Proper autophagy is indispensable for angiogenesis during chick embryo development

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Abstract

People have known that autophagy plays a very important role in many physiological and pathological events. But autophagy role on embryonic angiogenesis still remains obscure. In this study, we demonstrated that Atg7, Atg8 and Beclin1 were

expressed in the plexus vessels of angiogenesis at chick yolk sac membrane and chorioallantoic membrane. Interfering with autophagy with autophagy inducer or inhibitor could restrict the angiogenesis *in vivo*, which might be driven by the disorder of angiogenesis-related gene expressions, and also lead to embryonic hemorrhage, which was due to imperfection of endothelial cell-cell junctions including abnormal expressions of tight junction, adheren junction and desmosome genes. Using HUVECs, we revealed that cell viability and migration ability changed with the alteration of cell autophagy exposed to RAPA or 3-MA. Interestingly, tube formation assay showed that HUVECs ability of tube formation altered with the change of Atg5, Atg7 and Atg8 manipulated by the transfection of their corresponding siRNA or plasmids. Moreover, the F-actin labeled cell polarity lost and β-catenin absence in RAPA-treated cell membrane implied intracellular cytoskeleton alteration was induced by autophagy level. Taken together, our current experimental data reveal that autophagy is really involved in regulating angiogenesis during embryo development.

Key words: autophagy, angiogenesis, yolk sac membrane, chorioallantoic membrane, Atg.

Abbreviation: 3-MA, 3-Methyladenine; BECN1, beclin-1; CAM, chorioallantoic membrane DAPI, 49-6-Diamidino-2-phenylindole; DMSO dimethyl sulfoxide; HUVECs, human umbilical vein endothelial cells; GFP, green fluorescent protein; HN, Hensen's node; MAPILC3(LC3), microtubule-associated protein 1 light chain 3; mTOR, mammalian target of rapamycin; VE-Cad, VE-cadherin; RAPA, Rapamycin;

RT-PCR, reverse transcription PCR; YSM, yolk sac membrane; ZA, Zonula adherens.

Introduction

The functional vascularization undergoes three major processes including vasculogenesis, angiogenesis and arteriogenesis, i.e., new blood vessels are formed and remodeled. [1] The vasculogenesis is depicted as the two successive events of vascularization process. Firstly, angioblasts (vascular precursors) from mesoderm cells migrate to form blood islands, and then primary capillary plexuses form through connecting the blood islands. [2] The fundamental function for blood vessels is to deliver O_2 to all the cells round the body in order to maintain oxygen homeostasis in organism. Hypoxia occurs if O_2 supply and demand are out of balance and the hypoxia could be a physiological stimulus inducing the generation of angiogenic cytokines such as VEGF, which binds to its cognate receptors, VEGFRs on endothelial cells, to stimulate the cells to facilitate the angiogenesis. [1, 3] Although VEGF plays a crucial role for vascularization, it is not deemed to be efficient, so that other growth factors/cytokines including angiopoietins (Ang), SDF-1, PDGFb and FGF2 etc are also involved in sprouting angiogenesis. [4, 5] However, it is noteworthy that the angiogenesis in adult appears to be quite different from the vasculogenesis, the initial embryonic vascular plexus during embryo development. One of important distinctions is that the formation of the initial vascular plexus during embryo development is not completely driven by oxygen gradients, i.e., many other angiogenic growth factors/cytokines are implicated in the process. [1]

Autophagy is deemed to be a dynamic process of subcellular degradation, in which cell survival is under nutrient-deprived conditions. [6] In physiological conditions, autophagy is the process by which energy is supplied for embryonic development through the lysosomal degradation of cellular contents. [7] When autophagy is activated, some cellular components are sequestered in autophagosomes, and eventually degraded and fused with lysosomes for maintaining cell metabolism. [8] Thus, autophagy could act as a housekeeper by preventing dysfunctional protein accumulation in cells through removing dead or damaged organelles. [7] So far, people have found that autophagy-related genes (Atg) including Atg5, Atg7, and Atg8 (LC3) participate in the various stages of the autophagy process. [9] The importance of autophagy in embryo development has been confirmed by the experiment that the mice with either Atg5 or Atg7 mutation could survive in the embryonic period but die soon after birth. [10, 11] Therefore, the study on autophagy role in vertebrate development has been paid more and more attention at moment. Rapamycin (RAPA) has been employed as a pharmacological inducer of autophagy since it could provoke autophagy through mTOR-mediated pathway. [12] One of the other ways to promote autophagy is by inducing ER stress such as Tunicamycin, which is a glycosylation inhibitor. Another frequently-used pharmacological compound in autophagy study is 3-MA, [13, 14] which blocks the formation of LC3-II and p62 degradation, so that it could inhibit ATG5-induced autophagy.

The correlation between angiogenesis and autophagy has been gradually uncovered at moment. Ramakrishnan et al. reported that blocking angiogenesis with

kringle 5 (K5), a potent angiogenesis inhibitor, could induce autophagy in endothelial cells by knocking down Beclin-1 and increasing apoptosis simultaneously, which is independent of nutritional or hypoxic stress. [15] By blocking autophagy with 3-MA or knocking down ATG5 with small interfering RNA (siRNA) in bovine aortic endothelial cells (BAECs), Du et al. demonstrated that autophagy affected angiogenesis in aortic endothelial cells induced by nutrient deprivation. [16] However, the role of autophagy on embryonic angiogenesis is still obscure. In this study, we employed the combination of early embryo model and HUVECs to investigate the role of autophagy on vascularization and its cellular mechanism during embryo development.

Materials and methods

Chick embryos and exposure to compounds

Fertilized chick eggs were obtained from the Avian Farm of the South China Agriculture University. The eggs were incubated until the required HH stage [17] in a humidified incubator (Yiheng Instrument, Shanghai, China) at 38°C and 70% humidity.

