

Targeting CD93 in Infantile Hemangioma: Underlying Mechanisms of Angiogenesis and Therapeutic Intervention

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Abstract

Objective

To investigate the expression characteristics of CD93 in infantile hemangioma (IH) tissues, cells, and *in vivo* models, and to evaluate its biological function in IH angiogenesis as well as its potential value as a therapeutic target for anti-angiogenic therapy.

Methods

Clinical specimens from the proliferative and involuting phases of IH were collected. The expression levels of CD93 were detected using immunohistochemistry (IHC), immunofluorescence (IF), Western blotting (WB), and quantitative real-time PCR (qPCR). CD133⁺ hemangioma-derived stem cells (HemSCs) were isolated via collagenase digestion and CD133 immunomagnetic bead sorting. Stable cell lines with CD93 overexpression (CD93-OE) and knockdown (CD93-sh) were established using lentiviral transfection technology. The effects of CD93 and its polyclonal antibody on angiogenesis were assessed through *in vitro* tube formation assays. A subcutaneous xenograft model in BALB/c nude mice was constructed, and microvessel density (MVD) counting was performed to evaluate the regulatory role of CD93 in angiogenesis *in vivo*.

Results

CD93 was highly expressed in both proliferative-phase IH tissues and CD133⁺ HemSCs. *In vitro* experiments demonstrated that CD93 overexpression significantly enhanced the tube-forming ability of HemSCs, evidenced by increased total length, number of junctions, and number of meshes ($P < 0.05$). Conversely, silencing CD93 or blocking with a CD93 polyclonal antibody significantly suppressed this function ($P < 0.05$). The animal experiments successfully established a nude mouse model simulating IH proliferation and involution. *In vivo* angiogenesis results showed that the microvessel density (MVD) in the CD93 overexpression group was significantly higher than that in the control group (113.00 ± 16.95 vs. 67.67 ± 17.11 vessels/mm², $P < 0.001$), whereas the MVD in the CD93 knockdown and antibody blocking groups was significantly reduced ($P < 0.05$).

Conclusion

CD93 is highly expressed during the proliferative phase of IH and significantly promotes the *in vitro* and *in vivo* angiogenic capacity of HemSCs. Targeted blockade of CD93 effectively inhibits angiogenesis, suggesting that CD93 is a promising novel therapeutic target for the treatment of IH.

Introduction

Infantile haemangiomas (IH) constitute the most prevalent benign neoplasm of infancy, affecting approximately 4.5% of infants^[1]. The incidence is disproportionately higher among female infants, preterm neonates, and those with low birth weight, exhibiting a female-to-male ratio of up to 2.3–2.9^[1–3]. Furthermore, maternal gestational diabetes, gestational hypertension, multiple gestations, placental anomalies, progesterone therapy, and a positive family history have been identified as significant risk factors predisposing to the development of IH^[4–6].

IH are characterized by a unique natural history comprising a rapid proliferative phase followed by spontaneous involution. Although typically absent at birth, these lesions manifest within the first few days of life and expand aggressively. Subsequently, they enter a prolonged period of slow involution, ultimately resolving into residual fibrofatty tissue^[7]. However, spontaneous regression is rarely complete. Without active intervention, approximately 70% of lesions result in permanent disfigurement, characterized by residual telangiectasias, fibrofatty residue, scarring, or anetodermic skin laxity^[8].

Since the serendipitous discovery by Léauté-Labrèze et al. ^[9] in 2008 regarding the efficacy of propranolol for IH, it has established itself as the first-line therapeutic agent for complex IH globally^[10–12]. While extensive research and randomized clinical trials have corroborated its efficacy and relative safety, therapeutic challenges persist. Approximately 10% of patients exhibit resistance to propranolol therapy, and recurrence rates of 19–25% have been documented following drug cessation. Furthermore, oral propranolol administration is associated with a spectrum of adverse events, including hypoglycemia, bradycardia, hypotension, bronchospasm, and electrolyte disturbances^[7, 13]. Additionally, as a lipophilic compound capable of crossing the blood-brain barrier, the specific implications of propranolol on central nervous system function remain ill-defined^[14, 15]. Given that the precise molecular mechanisms underlying its therapeutic effect are not fully elucidated and adverse events remain a concern, the identification of novel therapeutic targets is of paramount importance in the management of IH.

Current hypotheses regarding the pathogenesis of IH are multifactorial, encompassing theories of placental origin, stem cell dysregulation, hypoxic stress, and angiogenesis^[1, 6, 16, 17]. Among these, angiogenesis is recognized as the pivotal driver of the rapid proliferative phase^[18, 19]. Indeed, IH serves as a unique and valuable natural model, offering critical insights into the mechanisms of tumoral angiogenesis. In recent years, accumulating evidence has highlighted the indispensable role of HemSCs in promoting angiogenesis and driving the proliferation of IH^[20–22].

CD93, also designated as C1qR1 or C1qRp, is a 625-amino acid transmembrane glycoprotein encoded by a gene located at chromosome 20p11.21. It is classified within the C-type lectin domain family, specifically group XIV^[23]. Research has demonstrated that the EGF-like domain of CD93 induces endothelial cell proliferation and migration in vitro, while promoting angiogenesis in vivo^[24]. Predominantly expressed on endothelial cells, CD93 functions as a pivotal adhesion molecule involved in vascular maturation^[25]. In the context of proliferating endothelium, CD93 mediates the activation of

diverse signaling pathways governing cell adhesion, migration, and vascular maturation through its interaction with the extracellular matrix ^[23].

In recent years, the role of CD93 in solid malignancies and its potential as a novel target for anti-angiogenic therapy have garnered increasing scientific attention. Identified as one of the top 20 genes associated with tumor angiogenesis^[26], CD93 plays a pivotal role in the angiogenic process^[27, 28]. Elevated expression of CD93 has been consistently observed across a variety of solid tumors^[25, 29, 30]. Furthermore, evidence indicates that in malignancies such as clear cell renal cell carcinoma, glioma, and nasopharyngeal carcinoma, CD93 serves not only as a significant prognostic factor but also as a potential biomarker^[25, 29, 30]. Consequently, CD93 represents a promising frontier in the field of anti-angiogenic therapy^[31–34]. This study aims to investigate the functional role of CD93 in angiogenesis using both in vitro assays and an in vivo IH animal model, thereby evaluating its potential value as a novel therapeutic target. This research is expected to deepen the understanding of IH pathogenesis and provide critical insights for the development of innovative therapeutic strategies.

Materials and Methods

1. Cell isolation, sorting, and culture

The study protocol was approved by the Medical Ethics Committee of Fujian Medical University Union Hospital (Fuzhou, China). Proliferative IH specimens were obtained from the Department of Plastic and Reconstructive Surgery, Fujian Medical University Union Hospital, in accordance with the institutional review board guidelines. The clinical diagnosis was confirmed by the Department of Pathology and the Department of Plastic and Reconstructive Surgery. Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki.

HemSCs were isolated from proliferative specimens using a previously reported protocol^[20] involving digestion with collagenase type I (Sigma, C0130) and magnetic sorting with CD133 MicroBeads (Miltenyi Biotec, 130-100-857). The cells were cultured in EGM-2 medium (Lonza, CC-3162) supplemented with 20% fetal bovine serum (Gibco, 10099-133) and 1% antibiotics (Thermo Fisher Scientific, 15140122).

