

# **Adrenomedullin in sinusoidal endothelial cells play protective roles against cold injury of liver**

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**Running Title: Adrenomedullin protects LSECs from cold injury**

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**Abstract**

Donor organ damage caused by cold preservation is a major problem affecting liver transplantation. Cold preservation most easily damages liver sinusoidal endothelial cells (LSECs), and information about the molecules modulating LSECs function can provide the basis for new therapeutic strategies. Adrenomedullin (AM) is a peptide known to possess anti-apoptotic and anti-inflammatory properties. AM is abundant in vascular endothelial cells, but levels are comparatively low in liver, and little is known about its function there. In this study, we demonstrated both AM and its receptors are expressed in LSECs. AM treatment reduced LSECs loss and apoptosis under cold treatment. AM also downregulated cold-induced expression of TNF $\alpha$ , IL1 $\beta$ , IL6, ICAM1 and VCAM1. AM reduced apoptosis and expression of ICAM1 and VCAM1 in an *in vivo* liver model subjected to cold storage. Conversely, apoptosis was exacerbated in livers from *AM* and *RAMP2* (AM receptor activity-modifying protein) knockout mice. These results suggest that AM expressed in LSECs exerts a protective effect against cold-organ damage through modulation of apoptosis and inflammation.

**Key words**

adrenomedullin (AM)

receptor activity-modifying protein (RAMP)

liver sinusoidal endothelial cell (LSEC)

vasoactive peptide

endothelial cell

liver

cold injury

## **1. Introduction**

Donor organ preservation remains one of the most important issues in liver transplantation. Cold injury, which contributes to both early and late liver graft dysfunction [20, 22, 30], is the main challenge that must be overcome. The process involves a set of interconnected events that include Kupffer cell activation, oxidative stress, cholestasis, sinusoidal microthrombosis, hepatocellular ballooning, neutrophil infiltration, and cell death of both liver sinusoidal endothelial cells (LSECs) and hepatocytes [1, 6, 16, 24]. Among these, cold preservation of liver grafts most easily damages LSECs [3, 14]. However, little is known about the molecules that modulate LSECs function. Precise information about these molecules could enable development of a new therapeutic strategies aimed at protecting the liver from injury.

Adrenomedullin (AM) is a multifunctional polypeptide initially isolated from human pheochromocytoma, which exerts a variety of effects, including stimulation of cell proliferation, differentiation and migration [9, 11, 13, 15]. During embryonic development, AM is indispensable for vascular morphogenesis [23]. Postnatally, AM is produced by a variety of tissues and cell types but is primarily secreted by vascular endothelial cells and functions as a local autocrine or paracrine mediator [12, 15, 17]. AM also reportedly exerts a protective effect against organ damage in several disease models [8, 18], and we speculated that AM and its receptors could be useful therapeutic targets for protection against cold-induced liver damage.

In the present study, we investigated AM and its receptor system in LSECs, focusing in particular on the possibility of their application to cold preservation of the liver.

## **2. Methods**

### **2.1 Animals**

C57BL/6J mice were obtained from Charles River Laboratories Japan, Inc. GFP mice were kindly provided by Prof. Sato of Kagoshima University. *AM* and *RAMP2* knockout mice [7, 23] were originally generated in our group. Both homozygous knockout mice of *AM* and *RAMP2* are embryonic lethal. Therefore, we used heterozygous knockout mice (*AM*<sup>+/-</sup> and *RAMP2*<sup>+/-</sup>) in this study. In both heterozygous knockout mice, we have shown that the expression of each gene is reduced by half of wild-type mice. All animal experiments were conducted in accordance with the ethical guidelines of Shinshu University.

### **2.2 Cell isolation and culture**

Primary adult mice liver sinusoidal endothelial cells (LSECs) and hepatocytes were isolated using a two step collagenase perfusion and centrifugation method [4, 26, 28]. LSECs were isolated from the supernatant from the first centrifugation. Hepatocytes were isolated from the precipitate from the second centrifugation through a 50% Percoll (MP Biomedicals, OH) gradient. The isolated LSECs were cultured in endothelial cell basal medium-2 (EBM-2) (Cambrex, MD), while the hepatocytes were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Equitech-Bio, TX).

### **2.3 RT-PCR and quantitative real-time RT-PCR**

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was carried out using Ex Taq DNA polymerase (Takara, Japan). Quantitative real-time PCR was carried out using an Applied Biosystems 7300 real time PCR System (Applied Biosystems, CA) with SYBR green (Toyobo, Japan). Values were normalized to mouse GAPD (glyceraldehyde-3-phosphate dehydrogenase) (Applied Biosystems, CA). PCR primers and the size of each PCR product are listed in Table 1.

### **2.4 Cell viability assay**

After seeding LSECs into 96-well plates, their viability was assessed using a WST-8 kit (Dojindo, Japan) according to the manufacturer's instructions.

### **2.5 Flow cytometric analysis of endothelial microparticles (EMP)**

LSECs were seeded into 24-well plates and incubated over night, after which they were washed, and 500 µl of medium with or without 0.1 µM *AM* was added to each well. The cells were then subjected to a cold storage protocol that entailed incubation at 4°C for 4 h and then at 37°C for 1 h. After the cold storage, 500 µl of supernatant was transferred from each well to a tube, and 1 µl of Annexin V-conjugated phycoerythrin (BD Pharmingen, CA) was added to label endothelial microparticles (EMPs), which were then analyzed by flow cytometry.

### **2.6 Isolation of Buffy coat**

Buffy coat (combined leukocyte and platelet fractions) was isolated from blood samples collected from GFP mice. Three ml of blood were collected from each mouse, after which heparin was added to prevent coagulation, and the samples were centrifuged at 2000 rpm for 5 min. The Buffy coat was collected as the pellet, washed with 0.9%

NH<sub>4</sub>Cl and centrifuged again at 2000 rpm for 5 min. The resultant pellet was diluted with 30 ml of medium and used as Buffy coat for experimentation.

