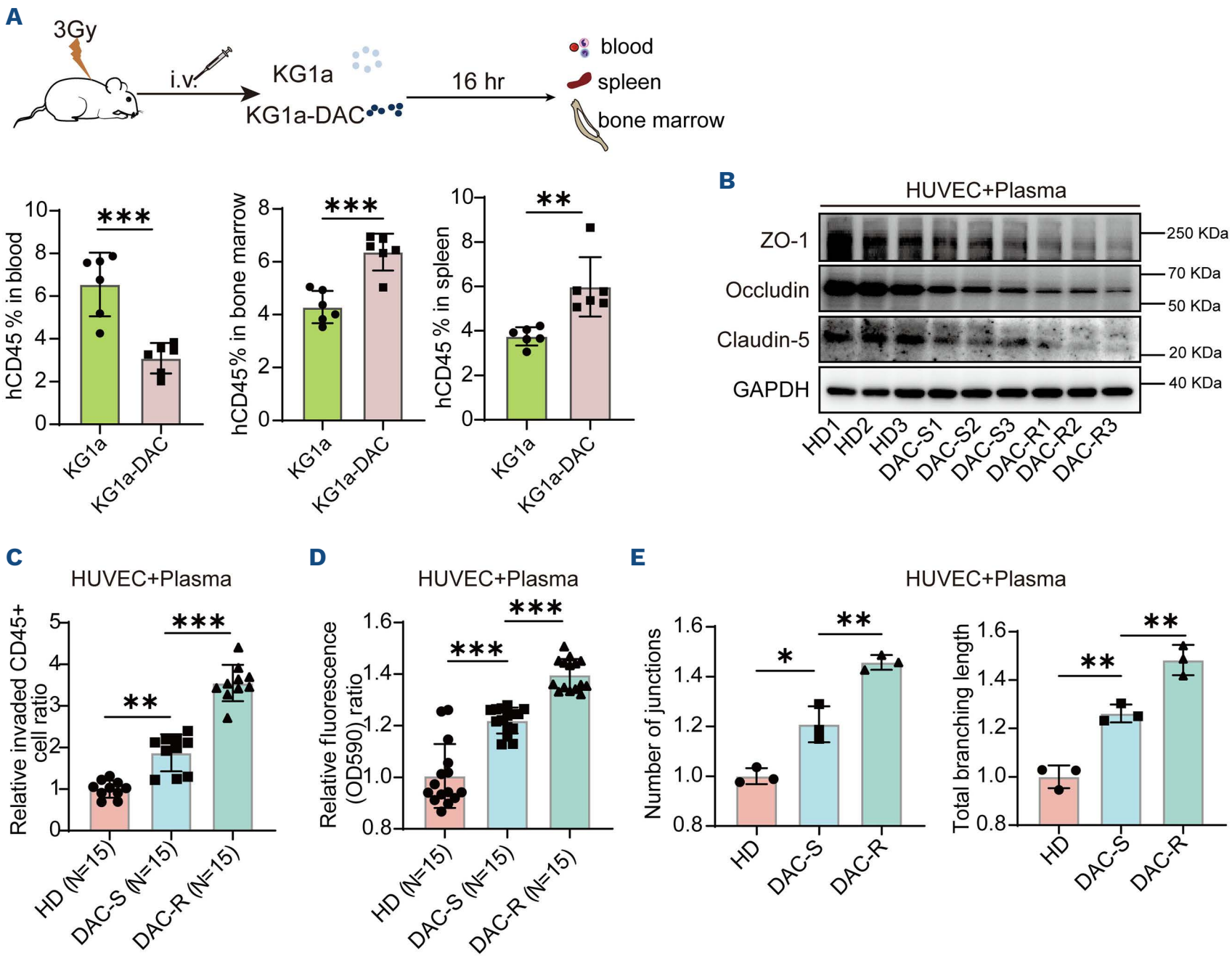

Figure 1. The effect of decitabine resistance on leukemic cell homing and endothelial permeability. (A) Xenotransplantation model. Mice were irradiated (3 Gy) and injected intravenously with KG1a or decitabine (DAC)-resistant KG1a cells (KG1a-DAC). Human CD45⁺ cells were quantified in blood, bone marrow, and spleen 16 hours (hr) after injection (N=6 mice per group). (B) Immunoblot analysis of tight junction proteins in human umbilical vein endothelial cells (HUVEC) incubated with plasma collected from healthy individuals (HD) and myelodysplastic syndromes/acute myeloid leukemia (MDS/AML) patients who were sensitive to DAC treatment (DAC-S) or refractory to DAC treatment (DAC-R). (C and D) HUVEC permeability to green fluorescent protein-positive (GFP⁺) KG1a cells and rhodamine B-labeled dextran were examined after 48 hr incubation with plasma from HD, DAC-S, and DAC-R MDS/AML patients. (E) Angiogenesis metrics, including junction count and total branching length, were quantified in HUVEC treated with the indicated plasma. Plasma samples: HD (N=15), DAC-S (N=15), DAC-R (N=15). Data are mean ± standard error of mean. Experiments were repeated three times with similar results. **P*<0.05, ***P*<0.01, ****P*<0.001.

Small extracellular vesicles released by decitabine-refractory leukemic cells demonstrate aberrant fucosylation

We previously reported the aberrant glycosylation pattern of sEV released by resistant leukemic cells.[18] Because patient plasma contains a mixture of vesicular and non-vesicular glycoconjugates, we next analyzed plasma N-glycan composition by MALDI-TOF/TOF-MS (*Online Supplementary Table S2, Online Supplementary Figure S3A-C*) to obtain an overview of systemic glycosylation changes among HD, DAC-S, and DAC-R patients. This global analysis suggested increased fucosylation in the plasma collected from DAC-R patients (*Online Supplementary Figure S4A-C*). In parallel, we isolated sEV from the KG1a and KG1a-DAC cells, respectively, and performed MALDI-TOF/TOF-MS–based N-glycomic profiling. Consistent with the plasma analysis, sEV derived from KG1a-DAC exhibited an overall increase in fucosylated N-glycans (*Online Supplementary Table S2, Online Supplementary Figure S4D, E*).