HUVECs were commercially purchased from Shanghai Saiyikang Biotechnology Co., Ltd. (Shanghai, China) and cultured in Endothelial Cell Complete Medium (PriMed-iCell-002) in a humidified incubator at 37°C with 5% CO₂.

2. Generation of CD93 Overexpression and Knockdown Stable HemSC Lines

To generate CD93-overexpressing cells, the human CD93 coding sequence (Gene ID: VB180313-1123tcw) was cloned into the mammalian lentiviral expression vector (pLV[Exp]-EGFP:T2A:Puro-EF1A>hCD93, Cyagen Biosciences, Suzhou, China). For knockdown experiments, short hairpin RNA

(shRNA) sequences targeting human CD93 were cloned into the lentiviral shRNA interference vector (pLV[shRNA]-EGFP:T2A:Puro-U6 > hCD93, Cyagen Biosciences).

HemSCs in the logarithmic growth phase were seeded into 24-well plates. Upon reaching approximately 50% confluence, the cells were transduced with the corresponding lentiviruses at a multiplicity of infection (MOI) of 80 for 24 hours. Subsequently, stable overexpression and knockdown cell lines were established by continuous selection with 0.8 µg/mL puromycin for 10 days, followed by validation using qPCR. Cells transduced with the corresponding empty vectors served as controls.

3. In vitro tube formation assay

A 30-µL aliquot of Matrigel (BD 356237) was dispensed into each well of a 96-well plate pre-chilled at 4°C. The plate was then incubated at 4°C for 30 min, followed by 15 min at room temperature, and finally at 37°C for 30 min to allow gelation. HemSCs were harvested by trypsinization, resuspended in EBM-2 complete medium, and counted. A 100µL cell suspension (1×10^5 cells/mL) was seeded onto the solidified Matrigel. After incubation at 37°C with 5% CO₂ for 6 h, tube formation was observed and images were captured under an inverted microscope.

CD93 Antibody Blocking Assay

The CD93-overexpressing stable cell line was expanded, digested with trypsin(25200-056, Gibco), and prepared as a single-cell suspension. Cells were seeded into 96-well plates at a density of 1×10^5 cells/mL (100 µL per well), with 4–5 replicates per group. The experimental group was treated with a CD93 polyclonal antibody (PA5-52664, Thermo Fisher Scientific) at a final concentration of 100 µg/mL for blocking, while the control group received an equal volume of PBS. The 96-well plates were incubated in a humidified incubator at 37°C with 5% CO₂. Microscopic images were captured at 1, 2, 4, 6, 8, and 10 h to observe and record changes in in vitro tube formation.

4. Western Blot

Stable cell lines were lysed on ice using RIPA lysis buffer (P0013, Beyotime Biotechnology, Jiangsu, China) supplemented with PMSF. Following sonication, the lysates were centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatants were collected. Protein concentrations were determined using a BCA assay kit (P0012, Beyotime Biotechnology). Equal amounts of denatured protein samples (boiled at 100°C for 5 min) were separated by SDS-PAGE (Beyotime Biotechnology) using a 10% separating gel and a 5% stacking gel. Electrophoresis was performed at 100 V for the stacking gel and 150 V for the separating gel. Proteins were then transferred onto methanol-activated PVDF membranes (IPVH00010, Millipore, USA) via wet transfer at a constant current of 200 mA for 60 min.

The membranes were blocked with 5% non-fat dry milk in TBST at room temperature for 2 h, followed by incubation with primary antibodies overnight at 4°C. The primary antibodies used were as follows: anti-

CD93 (ab134079, Abcam, Cambridge, UK), anti- β -Actin (HC201-01, TransGen Biotech, Beijing, China), and anti-GAPDH (AF1186, Beyotime Biotechnology). After washing with TBST, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 2 h, including HRP-conjugated goat anti-rabbit IgG (H + L) (GB23303, Servicebio, Wuhan, China) and HRP-conjugated goat anti-mouse IgG (H + L) (GB23301, Servicebio). Immunoreactive bands were visualized using an ECL chemiluminescence detection system. Band intensities were quantified using ImageJ software, and the relative expression levels of CD93 were calculated and normalized to the internal controls, GAPDH or β -actin.

5. Quantitative Real-Time PCR (qRT-PCR)

Quantitative real-time PCR was performed using the ChamQ Universal SYBR qPCR Master Mix kit (Q711-02, Vazyme Biotechnology, Nanjing, China). Each 20 μ L reaction system consisted of 10 μ L of 2 $\times$ Master Mix, 1 μ L of cDNA template, 0.4 μ L each of forward and reverse primers (10 μ M), and 8.2 μ L of RNase-free ddH₂O. The thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min; followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The primer sequences used in this study were as follows: CD93 (forward: 5'-GGGGCTACTGGTCTATCGCA-3', reverse: 5'-CCCAGGTGTCGGACTGTACT-3');

GAPDH (forward: 5'-GGTGTGAACCATGAGAAGTATGA-3', reverse: 5'-GAGTCCTTCCACGATACCAAAG-3'); and β -actin (forward: 5'-TGGCACCCAGCACAATGAA-3', reverse: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'). All samples were analyzed in triplicate. GAPDH or β -actin served as the internal control, and the relative expression levels of CD93 were calculated using the $2^{-\Delta\Delta C_t}$ method.

6. Immunohistochemistry

Deparaffinized and rehydrated tissue sections were subjected to heat-induced antigen retrieval using citrate buffer (pH 6.0; MVS-0100, Maixin Bio, Fuzhou, China) under high pressure. After natural cooling, sections were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity, followed by blocking with 5% BSA (A8412, Sigma, USA) at 37°C for 30 min. The sections were then incubated overnight at 4°C with the following primary antibodies: anti-CD93 (ab134079, Abcam) and anti-GLUT1 (21829-1-AP, Proteintech, Wuhan, China). After washing, the sections were incubated with HRP-conjugated goat anti-rabbit IgG (H + L) secondary antibody (GB23303, Servicebio) at 37°C for 30 min. Immunoreactive signals were visualized using a DAB Substrate Kit (CW0125M, Cowin Biotech, Jiangsu, China), and nuclei were counterstained with hematoxylin. Finally, the sections were dehydrated, cleared, and mounted for microscopic examination.

7. Immunofluorescence Staining

Paraffin-embedded sections of IH specimens were obtained from the Department of Pathology at Union Hospital, Fujian Medical University. These samples were collected over the past decade from patients who underwent surgical resection due to clear surgical indications. The lesions were predominantly located in the head, face, or chest regions and were selected based on unsatisfactory therapeutic outcomes following pharmacological treatment or spontaneous involution.