### **2.7 Flow cytometric analysis of leukocyte adhesion to LSECs**

Cell adhesion was assayed as described previously [29]. LSECs were seeded into 35-mm dishes, after which Buffy coat from GFP mice was added to the medium, and the cells were incubated for 12 h with or without 0.1  $\mu$ M AM. The cells were then washed three times with PBS and treated with 0.25% trypsin. LSECs and the Buffy coat cell fraction were collected, centrifuged at 2000 rpm for 5 min, diluted with 500  $\mu$ l PBS and analyzed by flow cytometry.

### **2.8 Platelet/leukocyte-LSEC adhesion assay**

LSECs were seeded into a 96-well plate and incubated for 9 h with or without 0.1  $\mu$ M AM. The cells were then washed with PBS, the medium was exchanged, and Buffy coat extracted from GFP mice was added. After incubation for an additional 2 h, the cells were washed three times with PBS and photographed. Total and GFP-positive cells were measured.

### **2.9 Cold storage of the liver**

Cold storage of the liver was carried out as described previously [21, 27]. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). A midline abdominal incision was then made, the portal vein was cannulated using 26 G needle, and the inferior vena cava was incised. The liver was then perfused with cold (4°C) 50 ml University of Wisconsin solution for 15 min, with or without AM (0.1  $\mu$ M) using Perista pump (ATTO). After sufficient perfusion, the liver was removed and stored at 4°C for 30 h.

### **2.10 Histological examination**

Livers were fixed in 10% formalin neutral buffer solution, embedded in paraffin, and cut into 5- $\mu$ m-thick sections. Apoptosis was detected by TUNEL using an Apoptosis In Situ Detection Kit (Chemicon, CA) and a tyramide-rodamine system (TSA<sup>TM</sup>-Plus Tetramethylrhodamine System, PerkinElmer, MA). Cellular nuclei were counterstained using DAPI (4',6-diamidino-2-phenylindole). For the immunohistochemical staining, labeling with anti-ICAM-1 and anti-VCAM-1 antibodies (Santa Cruz Biotechnology) was visualized using anti-goat HRP (Invitrogen, CA) as the secondary antibody.

### 3. Results

#### 3.1 LSECs express AM and its receptor system

We initially analyzed the expression of AM, and its heterodimeric receptors, which are comprised of calcitonin receptor-like receptor (CRLR) and receptor activator-modifying protein (RAMP) -2 or -3. To determine which cells within liver express AM and its receptors, we separated the LSECs and hepatocytes. We found that AM, CRLR and RAMP2 and -3 were readily detectable in LSECs, whereas we detected little expression in hepatocytes (Fig. 1A).

To confirm the quality of the cell purification, we isolated cells from GFP transgenic mouse line. This makes easy to identify the morphological feature of fenestrations, which are specifically detected in LSECs (Fig. 1B). We estimate that more than 90 % of cells used in this study were LSECs.

In LSECs, cold stress increased the expression of AM. In contrast, CRLR, RAMP2 and RAMP3 were downregulated (Fig. 1C).

#### 3.2 AM reduces apoptotic damage to LSECs caused by cold storage

We next assessed AM's ability to protect LSECs against the effects of cold storage. We found that AM-treated LSECs showed significantly greater rates of survival than untreated LSECs (Fig. 1D). Flow cytometric analysis showed that AM also suppressed cold-induced release of annexin V-positive endothelial microparticles (EMPs) from apoptotic LSECs (Fig. 1E).

#### 3.3 AM attenuates cell adhesion and inflammatory reactions in LSECs

Cold injury stimulates innate immunity and cell adhesion reactions [2]. To evaluate the adhesion of leukocytes, LSECs were incubated in the cold, with or without 0.1  $\mu$ M AM, in the presence of Buffy coat obtained from GFP mice. Using the GFP signal as a marker, flow cytometric and microscopic analyses showed that AM-treatment significantly reduced the adhesion of leukocytes and platelets to LSECs (Fig. 2A, B).

Moreover, quantitative real time PCR analysis showed AM significantly suppressed the expression of inflammatory cytokines and adhesion molecules, including TNF $\alpha$ , IL1 $\beta$ , IL6, ICAM1, and VCAM1 in LSECs after cold treatment (Fig. 3). These data suggest that AM-treatment attenuates cell adhesion and inflammatory reactions in LSECs under cold injury.

#### 3.4 AM shows protective effects on cold stored liver.

We assessed the ability of AM to protect against the adverse effects of cold storage in an *in vivo* model. Livers of wild-type mice were perfused with cold (4°C) University of Wisconsin solution with or without 0.1  $\mu$ M AM and then stored at 4°C. Immunohistochemical analysis of ICAM-1 and VCAM-1 showed that the cold injury upregulates the expression of inflammatory adhesion molecules around the vascular regions, while AM perfusion reduced it (Fig. 4A).

We next evaluated the effect of AM on the incidence of apoptosis in livers. After the cold storage, TUNEL-positive, apoptotic cells were detected. Notably, AM-treatment reduced the cellular apoptosis in livers, whereas apoptosis was actually enhanced in livers from both AM $\pm$  mice and RAMP2  $\pm$  mice (Fig. 4B).

#### 4. Discussion

Adrenomedullin (AM) is a multifunctional peptide that has attracted much attention as a result of its potential for clinical application. AM receptors are heterodimeric G protein-coupled receptors comprised of CRLR and one of two RAMP accessory proteins. So far, three RAMP subtypes (RAMP1, 2 and 3) have been identified, and the complex of CRLR with RAMP2 or -3 functions as an AM receptor. AM and its receptors are detected in the vasculature, where they are thought to regulate vascular homeostasis. By contrast, their expression is relatively low in liver, and the function of AM in the liver remains largely unexplored.

