Induction of LYVE-1/stabilin-2-positive liver sinusoidal endothelial-like cells from embryoid bodies by modulation of adrenomedullin-RAMP2 signaling

Takuma Arai [a,c], Takayuki Sakurai [a], Akiko Kamiyoshi [a], Yuka Ichikawa-Shindo [a], Nobuyoshi Iinuma [a,c], Yasuhiro Iesato [a,b], Teruhide Koyama [a], Takahiro Yoshizawa [a], Ryuichi Uetake [a], Akihiro Yamauchi [a], Lei Yang [a], Hisaka Kawate [a], Shinichiro Ogawa [c], Akira Kobayashi [c],Shinichi Miyagawa [c], Takayuki Shindo [a]

[a] Department of Organ Regeneration, Shinshu University Graduate School of Medicine, Japan
[b] Department of Ophthalmology, Shinshu University Graduate School of Medicine, Japan
[c] Department of Surgery, Shinshu University Graduate School of Medicine, Japan

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Address for correspondence
Takayuki Shindo, MD, PhD
Department of Organ Regeneration,
Shinshu University Graduate School of Medicine
Asahi 3-1-1, Matsumoto, Nagano, 390-8621, Japan
Tel: +81-263-37-3192
Fax: +81-263-37-3437
Email: tshindo@shinshu-u.ac.jp

Abstract

Embryonic stem cells (ESCs) are a useful source for various cell lineages. So far, however, progress toward reconstitution of mature liver morphology and function has been limited. We have shown that knockout mice deficient in adrenomedullin (AM), a multifunctional endogenous peptide, or its receptor-activity modifying protein (RAMP2) die in utero due to poor vascular development and hemorrhage within the liver. In this study, using embryoid bodies (EBs)-culture system, we successfully induced liver sinusoidal endothelial-like cells by modulation of AM-RAMP2. In an EB differentiation system, we found that co-administration of AM and SB431542, an inhibitor of transforming growth factor β (TGFβ) receptor type 1, markedly enhanced differentiation of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1)/stabilin-2-positive endothelial cells. These cells showed robust endocytosis of acetylated low-density lipoprotein (Ac-LDL) and upregulated expression of liver sinusoidal endothelial cells (LSECs)-specific markers, including factor 8 (F8), Fc-γ receptor 2b (Fcgr2b), and mannose receptor C type 1 (Mrc1), and also possess fenestrae-like structure, a key morphological feature of LSECs. In RAMP2-null liver, by contrast, LYVE-1 was downregulated in LSECs, and the sinusoidal structure was disrupted. Our findings highlight the importance of AM-RAMP2 signaling for

development of LSECs.

Key words

Adrenomedullin (AM)

Receptor activity-modifying protein (RAMP)

Lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1)

stabilin-2

Liver sinusoidal endothelial cells (LSEC)

Embryonic stem cells

Abbreviations

ESCs; Embryonic stem cells

EBs; Embryoid bodies

Ac-LDL; Acetylated low-density lipoprotein

AM; Adrenomedullin

RAMP; Receptor activity-modifying protein

CRLR; Calcitonin receptor-like receptor

GPCR; G protein-coupled receptor

TGF-β; Transforming growth factor β (TGFβ)

LYVE-1; Lymphatic vessel endothelial hyaluronan receptor-1

LSEC; Liver sinusoidal endothelial cells

LECs; Lymphatic endothelial cells

VEGF-A; Vascular endothelial growth factor A

VEGFR; Vascular endothelial growth factor receptor

ALK1, 5; Activin receptor-like kinase 1, 5

F8; Factor 8

Fcgr2b; Fc-γ receptor 2b

Mrc1; Mannose receptor C type 1

1. Introduction

Liver regeneration has long been desired as an alternative to transplantation of the organ. But while the pluripotency of embryonic stem cells (ESCs) has been exploited to obtain a variety of cell lineages for medical and research applications, progress toward reconstitution of mature liver morphology and function has been limited. Matsumoto et al. showed that primitive endothelial cells localized in the septum transversum are crucial for induction of the initial liver bud and for subsequent liver development[19]. In addition, Ogawa et al. recently reported that the emergence of cardiomyocytes and expansion of an endothelial cell network derived from ESCs plays an important role in the proliferation of hepatocytes and in liver organogenesis[26]. This suggests that differentiation of endothelial cells and reconstitution of the vasculature are key elements necessary for regeneration of mature liver.

Liver sinusoidal endothelial cells (LSECs) have unique structural and functional characteristics, among which are fenestrae and robust endocytic activity[5, 30]. Otherwise these cells are characterized physiologically as highly specialized scavenger endothelial cells that express such scavenger receptors as the mannose receptor, the Fc-γ receptor and stabilin-2[6, 24, 30]. The mechanism underlying the development of LSECs remains largely unknown, but several similarities between lymphatic endothelial

cells (LECs) and LSECs have been noted. For example, both LECs and LSECs have minimal basement membranes and loose cell-cell junctions, and both express lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1)[17]. This suggests that differentiation of LECs and LSECs is regulated to some degree via the same signaling pathways. Consistent with that idea, recent reports have shown that inhibition of endogenous transforming growth factor β (TGFβ) signaling enhances lymphangiogenesis and differentiation of fetal sinusoidal endothelial cells[27, 34].

Adrenomedullin (AM) is a multifunctional polypeptide originally isolated from human pheochromocytoma[14]. A noteworthy feature of AM is the unique system controlling its signaling[16, 20, 22, 28]. The AM receptor is a 7-transmembrane domain G protein-coupled receptor (GPCR) named calcitonin receptor-like receptor (CRLR), which associates with an accessory protein, receptor activity-modifying protein (RAMP). Three RAMP subtypes (RAMP1, 2, 3) have been identified. By interacting with RAMP1, CRLR acquires a high affinity for calcitonin gene-related peptide (CGRP), whereas by interacting with either RAMP2 or RAMP3, CRLR acquires a high affinity for AM. Homozygous AM and RAMP2 knockout (AM-/-, RAMP2-/-) mice die midgestation, on embryonic day (E)13.5 and E14.5, respectively. These AM-/- and RAMP2-/- mice share highly conserved phenotypes that include

generalized edema, as well as severe hemorrhagic changes within the liver and poor vascular formation[11, 29]. These phenotypes suggest that the AM-RAMP2 system is required for blood and lymphatic vessel function throughout embryogenesis, and that LSEC differentiation and sinusoidal morphogenesis may be regulated by the AM-RAMP2 system[4, 8, 11-13, 29].

The purpose of the present study is to generate LSECs using ESCs-derived embryoid bodies (EBs). To accomplish this, we focused on modulation of the AM-RAMP2 system.

2. Materials and methods

2.1. Culture of mouse embryonic stem cells

E14-1 ES cells derived from 129/Ola were grown on mitomycin C-treated mouse embryonic fibroblast (MEF) feeder layers to maintain them in an undifferentiated state. The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS) (MBL, Japan), 1 mM sodium pyruvate (Invitrogen), 100 μM nonessential amino acids (Invitrogen), 100 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) and 10^3 U/ml leukemia inhibitory factor (LIF) (Chemicon, CA). The medium was replaced daily.

Prior to differentiation, ES cells were first passaged onto gelatin coated plates for 30 min to remove the MEFs, and then resuspended in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen) containing 20% FBS, 1mM sodium pyruvate, 100 μM nonessential amino acids and 100 μM 2-mercaptoethanol, without LIF, and then formed into a hanging drop at a concentration of 1,500 cells per 50 μl. The hanging drop was cultured for 4 days at 37°C under an atmosphere of 5% CO_2 . The 15 EBs formed in the drops were transferred onto a 35-mm dish coated with collagen type I (Iwaki, Japan), and were cultured in differentiation medium consisting of IMDM supplemented with 10% FBS, 1 mM sodium pyruvate, 100 μM nonessential amino acids and 100 μM

2-mercaptoethanol. The following growth factors or inhibitors were added to the differentiation medium as indicated: 20 ng/ml human vascular endothelial growth factor A (VEGF-A) (R&D systems, Minneapolis, MN), 10^{-6} or 10^{-7} M human recombinant AM (Shionogi, Japan), 10^{-6} M SB431542 (Sigma-Aldrich). SB431542 was dissolved in 100% dimethyl sulfoxide (DMSO) at a stock concentration of 10 mmol/L. This stock was then diluted in medium, and 0.01% DMSO was used as the vehicle for SB431542 in each experiment. As a control, 0.01% DMSO was also added to the AM group. The medium was replaced every other day.

2.2. Primary culture of fetal mouse liver cells

Fetal mouse livers at E14.5 were dissected free of adhering tissue under a stereomicroscope. The livers were then minced and dissociated using collagenase (Wako, Japan) in Hank's buffer (Invitrogen), after which the cells were seeded onto a collagen type I-coated dish and maintained in DMEM supplemented with 10% FBS and 100 U/ml penicillin-100 μg/ml streptomycin.

2.3. Primary culture of adult mouse liver sinusoidal endothelial cells

Primary adult mouse LSECs were isolated using a two step collagenase perfusion

and centrifugation protocol.[3] The isolated sinusoidal endothelial cells were cultured in EGM2-MV (Cambrex, Walkersville, MD) at 37°C under a 5% CO_2 atmosphere.

2.4. Animals

C57BL/6J mice were obtained from Charles River Laboratories Japan, Inc. RAMP2-/- mice were originally generated in our group[11]. Because RAMP2-/- mice die in utero at E14.5, we analyzed embryonic liver from these and wild-type mice at E14.5. All animal experiments were conducted in accordance with the ethical guidelines of Shinshu University.

2.5. RNA extraction and RT-PCR analysis

Total RNA was extracted from the outgrowths of the EBs using Trizol Reagent (Invitrogen, Carlsbad, CA), after which it was treated with DNA-Free (Ambion, Austin, TX) to remove contaminating DNA, and 2-μg samples were subjected to reverse transcription using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlbad, CA). Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) was then carried out using Ex Taq DNA polymerase (Takara, Japan). PCR primers are listed in Table 1.

2.6. Quantitative real-time RT-PCR analysis

Quantitative real-time RT-PCR was carried out using an Applied Biosystems 7300 real time PCR System (Applied Biosystems) with SYBR green (Toyobo, Japan) or Realtime PCR Master Mix (Toyobo) and TaqMan probe (MBL). Values were normalized to mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Pre-Developed TaqMan®assay reagents, Applied Biosystems). PCR primers are listed in Table 2.

2.7. Immunohistochemical analysis

Cultured EBs were fixed with 4% paraformaldehyde/PBS for 20 min and then permeabilized with 0.1% Triton X for 10 min at room temperature. Embryos and livers were embedded in OCT compound (Sakura Finetek Japan Co., Tokyo, Japan), after which 6-μm sections were cut with a cryostat and mounted on glass slides. The sections were then fixed with 4% paraformaldehyde/PBS for 15 min and permeabilized with 0.1% Triton X for 10 min at room temperature. The fixed samples were incubated first for 30 min at room temperature in blocking buffer containing 4% goat serum (DAKO, Denmark) or 4% donkey serum (Jackson Immunoresearch, West Grove,

PA), and then with a primary Ab overnight at 4°C followed by a secondary Ab for 1 h at room temperature. The Abs used were rat anti-mouse CD31 (BD Pharmingen, San Jose, CA), rabbit anti-mouse LYVE-1 (RELIA Tech, Braunschweig, Germany), rat anti-mouse stabilin-2 (a kind gift from A. Miyajima, Tokyo University, Japan), goat anti-mouse albumin (Bethyl Laboratories, Montgomery, TX), Alexa 488-conjugated anti-rat or rabbit and Alexa 568-conjugated anti-rabbit or goat (Molecular Probes, Eugene, OR). For nuclear staining, the cells were incubated for 5 min at room temperature with 4',6-diamidino-2-phenylindole (DAPI).

2.8. Cellular uptake of scavenger ligands analysis

For cellular uptake of acetylated low-density lipoprotein (Ac-LDL), EBs were incubated with 10 μg/ml Alexa 488-conjugated Ac-LDL (molecular probes) at 37°C for 4 h. After rinsing the dish three times with phosphate-buffered saline (PBS), the cellular uptake of Alexa488-conjugated Ac-LDL was examined using a fluorescence microscope (BZ-9000, Keyence, Japan).

2.9. Scanning electron microscopy

Cultured EBs and primary adult mouse LSECs were fixed with 2.0%

glutaraldehyde. Samples were postfixed with 1.0% osmium tetroxide, freeze-dried with t-butyl alchol, sputter-coated with gold, and examined with JSM6510LV scanning electron microscope (JEOL, Tokyo, Japan).

3. Results

3.1. Gene expression in embryonic stem cells (ESCs)-derived embryoid bodies (EBs) during differentiation

We first used quantitative real-time RT-PCR to analyze the time course of brachyury, goosecoid and Flk-1 expression during EB formation in hanging drops (data not shown). Brachyury and goosecoid expression, which defines early mesoderm induction and development, was highest from day 3 to day 4 of EB formation and then declined. By contrast, expression of the early endothelial cell differentiation marker Flk-1 was first detected on day 3 and gradually increased until day 5. We therefore estimated that early mesodermal differentiation in EBs gave way to endothelial cell differentiation on day 4, and so we transferred EBs onto collagen type I dishes for expansion and further induction of the endothelial cell lineage on that day. We mainly observed the outer regions of the cultured EBs on the matix (EB outgrowth) because in

those areas vascular and cellular network formation was easily observed.

To assess expression of AM, TGFβ1 and their receptors during differentiation of EBs, we carried out a RT-PCR analysis using RNA extracted from undifferentiated ESCs and from ESC-derived EBs collected every 4 days from day 0 to day 20 (Fig. 1A). RAMP2 and RAMP3 were already expressed in the undifferentiated ESCs (day 0). Type I TGFß receptor, activin receptor-like kinase (ALK)5 was also dected in day 0. CRLR, VEGF and another type I TGFß receptor, ALK1 expression was first detected on day 4 and was sustained until day 20. RAMP3 expression was detected in the undifferentiated ESCs; it then disappeared by day 4 but reappeared on day 8, and its levels increased thereafter. By contrast, AM expression was not detected until day16, well after expression of its receptors.

3.2. AM enhances induction of CD31-positive endothelial cells in EBs during an early phase of differentiation

To assess the roles of VEGF and AM in vasculogenesis during early endothelial cell development, recombinant VEGF (20 ng/ml) and AM (10^{-7} M, 10^{-6}M) were added to the cultures from day 4 to day 14 (Fig. 1B), and the number of CD31-positive cells in

EB outgrowths was quantified by immunohistochemical analysis on day14 (Fig. 1C). We found that the number of CD31-positive endothelial cells increased in the VEGF-treated group. Moreover, adding AM in combination with VEGF significantly enhanced induction CD31-positive endothelial cells, as compared to VEGF alone (Fig. 1C, D).