Immunofluorescence staining was performed on both paraffin-embedded tissue sections and cultured cells (HemSCs and HUVECs). Tissue sections were deparaffinized and rehydrated, while cells were seeded onto sterile glass coverslips. Both sample types underwent the same antigen retrieval, blocking, and incubation procedures. Samples were incubated overnight at 4°C with anti-CD93 (PA5-52664, Invitrogen, USA) or anti-GLUT1 (21829-1-AP, Proteintech) antibodies, followed by incubation with Cy3-conjugated goat anti-rabbit IgG (H + L) secondary antibody (AS007, ABclonal, Wuhan, China) for 45 min at 37°C. Nuclei were counterstained with DAPI (D9542, Sigma-Aldrich, USA), and images were acquired using a fluorescence microscope.

8. Establishment of the IH In Vivo Model

All animal experiments were approved by the Animal Ethics Committee of Fujian Medical University (Approval No. IACUC FJMU 2023 – 0118). Four-week-old female BALB/c nude mice were obtained from GemPharmatech Co., Ltd. (Nanjing, China; License No. SCXK (Su) 2023-0009) and maintained under controlled temperature (20–26°C) and humidity (40–70%) with a natural light-dark cycle. Following a one-week acclimatization period, the *in vivo* model was established by modifying a previously reported method^[35]. Briefly, HemSCs (1×10^6) and HUVECs (7×10^5) were suspended in 100 μ L of ice-cold Matrigel and subcutaneously injected into the right dorsal flank near the axilla of each mouse ($n = 4/\text{group}$). Tumor growth and body weight were monitored daily, and tumor dimensions (length and width) were measured using a vernier caliper. The successful establishment of the IH model was confirmed by histopathological evidence of significant microvascular proliferation.

In Vivo Tumor Formation Assay with CD93 Antibody Blocking:

CD93-overexpressing HemSCs and HUVECs in the logarithmic growth phase were harvested and counted. The *in vivo* tumor formation assay was performed following the protocol described above. Mice were divided into two groups: the CD93 antibody group, in which the cell-Matrigel mixture was supplemented with CD93 antibody to a final concentration of 50 μ g/mL, and the control group, which received the mixture with an equivalent volume of PBS. After thorough mixing, the suspensions were subcutaneously injected into the right dorsal flank of BALB/c nude mice. Tumor formation was monitored and recorded daily.

9. Microvessel Density (MVD) Quantification

To evaluate angiogenesis in the IH *in vivo* models, paraffin-embedded tumor tissue sections were stained with H&E staining. Microvascular formation was examined under a microscope. Any lumen-like structure formed by one or more endothelial cells was identified and counted as a single microvessel. For each specimen, three randomly selected high-power fields (HPFs, $\times 200$) were selected for imaging. Microvessels were quantified using ImageJ software. The average number of microvessels per field was calculated to determine the MVD (vessels/mm²).

10. Statistical Analysis

Quantitative analysis of *in vitro* tube formation (total length, junctions, meshes) and *in vivo* MVD was performed using ImageJ software. Data are expressed as mean $\pm$ standard deviation (SD) and analyzed using GraphPad Prism. The unpaired two-tailed Student's t-test was applied for comparisons between two groups. Statistical significance was defined as $P < 0.05$ ($P < 0.05$, $*P < 0.01$, $**P < 0.001$).

Results

1. CD93 is highly expressed in proliferative IH tissues

Immunohistochemical staining demonstrated high expression of CD93 in proliferative IH tissues, whereas only minimal expression was observed in involuting IH and normal tissues (Fig. 1A). Consistently, immunofluorescence staining revealed significantly higher CD93 expression in IH tissues than in normal skin (Fig. 1B). Furthermore, Western blot and qPCR analyses were performed to quantify CD93 protein and mRNA levels in proliferative IH specimens ($n = 6$) versus adjacent non-lesional tissues ($n = 6$) (Fig. 1C). Western blot analysis indicated a significant upregulation of CD93 protein in proliferative IH compared to adjacent tissues ($P = 0.0003$), with a mean fold change of 33.78 (Fig. 1D-E). Similarly, qPCR results showed that CD93 mRNA levels were significantly elevated in proliferative IH tissues compared to adjacent controls ($P = 0.0133$), with a mean fold change of 26.07 (Fig. 1F-G). Collectively, CD93 was markedly upregulated at both the protein and mRNA levels in proliferative IH tissues, suggesting its potential involvement in IH progression.

2. CD93 is highly expressed in CD133 HemSCs with enhanced tube-forming capacity

CD133⁺ HemSCs and CD133⁻ cells were isolated from hemangioma tissues of different patients via collagenase digestion and CD133 immunomagnetic separation [23]. After serial passage, the differential expression of CD93 between these two cell populations was evaluated. Immunofluorescence staining showed higher CD93 expression in CD133⁺ HemSCs than in CD133⁻ cells (Fig. 2A), with predominant localization in the cytoplasm and cell membrane. We further assessed the tube-forming capacity of CD133⁺ HemSCs cells and CD133⁻ cells by analyzing three parameters: total tube length, number of nodes, and number of tubes. CD133⁺ HemSCs cells exhibited significantly enhanced angiogenic

potential compared to CD133⁺ cells, with statistically significant differences observed in total tube length ($P < 0.01$), number of nodes ($P < 0.0001$), and number of tubes ($P < 0.0001$) (Fig. 2B-E).

3. CD93 and its polyclonal antibody modulate the tube formation of stable cell lines

3. CD93 and its polyclonal antibody modulate the *in vitro* tube formation of stable cell lines
CD93 knockdown and overexpression stable cell lines were established via lentiviral transduction. HemSCs at passages 4–6 were transduced and subsequently selected with 0.8 µg/mL puromycin, resulting in high transfection efficiency (Fig. 3A). Western blot analysis confirmed robust CD93 overexpression in CD93^{OE} HemSCs group and significant downregulation across the three knockdown groups, indicating effective transduction (Fig. 3B-C). qPCR analysis further validated the successful construction of the stable lines (Fig. 3D). Among the knockdown candidates, the second group (sh-2) exhibited the lowest mRNA levels and was therefore selected as the representative CD93sh-HemSCs line for all subsequent experiments.

Statistical analysis using Student's t-test revealed that, compared to null-HemSCs, the CD93^{OE} HemSCs group exhibited significantly increased total tube length, number of nodes, and number of meshes ($P = 0.0249$, $P = 0.0325$, and $P = 0.0108$, respectively). Conversely, the CD93^{sh} HemSCs group showed significant reductions in these parameters ($P = 0.0294$, $P = 0.0396$, and $P = 0.0288$, respectively) (Fig. 3E-F). These results indicate that CD93 promotes angiogenesis in IH, while its knockdown significantly attenuates this effect.

To further investigate the pro-angiogenic function of CD93, we performed a functional blocking assay using a CD93 polyclonal antibody in CD93^{OE} HemSCs. The experimental group was treated with the CD93 antibody (100 µg/mL), while the control group received an equivalent volume of PBS.

Following a 6-hour incubation period, images were acquired and analyzed using ImageJ to quantify total tube length, number of junctions, and number of meshes. Statistical analysis was performed using Student's t-test. The results demonstrated that the antibody-treated group exhibited significant reductions in total tube length ($P < 0.05$), number of junctions ($P < 0.01$), and number of meshes ($P < 0.01$) compared to the control (Fig. 3G-I). These findings indicate that the CD93 polyclonal antibody (100 µg/mL) significantly inhibits the *in vitro* tube-forming capacity of CD93^{OE} HemSCs, further validating CD93 as a promising therapeutic target for inhibiting IH angiogenesis.