The chick embryos at HH9 stage were exposed to control 200μL 0.1% DMSO (Sigma-Aldrich, MO, USA) or 40nM of RAPA (LC Labs, USA) or 5mM of 3-MA(3-MA; Sigma, M9281) for 7.5 days. Briefly, approximately 200 μL of DMSO or RAPA or 3-Methyladenine of above concentrations were carefully injected into a small hole made in the air chamber of the 1.5-day incubated eggs as previously

described. [18] The experiments were performed in triplicate with 20 eggs assigned for each treatment group. After the treatment, the embryos were further incubated for 7.5 days before they were fixed with 4% paraformaldehyde for analysis of morphology and gene expression.

Immunofluorescent staining

The chick embryos were harvested after a given time incubation and fixed in 4% PFA overnight at 4°C. For the HUVECs, after exposure to 3-MA, RAPA or a combination of 3-MA and RAPA for 8 hours, the cells were fixed for 30 min in 4% PFA in PBS, rinsed and transferred into normal goat serum. Immunofluorescent staining on either whole-mount embryo or cultured cells was performed against the following antibodies: Atg7, Atg8, and Beclin-1. Briefly, the fixed embryos (cultured cells) were then incubated with Atg7 (1:200) (A2856, Sigma, USA), Atg8 (1:200, Sigma, L7543, USA) and Beclin-1 (1:200) (B6061, Sigma, USA), primary antibody at 4°C overnight (whole-mount) or 36 hours (cultured cells) on a shaker. F-actin (1:2000) (P5282, Sigma, USA) staining was performed after normal goat serum incubation without the secondary antibody. Following extensive washing, the embryos/cultured cells were incubated with either anti-rabbit IgG conjugated to Alexa Fluor 488 or 555 overnight at 4°C on a rocker. All the embryos/cultured cells were later counterstained with DAPI(4'-6-Diamidino-2-phenylindole) (1:1000) (AMEP4650, Invitrogen, USA) at room temperature for 1 hour.

RNA isolation and RT-PCR

Total RNA was isolated from CAM or YSM of chick embryos using a Trizol kit

(Invitrogen, USA) according to the manufacturer's instructions. Gene expressions were semi-quantitatively assessed utilizing reverse transcription-polymerase chain reaction (RT–PCR) as previously reported. [19] Following reverse transcription, PCR amplification of the cDNA was performed using chick specific primers. The primers sequences are provided in Supplementary Fig. S1. Briefly, 5μg amount of total RNA was reversely transcribed into cDNA at 42℃ for 1 hour in 20μL of reaction mixture containing iScript Reverse Transcriptase (BIO-RAD, Hercules, CA) with oligo (dT) and random hexamer primers (BIO-RAD, Hercules, CA) and followed by PCR amplification. PCR was carried out with 1μL of cDNA, 12.5 mL of DreamTaq Green PCR master mix(2X)(Thermo scientific, Foster City, California), containing dream-Taq DNA polymerase, dATP, dCTP, dGTP, dTTP and MgCl2, mixed with 1 mM forward primer, 1 mM reverse primer in a total volume of 25μL. The cDNA was amplified using specific primers with 30 cycles at 94℃ for 30 s, an annealing temperature of 60℃ for 30 s, and then 72℃ for 30 s, with final incubation at 72℃ for 7 min. The sets of primers used for RT-PCR are provided in the Supplementary Fig. 1. The PCR products (20 μl) were resolved using 2% agarose gels (Biowest, Spain) in 1× TAE buffer (0.04 M Trisacetate and 0.001 M EDTA) and 10,000x GeneGreen Nucleic Acid Dye (TIANGEN, China) solution. The resolved products were visualized using a transilluminator (SYNGENE, UK), and photographs were captured using a computer-assisted gel documentation system (SYNGENE).

Western blot

Chick embryo CAM tissues or human umbilical vein endothelial cells (HUVECs)

after treatment were collected and lysed with CytoBuster™ Protein Extraction Reagent (#71009, Novagen). Total protein concentrations were assessed via a BCA quantification kit (BCA01, DingGuo BioTECH, CHN). Samples containing identical amounts of protein were fractionated by SDS-PAGE, and then transferred to PVDF membranes (Bio-Rad). Membranes were blocked with 5% Difco™ skim milk (BD) and subsequently incubated with primary and secondary antibodies, then bands of interest protein were visualized using the ECL kit (#34079,Thermo) and GeneGnome5 (SYNGENE). Gray scale of bands was analyzed using Quantity One software (Bio-Rad). Antibodies: LC3B(1:1000) (A2856 , Sigma, USA); Atg7 (1:1000) (L7543, Sigma, USA), Beclin-1 (1:500) (B6061, Sigma, USA), VEGFR2 (D5B1,Cell Signaling Technology, USA); HIF2α (1:400) (10P26, Boster, CHN); VEGFA (1:200) (990489W, Boster, CHN) and β-actin (1:2000) (60008-1-Ig, Proteintech, USA); Atg5 (1:400) (13MK43, Boster, CHN) and Mtor (1:2000) (BS3611, Bioworld Technology, USA). HRP-conjugated anti-mouse IgG, HRP-conjugated anti-rabbit IgG (Cell Signaling Technology, USA). All primary antibodies were diluted in 5% skim milk, and secondary antibodies were 2000-fold diluted.

Assessment of angiogenesis using chick YSM

As previously described, [20] fertilized eggs were incubated for 2.5 days and then placed into a sterilized glass dish. The YSM containing blood vessels were orientated facing upward. Two silicone rings were placed directly on top of the leading edge of the blood vessels. To avoid developmental differences between different embryos, 40μL of PBS with 1% DMSO (control) was introduced into the ring located on the left

side of the YSM. Meanwhile, 40μL of 3-MA (5mM) or RAPA (40nM) was introduced into the ring on the right side of the same embryo. The rings were marked with red ink in the control side and black ink in the experiment side to indicate the starting position of the YSM within the ring. The extent of growth and expansion of the blood vessel plexus inside the silicone rings were determined and photographed (Olympus MVX10) after 12 - 48 hour incubation. Ten embryos in each treatment group were examined.