We also analyzed lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) expression during the early phase of EB development (days 4 to 14); however, no LYVE-1 positivity was detected in any group at this stage (data not shown). Therefore, to identify the stage at which LYVE-1-positive endothelial cells emerge, at a later phase (days 17 to 24) we double-immunostained cells in EB outgrowths for CD31 and LYVE-1 (Fig. 1E). A few CD31/LYVE-1 double positive endothelial cells were detected on days 17-20, but the numbers gradually increased up to day 24. Interestingly, the emergence of LYVE-1-positive endothelial cells occurred at about the same time as the upregulation of AM expression (Fig. 1A).

3.3. AM and SB431542 enhance the induction of LYVE-1-positive endothelial cells during late phase differentiation

Next, we examined the expression of lymphatic endothelial cells (LECs) and liver sinusoidal endothelial cells (LSECs)-specific genes in the EB outgrowths during later phase differentiation. Stabilin-2 is a fasciclin-like hyaluronan receptor and specific LSEC marker used to distinguish LSECs from LECs. Inhibition of TGFβ receptor type 1, using its specific inhibitor, SB431542, reportedly promotes stabilin-2-positive endothelial cell differentiation in monolayer cultures of ESCs[25]. In the present study, we compared the effect of AM on LSEC differentiation with that of SB431542. Recombinant AM or SB431542 was added to later phase EBs (days 14 to 20) attached to collagen I-coated dishes (Fig. 2A). Quantitative RT-PCR analysis showed that LYVE-1 gene expression was significantly elevated about 2-fold, as compared to control, in both the AM- and SB431542-treated groups. Furthermore, when AM and SB431542 were added together, they acted synergistically to upregulate LYVE-1 about 8-fold (Fig. 2B).

AM and SB431542 each significantly upregulated stablin-2 expression and, as with LYVE-1, their co-administration synergistically enhanced stabilin-2 expression. Vascular endothelial growth factor receptor (VEGFR)3 and CD31, which are expressed in fetal LSECs[24], were also upregulated by AM and SB431542. By contrast, expression of Prox-1 and podoplanin, LECs-specific markers, was unaffected. Thus

AM and SB431542 promote cell differentiation that is much more toward LSECs than LECs.

Immunohistochemical detection of CD31 and LYVE-1 in day 20 EB outgrowths revealed that the numbers of CD31/LYVE-1 double-positive endothelial cells were significantly increased by treatment with AM or SB431542 (Fig. 2C, D). And consistent with the quantitative RT-PCR results, AM and SB431542 acted synergistically to increase numbers of LYVE-1-positive endothelial cells (Fig. 2D). The proportion of LYVE-1-positive cells among the CD31-positive cells was nearly 70%, whereas no LYVE-1-positive cells were found among the CD31-negative cells.

3.4. AM- and SB431542-induced, EB-derived, LYVE-1-positive endothelial cells possess the characteristics of LSECs

To characterize the phenotype of EB-derived, LYVE-1-positive endothelial cells treated with AM and SB431542 in more detail, we initially carried out an immunohistochemical analysis in EBs treated with vehicle or AM+SB431542. Co-administration of AM and SB431542 promoted the appearance of LYVE-1 and stabilin-2 double-positive cells compared with control (Fig. 3A). This double

positivity of LSECs for LYVE-1 and stabilin-2 was also deteced in primary cultures of LSECs from adult mouse liver (lower panel of Fig. 3A). Higher magnification of the same condition well-demonstrated this double-positivity in AM-SB431542-treated group (lower panel of Fig. 3B).

We also assessed the expression of LSEC-specific markers known to be expressed in mature liver. Using quantitative RT-PCR , we determined that the LSEC markers, factor (F8), Fc-γ receptor 2b (Fcgr2b) and mannose receptor C type 1 (Mrc1), were all significantly upregulated by AM and SB431542 (Fig. 3C). To then evaluate the functional properties of EB-derived endothelial cells, we assessed endocytosis of fluorescently labeled (Alexa 488-conjugated) acetylated low-density lipoprotein (Ac-LDL). AM- and SB431542-treated cells showed higher existence of the fluorescent-positive cells than control, which means greater endocytotic activity (Fig. 3D). At 4 hrs, AM- and SB431542-treated cells exhibited significantly greater endocytotic activity than untreated cells (Fig. 3E). On day 24 of the culture, morphological features of EB-derived LYVE-1-positive endothelial cells were compared with primary-cultured adult LSECs in the higher magnification (Fig. 3F upper panel) and in scanning electron microscopy (Fig. 3F lower panel). AM- and

SB431542-treated EB-derived LYVE-1-positive endothelial cells revealed the presence of fenestrae-like structure, which are the most prominent feature of mature LSECs.

3.5. The crucial role played by the AM-RAMP2 system during hepatic sinusoidal development and morphogenesis in vivo

To study hepatic sinusoidal endothelial development and morphogenesis, we carried out an immunohistochemical analysis to detect CD31 and LYVE-1 in liver from E14.5-embryos and adults (Fig. 4A). We found that whereas fetal LSECs expressed CD31 and LYVE-1 equally (Fig. 4A upper panel), adult LSECs showed much stronger expression of LYVE-1 than the fetal cells and less expression of CD31 (Fig. 4A lower panel). In addition, the CD31-positive cells were limited to the larger vessels in the adult liver. LYVE-1-positive LSECs already showed capillary-like structures and ductal formation in the E14.5 liver.

Analysis of hepatic gene expression revealed that AM, CRLR and RAMP2 are expressed during mid-to-late gestation and in newborns, and that AM expression gradually increases during development until it peaks in newborns. On the other hand, TGFβ1 expression is downregulated after birth (Fig. 4B).

We have demonstrated that RAMP2, one of the AM-receptor modulating proteins, is a crucial determinant of AM's vascular function during development. AM-/- and RAMP2-/- mice die at midgestation because of vascular abnormalities [11, 29]. Immunohistochemical analysis using anti-CD31 and anti-LYVE-1 antibodies in E14.5 wild-type and RAMP2-/- embryo liver revealed downregulation of LYVE-1 expression in the sinusoidal endothelial cells (Fig. 4C). Quantitative RT-PCR analysis also revealed significant downregulation of LYVE-1 in the RAMP2-/- liver (Fig. 4D).

4. Discussion

The purpose of this study was to induce liver sinusoidal endothelial cells (LSECs) differentiation for the reconstitution of liver morphogenesis.

LSECs have unique features not seen in other vascular endothelial cells that make them more similar to lymphatic endothelilal cells (LECs). For example, both LECs and LSECs have minimal basement membranes and loose cell-cell junctions, and both express lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1)[17, 24]. Moreover, both LECs and LSECs originate from veins or mesenchyme[2, 10, 33]. That said, these two cell types clearly differ in some ways. For example, stabilin-2, FcγRs, Mrc1 and F8 are expressed in LSECs but not LECs, whereas podoplanin and Prox-1 are expressed in LECs, but not LSECs. LSECs also exhibit greater endocytotic activity than other types of endothelial cells[24]. In our study, LYVE-1-positive endothelial cells in EBs treated with AM and SB431542 expressed stabilin-2, and some exhibited fenestrae-like structures. In addition, transcription of the LSEC markers F8, Fcgr2b and Mrc1 was significantly upregulated, and the cells exhibited more robust endocytotic activity. On the other hand, expression of the specific LEC markers Prox-1 and podoplanin was unaffected. EB-derived, LYVE-1-positive endothelial

cells induced with AM and SB431542 thus appear to possess the characteristics of LSECs.

Matsumoto et al. showed that primitive endothelial cells localized in the septum transversum play a crucial role in the induction of the initial liver bud and in subsequent liver development[19]. Recently, Ogawa et al. reported that the emergence of cardiomyocytes and the expansion of the endothelial cell network derived from ESCs stimulate the proliferation of hepatocytes and liver organogenesis[26]. In addition, Fujimori et al. reported that VEGF increases proliferation of endothelial and hepatocyte-like cells in EBs[9]. In our study, LYVE-1-positive endothelial cells migrated to and made contact with albumin-positive cells in EB outgrowths, after which expression of mature hepatocyte markers increased (data not shown). This suggests that through the induction of LSECs we can promote mature hepatic morphogenesis, and that LSECs could be a crucial therapeutic target for regeneration of the liver. Consistent with that idea, it was recently shown that transplanted sinusoidal endothelial cells can repopulate the liver endothelium and correct the phenotype of hemophilia A mice[7, 15], and several reports have shown that endothelial progenitor cell transplantation ameliorates acute liver injury and liver cirrhosis in rats[18, 23, 31, 32].

Our second major finding is that AM-RAMP2 system is the potentital target for the induction of LSECs. From our observation of brachyury, goosecoid and Flk-1 expression, we estimated that by day 4 early mesoderm differentiation in EBs gave way to endothelial cell differentiation. In addition, expression of CRLR and RAMP2, which together form an AM receptor, also started on day 4. These observations prompted us to test the effect AM on VEGF-induced endothelial differentiation beginning on day 4. Our finding that AM dose-dependently enhanced induction of CD31-positive endothelial cells during the early differentiation phase (days 4-14) is consistent with earlier observations made using Flk-1-positive cells sorted from ESCs[35]. By contrast, LYVE-1-positive endothelial cells were not seen at this stage in any group. We therefore suggest that at this stage CD31-positive endothelial cells had not yet expressed differentiation factors for specific endothelial cellular lineages. Therefore, we next determined the stage at which LYVE-1-positive endothelial cells emerged in EB outgrowths. A few CD31/LYVE-1 double-positive cells were detected on days17-20, and their numbers gradually increased on days 22-24. Interestingly, the emergence of LYVE-1-positive endothelial cells and the upregulation of AM occurred at about the same time (day17). From these observations, we estimated that days14-17 is a critical period during which CD31-endothelial cells in EB outgrowths begin to

respond to differentiation factors for specific cellar lineages. We therefore applied differentiation stimuli from day 14. SB431542, a TGF-β receptor type 1 inhibitor, reportedly promotes CD31/LYVE-1/stabilin-2 positive endothelial cell differentiation in monolayer cultures of ESCs [25]. In the present study, we found that AM-treatment from day 14 to day 20 also promoted the appearance of CD31/LYVE-1/stabilin-2-positive endothelial cells. Moreover, when administered together, AM and SB431542 acted synergistically to promote differentiation of CD31/LYVE-1/stabilin-2-positive endothelial cells. This result suggests, in the differentiation of LSECs, AM and TGFβ-signaling show reverse correlation; TGFβ-TGFβ receptor system suppresses LSEC differentiation. On the other hand, AM-RAMP2 system promotes it. TGFβ1 and type I TGFß receptor (ALK1 and ALK5) were expressed in EBs and their expanded culture on the collagen dish. In addition, TGFβ1 was rather uniformly expressed in the liver of embryo, new born, and adult mice. In contrast, the AM expression level is temporally regulated and appears at relatively later stage during embryogenesis. Montuenga et al. showed that during rodent embryogenesis, the expression of TGFβ1 and AM is spatially and temporally regulated such that their expression patterns overlap at the same stage of development in several tissues and in the same cellular locations[21]. It has also been shown that there is less

AM expression in tissues from embryonic TGFβ1-null mice than in tissues from wild-type mice, but that AM expression increases during postnatal development, even in TGFβ1-null mice[1]. Taken together, critical balance between TGFβ-TGFβ receptor system and AM-RAMP2 system during development may determine the differentiation direction of endothelial cells. By a microarray analysis of AM and SB431542 co-administration condition in EBs, we confirmed the upregulation of LYVE-1. In contrast, we cannot detect specific changes in other angiogenic factors (Supplemental Table 1-3). This may suggest that in the co-administration of AM and SB431542, LYVE-1 upregulation is a critical determinant of LSEC differentiation.

It also has been previously reported that AM/cAMP is a novel signaling pathway that leads to activation of Notch signaling in differentiating endothelial cells, and is required for induction of arterial endothelial cells from Flk-1-positive cells sorted from ESCs in a monolayer culture system[35]. Our results are at variance to this report, and we suggest that the inconsistency reflects the difference in the culture systems used in the two studies: monolayer vs. semispheroid cultures and sorted purified cells vs. EBs that include endodermal and ectodermal cell lineages. Cell-cell interactions and unknown factors from endodermal and/or ectodermal cells may affect the endothelial cells in our culture system.

Analysis of gene expression in the embryonic liver revealed that AM, CRLR and RAMP2 are expressed throughout liver development. AM expression increased gradually during development and peaked in newborns. In E14.5 RAMP2-/- liver, LYVE-1 expression was downregulated in sinusoidal endothelial cells, and the capillary network and sinusoidal structure were disrupted. This finding, together with the observed morphology and functionality of the cells in our EB culture system, suggests the AM-RAMP2 system plays a critical role in the differentiation of LSECs and in sinusoidal morphogenesis. We suggest that these results could serve as the basis for development of techniques for the regeneration of liver, and could also be useful in a variety of other medical and research applications, including bioartificial liver systems and drug metabolism assays.

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Figure legends

Fig. 1

(A) Semiquantitative RT-PCR analysis of gene expression in ESC-derived EBs. RNA was extracted from undifferentiated ESCs (day 0, D 0) and from EBs on the indicated day and analyzed for expression of VEGF, AM, CRLR, RAMP2, RAMP3, TGFβ1, ALK1, ALK5 and HPRT. EBs were seeded onto collagen type I-coated dishes on day 4 and cultured in the absence of growth factors. (B) Protocol for the treatment of early phase EBs for endothelial cell differentiation. The medium was supplemented with VEGF and AM from day 4 to day14. (C) Immunohistochemical detection of CD31 (green) in EB outgrowths on day14. Scale bar = 200 μm. (D) Quantitative analysis of CD31-positive areas of EB outgrowths on day 14. Analyzed were 8 selected microscope fields. Bars are means ± SE, *; $p<0.01$, **; $p<0.001$. (E) Double immunostaining of CD31 (green) and LYVE-1 (red) in EB outgrowths cultured in the absence of growth factors on the indicated day. A few CD31/LYVE-1 double-positive endothelial cells were detected on days 17-20, but the number was increased on days 22-24. Scale bar = 200 μm.