We then performed lectin blot assays using *Lens Culinaris* Agglutinin (LCA) and *Aleuria Aurantia* Lectin (AAL), which specifically recognize core α1,6-fucose and terminal α1,3/α1,4/α1,6-fucose, respectively. Both LCA and AAL signals were increased in sEV from DAC-R patients compared with DAC-S, with AAL signal being the highest signal (Figure 3A). Consistently, the Lectin-based ELISA assay demonstrated increased AAL plasma levels (*Online Supplementary Figure S4F*), which were also detected in sEV (Figure 3B) when collected from the plasma of DAC-R compared to DAC-S patients and from HD. In line with patient findings, AAL reactivity was strongly enhanced in KG1a-DAC cells and their sEV (Figure 3C, D).

Fucosyltransferase (*FUT*) genes encode enzymes that transfer an L-fucose sugar from a GDP-fucose (guanosine diphosphate-fucose) donor substrate to an acceptor substrate. Quantitative reverse transcription PCR (qRT-PCR) analyses detected an increased expression of several genes of the *FUT* family, including *FUT3*, *FUT4*, *FUT6*, *FUT7* and *FUT8*, in KG1a-DAC cells compared to KG1a parental cells, contrasting with a significant decrease in the expression of *FUT5* and *FUT9* genes (Figure 3E). Among these genes, we selected the *FUT4* gene, whose expression was the

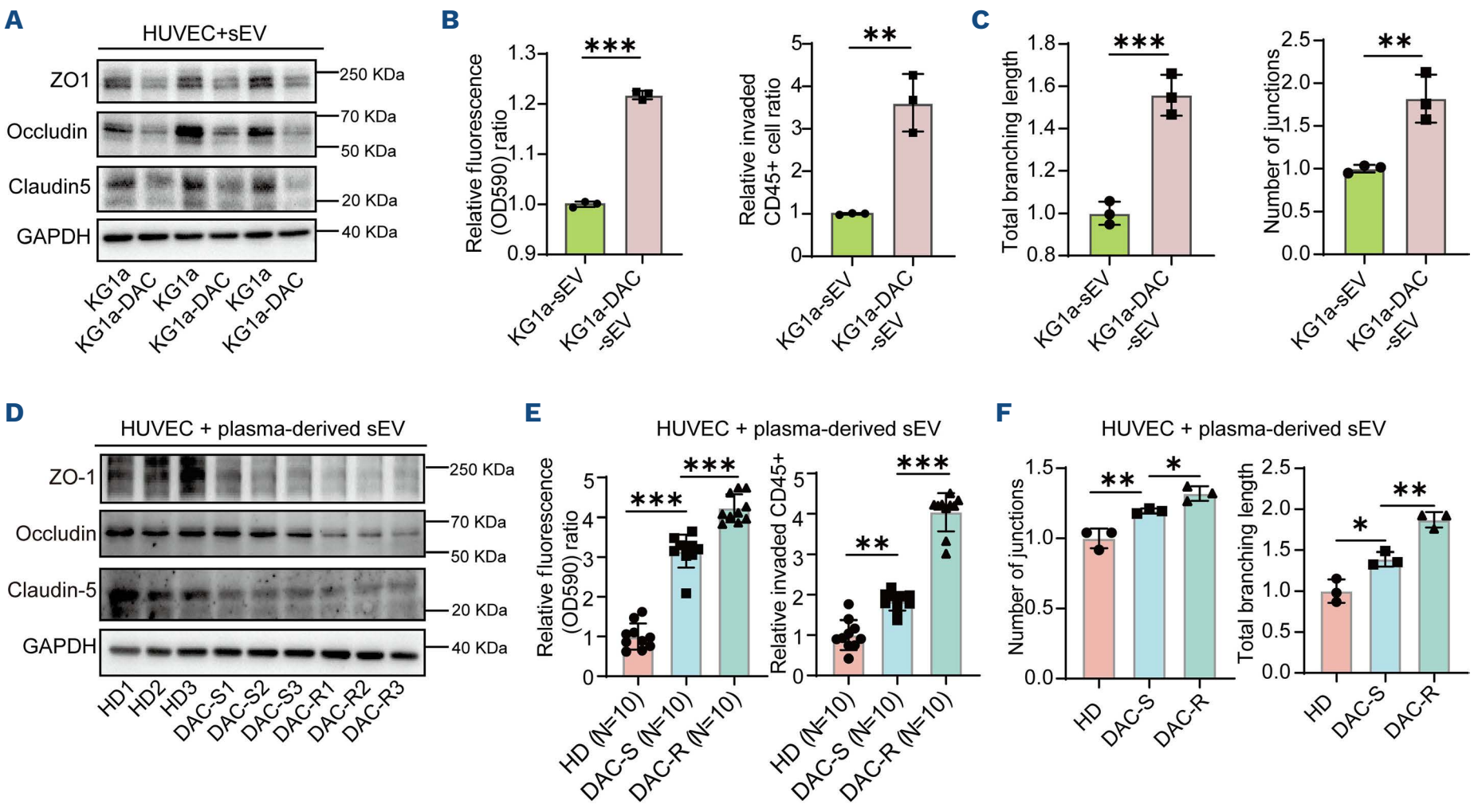

Figure 2. The effect of small extracellular vesicles released by decitabine-resistant cells on leukemia cell homing and endothelial permeability. (A) Immunoblot analysis of tight junction-related proteins in human umbilical vein endothelial cells (HUVEC) after exposure to small extracellular vesicles (sEV) from KG1a or KG1a-DAC. (B) HUVEC permeability after exposure to the indicated sEV. (C) Angiogenesis metrics, including junction count and total branching length, were quantified in HUVEC treated with the indicated sEV. (D) Expression of tight junction-related proteins in HUVEC after exposure to sEV from healthy individuals (HD) and myelodysplastic syndromes/acute myeloid leukemia (MDS/AML) patients who were sensitive to DAC treatment (DAC-S) or refractory to DAC treatment (DAC-R). (E) HUVEC permeability after exposure to the indicated sEV. (F) Angiogenesis metrics, including junction count and total branching length, were quantified in HUVEC treated with the indicated sEV. Plasma-derived sEV samples: HD (N=10), DAC-S (N=10), DAC-R (N=10). Data are mean ± standard error of mean. Experiments were repeated three times with similar results. **P*<0.05, ***P*<0.01, ****P*<0.001.

highest detected in resistant cells (*Online Supplementary Figure S4G*).