Assessment of angiogenesis using chick CAM

As previously described, [20] chick embryos were incubated until 7.5 days when the chorioallantoic membrane (CAM) is well developed. The embryos were treated with 200μL of 3-MA (5mM) or RAPA (20nM, 40nM, 80nM) or PBS (DMSO 0.1%) or tunicamycin (1μg/mL) for 48 hours and all surviving embryos were harvested for analysis. The CAM and accompanying blood vessels in the control and 3-MA- or RAPA-treated embryos were photographed using a Canon Powershot SX130 IS digital camera (12.1M Pixels). Ten embryos in each experimental group were examined. The CAMs from eight embryos in each group were embedded, sectioned and stained with hematoxylin & eosin. The BVD and microvasculature were quantified and analyzed as described above for assessing angiogenesis in YSM. The CAMs were also harvested for different biochemical assays as described below.

Assessment of blood vessel integrality

The assay of Evans blue (EB) (Sigma, US) leakage was used to evaluate blood vessel disruption following 40nM RAPA or 5mM 3-MA or 0.1% DMSO treatment as described previously. The EB measurement was performed according to the previous

study by Lenzser. [21] Briefly, 2 % EB (4 mL/kg) was injected via YSM. At 2h after EB injection, YSM and CAM were harvest. The embryos for detecting the Evans blue contents were reperfused with PBS before harvest. YSM samples were then homogenized by 80% trichloracetic acid and centrifuged. Then the supernatants were mixed with ethanol (1:4). The absorbance of supernatants was measured at 630 nm with microplate reader (Biotek, ELX800, US).

For YSM, they were fixed by 4% paraformaldehyde overnight, and photographed using stereoscope fluorescence microscope (Olympus MVX10) with imaging software (Image-Pro Plus 7.0).

Photograph

After immunofluorescent staining, the whole-mount embryos were photographed using stereoscope fluorescence microscope (Olympus MVX10) with imaging software (Image-Pro Plus 7.0). The sections of the embryos were photographed using an epi-fluorescent microscope (Olympus IX51, Leica DM 4000B) at 200 or 4006 magnification with the Olympus software package Leica CW4000 FISH.

Transmission electron microscopy

The treated chick embryos were fixed with 2.5% glutaral in 0.1 M PBS for 2 hours, and then yolk sac membranes (YSM) were dissected. The samples were sent to the TEM Laboratory of Sun Yat-sen University. The embedding, ultrathin sectioning and staining were performed by professional technicians and examined using a Tecnai G2 Spirit Twin (FEI, USA).

Cell lines and culture

Human umbilical vascular endothelialcells (HUVECs) were a kindly gift from Zhi Huang's lab, and cultured in Dulbecco's modified Eagle's medium (DMEM) medium (Gibco, shanghai, China) supplemented with 10% fetal bovine serum (FBS), and incubated at 37 °C and 5% CO_2

Migration assay

HUVECs were seeded in 6-well plates with DMEM (10% FBS) medium. At confluency, a wound was induced by scratching the monolayer with a 10-μl pipette tip. The cells were then washed 3 times with sterile PBS. HUVECs were incubated in serum-free DMEM medium with 5mM 3-MA, 40nM RAPA or 0.1% DMSO，under 5% CO_2 conditions. Images were acquired at 12 h, 24h, 36h and 48h post‑scratching. At least 3 wells were analyzed in each treatment group and the images were taken using an inverted microscope (Nikon Eclipse Ti-U, Japan).

Cell transfection

siRNA against ATG7 or control were purchased from Ribo Bio. Cells were transfected with 25 nM siRNAs using Lipofectamine 2000. For overexpression, cells were transfected with 14μg of control vector pIRES2-EGFP, pCMV-Myc or pEGFP-C2, and experiment groups with pIRES2-EGFP-ATG5, pCMV-Myc-ATG7 or pEGFP-C2-LC3 using Lipofectamine 2000. pIRES2-EGFP-ATG5 （NM_001286106.1） plasmids was purchased from Hanbio Biotechnology. pCMV-Myc-ATG7（Genbank：BC000091） and pEGFP-C2-LC3 （Genbank：BC067797）plasmids were kindly gifts from Toren's lab. The experiment procedure was according to the specification of Lipofectamine 2000. Briefly, For each well of

6-plate, 15μL Lipofectamine® 2000 Reagent was added into 150μL serum-free DMEM medium. SiRNA or plasmids were added into 150μL serum-free DMEM medium. Mixed them and incubated for 5 minute. At last, added 250μL mixture into each well. After 24h, the transfected cells were photographed using an inverted microscope (Nikon Eclipse Ti-U, Japan) then it used for subsequent experiments 48h later.

Tube assay

The tube formation assay was performed as follows. Each well of 12-well plates were coated with 200μL of the mixture of Matrigel (BD Biosciences，USA) and serum-free DMEM medium and incubated at 37℃ for 30 min to promote gelling. HUVECs were resuspended in DMEM medium (serum concentration 10%) and added to each well，and add the DMEM medium each well in a final volume of 1ml. After 24h, the plates were fixed with 4% paraformaldehyde. Then the images were taken using an inverted microscope (Nikon Eclipse Ti-U, Japan) at the middle version of the each well. Each well was tested in triplicate and each experiment was repeated at least three times. The average number of tubules was calculated from examination of six separate microscopic fields. Tube formation in the presence of 3-MA or RAPA, or a combination of 3-MA and RAPA or transfection group were compared to tube formation in media with 0.1% DMSO as control or the control vector.

Data analysis

Statistical analysis for all the experimental data generated was performed using a SPSS 13.0 statistical package program for windows. The data were presented as mean

± SD. Statistical significance were determined using paired T pest, independent samples T test. *p<0.05, **p<0.01 and ***p<0.01 indicate statistically significance between control and drug-treated groups.

The diagram of mechanism of autophagy on angiogenesis was drawn by Pathway Builder Tool software.

Results

The autophagy-related genes are expressed in chick YSM and CAM during embryo development.

Atg7, Atg8 and Beclin1 are deemed to be the genes related to autophagy activities.[22] In this study, we demonstrated that they were all strongly expressed in the developing vessel plexuses in yolk sac membranes (YSM), which could not only seen in whole-mount immunofluorescent staining against Atg7, Atg8 and Beclin1 （BECN1） (Fig. 1A-C), but also obviously seen in the transverse sections of those YSM tissues (Fig. 1A1-C1, A2-C2). To further confirm this observation, we performed Atg7 and Atg8 immunofluorescent staining on chorioallantoic membrane (CAM) again since it was full of newborn blood vessels as well. From the transverse sections of CAM tissues, we could see that both of Atg7 and Atg8 were apparently expressed in the epithelial cells (Fig. 1D-E, D1-E1, D2-E2). It suggests that autophagy is probably involved in the angiogenesis during embryo development.