Fig. 2

(A) Protocol for the treatment of late phase EBs for specific endothelial cell differentiation. To analyze the effect of AM and the TGFβ inhibitor SB431542 on late phase endothelial cell development, the medium was supplemented with recombinant AM (10^{-6} M) and/or SB431542 (10^{-6} M) from day 14 to day 20. (B) Quantitative RT-PCR analysis of CD31, LEC-specific genes (LYVE-1, Prox-1, podoplanin, VEGFR3) and LSEC-specific genes (LYVE-1, stabilin-2, VEGFR3) in the outgrowths of day 17 EBs treated with vehicle, SB431542, AM and AM+SB431542. mRNA levels was normalized to that of GAPDH mRNA. Bars are means ± SE, n=3 (each sample includes 15 EBs); *p<0.05, **p<0.001, ***p<0.0001 vs. control, †p<0.05, ††p<0.0001 vs. SB431542+AM. (C) Immunohistochemical detection of CD31 (green) and LYVE-1 (red) in 20 day EB outgrowths. Scale bar = 200 μm. (D) Quantitative analysis of the CD31- and LYVE-1-positive areas in 20 day EB outgrowths in 8 selected microscope fields. Bars are means ± SE, *p<0.05, **p<0.01, ***p<0.001 vs. control, †p<0.01 vs. AM.

Fig. 3

Phenotypic characterization of EB-derived, LYVE-1-positive endothelial cells treated with AM+SB431542. (A) Immunohistochemical detection of stabilin-2 (green) and

LYVE-1 (red) in day 20 EBs treated with vehicle, AM+SB431542 and in primary cultured adult LSECs. Scale bar = 100 μm. (B) In high power field. Scale bar= 100 μm. (C) Quantitative RT-PCR analysis of LSEC-specific genes in the outgrowths of day17 EBs treated with vehicle or AM+SB431542. □Control, ■ AM+SB431542. mRNA levels was normalized to that of GAPDH mRNA. Bars are means ± SE, n=3 (each of the sample includes 15 EBs); *p<0.05, **p<0.01. (D) Endocytotic activity in cultured EB outgrowths treated with vehicle or AM+SB431542 was estimated based on cellular uptake of Alexa 488-conjugated Ac-LDL (green) for 4 hrs and 24 hrs exposure. Scale bar = 300 μm. (E) Quantitative analysis of Alexa488-positive areas that were exposed for 4hours estimated in 8 selected microscope fields. □Control, ■AM+SB431542. Bars are means ± SE; **p<0.01. (F) Morphological analysis of EB-derived LYVE-1-positive endothelial cells treated with vehicle, AM and SB431542 on day 24 (left) and primary-cultured adult LSECs (right) using immunostaining of anti-LYVE-1 antibody (red), and using scanning electron microscopy. Scale bar = 50 μm (upper panel), 1 μm (lower panel).

Fig. 4

(A) Immunohistochemical detection of CD31 (green) and LYVE-1 (red) in E14.5 and

adult liver. Scale bar = 100 μm. (B) AM, CRLR, RAMP2, RAMP3, TGFβ1 and HPRT gene expression in E12.5, E14.5, E18.5, neonatal and adult livers. HPRT was amplified to normalize for the amount of RNA used as starting material. (C) Immunohistochemical detection of CD31 (green) and LYVE-1 (red) in E14.5 wild-type and RAMP2-/-embryo liver. Scale bar = 100 μm. (D) Quantitative RT-PCR analysis of AM, CRLR, RAMP2, RAMP3, LYVE-1 and CD31 in E14.5 wild-type and RAMP2-/- embryo liver. □Wild, ■RAMP2-/-. mRNA levels were normalized to that of GAPDH mRNA. Bars are means ± SE, n=4 per group; *$p<0.05$.

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Figure

A.

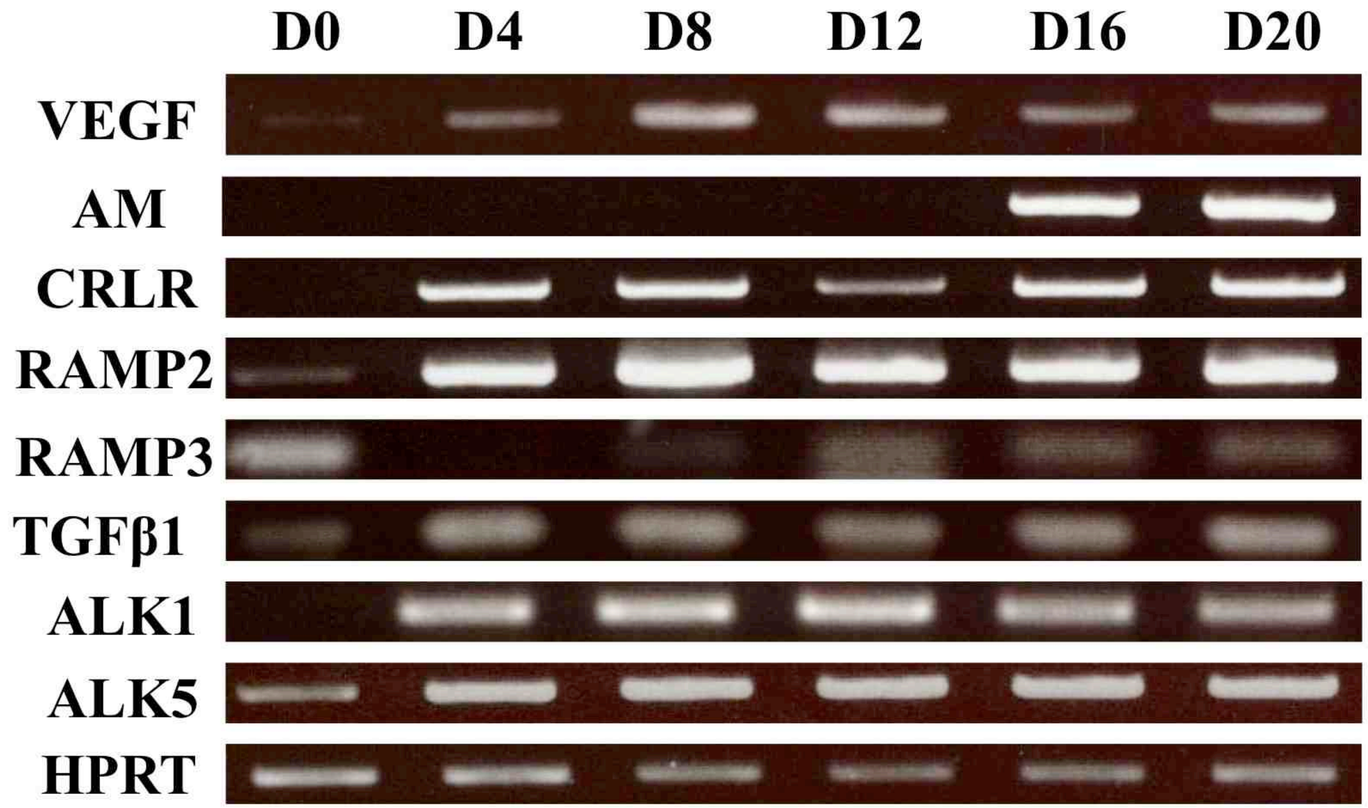

Fig 1.

B.

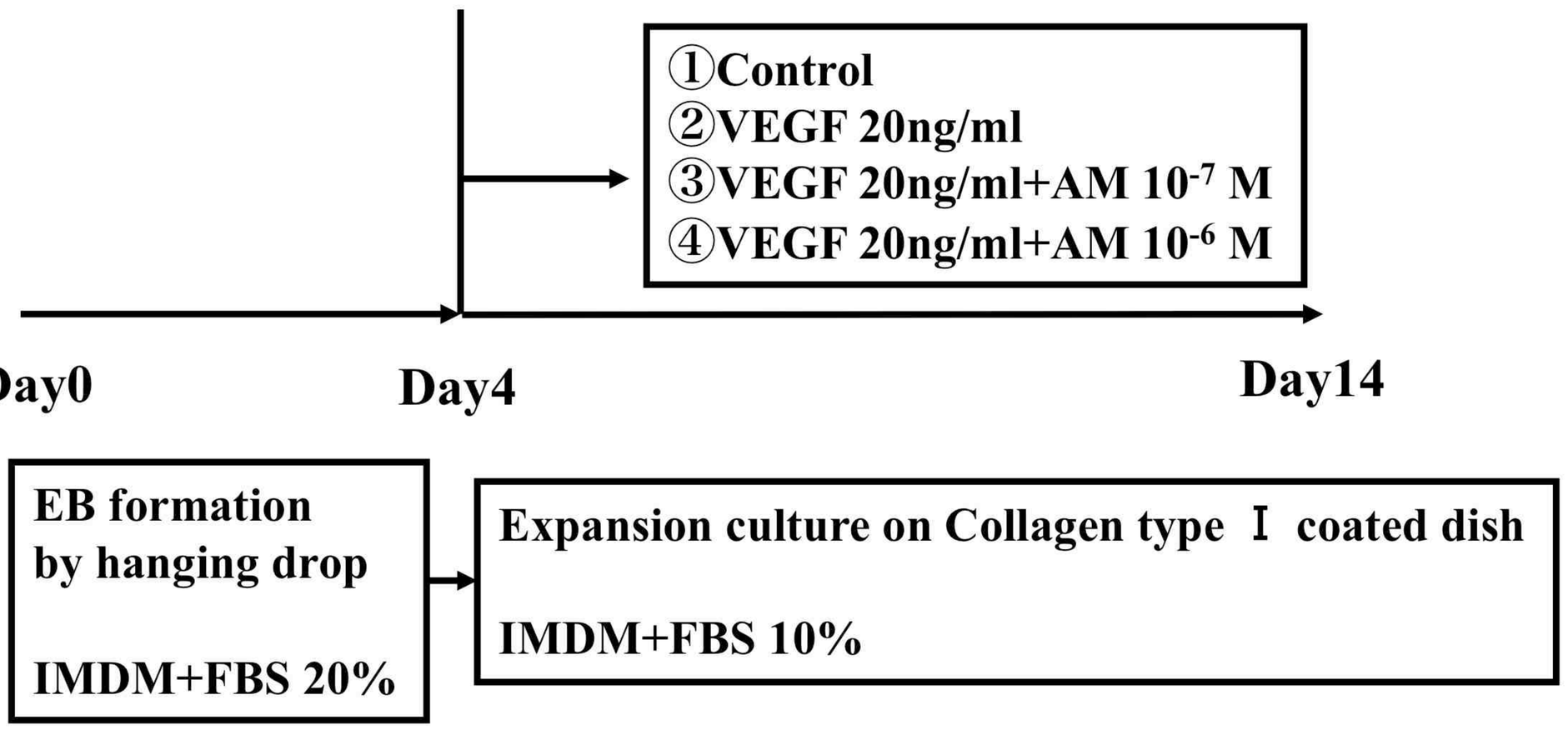
①Control
②VEGF 20ng/ml
③VEGF 20ng/ml+AM 10^{-7} M
④VEGF 20ng/ml+AM 10^{-6} M
Day0
Day4
Day14
EB formation
by hanging drop
IMDM+FBS 20%
Expansion culture on Collagen type Ⅰ coated dish
IMDM+FBS 10%

Fig 1.

C.

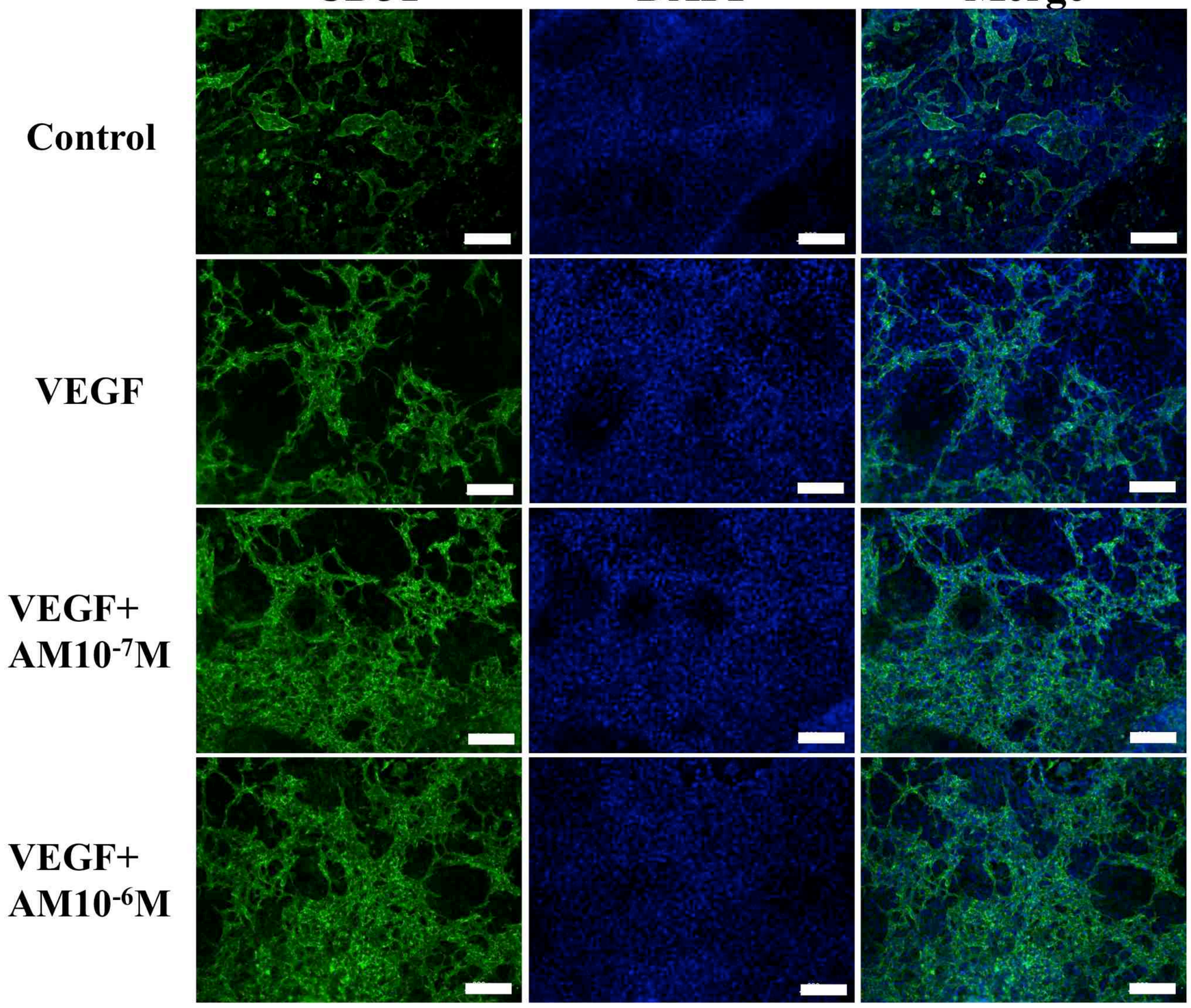

Fig 1.

D.