We initially showed that AM and its receptor system are abundantly expressed in primary cultured LSECs, whereas their expression was rarely detected in hepatocytes (Fig 1A). This suggests that AM and its receptor system function within the liver through regulation of LSECs.

Cold injury is a major problem affecting liver transplantation by contributing significantly to the dysfunction of liver grafts. Cold injury reportedly begins with damage to the LSECs [3, 14], which suggests that protection of LSECs from the injury could preserve liver function and increase the likelihood of a successful outcome after transplantation. To test the applicability of AM, we assessed its effect on damage done by cold-treatment to LSECs. Recently, EMPs were shown to be a sensitive early marker of endothelial cell damage [10]. EMPs are small (0.1-1.5  $\mu\text{m}$ ) vesicles comprised of plasma membrane and cytosolic proteins from endothelial cells. We found that AM treatment diminished the release of EMPs from LSECs subjected to cold storage (Fig 1E), suggesting AM protects LSECs from cold injury. Consistent with the idea, perfusion with AM also protected livers from cold-induced damage in *in vivo* model. Conversely, cold-induced organ damage was exacerbated in *AM* and *RAMP2* knockout mice (Fig 4B).

The process of cold injury involves a series of interconnected events leading to oxidative stress, sinusoidal thrombosis and activation of innate immune responses, which collectively act to stimulate apoptosis. Notably, a number of studies have shown that AM has both anticoagulation and anti-inflammatory effects [5, 13, 19, 25, 31, 32]. Using Buffy coat derived from GFP mice, we clearly showed in the present study that AM suppresses the adhesion of leukocytes and platelets to LSECs (Fig 2A, B). We also found that AM suppresses the expression of ICAM-1 and VCAM-1 in LSECs (Fig 3, 4A). ICAM-1 and VCAM-1 are adhesion molecules expressed on endothelial cells and mediate the adhesion of inflammatory cells. AM also suppressed the expression of inflammatory cytokines, including  $\text{TNF}\alpha$ ,  $\text{IL1}\beta$  and  $\text{IL6}$  (Fig 3). This suppression of inflammatory adhesion molecules and cytokines in LSECs may also contribute to the protective effect of AM against cold injury. The half life of AM is short and is reported as 30 min in *in vivo*, therefore it is possible that suppression of initial inflammations is the most important protective effects of AM in the cold injury.

In conclusion, our results suggest that AM and its receptor system are expressed in liver by LSECs and exert protective effects through the modulation of apoptosis, immune responses, and cellular adhesion. Based on these findings, we suggest that AM protects LSECs and has the potential to improve the preservation of donor organs for liver transplantation.



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## Figure legends

### Fig. 1A

RT-PCR analysis of AM, CRLR, RAMP2 and RAMP3 expression in liver sinusoidal endothelial cells (LSEC) and hepatocytes (HC). Albumin and stabilin2 were used as markers of HC and LSEC, respectively. Expression of HPRT served as an internal control.

### Fig. 1B

Fluorescence photomicrograph of primary cultured LSECs from GFP transgenic mouse liver. More than 90 % of cells used in this study were considered to be LSECs, which possess typical fenestrations. Scale bar = 200  $\mu$ m.

### Fig. 1C

Quantitative real-time PCR analysis of the effect of cold stress on the expression of AM and its receptor proteins in LSECs. Control cells were cultured for 13 h at 37°C. Cold group cells were incubated for 12 h at 4°C and then for 1 h at 37°C. The cold stress increased the expression of AM. In contrast, CRLR, RAMP2 and RAMP3 were downregulated. Bars are means  $\pm$  SE, n=4, \*\*P<0.01, \*\*\*P<0.001 vs. control.

### Fig. 1D

Protective effect of AM against cold-storage in LSECs. LSEC viability was assessed after incubation for 12 h at 4°C and for 3 h at 37°C. AM-treated LSECs showed significantly greater survival than untreated cells. Bars are means  $\pm$  SE, n=6, \*\*p<0.01.

### Fig. 1E

Effect of AM on endothelial microparticles (EMP) release from cold-treated LSECs. Supernatants from cultured LSECs were incubated with annexin V-labeled phycoerythrin (PE) and analyzed by flow cytometry. The dot plots show the result of a representative experiment. The bar graph shows the percent of annexin V-positive particles among the total particles in each group. The annexin V-positive particle is indicative of EMP-release. Bars are means  $\pm$  SE, Cold store; n=6, Cold store + AM; n=7; \*p< 0.05. 100% control is the total particles in each dot plot.

### Fig. 2A

Effect of AM on leukocyte adhesion to LSECs. LSECs were incubated for 12 h with or without 0.1  $\mu$ M AM and Buffy coat cells (combined leukocyte/platelet fraction) and then analyzed by flow cytometry.

(a) Dot plot of Buffy coat from GFP mice. (b) Dot plot of LSECs from wild-type mice. (c) Dot plot from cell culture of LSECs and GFP Buffy coat combination. (d) Dot plot from cell culture of AM-treated LSECs and GFP Buffy coat.

(e) Histogram, which shows the distribution of the dots shown in (a)-(d). M1 was designated to count cells, which show higher FITC-fluorescence (FL1-Hight >10<sup>2</sup>). These cells are regarded to be leukocytes derived from GFP mice. AM-treatment reduced the adhesion of leukocytes to LSECs, which is shown in the histogram shift (from blue to red line).