The disturbance of autophagy causes angiodysplasia in developing chick embryos.

To investigate whether or not autophagy wasz implicated in angiogenesis in

embryo development, we exposed early chick embryos for 7.5 days with either 40nM RAPA or 5mM 3-MA, which could induce or inhibit autophagy respectively (Fig. 2B-B2, C-C2). [13, 23] The embryos treated with 0.1% DMSO acted as control group (Fig. 2A-A2). In terms of the appearance of embryos, we could observe that there is a lot of hemorrhage in the embryos that was treated with either RAPA (n=8/16) (Fig. 2B) or 3-MA (n=4/16) (Fig. 2C) in comparison to the control embryos (Fig. 2A). Interestingly, we found that these hemorrhage was probably lack of integraty of blood vessels in the RAPA- and 3-MA-treated embryos compared to control one as schematically shown in Fig. 2A2-C2, which was based on the observations for transverse sections of those embryos (Fig. 2A1-C1). The hemorrhage phenotype dose actually not only exists in head region but also in trunk region of embryos treated with either RAPA (n=12/16) or 3-MA (n=6/16) (Fig. 2D). Electronic microscopy showed that the numbers of autophagosome increased in RAPA group while decreased in 3-MA group (Fig. 2E-G1), which suggests that the imperfection of blood vessels was associated with the alterations of cell autophagy level.

The disturbance of autophagy with autophagy activator or inhibitor affects the angiogenesis in chick YSM.

Chick YSM was employed to further study the role of autophagy on angiogenesis through disturbing autophagy with inducer or inhibitor since YSM had been mature angiogenesis model to investigate embryonic angiogenesis in our laboratory as previously described. [20, 24-27] Compared with the extension of leading edges of vessel plexuses in control embryos (Fig. 3A-A3), the extension of vessel plexuses in

3-MA-treated YSM (Fig. 3C-C3) was much slower while significantly faster in RAPA-treated YSM (Fig. 3B-B3), suggesting 3-MA treatment inhibited the angiogenesis and RAPA treatment promoted angiogenesis in YSM. The density of the blood vessel in RAPA treated group was 0.33±0.02 (n=6, $P<0.001$), which was 20% higher than in the control group (0.27±0.02, n=6), while it was 26% reduced in the 3-MA treated group (0.20±0.03, n=6) ($P<0.01$) (Fig. 3G). Meanwhile, the blood numbers increased in presence of RAPA (7.00±1.059, n=12) ($P<0.05$) and reduced in presence of 3-MA (2.50±0.548, n=12) ($P<0.001$) (Fig. 3D-F, H) compared with the control (4.67±0.516, n=12), which also implied the alteration of angiogenesis in presence of 3-MA/RAPA. Since the angiogenesis in YSM was not only presented in the plane extension of vessel plexuses but also into yolk sac, so that we could see the blood vessel depths became deeper when exposed to RAPA (525.76±108.44μm, n=6) ($P<0.05$) and became shallower when exposed to 3-MA (285.88±37.18μm, n=6) ($P<0.01$) compared with the control (407.75±43.90μm, n=6) (Fig. 3D1-F1, I). Next, the angiogenesis-related gene expressions in YSM, following the treatment of RAPA or 3-MA, were determined by using RT-PCR (Fig. 3J). The up-regulated genes comprised HIF 1α, HIF 2α, ANG1, ANG2, VE-cadherin (VE-Cad), FGF2, TEK, VEGFR1, VEGFR3 following the treatment of RAPA (Fig. 3J). And in presence of 3-MA, the genes of down-regulated expressions included HIF 1α, HIF 2α, ANG1, ANG2, FGF2, VEGFR1, VEGFR3 and others not changed dramatically (Fig. 3J). Compared with the control group, VE-Cad, VEGFA, VEGFR1, VEGFR2, VEGFR3, HIF 1α, HIF 2α, ANG1, ANG2, TEK and FGF2, the grey value of them in RAPA

group were significantly difference. However, in 3-MA group, the grey value of VE-Cad, VEGFR1, VEGFR3, HIF 1α, HIF 2α, ANG1, ANG2, TEK and FGF2 were significantly different, while VEGFA and VEGFR2 were not significantly different. (Supplementary Fig. 2). Those data suggest that any disturbance for autophagy homeostasis could lead to angiogenesis disorder.

The disturbance of autophagy with autophagy activator or inhibitor affects the angiogenesis in chick CAM.

To verify the observation above in chick YSM model, chick CAM, another angiogenesis model in early chick embryo development, was employed to determine the role of autophagy on angiogenesis again (Fig. 4). Likewise, we found that the blood vessel density in chick CAM was promoted following the exposure to 40nM RAPA (0.113 ±0.023 n=6) ($P<0.05$) compared with the control (0.100±0.023, n=6) (Fig. 4A-B, 4A1-B1, 4D). In contrast, 5mM 3-MA treatment (0.086±0.024, n=6) ($P<0.05$) could promote the blood vessel density in CAM compared with control (0.100±0.023, n=6) (Fig. 4A-C, 4A1-C1, 4D). Actually, RAPA showed the simulative effect of angiogenesis with a dose-dependent manner from 20nM to 80nM (0.057±0.023, 0.0933±0.0226, 0.0819±0.0145, n=8), but maximum effect on blood vessel density appeared at the concentration of 40nM (0.0933±0.0226, n=8) ($P<0.05$) (Supplementary Fig. 3A-D, A1-D1, A2-D2, E), and so was the data of the blood vessel diameters (Supplementary Fig. 3A3-D3). Therefore, the concentration of RAPA was chosen to be 40nM in this study. In addition, the alteration of blood vessel diameters in presence of RAPA (150.9±38.3μm, n=24) ($P<0.001$) or 3-MA

(46.7±15.7μm, n=24) (P<0.01) compared to the control (70.6±36.6μm, n=24) were presented in the same tendency as well (Fig. 4E).