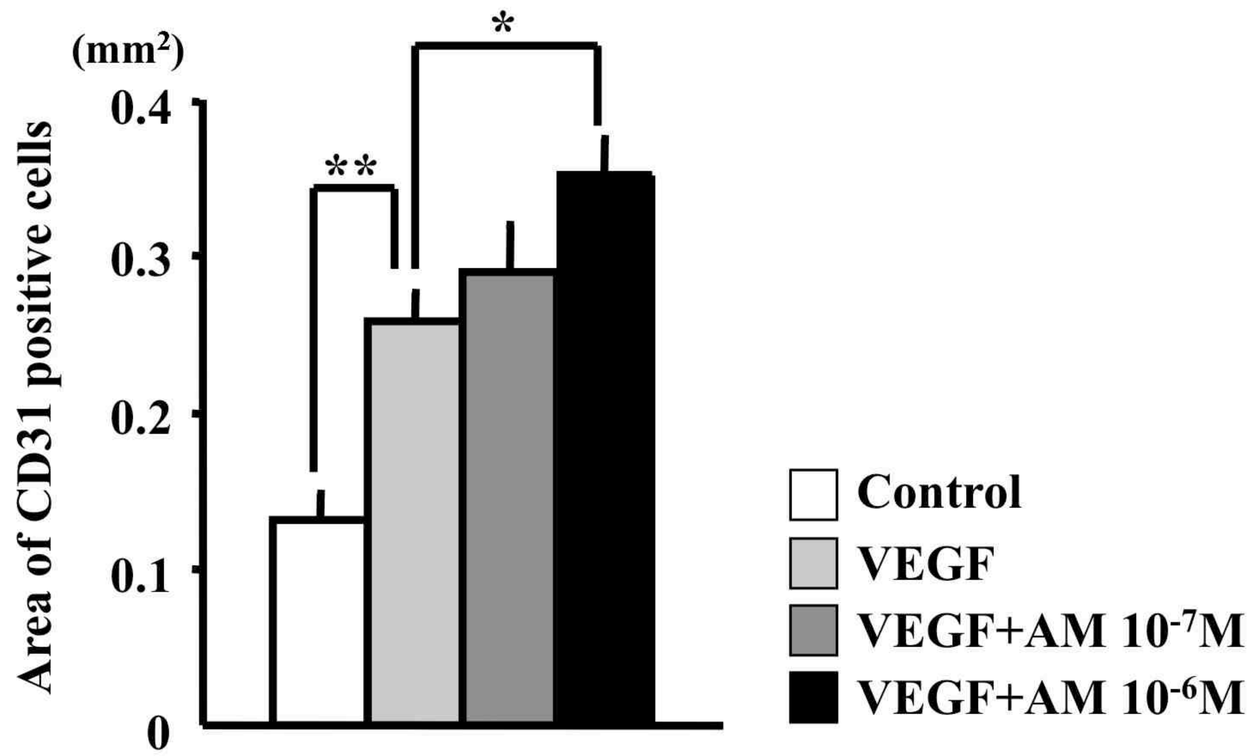
(mm²)
Area of CD31 positive cells
0.4
0.3
0.2
0.1
0
**
*
Control
VEGF
VEGF+AM 10-7M
VEGF+AM 10-6M

Fig 1.

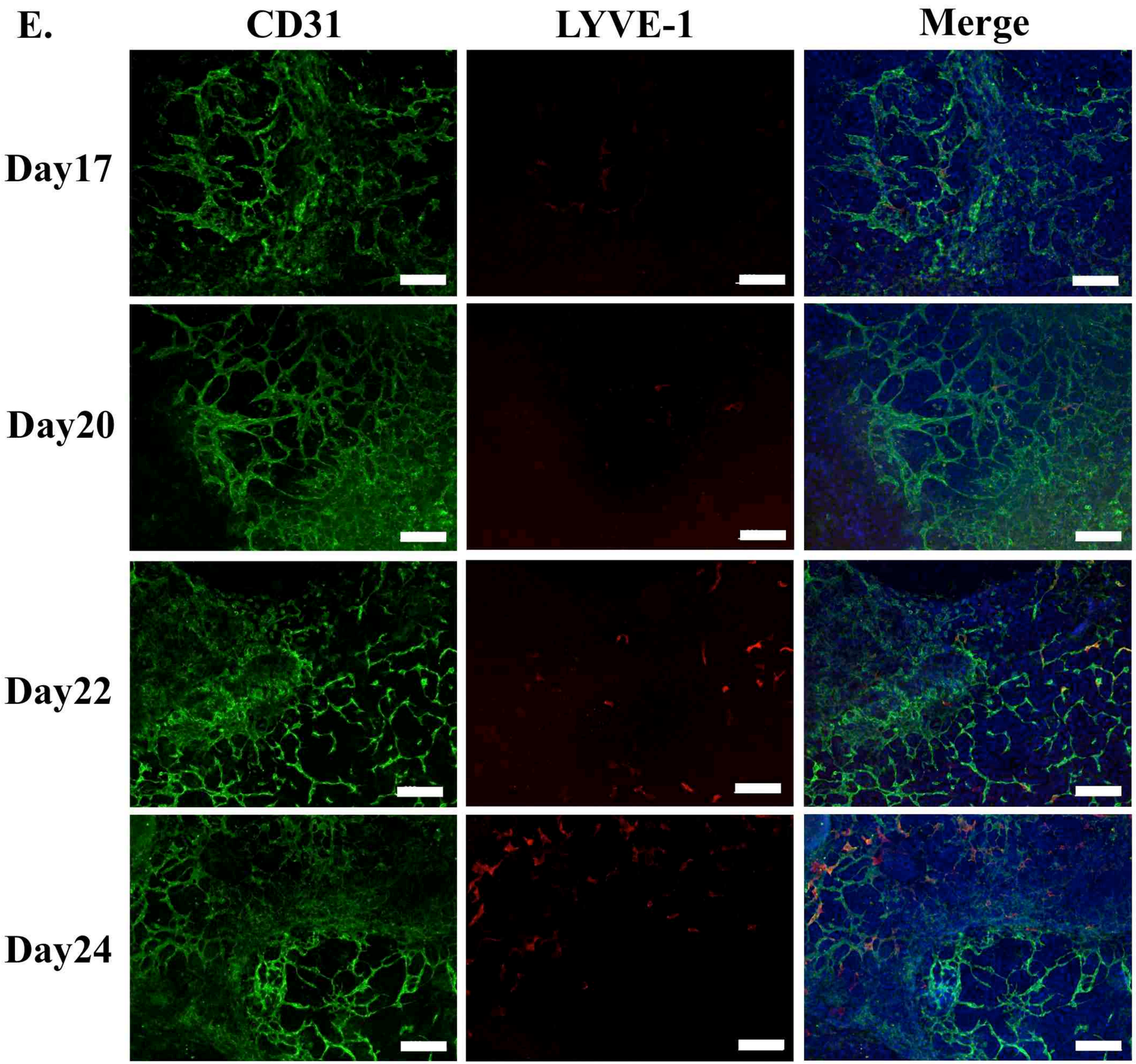

Fig 1.

A.

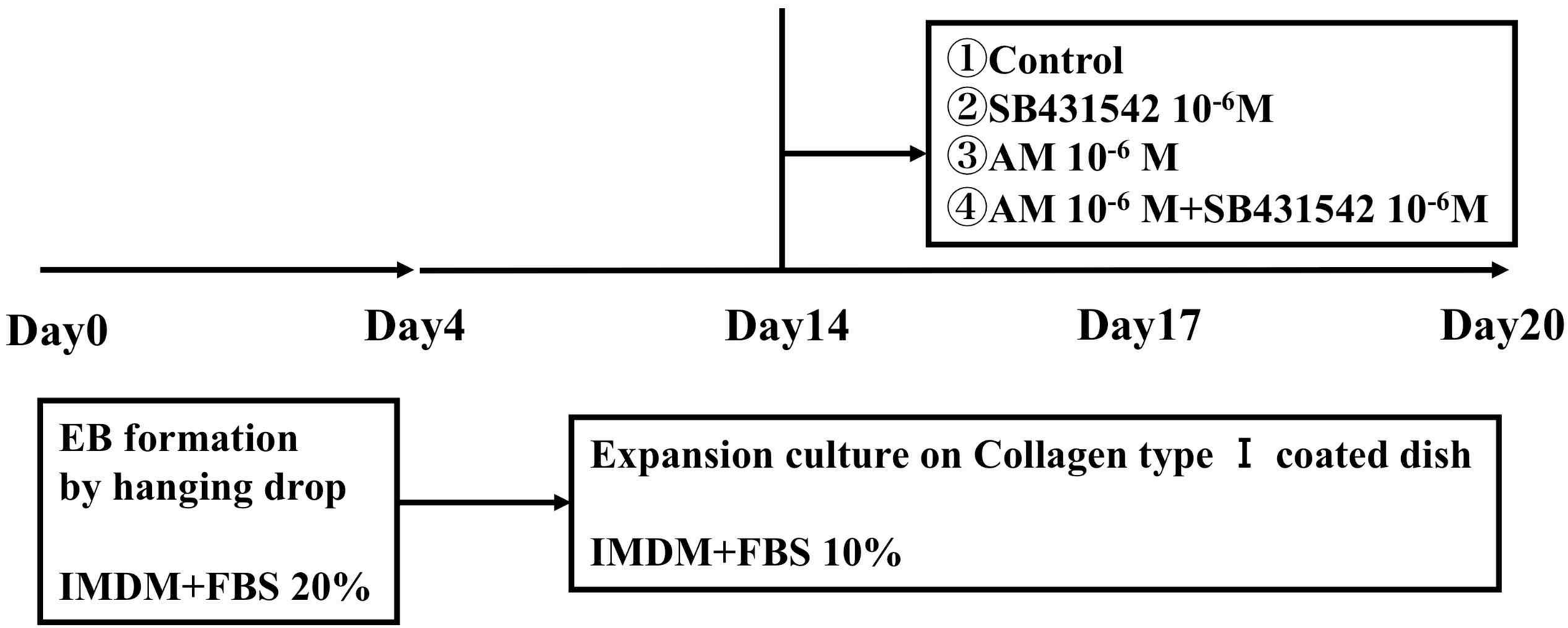
①Control
②SB431542 10-6M
③AM 10-6 M
④AM 10-6 M+SB431542 10-6M
Day0
Day4
Day14
Day17
Day20
EB formation
by hanging drop
IMDM+FBS 20%
Expansion culture on Collagen type Ⅰ coated dish
IMDM+FBS 10%

Fig 2.

B.

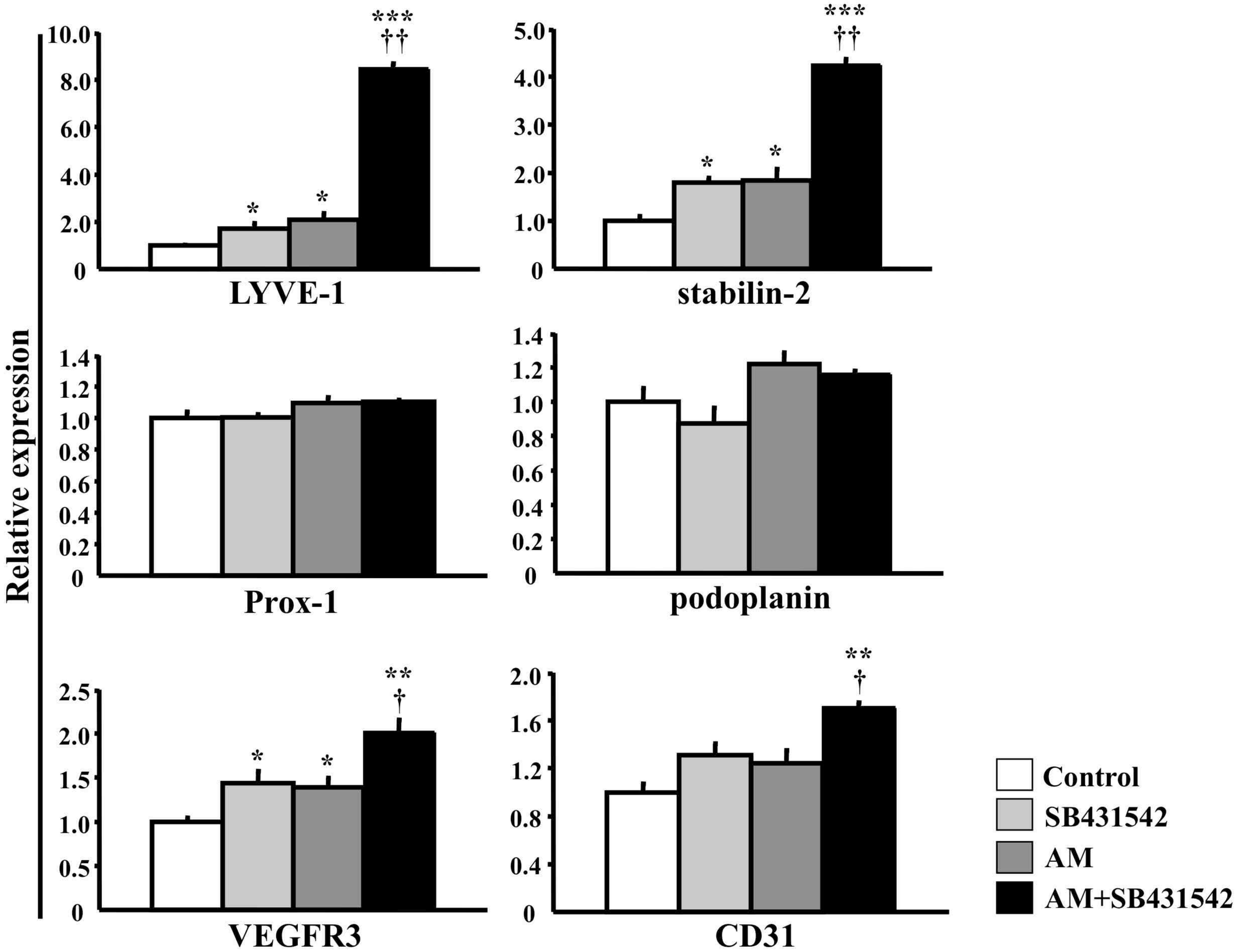
Relative expression
LYVE-1
stabilin-2
Prox-1
podoplanin
VEGFR3
CD31
Control
SB431542
AM
AM+SB431542

Fig 2.

C.