Lewis x enhances leukemic cell homing and vascular permeability

FUT4 encodes a fucosyltransferase that primarily catalyzes the biosynthesis of non-sialylated Lewis x (Le^x, also known as CD15) (Figure 4A). Le^x is expressed on a variety of glycolipids and glycoproteins on the cell surface of neutrophils, monocytes, eosinophils and non-hematopoietic tissues, and in our system was abolished by PNGase F treatment (*Online Supplementary Figure S4H*).[19] Le^x levels were markedly increased in sEV isolated from DAC-R patient plasma compared with DAC-S and HD samples (*Online Supplementary Figure S4 I*). Compared to KG1a parental cells, Le^x expression was increased at the surface of KG1a-DAC (Figure 4B, *Online Supplementary Figure S4J*) as well as in their derived sEV (Figure 4B). We over-expressed *FUT4* gene in KG1a cells (designated as KG1a-FUT4), which resulted in a significant increase in Le^x levels (Figure 4C), A similar trend was also observed in the corresponding sEV (Figure 4C). Treatment of HUVEC monolayers with sEV or CM from KG1a-FUT4 cells reduced ZO-1, occludin and claudin5 expression (Figure 4D, *Online Supplementary Figure S5A*), along with increased permeability (Figure 4E, *Online Supplementary Figure S5B, C*).

In contrast, knockdown of *FUT4* in KG1a-DAC cells (KG1a-DAC-shFUT4) significantly decreased Le^x levels in both cells and in derived sEV (*Online Supplementary Figure S5E, F*). Treatment of HUVEC monolayers with sEV or CM from KG1a-DAC-shFUT4 cells up-regulated ZO-1, occludin and claudin5 expression (*Online Supplementary Figure S5G, H*), and decreased permeability (*Online Supplementary Figure S5 I, J*). Furthermore, sEV or CM from KG1a-FUT4

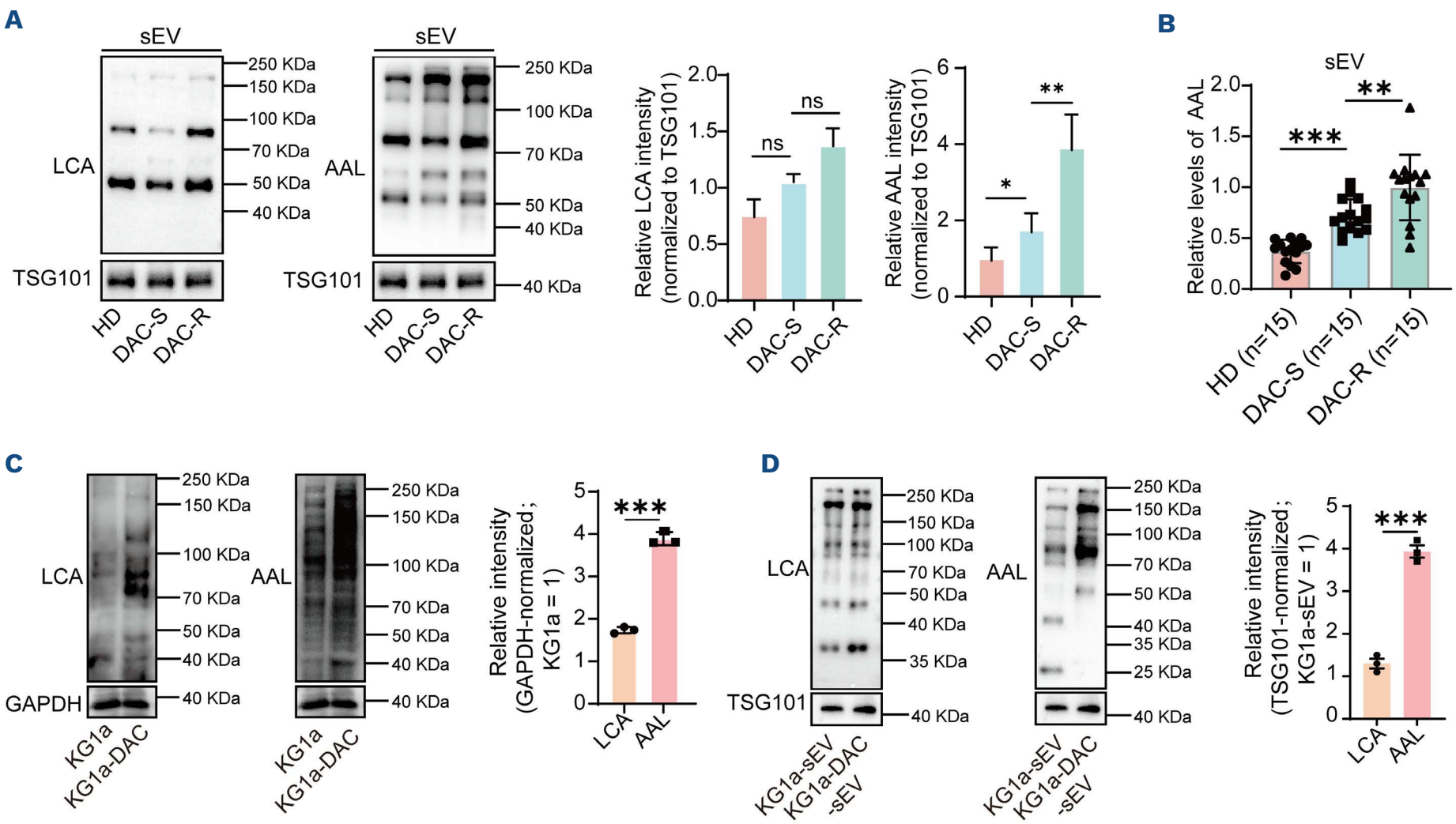

Figure 3. Analysis of fucosylation and fucosyltransferase expression in myelodysplastic syndromes/acute myeloid leukemia patients who were sensitive to decitabine treatment or refractory to decitabine treatment. (A) Lectin blotting analysis of core fucose (LCA) and terminal fucose (AAL) in plasma-derived small extracellular vesicles (sEV) from healthy individuals (HD), myelodysplastic syndromes/acute myeloid leukemia (MDS/AML) patients who were sensitive to DAC treatment (DAC-S) and refractory to DAC treatment (DAC-R). Band intensities were normalized to TSG101. (B) Relative levels of AAL on sEV determined by ELISA. (C and D) Lectin blotting analysis of LCA and AAL in KG1a, KG1a-DAC cells and their derived sEV. Band intensities were normalized to GAPDH (cell lysates) or TSG101 (sEV), as indicated. (E) Relative expression of fucosyltransferases (FUT) in KG1a and KG1a-DAC measured by quantitative reverse transcription PCR (qRT-PCR). Plasma-derived sEV samples: HD (N=15), DAC-S (N=15), DAC-R (N=15). Data are mean ± standard error of mean. Experiments were repeated three times with similar results. ns: not significant, **P*<0.05, ***P*<0.01, ****P*<0.001.

cells enhanced tube formation (Figure 4F, *Online Supplementary Figure S5D*), while KG1a-DAC-shFUT4 sEV or CM reduced it (*Online Supplementary Figure S5K, L*). Furthermore, treatment of HUVEC with OptiPrep-purified sEV from KG1a-DAC or KG1a-FUT4 cells reduced ZO-1, occludin and claudin5 expression, while KG1a-DAC-shFUT4 sEV restored them (*Online Supplementary Figure S5M*), confirming the sEV-specific endothelial effects.