4. Role of CD93 in the In Vivo IH Model

Next, we investigated the role of CD93 in an *in vivo* model. An infantile hemangioma (IH) model was established by subcutaneously injecting a mixture of HemSCs, HUVECs, and Matrigel into female BALB/c nude mice. The successful construction of the model was confirmed by verifying GLUT-1 (an

exclusive marker for IH) expression in the explants (Fig. 4A). Histological analysis revealed angiogenesis with erythrocyte-containing lumens in the Matrigel plugs on day 4, whereas fibroadipose tissue structure and signs of involution were observed by day 5 (Fig. 4B-E). Therefore, day 4 was selected as the endpoint for subsequent experiments.

To evaluate the impact of CD93 modulation on the IH model and exclude potential effects from the vector, stable cell lines for overexpression control (CD93-OENC) and knockdown control (CD93-shNC) were constructed and validated via Western blot and qPCR (Fig. 4I-K). On day 4, angiogenesis with erythrocyte-containing lumens was observed in all groups (Fig. 4F). The microvessel density (MVD) in the CD93 overexpression group (CD93-OE) was 113.00 ± 16.95 vessels/mm², which was significantly higher than that in the overexpression control group (CD93-OENC) (67.67 ± 17.11 vessels/mm²). This 1.68-fold increase indicates that CD93 overexpression promotes *in vivo* angiogenesis in the IH nude mouse model ($P < 0.001$). Conversely, the CD93 knockdown group (CD93-sh) exhibited an MVD of 67.24 ± 4.19 vessels/mm², which was significantly lower than the knockdown control group (CD93-shNC) (78.69 ± 4.19 vessels/mm²). This 0.85-fold reduction suggests that CD93 knockdown suppresses angiogenic capacity *in vivo* ($P < 0.05$) (Fig. 4G-H).

5. CD93 Antibody Inhibits Angiogenesis in the IH In Vivo Model

To investigate the therapeutic potential of targeting CD93, CD93-OE HemSCs were pre-treated with a CD93 antibody (10 µg/mL) or PBS (control) prior to co-injection with HUVECs into nude mice. Macroscopic examination revealed that tumor growth was visibly attenuated in the CD93 antibody-treated group compared to the PBS control group (Fig. 5A). Histological evaluation via HE staining at day 4 confirmed the presence of microvessels within the explants (Fig. 5B). Quantitative analysis demonstrated a significant reduction in MVD in the CD93 antibody group (46.36 vessels/mm²) compared to the PBS control group (184.88 vessels/mm²) ($P < 0.0001$) (Fig. 5C). These results indicate that the CD93 antibody effectively inhibits angiogenesis in the IH *in vivo* model, validating CD93 as a functional therapeutic target for anti-angiogenic therapy in infantile hemangioma.

(C) MVD analysis showing significantly lower MVD in the CD93 antibody group compared to the PBS group ($P < 0.0001$).

Discussion

IH represents the most common benign tumor of infancy, yet its precise pathogenesis remains elusive. Currently, oral propranolol serves as the first-line pharmacotherapy for complicated IH. However, residual sequelae such as telangiectasia, fibrofatty tissue, and erythema persist in approximately 72.4% of patients following propranolol therapy. Furthermore, 37.5% of patients ultimately require surgical intervention due to residual fibrofatty tissue or incomplete hemangioma regression^[36]. Consequently, elucidating the molecular pathogenesis of IH and developing targeted therapeutic strategies are of paramount clinical importance. In this study, we employed lentiviral transfection technology to

investigate the functional role of CD93 in IH tissues, cellular models, and in vivo models. Our in vitro assays demonstrated that CD93 overexpression significantly promoted angiogenesis. These findings were corroborated by our in vivo experiments, where the MVD in the CD93-OE group was significantly elevated compared to the CD93-OENC group. Conversely, CD93-sh group resulted in a marked reduction in MVD relative to the CD93-shNC group. These data collectively indicate that CD93 exerts a pro-angiogenic effect in the pathophysiology of IH. Furthermore, we validated that a CD93 antibody effectively inhibited angiogenesis, suggesting that CD93 represents a promising potential therapeutic target for anti-angiogenic treatment in IH.

The construction of an in vivo model for IH represents a pivotal prerequisite for investigating angiogenesis and associated pharmacological mechanisms, providing a robust experimental foundation for elucidating the pathogenesis of IH. HemSCs are recognized as the fundamental cellular drivers in the pathogenesis and progression of this vascular anomaly. During the proliferative phase, HemSCs predominantly differentiate into hemangioma-derived endothelial cells (HemECs) and pericytes, thereby fueling the rapid expansion of the lesion. Conversely, during the involution phase, these cells primarily undergo adipogenic differentiation, contributing to the regression of the tumor^[37]. In the context of in vivo modeling of IH, the subcutaneous implantation of HemSCs resuspended in Matrigel into immunodeficient mice has historically been the most widely utilized approach^[20, 38], however, this model is characterized by inherent instability. Notably, Mai et al. demonstrated that the co-injection of HemSCs with HUVECs facilitated the formation of CD31 + vasculature with a significantly increased vessel density^[35]. Accordingly, we utilized BALB/c nude mice to construct an in vivo IH model via the co-injection of HemSCs and HUVECs (at a specific ratio) resuspended in Matrigel.

Previous studies have established that Glucose transporter-1 (GLUT-1) is the most ubiquitous glucose transporter in humans. Physiologically, it is absent from the normal cutaneous and subcutaneous vasculature, being restricted to specific sites such as the blood-tissue barriers, placenta, retina, and central nervous system^[16]. GLUT-1 serves as a definitive diagnostic marker for IH, possessing high sensitivity and specificity that facilitate the differentiation of IH from other vascular anomalies, such as vascular malformations, pyogenic granulomas, and hemangioendotheliomas^[39–41]. In the present study, we evaluated the expression of GLUT-1 in Matrigel plugs derived from our established in vivo IH model using immunohistochemistry and immunofluorescence assays. The results demonstrated robust GLUT-1 expression throughout the Matrigel plugs. Crucially, this finding confirmed that the neovasculature originated from the implanted human HemSCs rather than the murine host cells, thereby validating the reliability of the model. Furthermore, we observed that abundant microvasculature emerged by day 4 post-injection, followed by signs of involution starting on day 5. HE staining revealed the presence of extensive residual fibrofatty tissue. Collectively, these findings indicate that the in vivo IH model constructed in this study successfully recapitulates both the proliferative and involutive phases of IH, rendering it a suitable platform for in vivo investigations into the pathogenesis and therapeutic mechanisms of IH.