CD31 | LYVE-1 | Merge

Control

SB431542

AM

AM+SB431542

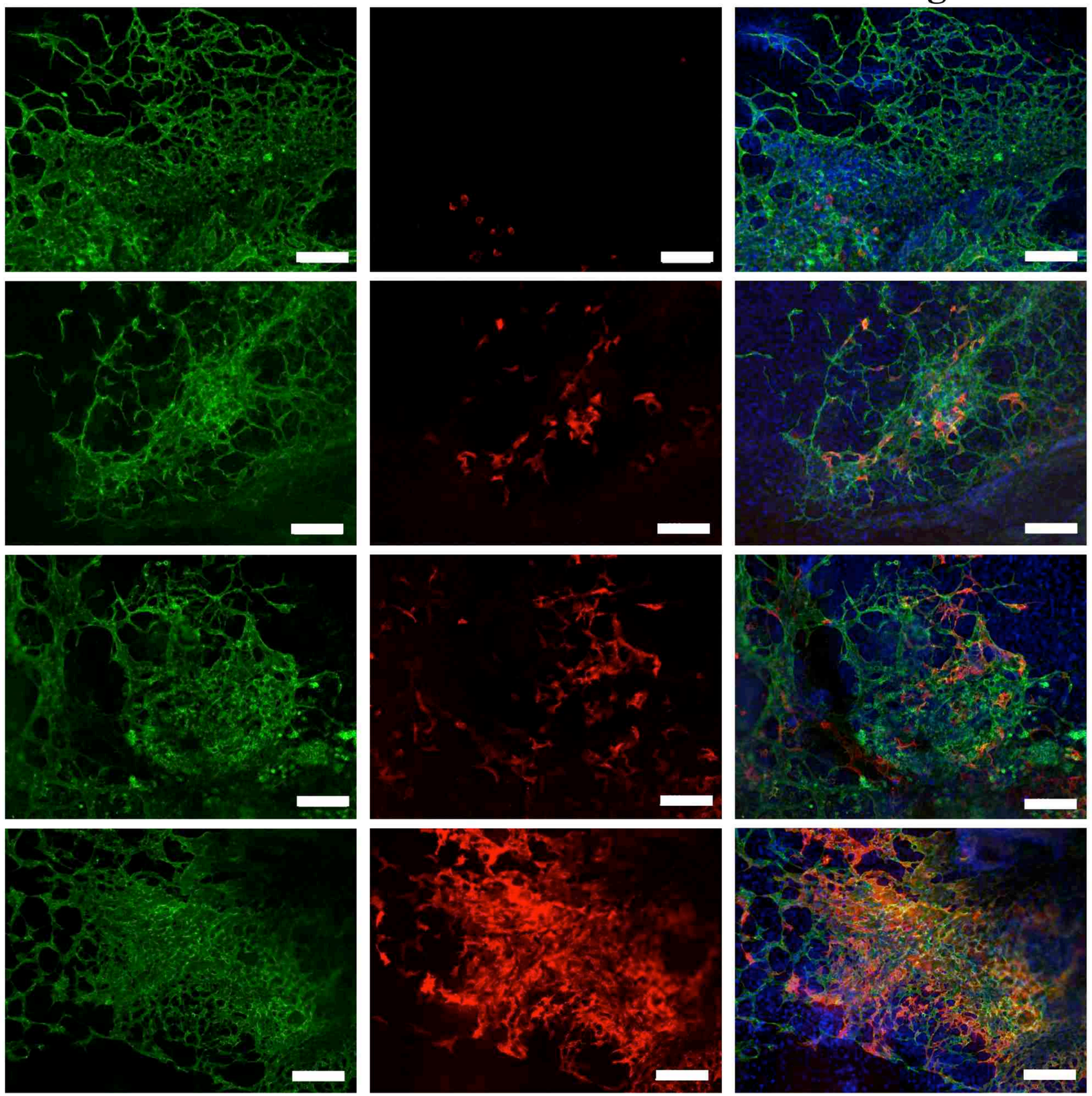

Fig 2.

D.

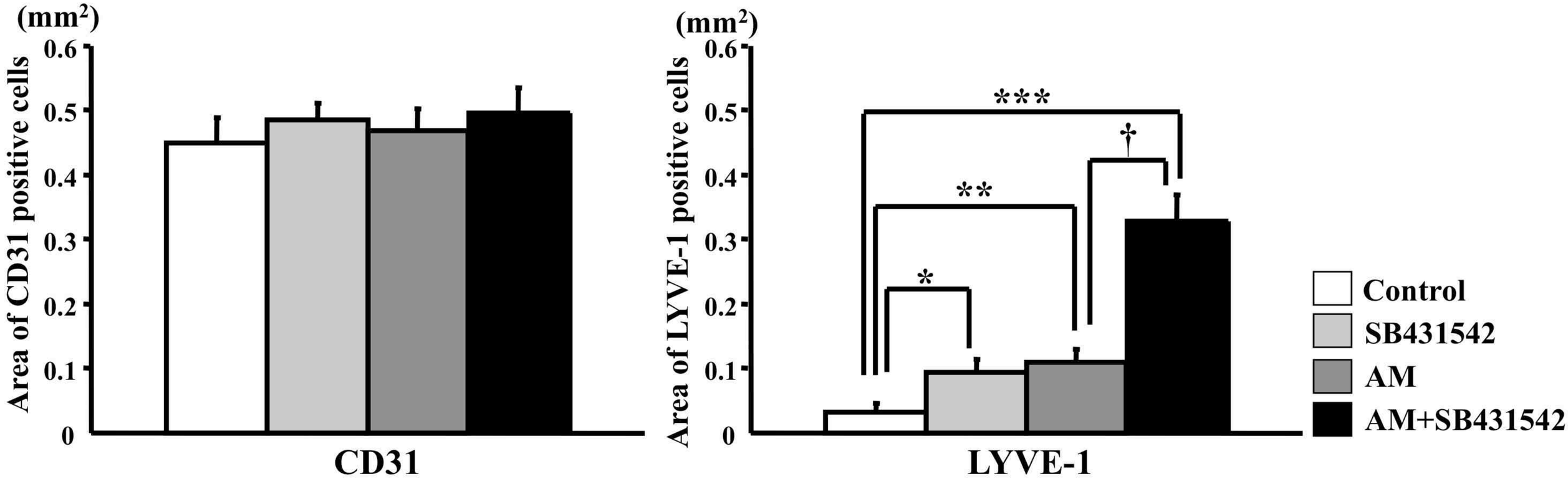
(mm²)
Area of CD31 positive cells
0.6
0.5
0.4
0.3
0.2
0.1
0
CD31
(mm²)
Area of LYVE-1 positive cells
***
**
*
†
LYVE-1
Control
SB431542
AM
AM+SB431542

Fig 2.

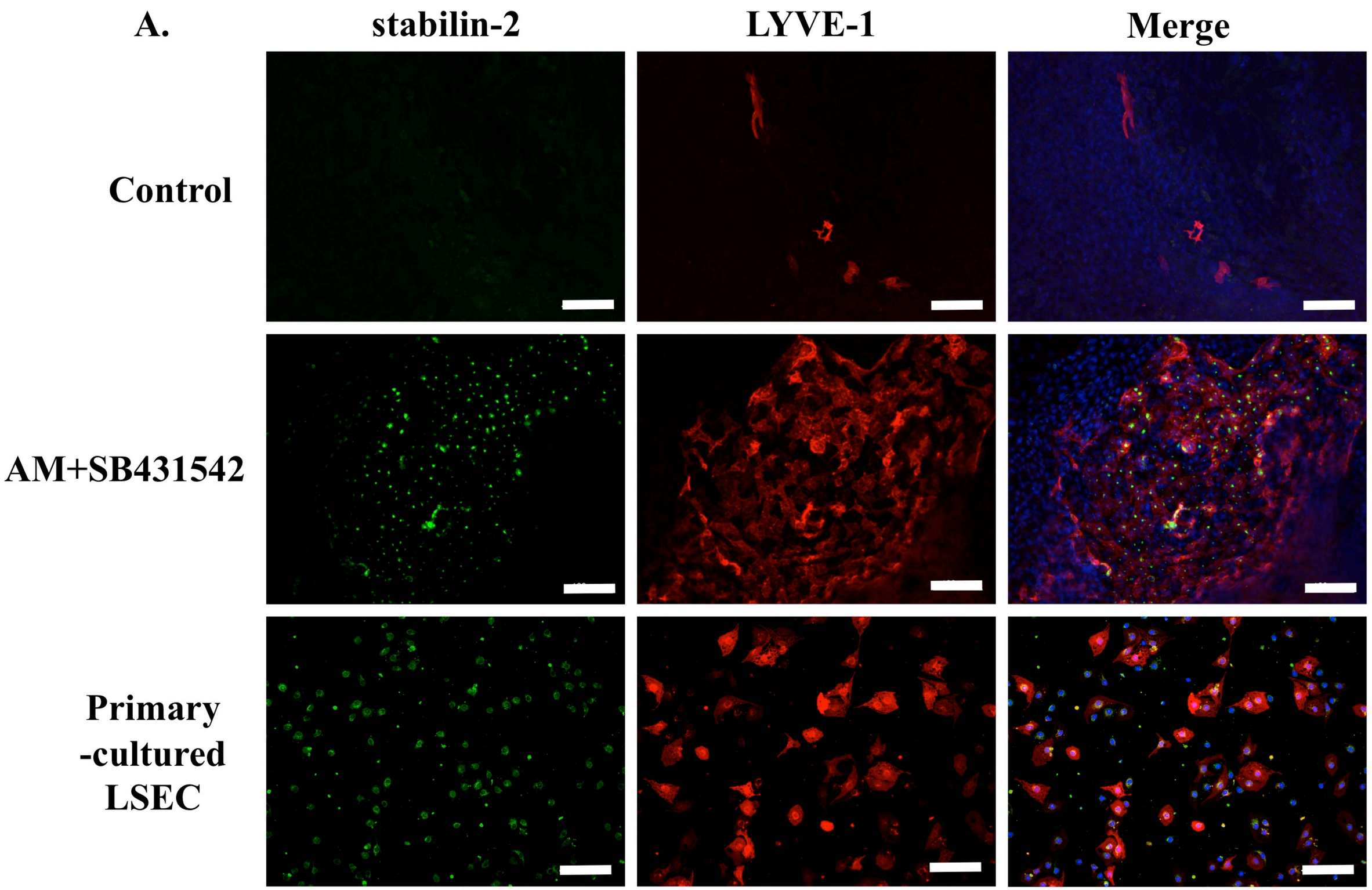
A.
stabilin-2
LYVE-1
Merge
Control
AM+SB431542
Primary
-cultured
LSEC

Fig 3.

B.

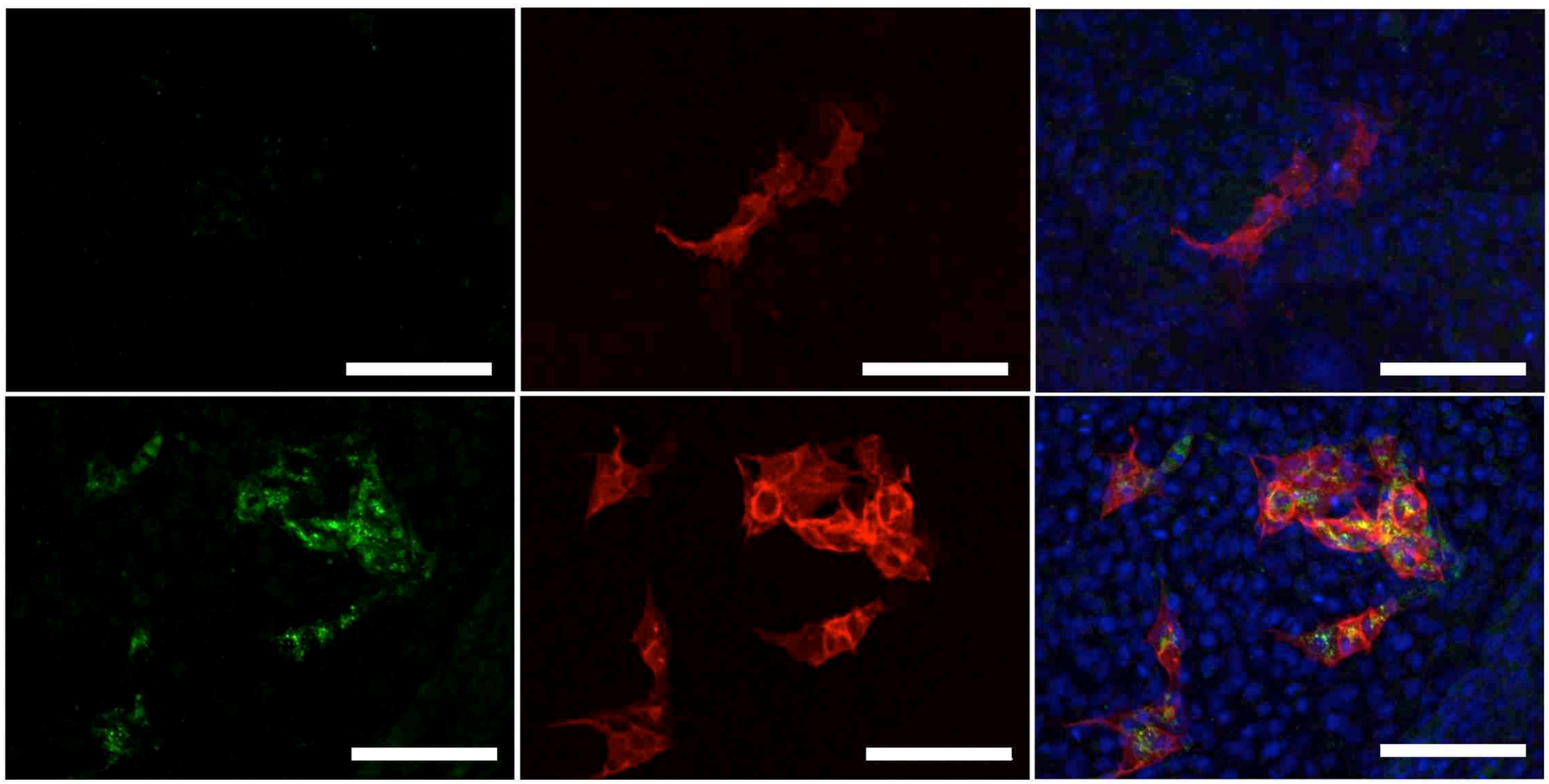

Fig 3.

C.