(f) Percentage of M1 fraction cells within the combination of Buffy coat and LSECs. 100% control is the total cells in each dot plot. AM-treatment reduced %M1, which means that adhesion of leukocytes to LSECs was reduced by the treatment (n=3). SSC (Side scatter). FL1 (fluorescence 1) =  $530 \pm 15$  nm.

**Fig. 2B**

Microscopic analysis of the effect of AM on adhesion of Buffy coat cells to LSECs. Cells incubated for 9 h with or without 0.1  $\mu$ M AM and then an additional 2 h in the presence of Buffy coat extracted from GFP mice. Upper panels: GFP-positive cells were considered to be LSECs with adherent leukocytes/platelets. Lower panel: ratios of GFP-positive to GFP-negative cells. AM-treatment significantly reduced the cellular adhesion. n=10, \*\*\*p<0.001 vs. control.

**Fig. 3**

Quantitative real-time PCR analysis of the effect of AM on cold-induced expression of inflammatory cytokines and adhesion molecules in LSECs. The cells were incubated for 12 h at 4°C with or without 0.1  $\mu$ M AM and then for 6 h at 37°C. Bars are means  $\pm$  SE, n=4, \*P<0.05, \*\*P<0.01 vs. control.

**Fig. 4A.**

Immunohistochemical analysis of the effect of AM on cold-induced ICAM1 and VCAM1 expression in mouse liver subjected to cold storage. AM perfusion reduced expression of both inflammatory adhesion molecules. Scale bars: low magnification, 200  $\mu$ m; high magnification, 50  $\mu$ m.

**Fig. 4B.**

TUNEL analysis of the effect of AM on the incidence of apoptosis in mouse liver subjected to cold storage. Apoptosis was suppressed by AM treatment and enhanced in AM or RAMP2 knockout mice. Scale bars = 200  $\mu$ m.

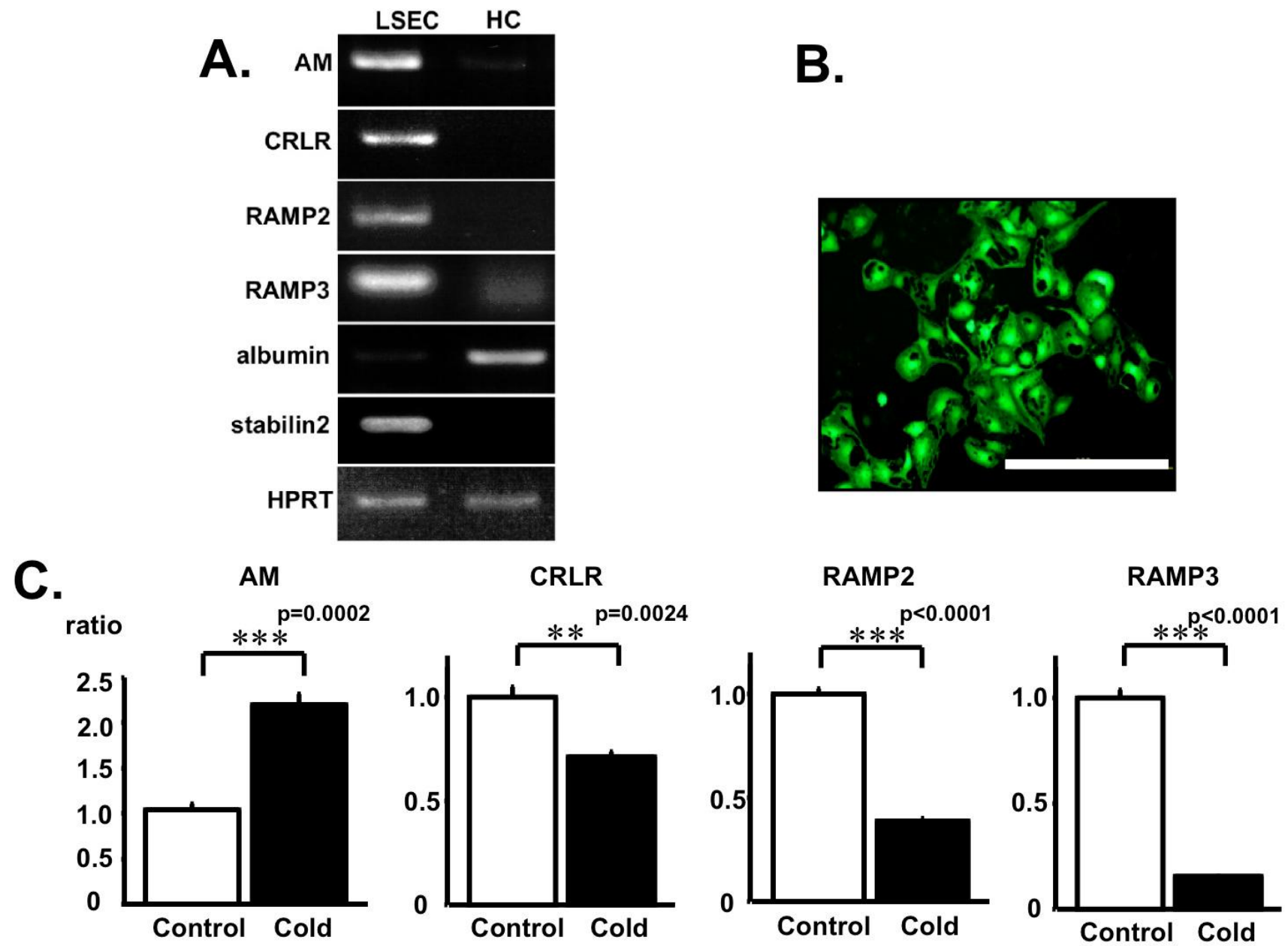
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**Fig.1**