Next, we investigated whether Le^x promotes leukemic cell homing by enhancing vascular permeability. NSG mice were

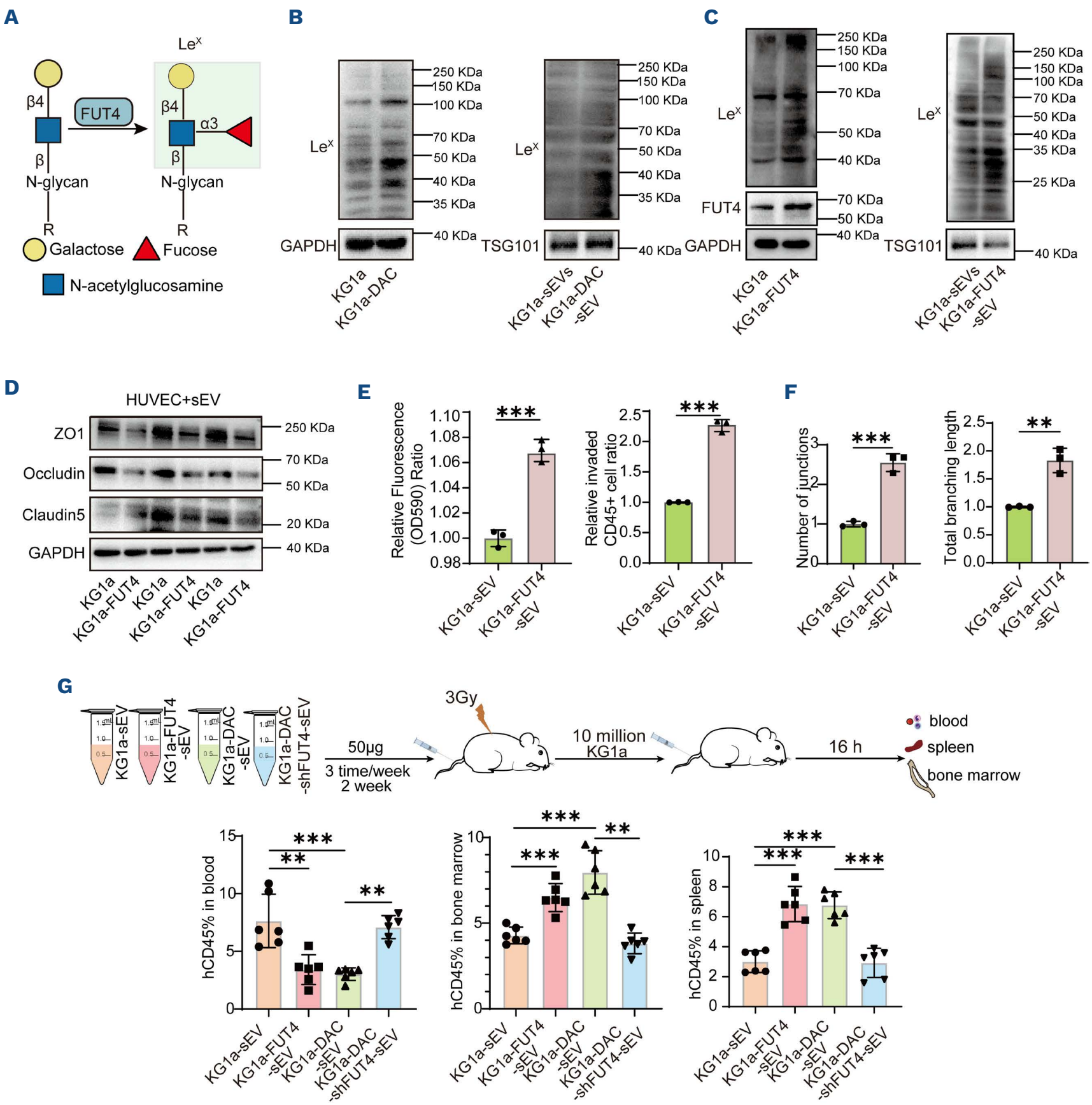

Figure 4. Impact of Lewis x expression on leukemic cell homing and vascular permeability. (A) Schematic illustration of Lewis x (Le^x) and fucosyltransferase 4 (FUT4)-mediated Le^x modification. (B) Le^x levels in KG1a and KG1a-DAC cells and their derived small extracellular vesicles (sEV). (C) FUT4 expression and Le^x levels in KG1a cells over-expressing FUT4 (designated as KG1a-FUT4) and their sEV. (D) Tight junction-related proteins in human umbilical vein endothelial cells (HUVEC) treated with sEV from KG1a and KG1a-FUT4. (E) HUVEC permeability and leukemic cell transendothelial migration after treatment with sEV from KG1a or KG1a-FUT4. (F) Angiogenesis metrics, including junction count and total branching length, were quantified in HUVEC treated with sEV from KG1a or KG1a-FUT4. (G) Schematic of the xenotransplantation mouse model and quantification of human $CD45^+$ cells in blood, bone marrow, and spleen. Data are mean ± standard error of mean. Experiments were repeated three times with similar results. **P*<0.05, ***P*<0.01, ****P*<0.001.

preconditioned with sEV from HD, KG1a, KG1a-DAC, KG1a-FUT4, or KG1a-DAC-shFUT4 cells, followed by intravenous injection of KG1a cells (Figure 4G, *Online Supplementary Figure S5N*). Mice pretreated with HD-sEV showed no significant enhancement of leukemic cell homing, as indicated by higher levels of circulating hCD45^{+} cells and fewer cells in bone marrow and spleen compared with the KG1a-sEV group (*Online Supplementary Figure S5N*). In contrast, KG1a-DAC-sEV and KG1a-FUT4-sEV significantly increased KG1a cell homing to the BM and spleen, accompanied by fewer circulating cells in peripheral blood. Conversely, KG1a-DAC-shFUT4-sEV reduced KG1a cell homing to the BM and spleen, with more cells being retained in the peripheral blood (Figure 4G).