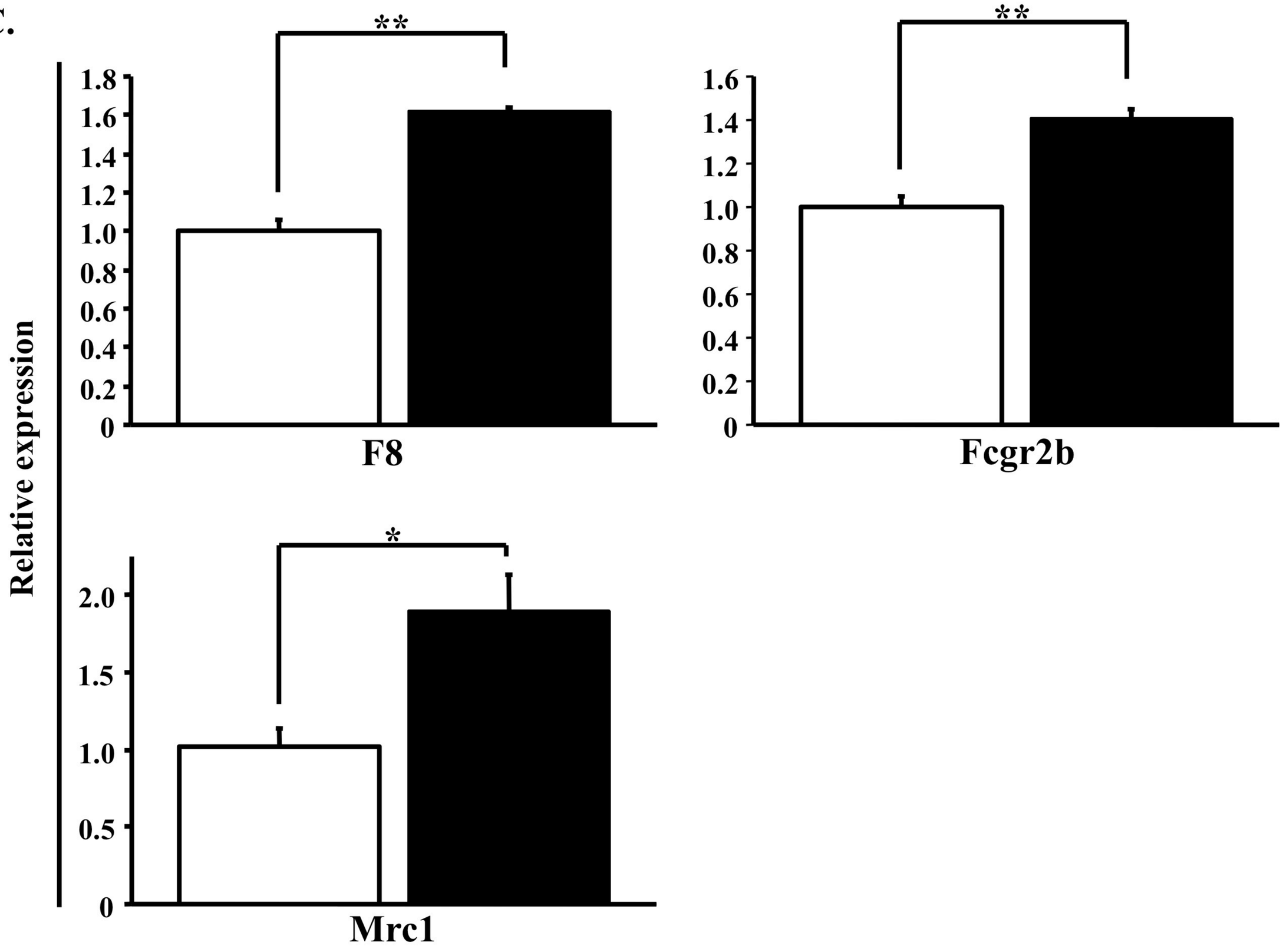
**
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
F8
**
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Fcgr2b
Relative expression
*
2.0
1.5
1.0
0.5
0
Mrc1

Fig 3.

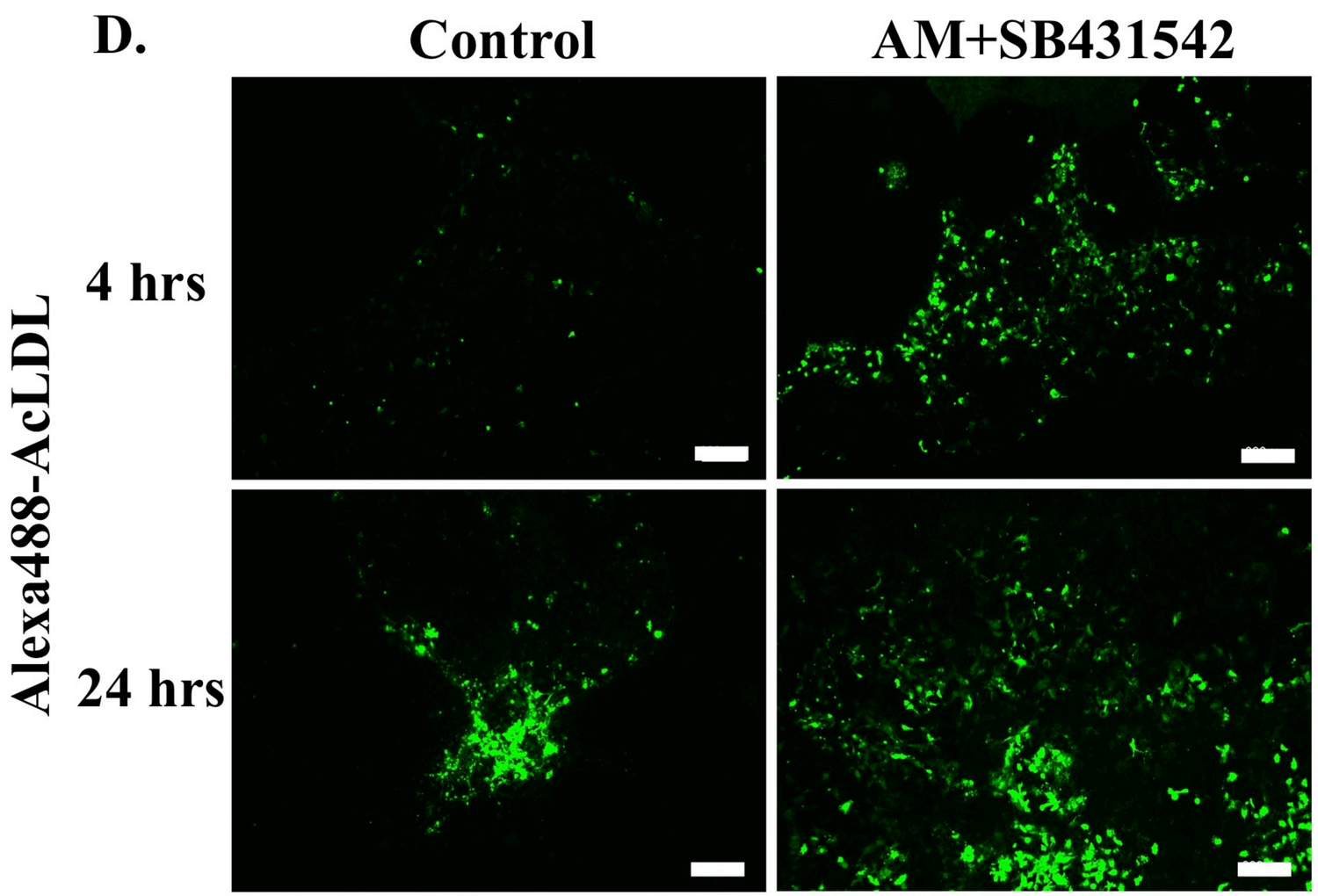

Fig 3.

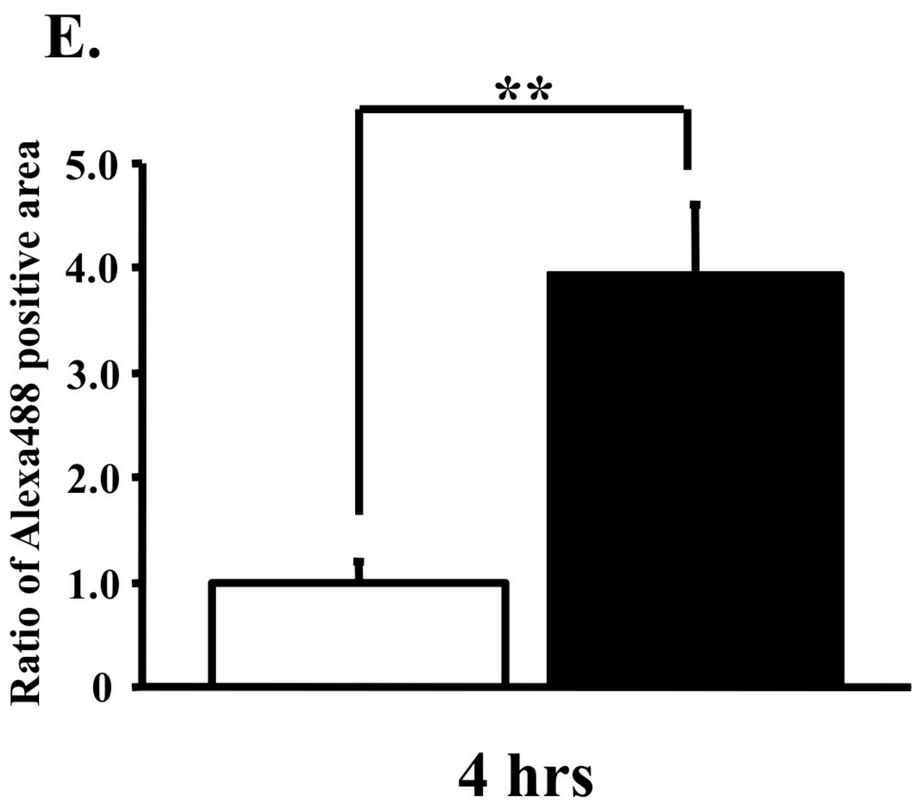

Fig 3.

F.

Control

AM+SB431542

Primary-cultured LSEC

LYVE-1

EM

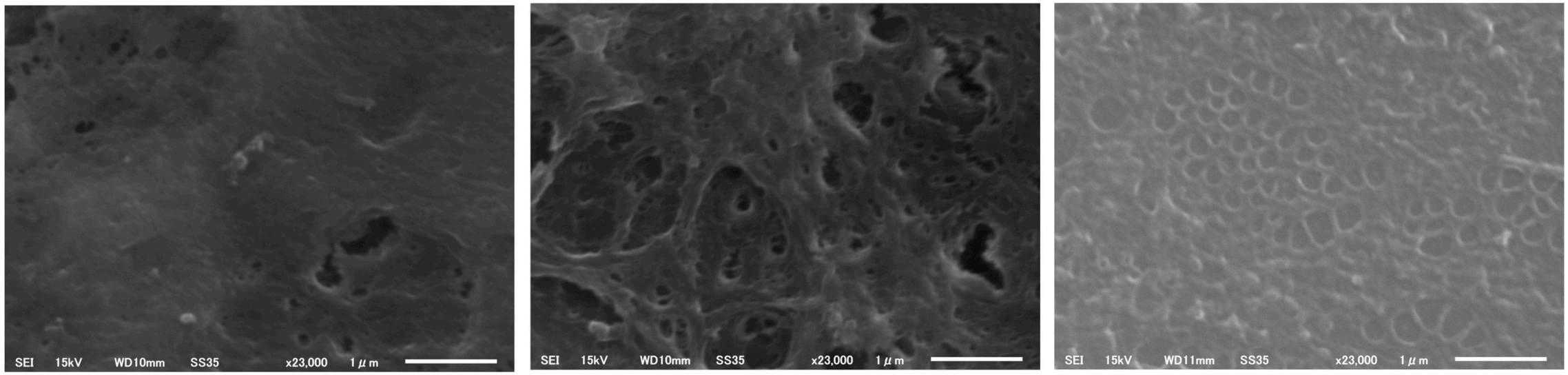

Fig 3.

A.

CD31

LYVE-1

Merge

E14.5 Liver

Adult Liver

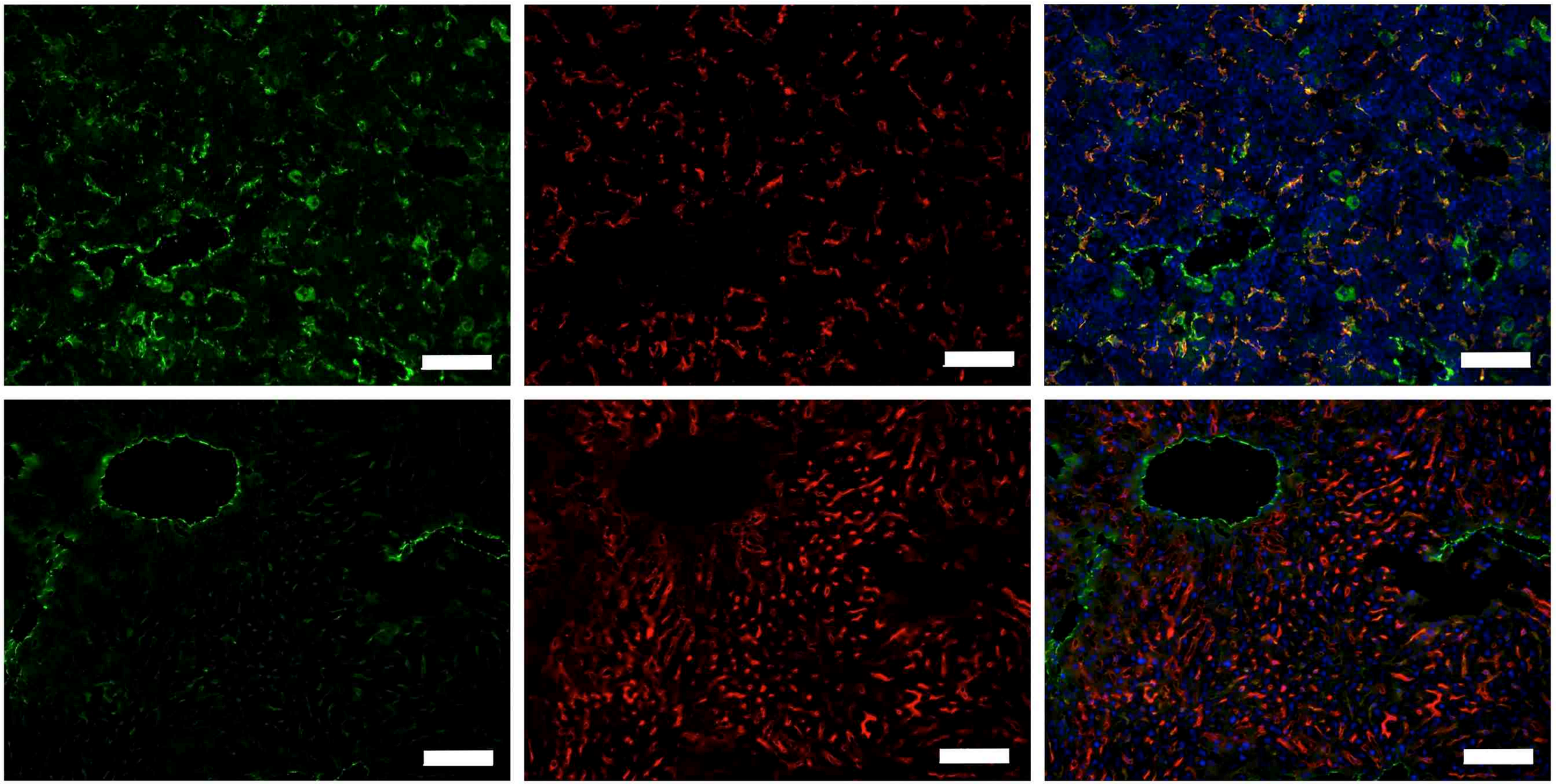

Fig 4.