Western blot data showed that the ratio of LC3-II/LC3-I dramatically increased following the treatment of RAPA (2.869±0.0823, n=3) (P<0.001) and reduced following the treatment of 3-MA (0.187±0.008, n=3) (P<0.01) compared to the control group (0.280±0.008, n=3) (Fig. 4F), suggesting that the autophagy homeostasis was interrupted by the exposure to 3-MA or RAPA in chick CAM. Meanwhile, RT-PCR data indicated that RAPA treatment up-regulated the expressions of HIF 2α, ANG1, TEK, VE-Cad, VEGFA, FGF2, VEGFR1 and VEGFR3, while 3-MA treatment down-regulated the expressions of ANG1, ANG2, VEGFA, TEK, FGF2, VE-Cadherin, VEGFA, VEGFR1 and VEGFR3 in the same tissues of chick CAM (Fig. 4G). Compared to the control group, the grey value of RAPA group of VE-Cad, VEGFA, VEGFR1, VEGFR2, VEGFR3, HIF 2α, ANG1, TEK and FGF2 were significantly different, while ANG2 was not significantly different. However, the 3-MA group of VE-Cad, VEGFA, VEGFR1, VEGFR2, VEGFR3, HIF 2α, ANG1, ANG2, TEK and FGF2 were significantly different. (Supplementary Fig. 4). It suggests that autophagy disturbance is probably involved in the angiogenesis disorder following the treatment of 3-MA or RAPA.

The integrality of blood vessels is damaged by the autophagy disturbance with the treatment of 3-MA or RAPA.

To investigate if the integrity of blood vessels was destroyed by the alteration of autophagy, we carried out the Evans blue perfusion experiments in chick YSM model.

The result showed that many leaks were seen in the vessel plexus of YSM treated with either RAPA or 3-MA as indicated by arrows (Fig. 5A-C1). And the fluorescent assessment of Evans blue demonstrated that more Evans blue accumulated in the tissues treated with both RAPA and 3-MA (Fig. 5D). The electronic microscopy showed that the more cleft of zonula adheren (ZA), one kind of adherens junctions, [28] appeared in both RAPA and 3-MA-treated YSM vessels in comparison with control one (Fig. 5E-G), indicating the lose of endothelial cell integrity following the interference of autophagy. Furthermore, β-catenin expression dropped at embryonic blood vessel endothelial cells in presence of 3-MA and RAPA (Fig. 5H-J). RT-PCR data displayed that RAPA treatment reduced the expressions of Claudin-5, Occludin, α-catenin, P120, and increased the expressions of Vinculin, Par3; 3-MA treatment increased the expressions of Par3, and decreased the expressions of Claudin-5, α-catenin, P120; for the other tight junction genes, adheren junction genes and desmosome genes (Claudin-1, Claudin-12, ZO1, ZO2, CGN, Plakoglobin, Desmoplakin), neither autophagy inducer nor autophagy inhibitor affected their expressions (Fig. 5J).

The HUVEC vitality reduces after autophagy disturbance with exposure to 3-MA or RAPA.

To further investigate the cellular mechanism of autophagy role on angiogenesis, we carried out LC-3 immunofluorescent staining on the *in vitro* 2-hour cultured human umbilical vein endothelial cells (HUVECs) in presence of RAPA or RAPA + 3-MA (Fig. 6A-C, A1-C1). The LC3 was not strongly expressed in HUVECs in

control group (Fig. 6A-A1); LC3 expression was dramatically upgraded by the exposure of RAPA (40nM) (Fig. 6B-B1) and the increased LC3 expression induced by RAPA exposure was inhibited by adding 3-MA (5mM) in the culture medium (Fig. 6C-C1). The expressions of Atg7, LC-3I, LC-3II and Beclin-1 at different cultured time point (2, 4, 8 hours) were determined using western blot (Fig. 6D-E). The results showed that RAPA treatment increased Atg7 and Belcin-1 expression, and decreased mTOR and P62 expressions (Fig. 6D), meanwhile, the 3-MA treatment inhibited Atg7 and Beclin-1 gene expressions, and increased mTOR and P62 expressions (Fig. 6E) with a time-dependent manner. On the expression of LC3II and LC3I, we could concluded that in RAPA treated group the gray value of LC3II/LC3I increased (0.567±0.057, 1.353±0.051, 0.903±0.049, n=3) ($P<0.01$ or 0.05) than the control at 0-hour (0.777±0.057, n=3), while that decreased in the treatment of 3-MA (0.108±0.004, 0.018±0.002, 0.600±0.010, n=3) ($P<0.01$) and decreased prominently in 4 hour, compared to control (1.007±0.030, n=3) (Fig. 6E).

After confirming the correlation between autophagy and angiogenesis in HUVECs, we determined the HUVECs vitality using scratch test in presence of 3-MA or RAPA（n=7）(Fig. 7). We could see that following the treatment RAPA in 12h, 24h, 48h promoted the cell migration distance toward midline or cell proliferation along with the extension of culture time in comparison to control (Fig. 7A-A3, B-B3, D-E); 3-MA treatment inhibited the cell migration distance toward midline or cell proliferation along with the extension of culture time in comparison with control (Fig. 7A-A3, C-C3, D-E). The number of the migrated cells toward the midline in RAPA

treated group in 12h, 24h and 48h, compared to the control, is increased significantly . And the number of the migrated cells toward the midline in 3-MA treated group compared to the control is reduced significantly. And the Western blot data showed that expressions of HIF 2α, VEGFR2 and VEGFA were suppressed by the exposure of 3-MA and RAPA for 8h. These data indicated that interference of autophagy indeed affected HUVEC vitality.

The overexpression or knock-down of autophagy genes dramatically influence on the tube formation of HUVECs in vitro.