## D. Cell survival



## E.

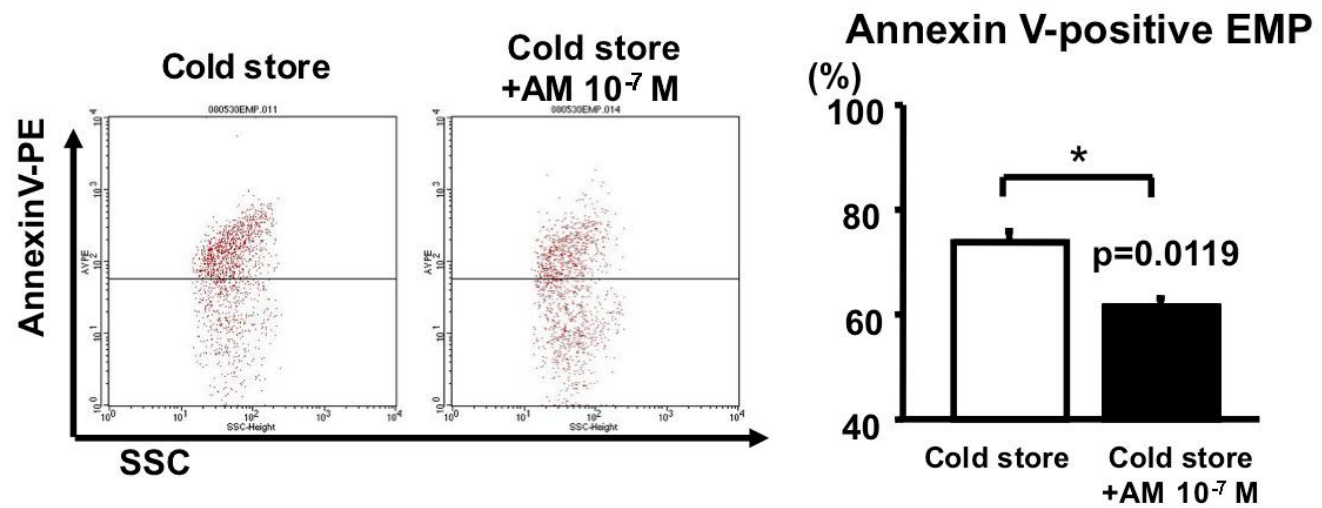
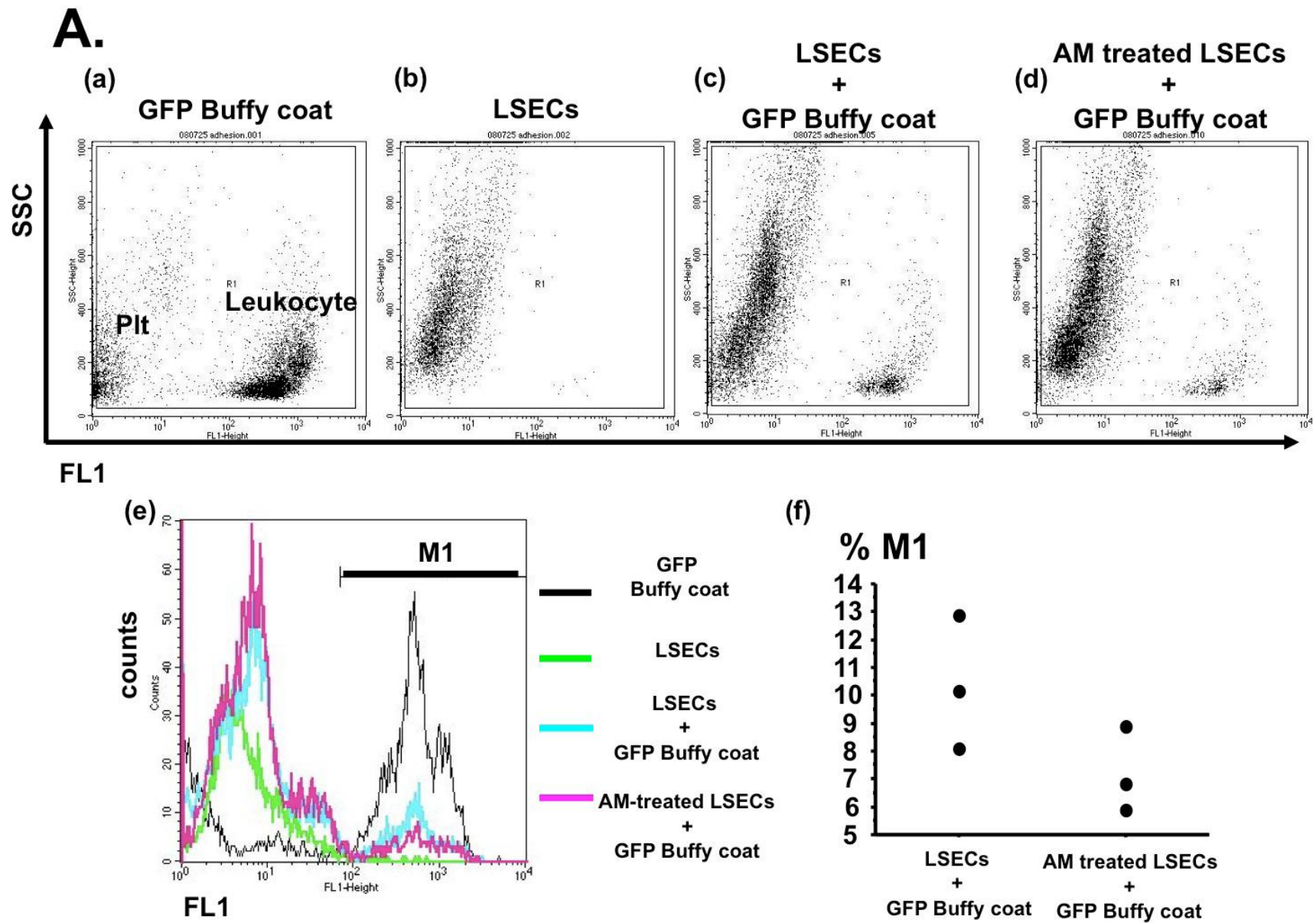