Excessive angiogenesis is intimately associated with the proliferative phase of IH^[18, 19, 42]. In recent years, accumulating evidence has highlighted the pro-angiogenic role of CD93^[24, 28, 29, 32, 33], which is highly expressed in the tumor vasculature. Masiero et al. ^[26] through a comprehensive analysis of data from 959 breast cancer, 170 renal cancer, and 121 head and neck cancer cases, identified CD93 as a pivotal gene in tumor angiogenic signaling. Furthermore, CD93 is upregulated in nasopharyngeal carcinoma and specific ocular tumors—including retinoblastoma and choroidal melanoma—where its expression correlates with a poor prognosis^[29, 31]. Consequently, the potential of CD93 as a novel target for anti-angiogenic therapy in solid tumors, such as colorectal, liver, and gastric cancers, has garnered increasing attention^[30, 43, 44]. Mechanistically, knocking down CD93 in human dermal microvascular endothelial cells has been shown to attenuate their proliferation, migration, and angiogenic capacity^[28]. Additionally, a human CD93-specific monoclonal antibody (4E1), which binds to the region between the C-type lectin-like domain (CTLD) and sushi-like domains, has demonstrated anti-angiogenic activity both in vitro and in vivo ^[45]. Consistent with findings in other solid tumors, our study revealed that CD93 is highly expressed in IH tissues. We previously demonstrated its pro-angiogenic function in vitro, showing that CD93 overexpression enhances tube formation, whereas its knockdown suppresses this capability. Building on this, the present study provides further in vivo evidence supporting CD93 as a promising anti-angiogenic target in IH.

Angiogenesis is pivotal for tumor progression, and current anti-angiogenic therapies have primarily focused on the VEGF/VEGFR axis, which is widely utilized in clinical practice. However, studies indicate that anti-VEGFR monoclonal antibodies significantly disrupt normal vascular homeostasis, in contrast, CD93 monoclonal antibody blockade appears to have no discernible impact on the normal vasculature, suggesting that CD93 may serve as a safer therapeutic target for tumor vessel normalization^[25]. To date, no reports have addressed the role of CD93 in IH. Consequently, elucidating the function of CD93 in IH will not only shed light on its pathogenesis but also lay the groundwork for developing novel therapeutic strategies.

CD93 possesses two key endogenous ligands: Multimerin-2 (MMRN2) and Insulin-like growth factor binding protein-7 (IGFBP7). These ligands bind to distinct domains of CD93, and CD93 can concurrently associate with both to form a complex^[25, 46]. On one hand, MMRN2 is a critical molecule for maintaining vascular stability, its ablation leads to destabilized inter-endothelial cell junctions and increased vascular permeability^[47]. The interaction between CD93 and MMRN2 is dependent on the C-type lectin-like domain (CTLD) ^[48], suggesting that specifically targeting CD93 to disrupt this interaction could significantly impact the angiogenic process. On the other hand, within the tumor vasculature, hypoxia, VEGF, and Transforming Growth Factor- β 1 have been shown to correlate with IGFBP7 expression levels^[25, 46, 49], and IGFBP7 upregulation is observed in both physiological and pathological angiogenesis^[46]. Therefore, the binding of CD93 to both IGFBP7 and MMRN2 likely constitutes a pivotal mechanism underlying its pro-angiogenic function.

Nevertheless, several limitations in this study should be acknowledged. First, the sample size was relatively small, and inter-individual variability among the mice was observed, therefore, validation in a larger cohort is warranted. Second, the specific signaling pathways and molecular mechanisms underlying CD93-mediated angiogenesis in IH were not fully elucidated. Future studies are required to further investigate these mechanisms and clarify whether the pro-angiogenic effects of CD93 are associated with MMRN2 or IGFBP7.

Conclusion

This study demonstrates that CD93 is enriched in proliferative IH tissues and CD133 + HemSCs and functionally promotes HemSC-mediated angiogenesis both in vitro and in vivo. Genetic silencing or antibody-mediated blockade of CD93 attenuates angiogenic activity, supporting CD93 as a potential anti-angiogenic therapeutic target in IH. Further studies are warranted to define the downstream CD93-associated signaling network and validate its translational feasibility.

Declarations

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Figures

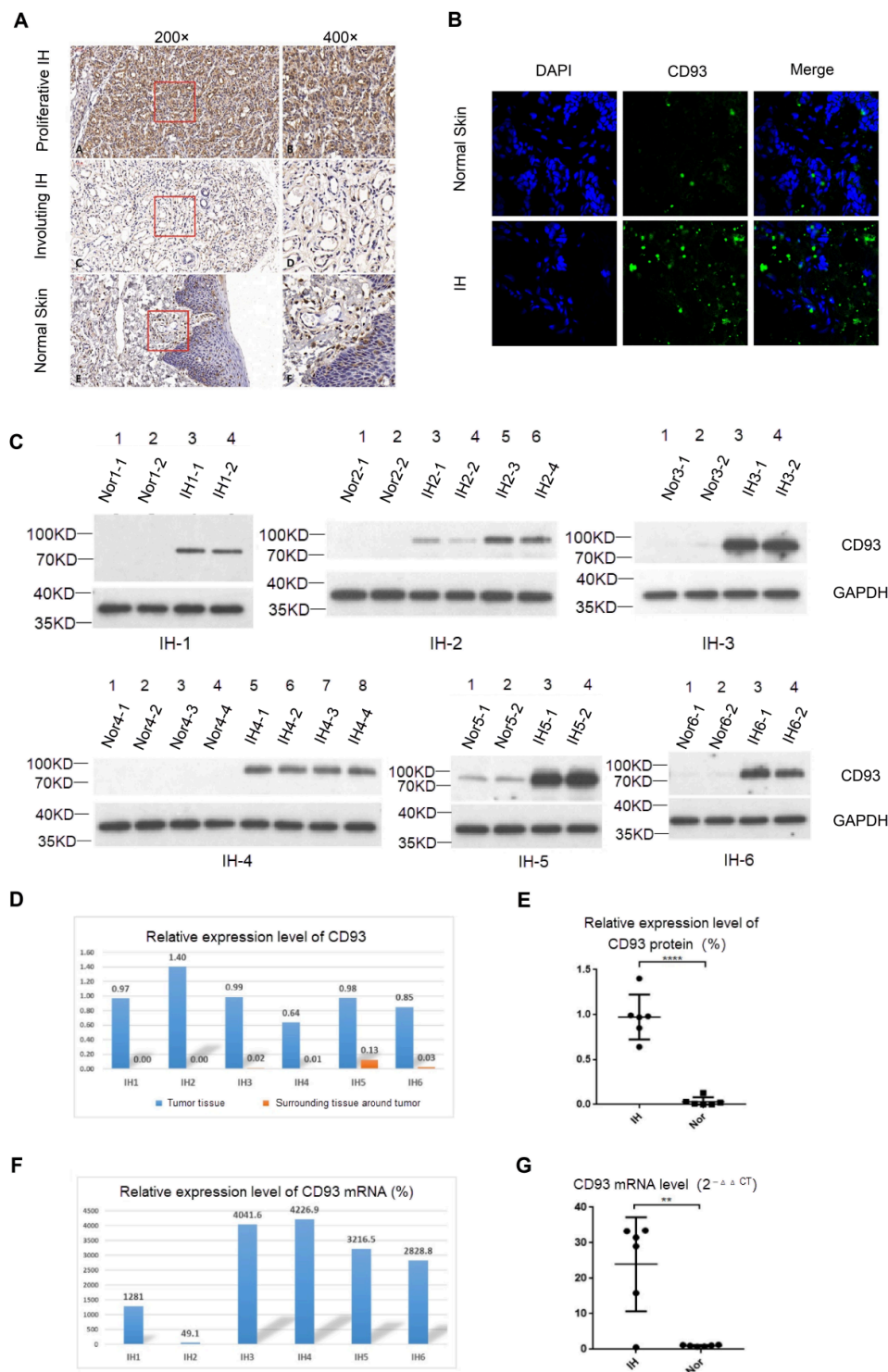


Figure 1

(A) Representative immunohistochemical staining of CD93 in proliferative IH, involuting IH, and normal skin tissues. (B) Immunofluorescence confocal microscopy images of IH tissue and normal skin, showing significantly higher CD93 expression in IH tissue compared to normal skin. (C) Western blot analysis of CD93 protein expression in proliferative IH tissues (n=6) and paired adjacent non-lesional tissues (each sample was repeated 2-4 times), demonstrating elevated CD93 protein levels in all