To further explore the role of autophagy on angiogenesis, we employed the HUVECs to carry on the tube formation experiment following the manipulating the Atg5, Atg7, Atg8 expression level by transfection of siRNA or plasmids. The tube formation assay showed that the overexpression of Atg5 with plasmids caused the less tube formation compared to control (Fig. 8A-D). Down-regulation of Atg7 or overexpression of Atg7 with siRNA/plasmids, suppressed the tube formation while the western blot confirmed the knock-down/overexpression worked (Fig. E-I). Overexpression of Atg8 with plasmids (approved by western blot) restricted the tube formation (Fig. 8J-M). Here, we repeated the *in vitro* experiment, tube formation, with the treatment of RAPA/3-MA in HUVECs, and obtained the suppressed effect for tube formation, and partially rescued when combination application of RAPA and 3-MA (Fig. 8N-R). Eventually, we determined F-actin expression in presence of RAPA/3-MA since F-actin is cytoskeleton and it is certainly associated with the cell-cell junctions and cell migration. The results showed that F-actin labeled

HUVECs lost their polarities in presence of RAPA/3-MA compared to control (Fig. 9A-C). After treatment of RAPA/3-MA the expression of β-catenin dropped and was lost at HUVECs membranes as indicated by arrows (Fig. 9D-F). The *in vivo* and *in vitro* experiment with autophagy expression level manipulation further indicated that there was a close correlation between autophagy level and angiogenesis.

Discussion

Avian and mouse vertebrate models are often employed to extrapolate from animal studies to explore gene functions during human fetal development. Utilizing the characteristics that angiogenesis is easily observed at chick yolk sac membrane (YSM) and chorioallantoic membrane (CAM) model, we firstly performed immunostaining against Atg7, Atg8 and Beclin1 [29] and demonstrated that all of those autophagy-related genes were expressed there (Fig. 1), implying the possibility that autophagy is involved in the angiogenesis process. Moreover, the hemorrhage phenotypes occurred when the developing chick embryos were exposed to either rapamycin (RAPA, autophagy inducer) or 3-MA (autophagy inhibitor) (Fig. 2A-D), which work well by the electronic microscopy examination of autophagosome (Fig. 2E-G1). The histological analysis showed that the hemorrhage was probably due to the imperfection of capillary endothelium (Fig. 2A1-C2). To some extent, those observations are similar to the report on pathological angiogenesis by Ramakrishnan et al., in which they showed that the autophagic response could be a novel target for inhibiting pathological angiogenesis through studying the effective mechanism of

kringle 5, a potent angiogenesis inhibitors. [15]

To further investigate the role of autophagy on angiogenesis, we carefully investigated the angiogenesis process after chick YSM and CAM were exposed to RAPA and 3-MA at different developmental stage of angiogenesis. The intuitive images of blood vessel plexus extension on YSM demonstrated that the angiogenesis was accelerated by exposing to RAPA, autophagy inducer; and was restricted by exposing to 3-MA, autophagy inhibitor (Fig. 3A-I). Using chick CAM model, we found the similar experimental results as it was in YSM (Fig. 4A-E) after confirming the cell autophagy manipulation by the altered ratios of LC3II and LC3I following the treatment of 3-MA/RAPA, indicating that 3-MA/RAPA exposure lead to the disturbance of autophagy (Fig. 4F), and RAPA-induced enhancement of angiogenesis showed the dose-dependent manner below 80nM (Supplementary Fig. 3A-F). Meanwhile, exposing to RAPA at both chick YSM and CAM increased the expressions of HIF 2α and angiogenesis-related genes including ANG1, ANG2, TEK, FGF2, VE-Cadherin, VEGFA, VEGFR1-3, while exposing to 3-MA, their expression were inhibited at both angiogenesis models (Fig. 3J)(Fig. 4G)(Supplementary Fig. 2) (Supplementary Fig. 4). Promoting autophagy by tunicamycin in the CAM leads to the similar results to the RAPA treatment. It suggests that the alteration of angiogenesis in presence of RAPA/3-MA/tunicamycin could be due to the fact that interference with autophagy changed the expression levels of angiogenesis-related genes. Or it could also be the result of activation/degradation of hypoxia-inducible factor (HIF), the proangiogenic factor, by autophagy inducer/inhibitor since HIF 2α

could act as a hypoxia-inducible transcription factor involved in vascular remodeling. [30]

We speculated that the reason for the hemorrhage at RAPA/3-MA-treated embryos was that the integrity of blood vessels was damaged during vasculature formation. To address this pathologic cause, we carried out the Evans blue perfusion experiments at chick CAM, and found that there were leaks from the blood vessels treated with either RAPA or 3-MA (Fig 5A-C1), implying the imperfection of blood vessels following the treatments of autophagy interference. As we know, maintaining the integrity of blood vessel relies on the endothelial cell-cell junctions including tight junctions, desmosome and adherens junctions etc. [28] In the current study, electronic microscopy showed the increased numbers of bigger clefts between epithelial cells in either RAPA or 3-MA-treated CAM vessels (Fig. 5E-G), and the expressions of corresponding tight junction genes and adherens junction genes were also altered by the treatments of autophagy inducer or inhibitor (Fig. 5K). This suggests that the autophagy inducer/inhibitor-induced tight/adherens junction and desmosome gene expression alteration might interfere with the formation of vessel endothelial cell integrity, so that hemorrhage occurred in RAPA/3-MA-treated embryos.

Given all that, it seems that the problem happens on endothelial cells of blood vessels. Using HUVECs (human umbilical vein endothelial cells) model, we tried to explore the effect of autophagy on endothelial cell biology. RAPA treatment could increased LC3 expression and completely restricted the enhanced LC3 expression after adding 3-MA at HUVECs (Fig. 6A-C1), and the ratio of LC3II/LC3I and the

expressions of other autophagy-related genes (Atg7, Beclin1, mTOR, P62) changed with RAPA/3-MA at time-dependent manner (Fig. 6D-E), indicating that autophagy level in the HUVECs could change with the treatment of autophagy inducer and inhibitor. In such case, scratch test demonstrated that autophagy inducement with RAPA could accelerate HUVECs migration ability, while autophagy inhibition with 3-MA could suppress HUVECs migration ability (Fig. 7). However, this is only the experiment that reflects the cell viability of HUVECs. To further confirm the possibility of autophagy involvement in angiogenesis, we assessed the tube formation ability of HUVECs after knocking-down Atg7 gene and over-expression Atg5, Atg7, Atg8 in HUVECs with tube formation assay. Generally, knock-down of Atg7 gene expression in HUVECs could suppress the tube formation ability, so could overexpressed of Atg5, Atg7 and Atg8 in HUVECs (Fig. 8), which directly indicates that autophagy is involved in the tube formation *in vitro*, the similar process *in vivo* – angiogenesis. For both cell variability and cell-cell junctions, β-catenin and cytoskeleton such as actin play very important functions to maintain cell structure and support cell migration. [28] Here, we also can see that β-catenin expression is lost in RAPA/3-MA-treated HUVECs membrane, and cell polarity disappeared in comparison with control cells (Fig. 9), suggesting that autophagy interference is indeed able to lead to dysfunctional cytoskeleton.