Enhanced vascular permeability involves TWIST1-driven *FUT4* gene expression

Since we previously associated MDS/AML cell resistance to DAC with an elevated expression of TWIST1,[12] a basic helix-loop-helix transcription factor, we explored TWIST1 impact on *FUT4* gene expression and Le^{X} level. TWIST1 was over-expressed in KG1a (KG1a-TWIST1) (Figure 5A, *Online Supplementary Figure S6A*) and was stably knocked down in SKM1(SKM1-shTWIST1) (*Online Supplementary Figure S6B, C*), an MDS cell line that spontaneously expresses high levels of TWIST1. When HUVEC monolayer was incubated with CM from KG1a-TWIST1 cells, we observed a decrease in ZO-1, occludin and claudin5 expression (Figure 5B), an increased permeability (Figure 5C) and an enhanced tube formation (Figure 5D). In contrast, CM from SKM1-shTWIST1 cells increased ZO-1, occludin and claudin5 expression (*Online Supplementary Figure S6D*), decreased permeability (*Online Supplementary Figure S6E, F*) and reduced tube formation (*Online Supplementary Figure S6G*).

TWIST1 binds preferentially to E-box DNA promoter sequences (-CANNTG-) in target gene promoters.[20,21] Analysis of the

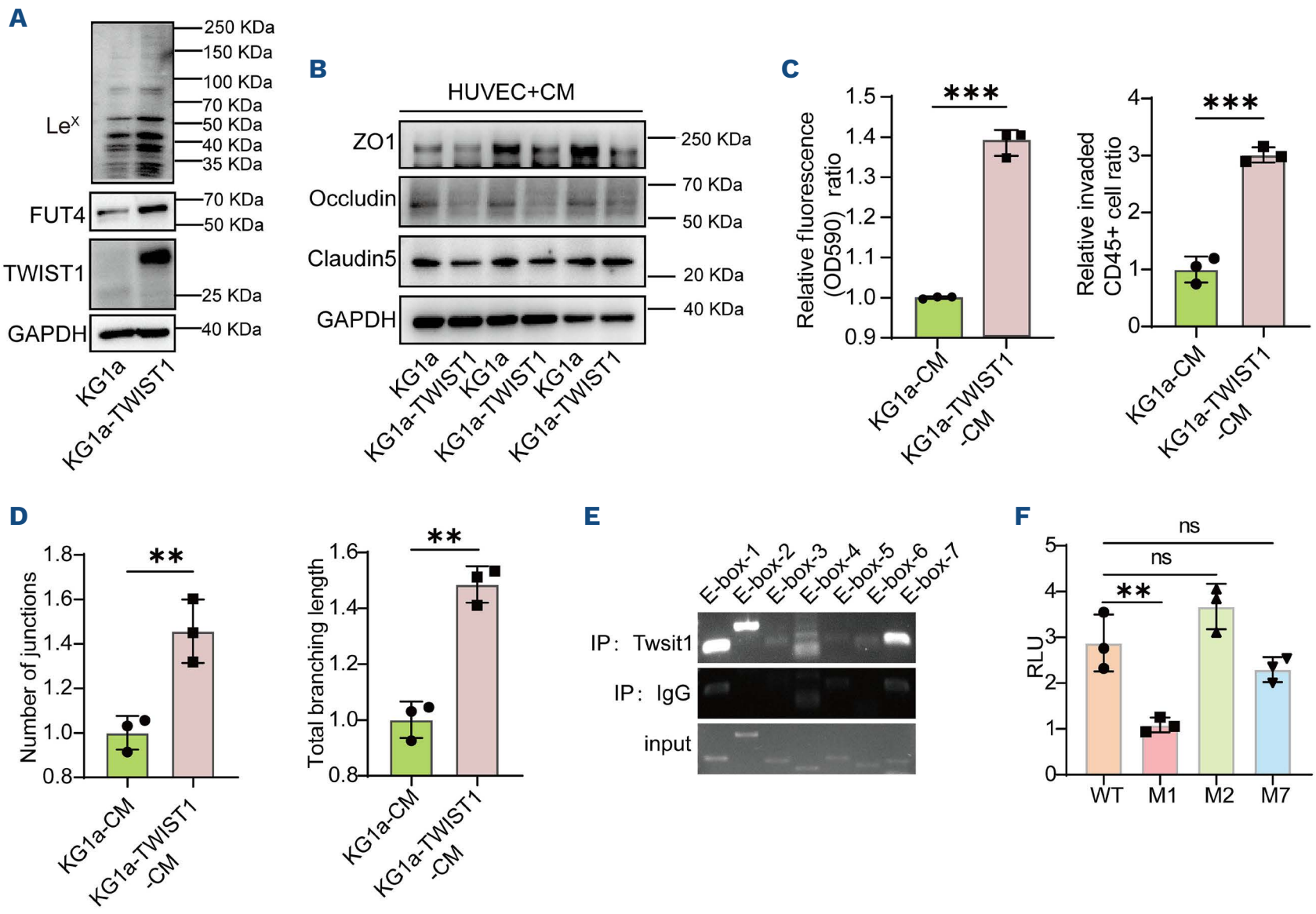

Figure 5. TWIST1 activated fucosyltransferase 4 transcription. (A) Expression of fucosyltransferase 4 (FUT4), TWIST1 and the levels of Le^{X} in KG1a and KG1a cells over-expressing TWIST1 (KG1a-TWIST1). (B) Tight junction–related proteins and (C) human umbilical vein endothelial cells (HUVEC) permeability and leukemic cell transendothelial migration after treatment with conditioned medium (CM) from KG1a or KG1a-TWIST1 cells. (D) Angiogenesis metrics, including junction count and total branching length, were quantified in HUVEC treated with CM from KG1a or KG1a-TWIST1 cells. (E) ChIP analysis of TWIST1 binding to E-box motifs within the *FUT4* promoter (0-2000 bp). GAPDH was used as negative control. (F) Dual luciferase assay of TWIST1 binding to wild-type and mutant E-box motifs. Data are mean ± standard error of mean. Experiments were repeated three times with similar results. ns: not significant, **P*<0.05, ***P*<0.01, ****P*<0.001.