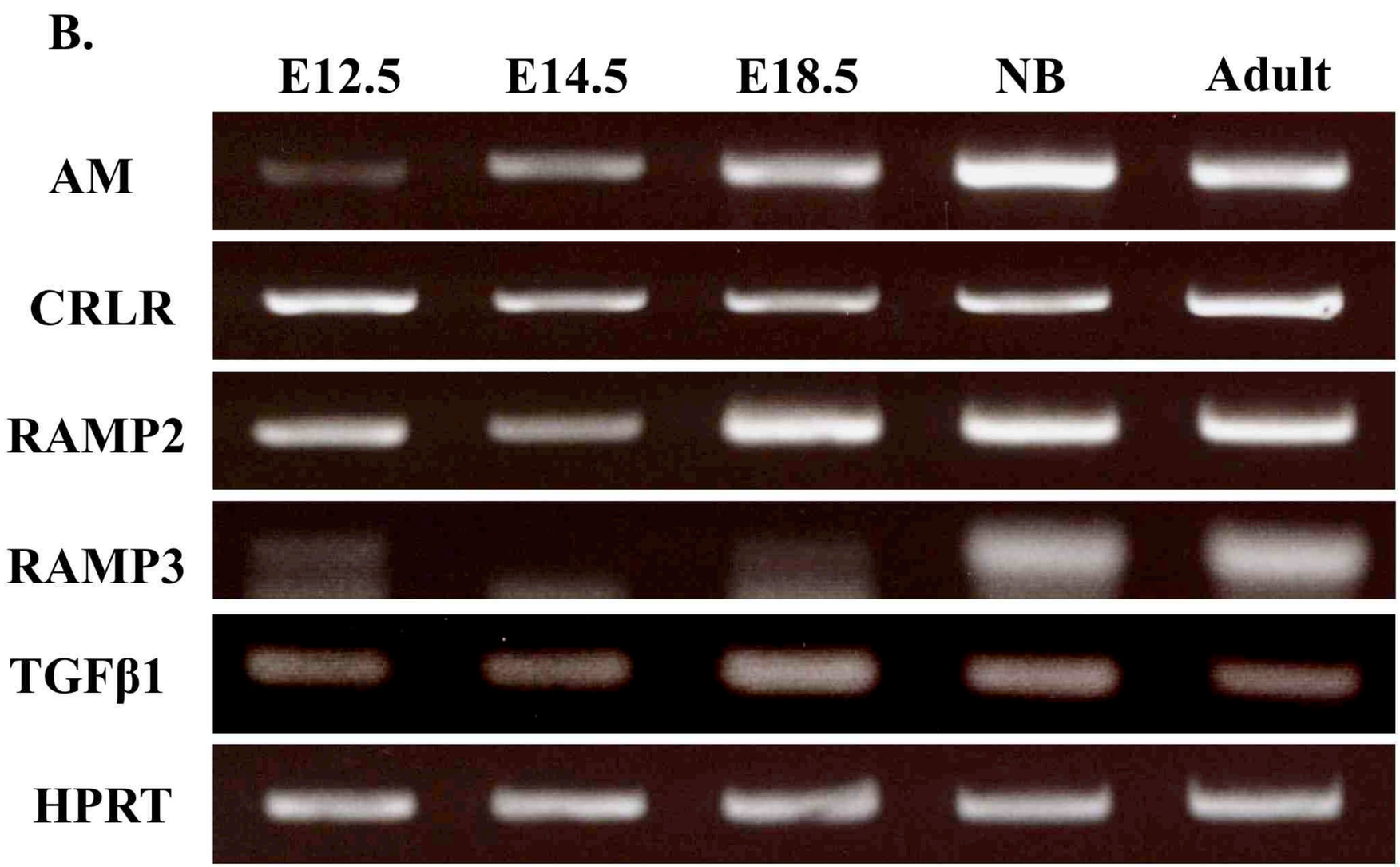

Fig 4.

C.

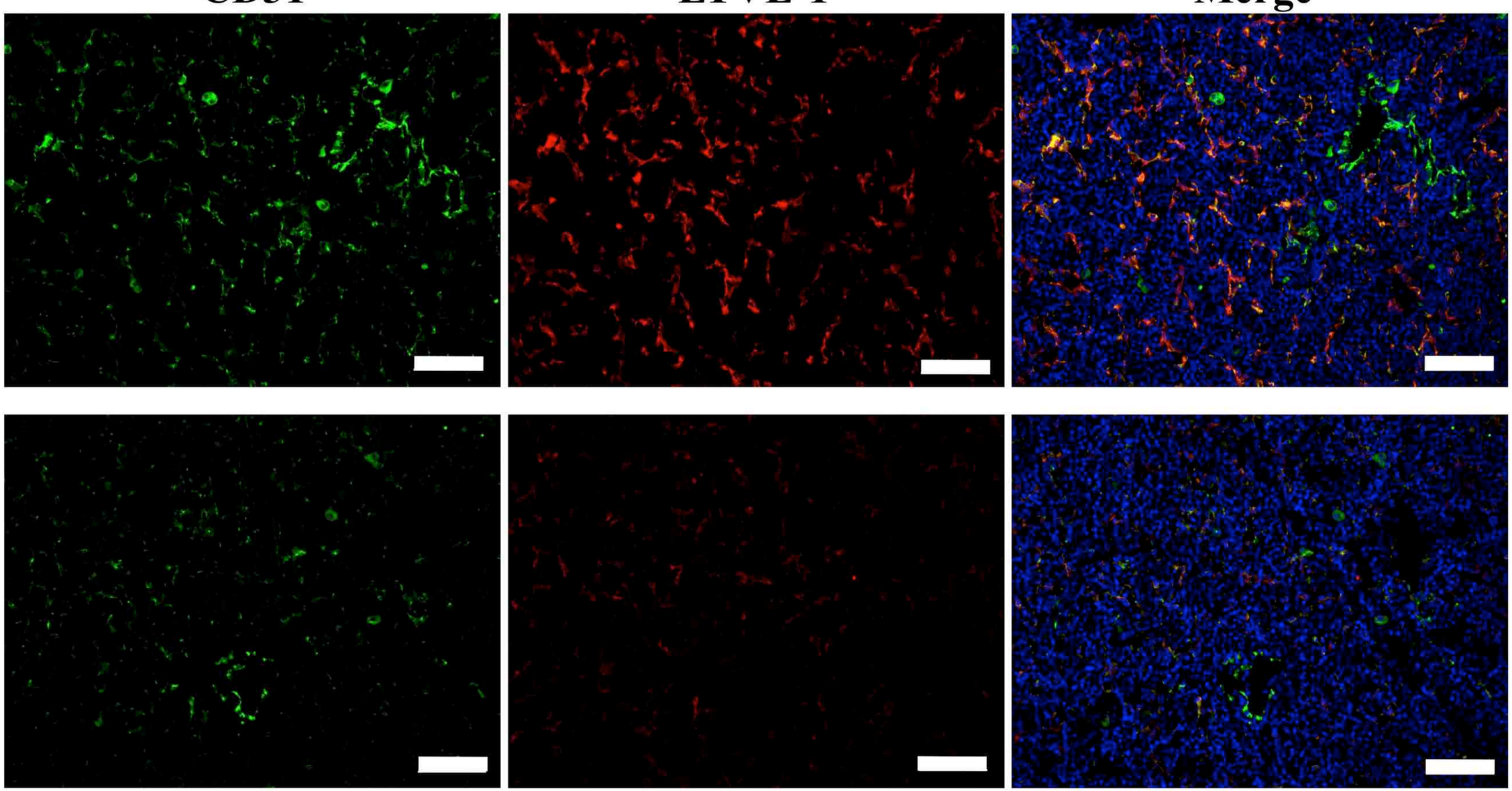

Fig 4.

D.

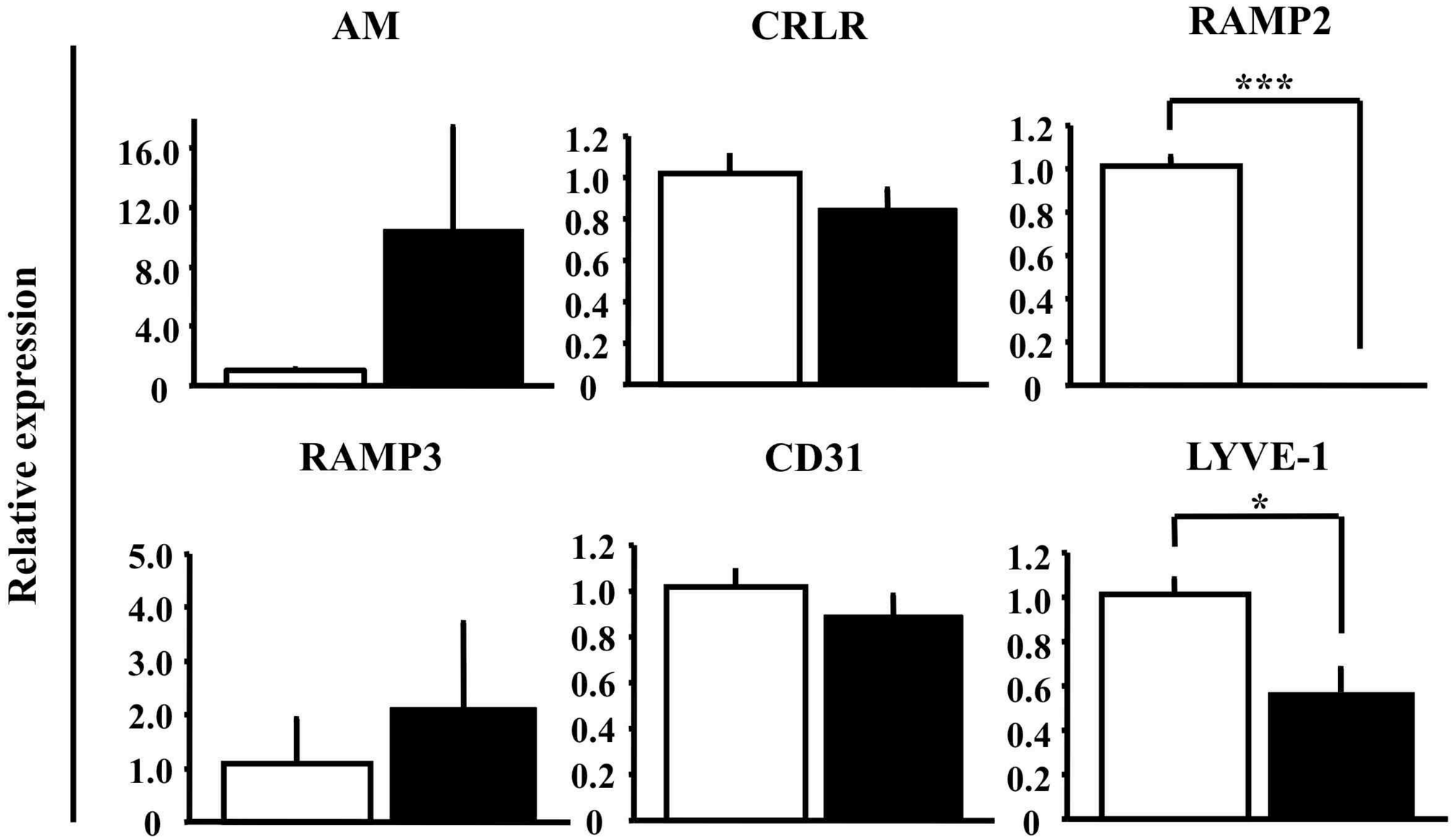
AM
CRLR
RAMP2
***
Relative expression
16.0
12.0
8.0
4.0
0
1.2
1.0
0.8
0.6
0.4
0.2
0
1.2
1.0
0.8
0.6
0.4
0.2
0
RAMP3
CD31
LYVE-1
*
5.0
4.0
3.0
2.0
1.0
0
1.2
1.0
0.8
0.6
0.4
0.2
0
1.2
1.0
0.8
0.6
0.4
0.2
0

Fig 4.

TABLE 1.

For qRT-PCR

LYVE-1	Forward	AAGCAGCTGGGTTTGGAGGT
	Reverse	CACCAAAGAAGAGGAGAGCCA
stabilin-2	Forward	GCTCGAGACAAAACCACTTAGTGA
	Reverse	CCCGATGAAAATGGATCTCTTC
Prox-1	Forward	CGGGTTGAGAATATCATTC
	Reverse	TCTTTCGTTTTCATTGCCCC
podoplanin	Forward	TGGCAAGGCACCTCTGGTA
	Reverse	TGAGGTGGACAGTTCCTCTAAGG
VEGFR3	Forward	AAGGCCTGCCCATGCA
	Reverse	TCGCCAGGGTCCATGATG
F8	Forward	TGCCTGACCCGCTATTATTC
	Reverse	AGCGTTGCATGTTCTCTGTG
Fcgr2b	Forward	CCCTGGGAACTCTTCTACCC
	Reverse	CAGCAGCCAGTCAGAAATCA
Mrc1	Forward	ATGCCAAGTGGGAAAATCTG
	Reverse	TGTAGCAGTGGCCTGCATAG

TABLE 2.