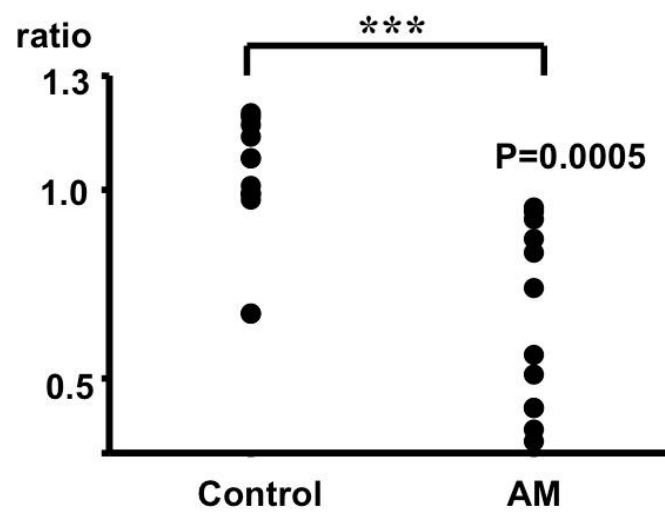
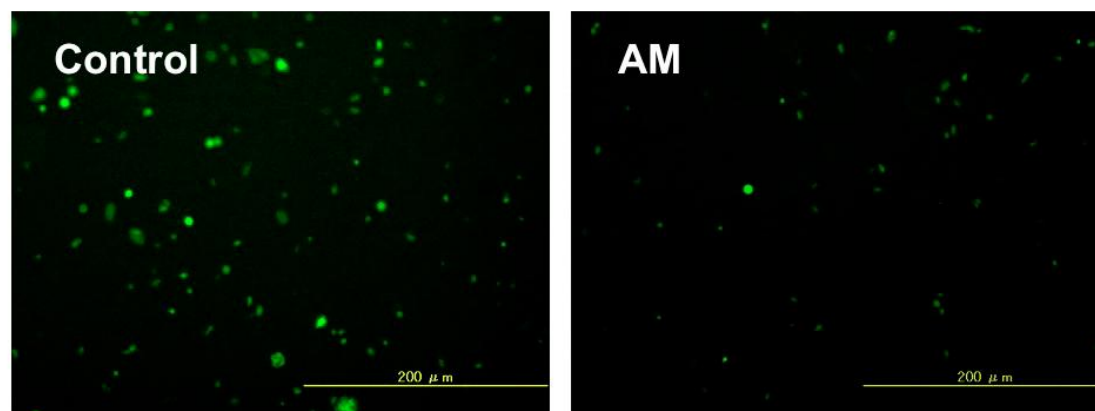
Fig.1