FUT4 promoter (-2.0 kb) identified seven E-box motifs (*Online Supplementary Figure S6H*). ChIP assays revealed a strong binding of TWIST1 to motifs 1, 2, and 7 while binding to motifs 3, 4, 5, and 6 was weak (Figure 5E, *Online Supplementary Table S3*). A luciferase reporter assay confirmed that TWIST1 overexpression strongly enhanced FUT4-WT promoter activity, whereas mutation of the first E-box motif (FUT4-M1) markedly reduced TWIST1-mediated transactivation (Figure 5F, *Online Supplementary Figure S6 I, Online Supplementary Table S4).* These findings demonstrate that TWIST1 enhances *FUT4* gene transcription by binding to the first E-box motif of its promoter.

Intercellular adhesion molecule 3 identified as key Lewis x-modified protein

Using quantitative proteomic analysis, we identified differentially expressed proteins in KG1a, KG1a-FUT4, KG1a-DAC, and KG1a-DAC-shFUT4 cells. A total of 77 proteins were differentially expressed (fold change >1.5 or <0.67; P<0.05) across these groups (Figure 6A). Notably, intercellular adhesion molecule 3 (ICAM3), a key adhesion molecule involved in promoting angiogenesis, was significantly increased in KG1a-DAC and KG1a-FUT4 while being decreased in KG1a-DAC-shFUT4 (Figure 6B). Immunoprecipitation (IP) assay confirmed enhanced Le^X modification on ICAM3 in KG1a-DAC and KG1a-FUT4 cells, whereas this modification was reduced in KG1a-DAC-shFUT4 cells (Figure 6C). Consistently, the presence of ICAM3 on sEV was clearly revealed by density gradient fractionation (*Online Supplementary Figure S7A*). Additionally, ICAM3 and Le^X expression was up-regulated in sEV from KG1a-DAC and KG1a-FUT4 cells but down-regulated in sEV from KG1a-DAC-shFUT4 (*Online Supplementary Figure S7B, C*). Inhibiting protein synthesis with cycloheximide (CHX) revealed

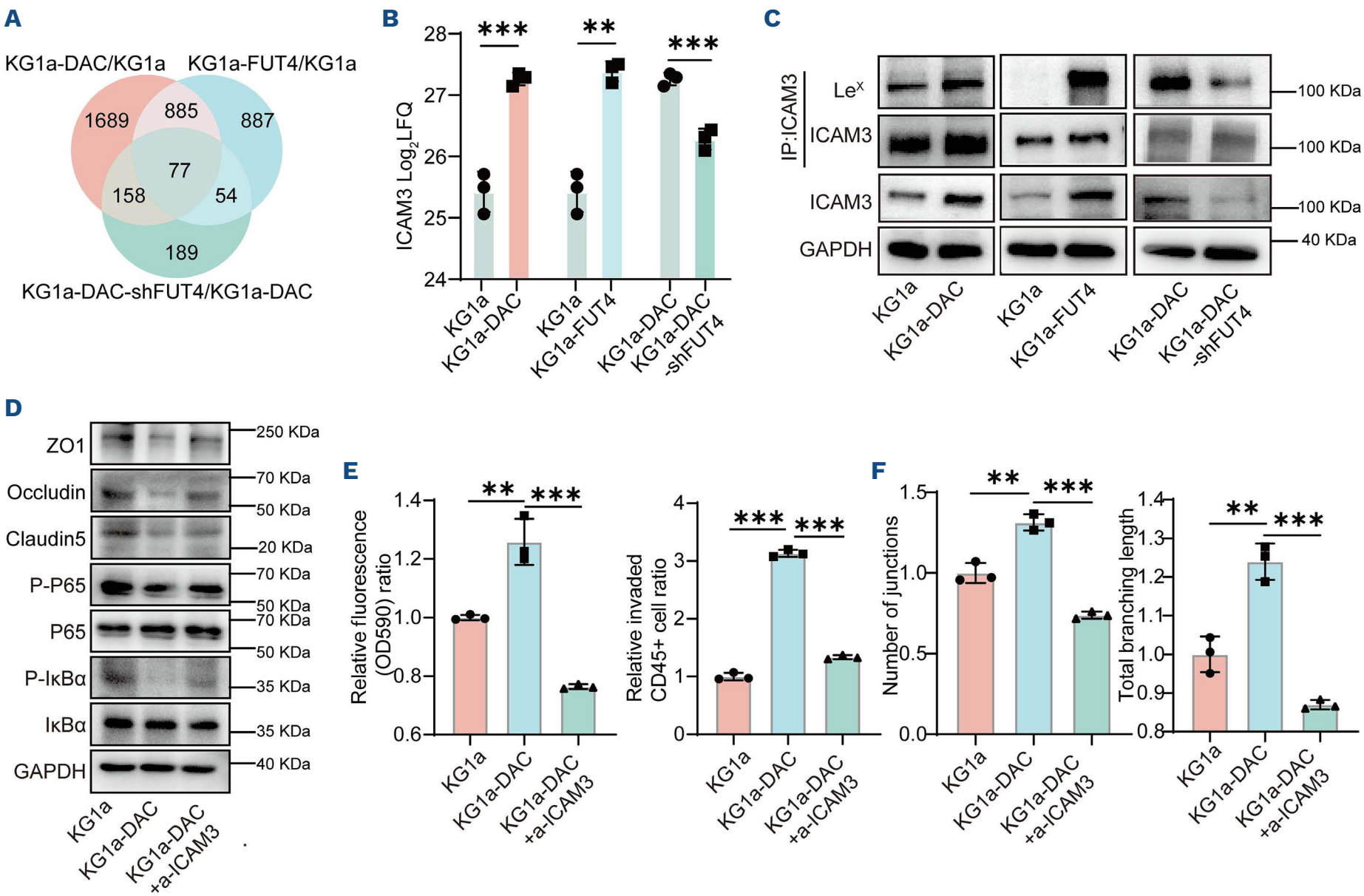

Figure 6. Effects of Lewis x-modified intercellular adhesion molecule 3 on vascular permeability and leukemic cell homing. (A) Venn diagram showing differentially expressed proteins among parental KG1a cells, decitabine (DAC)-resistant KG1a (KG1a-DAC) cells, fucosyltransferase 4-overexpressing KG1a (KG1a-FUT4) cells, and FUT4-silenced KG1a-DAC (KG1a-DAC-shFUT4) cells. (B) Liquid chromatography-mass spectrometry analysis of intercellular adhesion molecule 3 (ICAM3) in these cells. (C) Lewis x (Le^X) modification on ICAM3 evaluated by immunoprecipitation (IP)/western blotting. (D) Expression of tight junction-related proteins and NF-κB signaling components (p65, p-p65, IκBα, p-IκBα) in KG1a, KG1a-DAC and KG1a-DAC treated with anti-human intercellular adhesion molecule 3 antibody (a-ICAM3). (E) Cell permeability and (F) angiogenesis metrics of human umbilical vein endothelial cells (HUVEC) after co-culture with KG1a, KG1a-DAC or KG1a-DAC in the presence of a-ICAM3. Data are mean ± standard error of mean. Experiments were repeated three times with similar results. *P<0.05, **P<0.01, ***P<0.001.