proliferative IH specimens compared to adjacent controls. (D-E) Densitometric analysis of Western blot bands, indicating significantly higher CD93 protein expression in proliferative IH tissues versus adjacent tissues ($P=0.0003$). (F-G) qPCR analysis of CD93 mRNA expression in proliferative IH tissues ($n=6$) relative to adjacent controls (each sample was repeated twice), revealing significantly upregulated CD93 mRNA levels in proliferative IH tissues ($P=0.0133$).

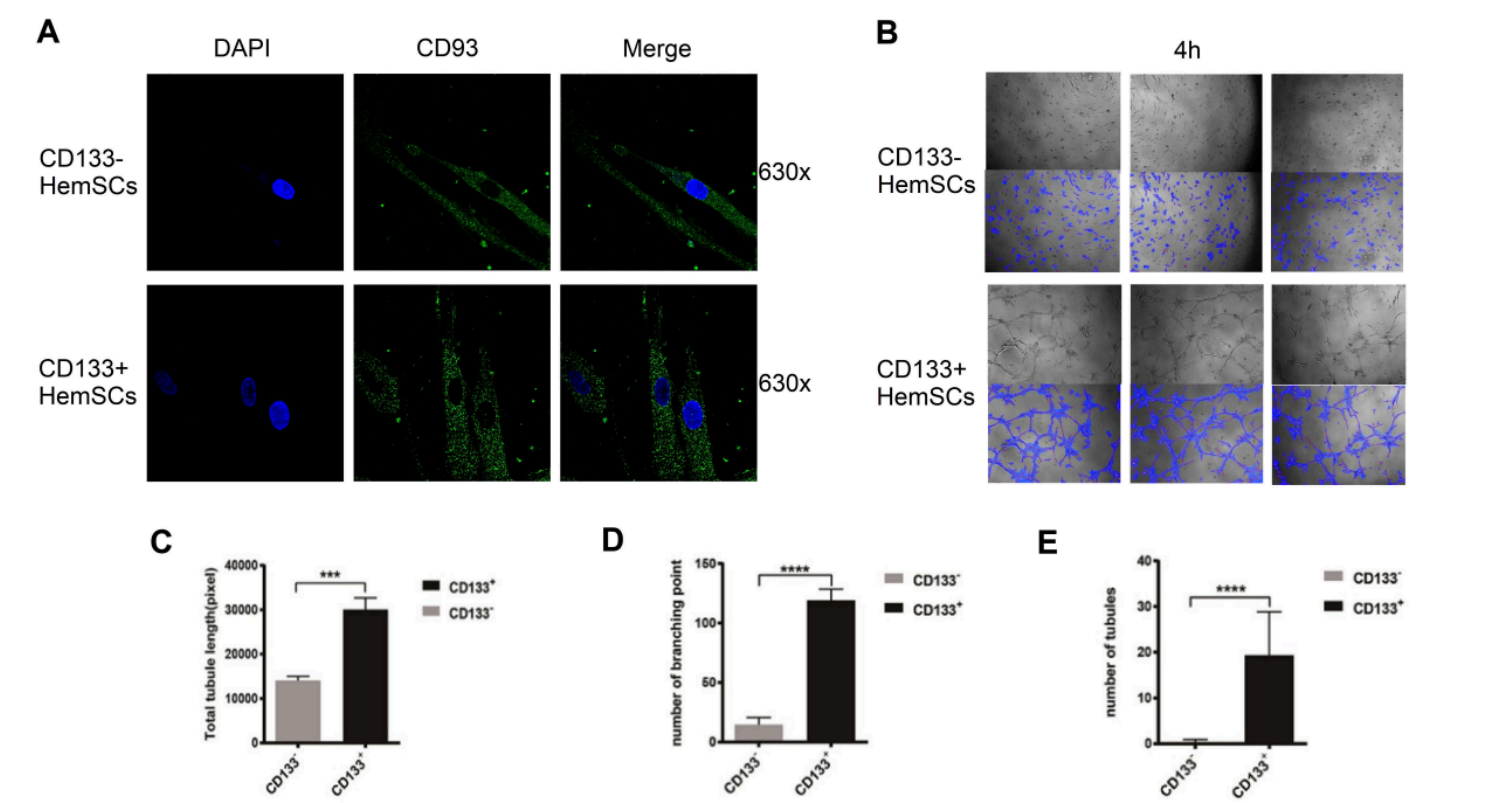


Figure 2

A) Representative immunofluorescence confocal microscopy images showing CD93 expression in CD133⁺ HemSCs and CD133⁻ cells. CD93 expression is significantly higher in CD133⁺ HemSCs compared to CD133⁻ cells, with predominant localization in the cytoplasm and cell membrane. **(B)** Representative images of in vitro tube formation assays for CD133⁺ HemSCs and CD133⁻ cells, alongside corresponding skeletonized images analyzed by ImageJ. The images illustrate the quantification of total tube length, number of nodes, and number of tubes, demonstrating the markedly enhanced tube-forming capacity of CD133⁺ HemSCs cells. **(C-E)** Quantitative analysis of tube formation. CD133⁺ HemSCs cells exhibited significantly greater total tube length (C), number of nodes (D), and number of tubes (E) compared to CD133⁻ cells ($P<0.01$, $P<0.0001$, and $P<0.0001$, respectively). Statistical significance was determined by Student's t-test.