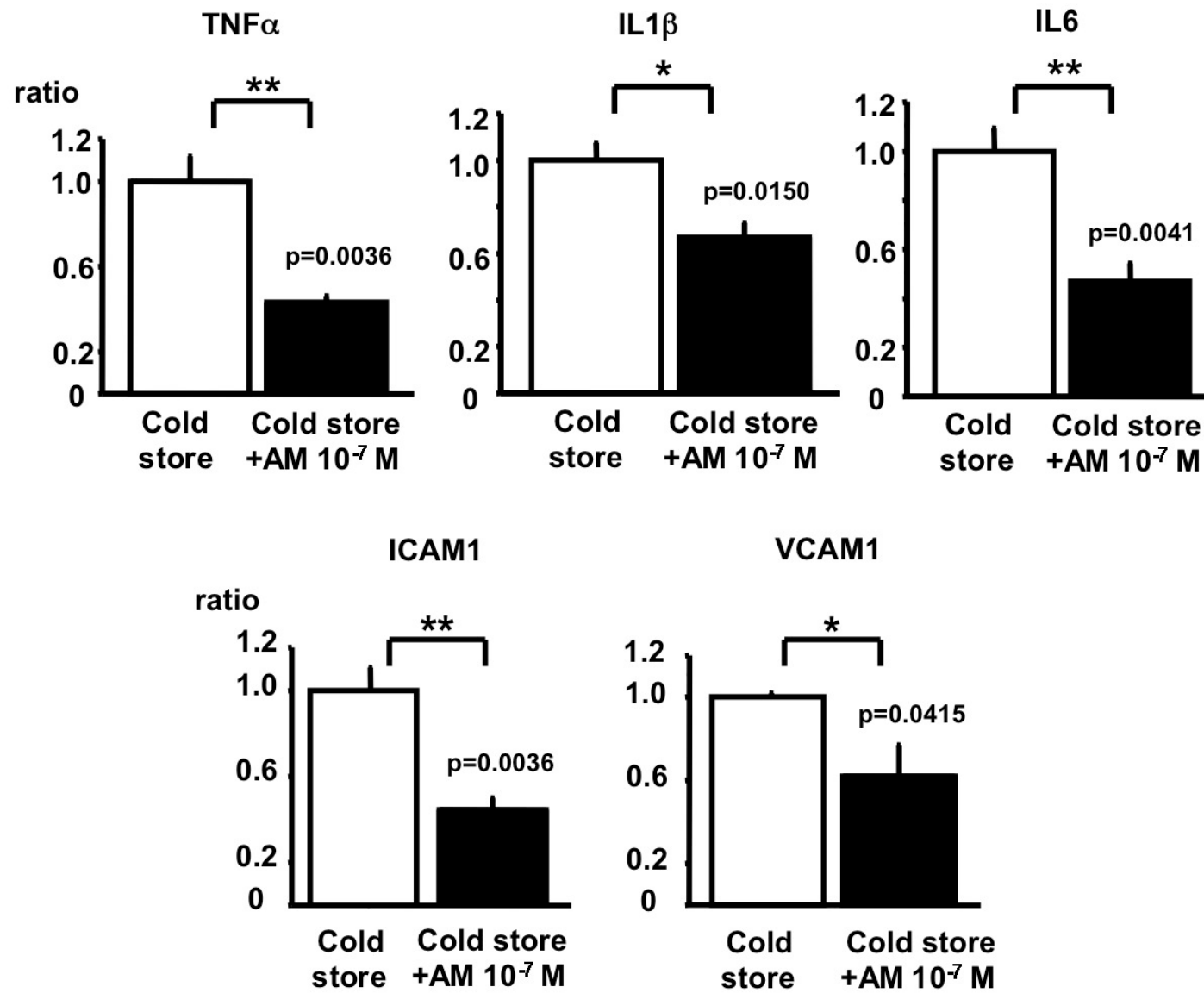


**Fig.2**

**B.**

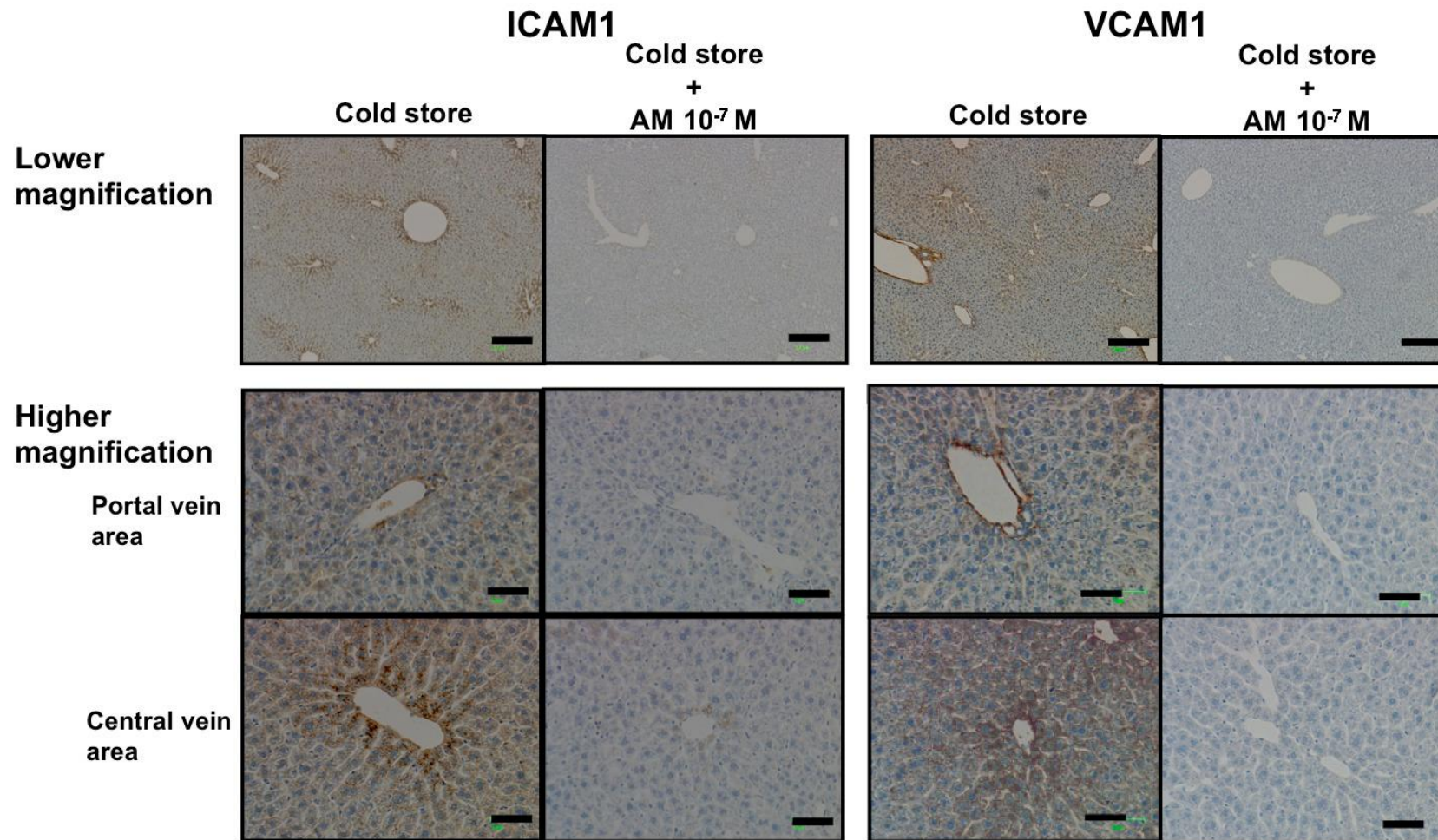


**Fig.2**



**Fig.3**

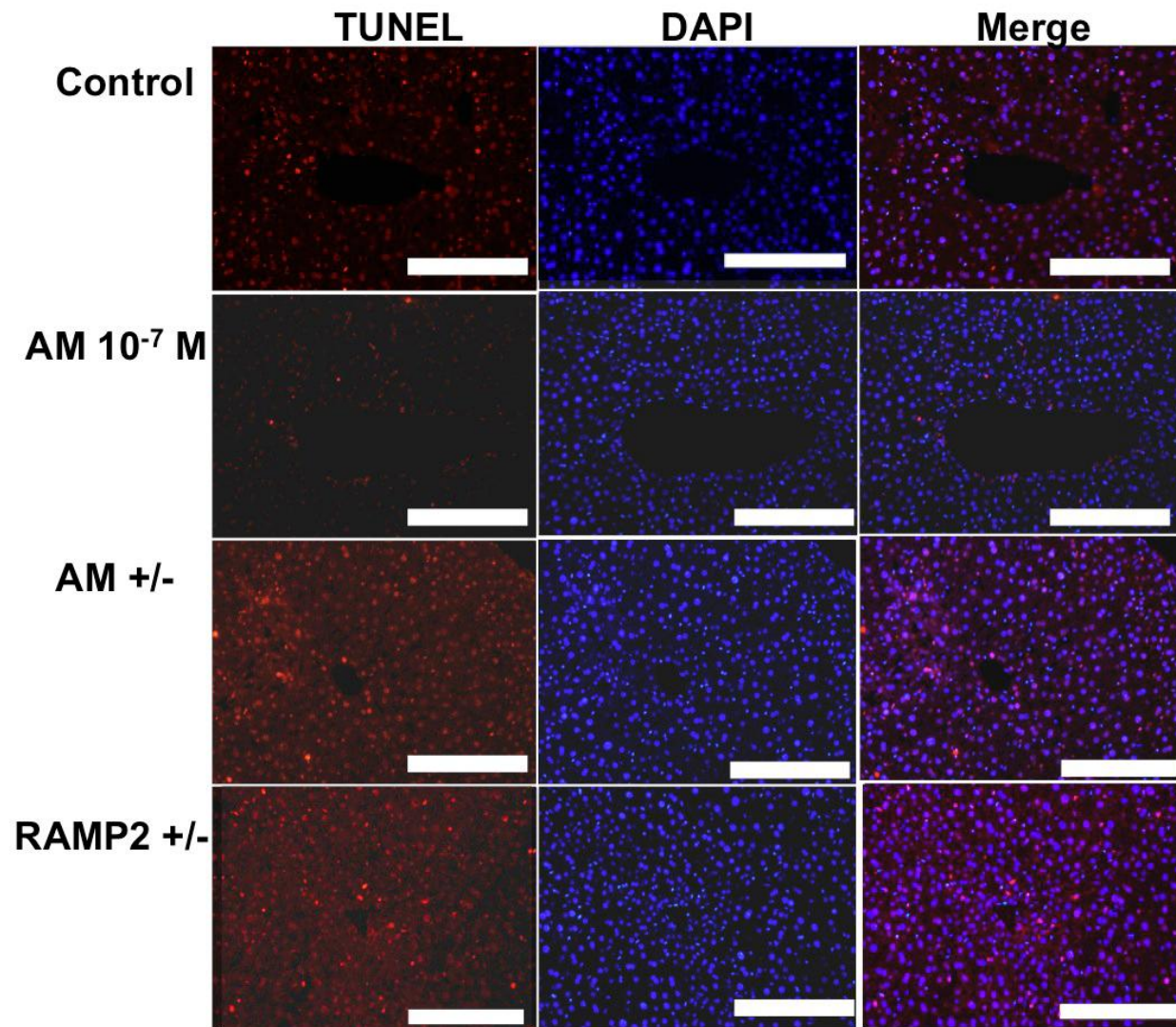
**A.**



**Fig.4**



**B.**



**Fig.4**

| RT-PCR    |              |                                |
|-----------|--------------|--------------------------------|
| AM        | Forward      | TCGAATTCATCGCCACAGAATGAAGCTGGT |
|           | Reverse      | TCGAATTCATATCCTAAAGAGTCTGGAGA  |
|           | Product size | 581 bp                         |
| CRLR      | Forward      | ACAGCACGCATGAGAAAGTG           |
|           | Reverse      | CCCTTGCATGTCCTGATTG            |
|           | Product size | 908 bp                         |
| RAMP2     | Forward      | CATCCCACTGAGGACAGCCT           |
|           | Reverse      | GATCATGGCCAGGAGCACAT           |
|           | Product size | 312 bp                         |
| RAMP3     | Forward      | TCGAATTCATCTTAGTTGGCCATGAAGAC  |
|           | Reverse      | ATACCTGGGCACACTCACCACAA        |
|           | Product size | 94 bp                          |
| stabilin2 | Forward      | GAGTGTCAGGAGGAGCAAG            |