slower degradation of ICAM3 in KG1a-FUT4 cells compared to KG1a cells (*Online Supplementary Figure S7D*). Further study demonstrated that Le^X modification stabilizes ICAM3 by inhibiting its degradation via the lysosomal pathway (*Online Supplementary Figure S7E*).

Lewis x-modified intercellular adhesion molecule 3 enhances vascular permeability via NF-κB signaling

Since the transcription factor NF-κB regulates the expression of genes that modulate the vascular barrier,[22] we treated HUVEC monolayers cells with the NF-κB signaling inhibitor BAY-11-7082, which decreased the expression of ZO-1, occludin and claudin5 (*Online Supplementary Figure S7F, G*). Conversely, stimulation of HUVEC monolayers with phorbol 12-myristate 13-acetate (PMA), an activator of NF-κB signaling, led to increased phosphorylation of P65 and IκBα, and a time-dependent increase in ZO-1, occludin and claudin5 expression (*Online Supplementary Figure S7H, I*). Furthermore, treatment of HUVEC monolayers with sEV from KG1a-DAC or KG1a-FUT4 reduced phosphorylation of p65 (p-p65) and IκBα (p-IκBα) levels in these cells, while sEV from KG1a-DAC-shFUT4 enhanced NF-κB activation (*Online Supplementary Figure S7J*). In HUVEC monolayers, treatment with sEV from KG1a-DAC or KG1a-FUT4 significantly down-regulated p-p65 and p-IκBα signaling and reduced the expression of ZO-1, occludin and claudin5, while these effects were partly reversed by anti-human ICAM3 antibody treatment (Figure 6D, *Online Supplementary Figure S8A*). These sEV also increased permeability and tube formation, and these effects were mitigated by α-ICAM3 treatment (Figure 6E, F, *Online Supplementary Figure S8B-D*). Conversely, sEV from KG1a-DAC-shFUT4 enhanced p-p65 and p-IκBα signaling, increased ZO-1, occludin and claudin5 expression, and reduced permeability and tube formation in HUVEC. These effects were partly reversed by recombinant ICAM3 protein (rICAM3) (*Online Supplementary Figure S8E-H*). Finally, stable overexpression of the ICAM3 gene in HUVEC (HUVEC-ICAM3) led to reduced phosphorylation of p65 and IκBα, accompanied by decreased expression of ZO-1, occludin and claudin5 (*Online Supplementary Figure S8 I, J*), which was associated with an increased permeability of HUVEC monolayer (*Online Supplementary Figure S8K, L*) and an enhanced tube formation (*Online Supplementary Figure S8M*).

Discussion

Primary resistance of leukemic cells to HMA, which is observed in approximately 50% of elderly patients with MDS or AML, remains a therapeutic challenge. The present study deciphers a pathway by which leukemic cells modulate their glycosylation profile and release sEV that alter surrounding endothelial cell functions, leading to the disruption on vascular wall integrity with increased permeability and enhanced leukemic cell homing. Such a toxic interaction between leukemic cells and their microenvironment may contribute to therapeutic resistance.

The integrity of the blood vessel endothelium serves as a barrier that regulates hematopoietic cell migration into tissues.[23] Leukemic cells actively remodel their microenvironment by disrupting vascular integrity, thereby facilitating their homing to tissues and promoting their survival.[5] Recent studies have highlighted the role of leukemic cell-derived sEV in mediating interaction between leukemic cells and their microenvironment, especially in the BM niche.[24] sEV from drug-resistant malignant cells were shown also to generate pre-metastatic niches into distant tissues by promoting vascular development and permeability to facilitate tumor cell dissemination.[25,26] Examples include sEV generated by glioma stem cells that promote angiogenesis through activating the VEGF/VEGFR2 signaling pathway,[27] and cancer cell-derived sEV that enhance tumor vascularization and trigger stroma remodeling through promoting CXCL8 secretion by mesenchymal stem cells.[28] We recently demonstrated that extracellular vesicles released from KG1a-DAC promote DAC-resistant through miR-4755-5p that reduces the expression of cyclin-dependent kinase inhibitor 2B (CDKN2B) in KG1a cells.[13] In the present study, we show that sEV released by DAC-resistant leukemic cells, which exhibit elevated homing ability in a xenotransplantation mouse model, down-regulate tight junction-associated proteins to increase vascular permeability.

We have previously shown that glycosylation modifications detected on leukemic cell-derived sEV contribute to reshaping their microenvironment.[18] Our glycomic analyses identify a significant upregulation of terminal fucosylation in DAC-R patients. More precisely, we found elevated levels of Le^X in sEV collected from DAC-R plasma, highlighting the potential role of FUT4-driven fucosylation in therapy resistance. Such an increased FUT4-driven fucosylation was previously detected in AML stem cells[29] and in metastatic colorectal cancer cells.[30]

Upstream of *FUT4* gene upregulation, we have identified TWIST1, an oncogenic transcription factor whose increased expression was depicted in chronic myeloid leukemia cells and involved in their resistance to the tyrosine kinase inhibitor imatinib.[31] TWIST1 expression was also detected in some MDS/AML cell lines, and its overexpression was related to leukemogenesis and drug resistance, including resistance to DAC.[32] The present study identifies one of the seven E-box depicted within the *FUT4* gene promoter as the primary binding site for TWIST1.