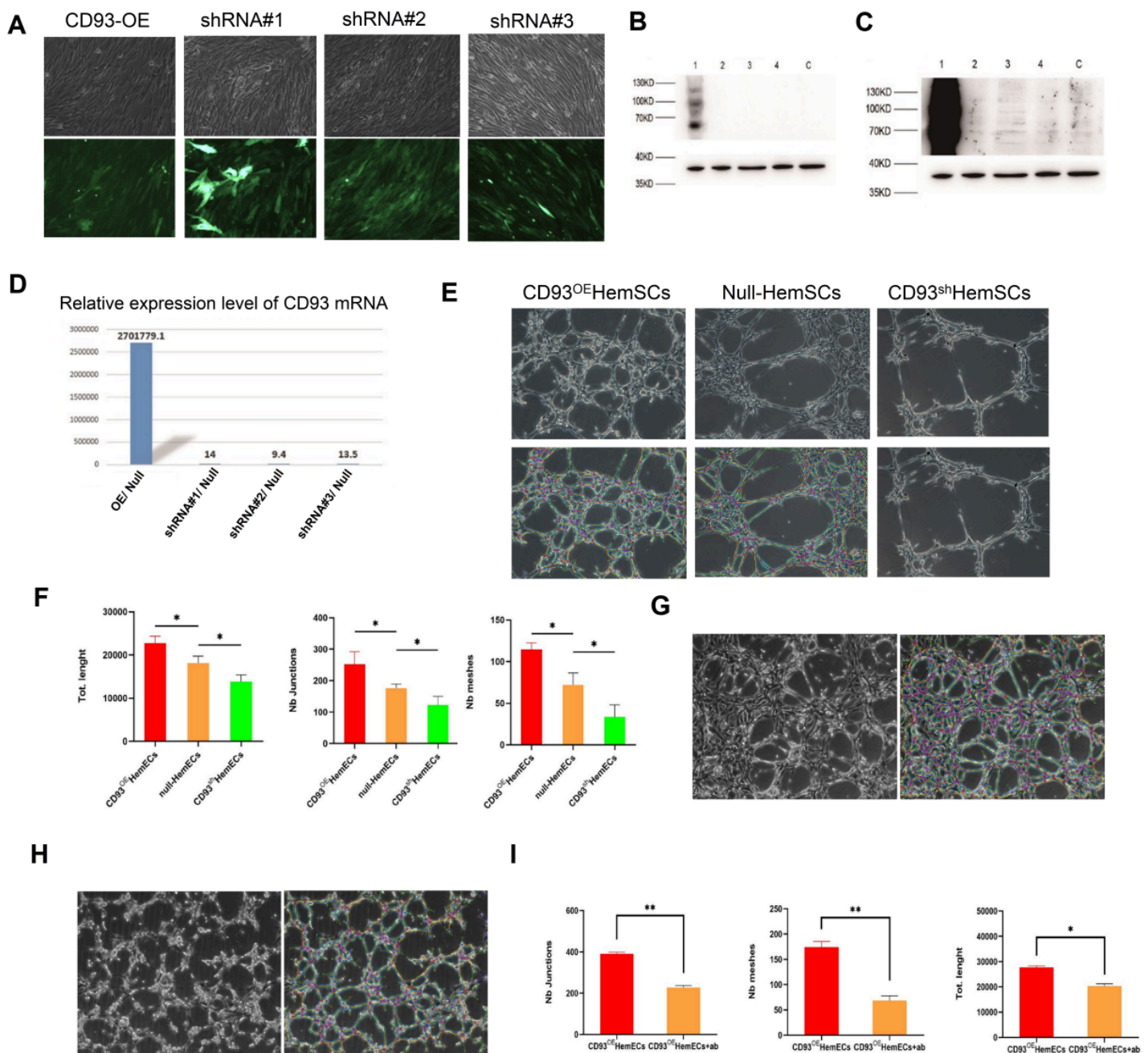


Figure 3

Functional characterization of CD93 in HemSCs *in vitro*. (A) Morphology and fluorescence microscopy images of stable HemSC cell lines (CD93^{OE} HemSCs, shRNA#1, shRNA#2, and shRNA#3) (×100). (B) Western blot analysis of CD93 expression. CD93 levels were elevated in the CD93^{OE} HemSCs group (1) compared to the Null-HemSCs (c) and CD93^{sh} HemSCs groups (2, 3, and 4 representing knockdown clones 1, 2, and 3, respectively). Note: The band intensity in the overexpression lane was saturated and could not be quantified. (C) Enhanced exposure of the Western blot shown in (B), demonstrating reduced CD93 expression in all three knockdown groups compared to Null-HemSCs. (D) qPCR analysis of CD93

mRNA expression. CD93 expression was significantly upregulated in the overexpression group compared to Null-HemSCs, while all knockdown groups showed decreased expression, with clone 2 exhibiting the lowest levels. **(E)** Representative images of in vitro tube formation assays for CD93^{OE} HemSCs, Null-HemSCs, and CD93^{sh} HemSCs. **(F)** Quantification of tube formation parameters. Compared to Null-HemSCs, the CD93^{OE} HemSCs group exhibited significantly increased total length, number of junctions, and number of meshes ($P=0.0249$, $P=0.0325$, and $P=0.0108$, respectively). Conversely, the CD93^{sh} HemSCs group showed significant reductions in these parameters ($P=0.0294$, $P=0.0396$, and $P=0.0288$, respectively). **(G)** Representative images of in vitro tube formation in the control group (CD93^{OE} HemSCs + PBS). **(H)** Representative images of in vitro tube formation in the experimental group (CD93^{OE} HemSCs + CD93 antibody). **(I)** Quantitative analysis showing that the CD93 antibody significantly inhibited total length, number of junctions, and number of meshes in CD93^{OE} HemSCs ($P<0.05$, $P<0.01$, and $P<0.01$).