|           | Reverse      | GTCTTTGCTGGAAGGTGAGC           |
|           | Product size | 318 bp                         |
| albumin   | Forward      | GCTACGGCAGTGCTTG               |
|           | Reverse      | CAGGATTGCAGACAGATAGCT          |
|           | Product size | 265 bp                         |
| HPRT      | Forward      | GTTGGATACAGGCCAGACTTTGTTG      |
|           | Reverse      | GAGGGTAGGCTGGCCTATAGGCT        |
|           | Product size | 352 bp                         |

| realtimePCR  |         |                       |
|--------------|---------|-----------------------|
| TNF $\alpha$ | Forward | ACGGCATGGATCTCAAAGAC  |
|              | Reverse | AGATAGCAAATCGGCTGACG  |
| IL6          | Forward | CCCAATTTCCAATGCTCTCC  |
|              | Reverse | TGAATTGGATGGTCTTGGTCC |
| IL1 $\beta$  | Forward | TCTCACAGCAGCACATCAAC  |
|              | Reverse | TCGTTGCTTGGTTCTCCTTG  |
| ICAM1        | Forward | CCTAAAATGACCTGCAGACGG |
|              | Reverse | TTTGACAGACTTCACCACCCC |
| VCAM1        | Forward | CCCTGAATACAAAACGATCGC |
|              | Reverse | CAGCCCGTAGTGCTGCAAG   |

**Table.1**