For RT-PCR

VEGF	Forward	CAGGCTGCTGTAACGATGAA
	Reverse	AATGCTTTCTCCGCTCTGAA
AM	Forward	TCGAATTCATCGCCACAGAATGAAGCTGGT
	Reverse	TCGAATTCTATATCCTAAAGAGTCTGGAGA
CRLR	Forward	TAAGTTGCCAACGGATCACA
	Reverse	CCCTTGCATGTCACTGATTG
Ramp2	Forward	CATCCCACTGAGGACAGCCT
	Reverse	GATCATGGCCAGGAGCACAT
Ramp3	Forward	TCGAATTCATCTTAGTTGGCCATGAAGAC
	Reverse	ATACCTGGGCACACTCACCACAA
TGF β 1	Forward	CCCGAAGCGGACTACTATGC
	Reverse	TAGATGGCGTTGTTGCGGT
ALK1	Forward	TGACCTCAAGAGTCGCAATG
	Reverse	CTCGGGTGCCATGTATCTTT
ALK5	Forward	GGCGAAGGCATTACAGTGTT
	Reverse	TGCACATACAAATGGCCTGT
HPRT	Forward	GTTGGATACAGGCCAGACTTTGTTG
	Reverse	GAGGGTAGGCTGGCCTATAGGCT

Supplemental Table 1.
Genes upregulated in EBs treated with AM and SB431542 (>three fold)

Gene	NCBI Ref.Seq.	Description	fold change
Sprr3	NM_011478	small proline-rich protein 3 [Source:MarkerSymbol;Acc:MGI:1330237]	12.20
Myl1	NM_021285	myosin, light polypeptide 1 [Source:MarkerSymbol;Acc:MGI:97269]	7.63
Adra2b	NM_009633	adrenergic receptor, alpha 2b [Source:MarkerSymbol;Acc:MGI:87935]	7.19
Krt4	NM_008475	keratin 4 [Source:MarkerSymbol;Acc:MGI:96701]	5.91
Tesp2	-	testicular serine protease 2 [Source:MarkerSymbol;Acc:MGI:1270857]	5.79
Tnnc2	NM_009394	troponin C2, fast [Source:MarkerSymbol;Acc:MGI:98780]	5.56
Slc4a1	NM_011403	solute carrier family 4 (anion exchanger), member 1 [Source:MarkerSymbol;Acc:MGI:109393]	5.22
Pax1	NM_008780	paired box gene 1 [Source:MarkerSymbol;Acc:MGI:97485]	5.18
Spt2	NM_009268	salivary protein 2 [Source:MarkerSymbol;Acc:MGI:98393]	4.57
IGKV4-57-1	XM_357683	Immunoglobulin Kappa light chain V gene segment [Source:IMGT/GENE-DB;Acc:IGKV4-57-1]	4.39
Ubash3a	NM_177823	ubiquitin associated and SH3 domain containing, A [Source:MarkerSymbol;Acc:MGI:1926074]	4.20
-	NM_010743	-	4.18
Rhox4e	NM_201236	reproductive homeobox 4E [Source:MarkerSymbol;Acc:MGI:3613390]	4.10
Pla2g2a	NM_011108	phospholipase A2, group IIA (platelets, synovial fluid) [Source:MarkerSymbol;Acc:MGI:104642]	4.02
Bank1	XM_143587	B-cell scaffold protein with ankyrin repeats 1 [Source:MarkerSymbol;Acc:MGI:2442120]	3.88
Gp9	NM_018762	glycoprotein 9 (platelet) [Source:MarkerSymbol;Acc:MGI:1860137]	3.85
-	NM_016956	-	3.81
Vsnl1	NM_012038	visinin-like 1 [Source:MarkerSymbol;Acc:MGI:1349453]	3.80
Pip5k1c	NM_008844	phosphatidylinositol-4-phosphate 5-kinase, type 1 gamma [Source:MarkerSymbol;Acc:MGI:1298224]	3.80
Olfr329	-	olfactory receptor 329 [Source:MarkerSymbol;Acc:MGI:3030163]	3.73
Myh3	XM_354614	myosin, heavy polypeptide 3, skeletal muscle, embryonic [Source:MarkerSymbol;Acc:MGI:1339709]	3.66
Nepn	NM_025684	nephrocan [Source:MarkerSymbol;Acc:MGI:1913900]	3.62
Nox1	NM_172203	NADPH oxidase 1 [Source:MarkerSymbol;Acc:MGI:2450016]	3.62
Olfr921	NM_146782	olfactory receptor 921 [Source:MarkerSymbol;Acc:MGI:3030755]	3.61
-	NM_024204	-	3.57
Zfp142	-	zinc finger protein 142 [Source:MarkerSymbol;Acc:MGI:1924514]	3.55
EG225416	XM_140295	-	3.53
Kcnt1	-	potassium channel, subfamily T, member 1 [Source:MarkerSymbol;Acc:MGI:1924627]	3.52
Q8C315_MOUSE	-	16 days neonate heart cDNA, RIKEN full-length enriched library, clone:D830014A20 product	3.45
-	NM_133245	-	3.42
Gpr115	-	G protein-coupled receptor 115 [Source:MarkerSymbol;Acc:MGI:1925499]	3.41
Q9QUL5_MOUSE	NM_010416	HemT-3 protein. [Source:Uniprot/SPTREMBL;Acc:Q9QUL5]	3.41
Atp1b2	NM_013415	ATPase, Na+/K+ transporting, beta 2 polypeptide [Source:MarkerSymbol;Acc:MGI:88109]	3.38
Hbb-y	NM_008221	hemoglobin Y, beta-like embryonic chain [Source:MarkerSymbol;Acc:MGI:96027]	3.37
Olfr963	-	olfactory receptor 963 [Source:MarkerSymbol;Acc:MGI:3030797]	3.35
Olfr958	NM_146330	olfactory receptor 958 [Source:MarkerSymbol;Acc:MGI:3030792]	3.34
Xlkd1(Lyve-1)	NM_053247	extracellular link domain-containing 1 [Source:MarkerSymbol;Acc:MGI:2136348]	3.32
Steap4	NM_054098	STEAP family member 4 [Source:MarkerSymbol;Acc:MGI:1923560]	3.31
Myog	NM_031189	myogenin [Source:MarkerSymbol;Acc:MGI:97276]	3.29
Olfr301	NM_212436	olfactory receptor 301 [Source:MarkerSymbol;Acc:MGI:3030135]	3.29
Zdhhc20	-	zinc finger, DHHC domain containing 20 [Source:MarkerSymbol;Acc:MGI:1923215]	3.28
Olfr303	NM_146619	olfactory receptor 303 [Source:MarkerSymbol;Acc:MGI:3030137]	3.28
Rhox4b	NM_021300	reproductive homeobox 4B [Source:MarkerSymbol;Acc:MGI:1930129]	3.24
-	NM_175462	-	3.23
Hba-a1	NM_008218	hemoglobin alpha, adult chain 1 [Source:MarkerSymbol;Acc:MGI:96015]	3.21
Clec1b	NM_019985	C-type lectin domain family 1, member b [Source:MarkerSymbol;Acc:MGI:1913287]	3.19
-	NM_145435	-	3.15
Slc22a8	NM_031194	solute carrier family 22 (organic anion transporter), member 8 [Source:MarkerSymbol;Acc:MGI:1336187]	3.12
Tnfsg5	-	TNF-stimulated gene 5 [Source:MarkerSymbol;Acc:MGI:3608660]	3.11
4833405L16Rik	NM_177197	RIKEN cDNA 4833405L16 gene [Source:MarkerSymbol;Acc:MGI:2444315]	3.08
Olfr993	NM_146435	olfactory receptor 993 [Source:MarkerSymbol;Acc:MGI:3030827]	3.01
Lrfn2	XM_128642	leucine rich repeat and fibronectin type III domain containing 2 [Source:MarkerSymbol;Acc:MGI:1917780]	3.01
-	NM_146565	olfactory receptor 837 (Olfr837), mRNA [Source:RefSeq_dna;Acc:NM_146565]	3.00

Supplemental Table 2.
Genes downregulated in EBs treated with AM and SB431542 (<three fold)

Gene	NCBI Ref.Seq.	Description	fold change
Lefty2	NM_177099	Left-right determination factor 2 [Source:MarkerSymbol;Acc:MGI:2443573]	0.05
Pga5	NM_021453	pepsinogen 5, group I [Source:MarkerSymbol;Acc:MGI:1915935]	0.08
Mesp1	NM_008588	mesoderm posterior 1 [Source:MarkerSymbol;Acc:MGI:107785]	0.12
NP_444473.1	NM_053243	protease, serine, 1 [Source:RefSeq_peptide;Acc:NP_444473]	0.14
Apoc2	NM_009695	apolipoprotein C-II [Source:MarkerSymbol;Acc:MGI:88054]	0.15
Lhx1	NM_008498	LIM homeobox protein 1 [Source:MarkerSymbol;Acc:MGI:99783]	0.15
Lefty1	NM_010094	left right determination factor 1 [Source:MarkerSymbol;Acc:MGI:107405]	0.18
Megf10	NM_001001979	multiple EGF-like-domains 10 [Source:MarkerSymbol;Acc:MGI:2685177]	0.20
Irs4	-	insulin receptor substrate 4 [Source:MarkerSymbol;Acc:MGI:1338009]	0.21
Apoa2	NM_013474	apolipoprotein A-II [Source:MarkerSymbol;Acc:MGI:88050]	0.21
Mixl1	NM_013729	Mix1 homeobox-like 1 (Xenopus laevis) [Source:MarkerSymbol;Acc:MGI:1351322]	0.22
Cyp26a1	NM_007811	cytochrome P450, family 26, subfamily a, polypeptide 1 [Source:MarkerSymbol;Acc:MGI:1096359]	0.22
-	NM_134249	-	0.22
Angptl7	-	angiopoietin-like 7 [Source:MarkerSymbol;Acc:MGI:3605801]	0.23
Fgg	NM_133862	fibrinogen, gamma polypeptide [Source:MarkerSymbol;Acc:MGI:95526]	0.23
Apoa4	NM_007468	apolipoprotein A-IV [Source:MarkerSymbol;Acc:MGI:88051]	0.24
Trh	NM_009426	thyrotropin releasing hormone [Source:MarkerSymbol;Acc:MGI:98823]	0.26
T	NM_009309	brachyury [Source:MarkerSymbol;Acc:MGI:98472]	0.28
Prss35	NM_178738	protease, serine, 35 [Source:MarkerSymbol;Acc:MGI:2444800]	0.28
-	XM_127824	-	0.28
Bcl11b	NM_021399	B-cell leukemia/lymphoma 11B [Source:MarkerSymbol;Acc:MGI:1929913]	0.29
Ccl17	NM_011332	chemokine (C-C motif) ligand 17 [Source:MarkerSymbol;Acc:MGI:1329039]	0.30
Tmepai	-	transmembrane, prostate androgen induced RNA [Source:MarkerSymbol;Acc:MGI:1929600]	0.30
Ptchd1	XM_142262	patched domain containing 1 [Source:MarkerSymbol;Acc:MGI:2685233]	0.31
Zic5	NM_022987	zinc finger protein of the cerebellum 5 [Source:MarkerSymbol;Acc:MGI:1929518]	0.31
Igfbp1	NM_008341	insulin-like growth factor binding protein 1 [Source:MarkerSymbol;Acc:MGI:96436]	0.32
Afp	NM_007423	alpha fetoprotein [Source:MarkerSymbol;Acc:MGI:87951]	0.32
1810049H19Rik	NM_001003405	RIKEN cDNA 1810049H19 gene [Source:MarkerSymbol;Acc:MGI:3045752]	0.32
Fgf5	NM_010203	fibroblast growth factor 5 [Source:MarkerSymbol;Acc:MGI:95519]	0.32

Supplemental Table 3.
Specific endothelial markers, early mesodermal markers and angiogenic factors in EBs treated with AM and SB431542

Gene	NCBI Ref.Seq.	Description	fold change
Xlkd1(Lyve-1)	NM_053247	extracellular link domain-containing 1 [Source:MarkerSymbol;AccMGI:2136348]	3.3
Stab1	NM_138672	stabilin 1 [Source:MarkerSymbol;AccMGI:2178742]	1.2
Stab2	NM_138673	stabilin 2 [Source:MarkerSymbol;AccMGI:2178743]	1.7
Pecam1	NM_008816	platelet/endothelial cell adhesion molecule 1 [Source:MarkerSymbol;AccMGI:975	1.4
Pdpn	NM_010329	podoplanin [Source:MarkerSymbol;AccMGI:103098]	0.9
Efnb2	NM_010111	ephrin B2 [Source:MarkerSymbol;AccMGI:105097]	0.8
Ephb4	NM_010144	Eph receptor B4 [Source:MarkerSymbol;AccMGI:104757]	0.9
F8	NM_007977	coagulation factor VIII [Source:MarkerSymbol;AccMGI:88383]	1.6
Mrc1	NM_008625	mannose receptor, C type 1 [Source:MarkerSymbol;AccMGI:97142]	1.7
Fcgr2b	NM_010187	Fc receptor, IgG, low affinity IIb [Source:MarkerSymbol;AccMGI:95499]	1.0
Oct3/4	NM_013633	POU domain, class 5, transcription factor 1 [Source:RefSeq_peptide;AccNP_038(	0.6
Nanog	XM_132755	Nanog homeobox [Source:MarkerSymbol;AccMGI:1919200]	0.7
T	NM_009309	brachyury [Source:MarkerSymbol;AccMGI:98472]	0.3
Gsc	NM_010351	goosecoid [Source:MarkerSymbol;AccMGI:95841]	0.5
Mesp1	NM_008588	mesoderm posterior 1 [Source:MarkerSymbol;AccMGI:107785]	0.1
Lefty1	NM_010094	left right determination factor 1 [Source:MarkerSymbol;AccMGI:107405]	0.2
Lefty2	NM_177099	Left-right determination factor 2 [Source:MarkerSymbol;AccMGI:2443573]	0.0
Vegfa	NM_009505	vascular endothelial growth factor A [Source:MarkerSymbol;AccMGI:103178]	0.9
Vegfb	NM_011697	vascular endothelial growth factor B [Source:MarkerSymbol;AccMGI:106199]	1.1
Vegfc	NM_009506	vascular endothelial growth factor C [Source:MarkerSymbol;AccMGI:109124]	0.7
Flt1	NM_010228	FMS-like tyrosine kinase 1 [Source:MarkerSymbol;AccMGI:95558]	0.8
Kdr	NM_010612	kinase insert domain protein receptor [Source:MarkerSymbol;AccMGI:96683]	1.2
Flt4	NM_008029	FMS-like tyrosine kinase 4 [Source:MarkerSymbol;AccMGI:95561]	1.1
Angpt1	NM_009640	angiopoietin 1 [Source:MarkerSymbol;AccMGI:108448]	0.9
Angpt2	-	angiopoietin 2 [Source:MarkerSymbol;AccMGI:1202890]	1.0
Angptl2	NM_011923	angiopoietin-like 2 [Source:MarkerSymbol;AccMGI:1347002]	0.9
Angptl3	NM_013913	angiopoietin-like 3 [Source:MarkerSymbol;AccMGI:1353627]	1.4
Angptl4	NM_020581	angiopoietin-like 4 [Source:MarkerSymbol;AccMGI:1888999]	1.3
Angptl4	-	angiopoietin-like 4 [Source:MarkerSymbol;AccMGI:1888999]	1.0
Angptl6	NM_145154	angiopoietin-like 6 [Source:MarkerSymbol;AccMGI:1917976]	1.1
Angptl7	-	angiopoietin-like 7 [Source:MarkerSymbol;AccMGI:3605801]	0.2
Tie1	NM_011587	tyrosine kinase receptor 1 [Source:MarkerSymbol;AccMGI:99906]	1.6
Tek(Tie2)	NM_013690	endothelial-specific receptor tyrosine kinase [Source:MarkerSymbol;AccMGI:986	1.8