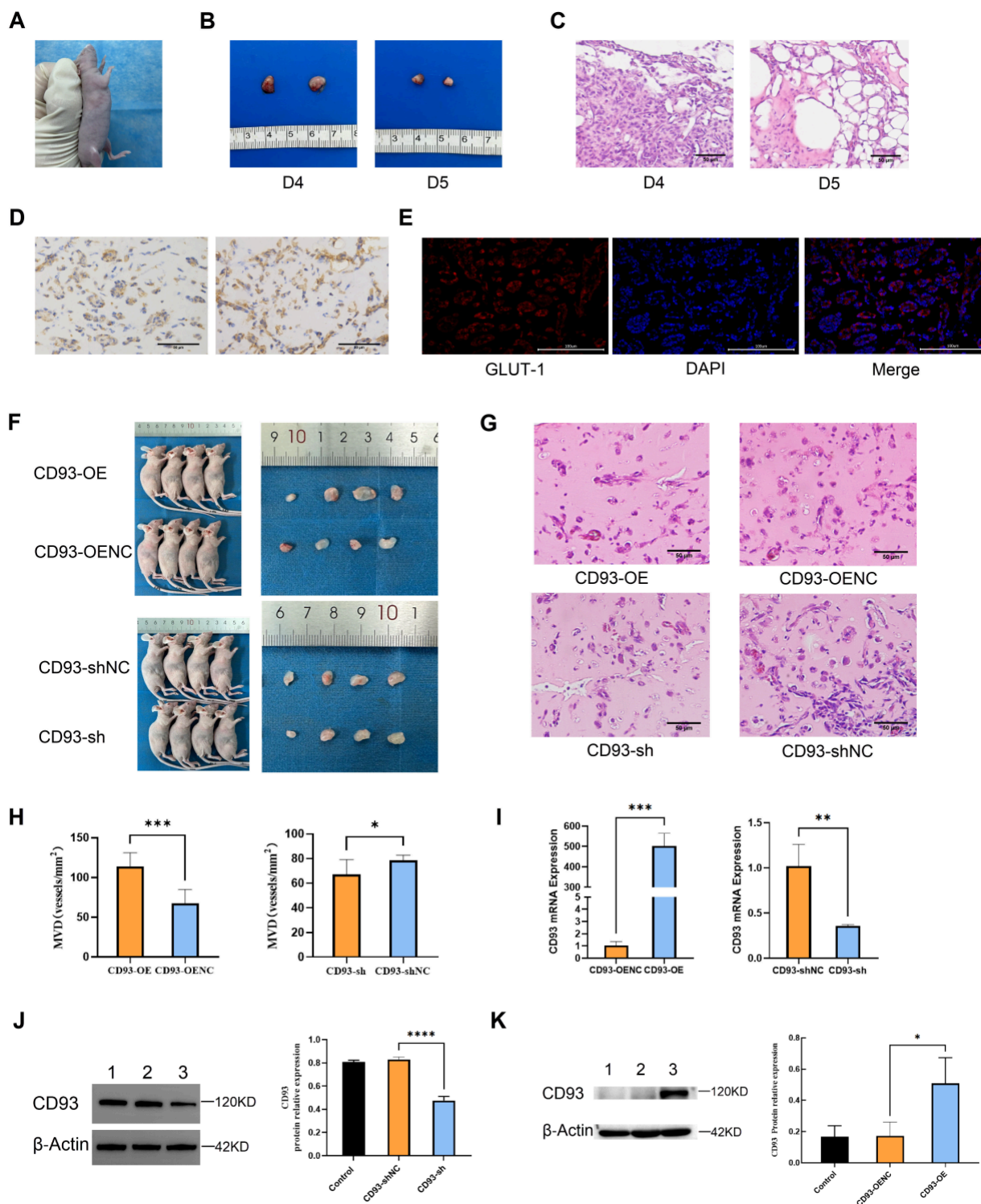


Figure 4

(A) Schematic illustration of the IH in vivo model. **(B)** Images of Matrigel plug on day 4 and day 5. **(C)** HE staining of Matrigel plugs on day 4 and day 5 (×200). **(D)** Immunohistochemical analysis of GLUT-1 expression in the Matrigel (×400). **(E)** Immunofluorescence staining for GLUT-1 in the Matrigel (×400). **(F)** Image of tumor formation in mice injected with CD93-OE, CD93-OENC, CD93-shNC, and CD93-sh stable cell lines. **(G)** HE staining of Matrigel derived from the four stable cell lines (×200). **(H)** The MVD in the

CD93-OE group was significantly higher than in the CD93-OENC group ($P<0.001$), while the MVD in the CD93-sh group was significantly lower than in the CD93-shNC group ($P<0.05$). **(I)** qPCR analysis of CD93 mRNA expression levels in the four stable cell lines ($*P<0.01$, $P<0.001$). **(J)** Western blot analysis showing that CD93 protein expression in the CD93-sh group (lane 3) was lower than in the Control (lane 1) and CD93-shNC (lane 2) groups ($P<0.0001$). **(K)** Western blot analysis showing that CD93 protein expression in the CD93-OE group (lane 3) was higher than in the Control (lane 1) and CD93-OENC (lane 2) groups ($P<0.05$).

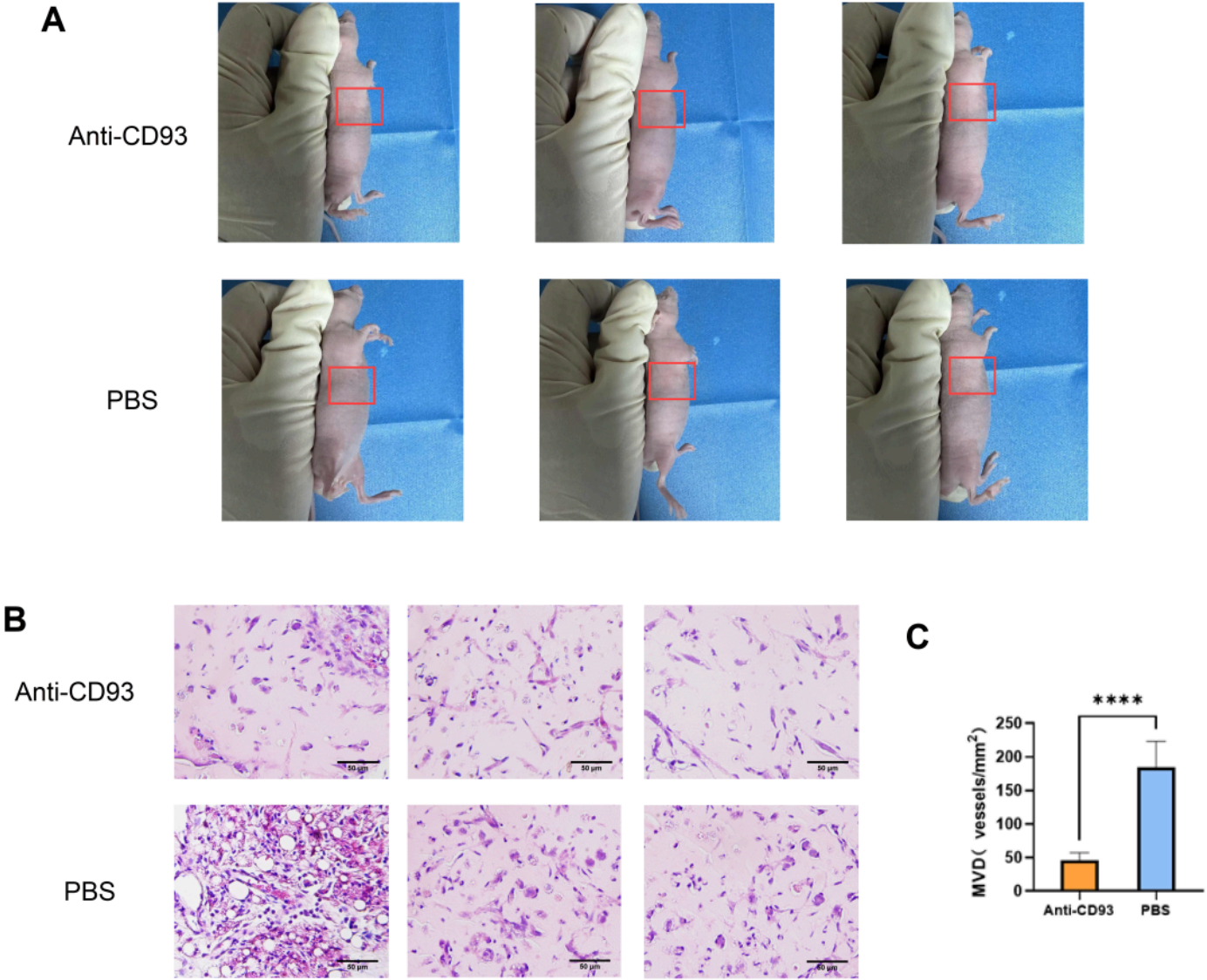


Figure 5

(A) Image of tumors in the IH in vivo model treated with CD93 antibody or PBS. **(B)** HE staining of Matrigel plugs from the CD93 antibody and PBS groups ($\times 200$).

(C) MVD analysis showing significantly lower MVD in the CD93 antibody group compared to the PBS group ($P<0.0001$).