Downstream of FUT4 overexpression, our quantitative proteomic analysis identifies ICAM3 as a Le^X-modified protein in DAC-resistant leukemic cells. Immunoprecipitation (IP) confirmed Le^X modification on ICAM3 in KG1a-DAC and KG1a-FUT4 cells, while this modification was decreased in KG1a-DAC-shFUT4. Mechanistically, Le^X modification could stabilize ICAM3 by preventing its lysosomal degradation. ICAM3 was shown to play a role in inflammatory signaling,

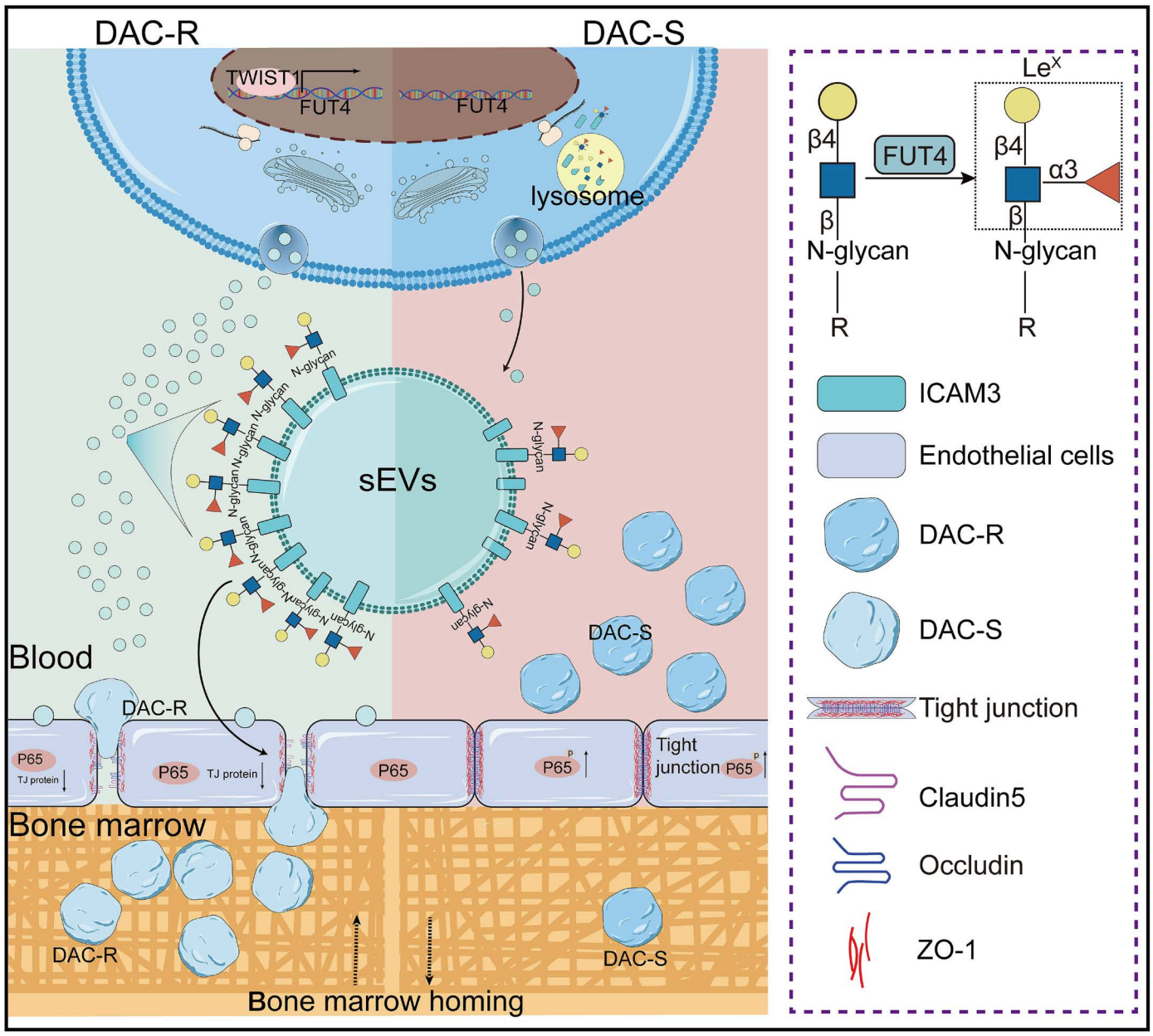

Figure 7. Lewis x-modified intercellular adhesion molecule 3 on small extracellular vesicle-mediated endothelial dysfunction promotes leukemic cell homing in decitabine-resistant myelodysplastic syndromes/acute myeloid leukemia: a conceptual model. DAC: decitabine; ICAM3: intercellular adhesion molecule 3; MDS/AML: myelodysplastic syndromes/acute myeloid leukemia; sEV: small extracellular vesicles.

cancer cell stemness and vascular permeability.[33,34] Increased ICAM3 delivery through leukemic cell-derived sEV to endothelial cells may inhibit NF-κB signaling, thereby disrupting vascular permeability,[22] thereby promoting MDS/AML cell tissue migration (Figure 7). In line with this, our results show that FUT4-high sEV suppress NF-κB activation by lowering p65 and IκBα phosphorylation in HUVEC, consequently down-regulating tight junction proteins ZO-1 and occludin and compromising endothelial barrier integrity. Together, this work deciphers a mechanism by which MDS/AML cells that are refractory to DAC release extracellular vesicles expressing modified proteins that reshape their microenvironment by increasing vascular permeability and leukemic cell migration into tissues. If this reshaped microenvironment affords stimulatory signaling that further enhance leukemic cell resistance to DAC and other anti-leukemic drugs, disruption of this stimulatory signaling may improve their therapeutic efficacy. In this context, therapeutic strategies aimed at inhibiting FUT4 activity or blocking Le^X modification of ICAM3 may serve as promising adjuncts to HMA, with the potential to normalize endothelial barrier function, limit leukemic cell homing, and reduce microenvironmental protection.

Disclosures

No conflicts of interest to disclose.

Contributions

XL conceptualized the study, designed the methodology, acquired funding, oversaw project administration, supervised the study, and contributed to reviewing and editing the manuscript; JF designed the methodology, curated the data, conducted the formal analysis, prepared the visualizations, carried out the investigation, wrote the original draft, and helped to review and edit the manuscript; KW, JGo, BH, WW, JGe and SF carried out the investigation; YW and YF provided resources; ES and FG supervised the study, and helped to review and edit the manuscript. All authors have reviewed and approved the final version of the manuscript for publication.

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Data-sharing statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request. Raw mass spectrometry data have been deposited in the GlycoPOST repository under accession GPST000674.

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