In sum, we demonstrated that autophagy-related genes express in the vascular plexus at chick YSA and CAM. The angiogenesis *in vivo* will be interfered dramatically when chick embryos are exposed to autophagy inducer or inhibitor.

Meanwhile, angiogenesis-related gene expression is in chaos as well. In such case, we could find hemorrhage occured in the exposed embryos as well. Further investigation showed that the hemorrhage occurred because of imperfection of blood vessel formation including damaged endothelial cell-cell junctions, endothelial cell viability and migration etc. Taken together, our data suggests that autophagy is indeed involved in the embryonic angiogenesis (Supplementary Fig. 6).

Though there are some reports about the relationship between angiogenesis-related genes and epithelial-related genes. For instance, Gurnik S et. al found that ang2 could increase the permeability of the vascular by depressing the tight junction and increasing the expression of caveolin-1, another vesicular permeability-related molecule.[31] However, ang1 and ang2 antagonized each other to modulate the permeability of the blood vessel, [32]and the imbalance of these two gene may result in the lost of the permeability of the vessels no matter we inhibit autophagy or activate it. Like autophagy, we speculate that angiogenesis and cell junctions are all a balanced-process in development relying on the coordination of all of these genes. Up-regulation and down-regulation of some of genes may lead to a comprehensive result. Any disturbance in cell junctions may result in the impermeability of the vessels, and as some of the functions of the genes related to angiogenesis remain obscure, it is certain that further experimentation is required to explore the precise molecular biological mechanism.

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Conflict of interest

The authors declare that they have no conflict of interest.

Figure legends

Fig 1. Atg7, Atg8 and BECN1 are expressed in chick YSM and CAM

A-C: Immunofluorescent staining against Atg7 (A), Atg8 (B) and ***BECN1*** (C) was performed on the YSM of HH13 chick embryos. **A1-C1:** The transverse sections at the sites shown with dotted lines in A-C respectively. **A2-C2:** DAPI staining + A1-C1 respectively. **D-E:** The transverse sections of Atg7 (D) and Atg8 (E) immunofluorescent staining from 10.5-day chick CAM. **D1-C1:** DAPI staining + D-E respectively. **D2-C2:** H&E staining on the next transverse sections of D and E respectively. Abbreviation: CAM, chorioallantoic membrane; YSM, yolk sac membrane; BECN1, beclin-1. Scale bars = 150 μm in A-C, 100 μm A1-C1, A2-C2, 100 μm in D-E, D1-E1, D2-E2.

Fig 2. The exposure of RAPA or 3-MA lead to hemorrhage in developing chick embryos.

A-B: The representive images showing the cranial regions of 9-day control (0.1%

DMSO) (A), RAPA-treated (B) and 3-MA-treated (C) chick embryos respectively. **A1-C1**: The H&E staining on the transverse sections at the levels indicated by white dotted lines in A-C respectively. **A2-C2**: The schematical drawings show the correlation between red blood cells and blood vessels among the different groups. **D**: The bar chart showing the comparison of phenotype incidences among control, RAPA-treated and 3-MA-treated groups. **F-G**: The transmission electronic microscope images from control (A1) and RAPA- or (B1)3-MA-treated groups (C1). **F1-G1**: The high magnification images from E-G respectively. Abbreviation: RAPA, rapamycin; 3-MA, 3-Methyladenine; DMSO, dimethyl sulfoxide. Scale bars = 5 mm in A-C, 50 μm in A1-C1, 0.6 μm in E-G and 2.5 μm in E1-G1.

Fig 3. The 3-MA exposure reduces and RAPA exposure promotes angiogenesis in chick YSM.

A-C: The representive images of leading edges of vessel plexuses within silicon rings at 0 hour from control (0.1% DMSO) (A), RAPA-treated (B) and 3-MA-treated (C) YSM respectively. **A1-C1, A2-C2, A3-C3**: The representive images at 12-hour, 24-hour, 36-hour incubation respectively. **D-F**: The transverse sections at the levels indicated by white dotted lines in A3-C3 respectively. **D1-F1**: The high magnification images from D-F respectively. **G-I**: The bar charts showing the comparison of blood vessel densities, numbers, depths among control, RAPA-treated and 3-MA-treated groups. **J**: The RT-PCR data showing the expressions of TEK, VE-Cad, HIF 2α, FGF2, VEGFA, VEGFR1, VEGFR2, VEGFR3, ANG1 and ANG2 in control (0.1% DMSO), RAPA-treated and 3-MA-treated YSM tissues. Abbreviation: VE-Cad, VE-cadherin.

Scale bars = 2.5 mm in A-C, A1-C1, A2-C2, A3-C3; 200 μm in D-F and 100 μm in D1-F1.

Fig 4. The 3-MA exposure reduces and RAPA exposure promotes angiogenesis in chick CAM.

A-B: The representive images of vessel plexuses in CAM, which were treated with DMSO (control, A), RAPA (B) and 3-MA (C) for 2 days. **D-E**: The bar chart showing the comparison of blood vessel densities and diameters among control, RAPA-treated and 3-MA-treated groups. **F**: Western blot data showing the expressions of LC3-I and LC3-II following the treatments of RAPA and 3-MA. **G**: The RT-PCR data showing the expressions of HIF 2α, ANG1, ANG2, VEGFA, TEK, VE-Cadherin, FGF2, VEGFR1, VEGFR2 and VEGFR3 in control, RAPA-treated and 3-MA-treated CAM tissues. Scale bars = 1 cm in A-C and 3 mm in A1-C1.

