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

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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 possessed 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.

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

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; TGFβ, Transforming growth factor β; 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.

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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 at 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, 1 mM 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 1500 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.

Table 1
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
TGFβ1	Forward	CCCGAAGCGGACTACTATGC
	Reverse	TAGATGGCGTTGTTGCGGT
ALK1	Forward	TGACCTCAAGAGTCGCAATG
	Reverse	CTCGGGTGCCATGTATCTTT
ALK5	Forward	GGCGAAGGCATTACAGTGTT
	Reverse	TGCACATACAAATGGCCTGT
HPRT	Forward	GTTGGATACAGGCCAGACTTTGTTG
	Reverse	GAGGGTAGGCTGGCCTATAGGCT

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.

Table 2
For qRT-PCR.

AM	Forward	CTACCGCCAGAGCATGAACC
	Reverse	GAAATGTGCAGGTCCCGAA
	Probe	CCCGCAGCAATGGATGCCG
CRLR	Forward	AGGCGTTTACCTGCACACACT
	Reverse	CAGGAAGCAGAGGAAACCCC
	Probe	ATCGTGGTGGCTGTGTTTGCGGAG
RAMP2	Forward	GCAGCCCACCTTCTCTGATC
	Reverse	AACGGGATGAGGCAGATGG
	Probe	CCCAGAGGATGTGCTCCTGGCCAT
RAMP3	Forward	TGCAACGAGACAGGGATGC
	Reverse	CATCATGTCAGCGAAGGC
	Probe	AGAGGCTGCCTCGCTGTGGGAA
CD31	Forward	CTGCAGGCATCGGCAAA
	Reverse	GCATTTCGCACACCTGGAT
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