Fig 5. Both exposures of RAPA and 3-MA restrict the integrality of endothelium in YSM and CAM.

A-C: The representative fluorescent images of blood vessels in YSM of 3-day incubated embryos from control (A), RAPA-treated (B) and 3-MA-treated (C) groups. The photographs were taken after two hours following the injection of Evans blue. **A1-C1**: The bright field images of A-C respectively. **E-G**: The transmission electronic microscope images from control and RAPA- or 3-MA-treated groups. **G**: The bar chart showing the comparison of evans blue contents among control, RAPA-treated and 3-MA-treated groups. **H-J**: The β-catenin immunofluorescent staining was performed on the transverse sections in the cranial regions of the 9d chick embryos

control (H), RAPA-treated (I) and 3-MA-treated (J) groups respectively. **K**: The RT-PCR data showing the expressions of Claudin-1, Claudin-5, Claudin-12, Occludin, ZO1, ZO2, α-catnin, Vinculin, CGN, Par3, CGN, P120, Plakoglobin, Desmoplakin and PPIA in control (0.1% DMSO), RAPA-treated and 3-MA-treated YSM tissues. Abbreviation: ZA, Zonula adherens. Scale bars = 300 μm A-C, A1-C1, 1 μm in E-G and 20 μm in E-G.

Fig 6. The exposure of RAPA promotes LC3 expression in cultured HUVECs.

A-C: The LC3 immunofluorescent staining was performed on 8-hour exposed HUVECs with 0.1% DMSO (control) (A), RAPA (B) and RAPA+3-MA. **A1-C1**: DAPI staining + A-C respectively. **D**: Western blot data showing the expressions of LC3-I/LC3-II, Atg7, Beclin-1, mTOR, P62 and β-actin at 2-hour, 4-hour and 8-hour incubation following the treatments of RAPA. **E**: Western blot data showing the expressions of LC3-I/LC3-II, Atg7, Beclin-1, mTOR, P62 and β-actin at 2-hour, 4-hour and 8-hour incubation following the treatments of 3-MA. Abbreviation: HUVECs, human umbilical vein endothelial cells. Scale bars = 20 μm in A-C, A1-C1.

Fig 7. The exposure of 3-MA suppresses, but RAPA doesn't affect HUVECs cell migration in scratch test.

A-C: The representative images of HUVECs scratch test at 0-hour incubation from control (A), 3-MA-treated (B) and RAPA-treated (C) groups respectively. **A1-C1, A2-C2, A3-C3**: The representative images of HUVECs scratch test at 12-hour (A1-C1), 24-hour (A2-C2) and 48-hour (A3-B3) incubation from control (A1-A3), 3-MA-treated (B1-B3) and RAPA-treated (C1-C3) groups respectively. **D**: The graph

showing the distances of HUVEC cell migration along with incubation time in presence/absence of RAPA or 3-MA. **E**: The graph showing the alteration of migrated HUVECs cell numbers along with incubation time in presence/absence of RAPA or 3-MA. **F**: Western blot data showing the expressions of HIF 2α, VEGFR2 and VEGFA following the treatments of RAPA and 3-MA. Scale bars = 100 μm in A-C, A1-C1, A2-C2, A3-C3 and 100 μm in A4-C4.

Fig 8. Inhibition of ATG7 or promotion of ATG5, ATG7 and ATG8 decreased the capillary tube formation of HUVECs

A-B: The bright field images of HUVECs after transfection of GFP (A) or ATG5 (B). **A1-B1**: The fluorescent images of A-B. **A2-B2**: The bright images of tube formation by HUVECs expressed ATG5 (A2) and GFP (B2) steadily. **C**: Western blot data showing the expressions of ATG5 after transfection of GFP or ATG5. **D**: The bar chart showing the average tube numbers in ATG5 or GFP transfected HUVECs. **E-G**: The bright images of tube formation by HUVECs after transfection of control vector (E), siATG7 (F) or ATG7 (G), respectively. **H**: Western blot data showing the expressions of ATG7 after transfection of control vector, siATG7 or ATG7, respectively. **I**: The bar chart showing the average tube numbers in siATG7, ATG7 or control plasimids transfected HUVECs. **J-K**: The bright field images of HUVECs after transfection of GFP (J) or ATG8 (K). **J1-K1**: The fluorescent images of J-K. **J2-K2**: The bright images of tube formation by HUVECs expressed ATG8 (K2) and GFP (J2) steadily. **L**: Western blot data showing the expressions of ATG8 after transfection of GFP or ATG8. **M**: The bar chart showing the average tube numbers in

ATG8 or GFP transfected HUVECs. **N-Q**: The representative images of tube formation by HUVECs treated by 0.1% DMSO as control (N), RAPA (O) 3-MA (P) and RAPA+3-MA (Q) groups respectively. Scale bars = 100 μm in A-B, A1-B, 50 μm in A2-B2, 50 μm in E-G, 100 μm in J-K, J1-K1, 50 μm in J2-K2 and 60 μm in N-Q.

Fig 9. Both exposures of RAPA and 3-MA impaired the integrality of HUVECs

A-C: The F-actin immunofluorescent staining was performed on the HUVECs after 8-hour incubation of 0.1% DMSO as control (A), RAPA-treated (B) and 3-MA-treated (C) groups respectively. **D-F**: The representative β-catenin fluorescent images + DAPI staining of HUVECs after 8-hour incubation of 0.1% DMSO as control (D), RAPA-treated (E) and 3-MA-treated (F) groups respectively. **G**: The RT-PCR data showing the expressions of Plakoglobin, β-catnin and GAPDH. Scale bars = 20 μm in A-C and 30 μm in D-F.

Supplementary Fig 1. The sets of primers used for PCR in this study.

Supplementary Fig 2. The bar chart showing the comparisons of gene expressions in Fig 3J.

Supplementary Fig 3. The exposure of various concentrations of RAPA uphold angiogenesis in chick CAM.

A-D: The representive images of vessel plexuses in CAM, which were treated with 0.1% DMSO (control, A), 20nM (B), 40nM (C) and 80nM (D) RAPA for 2 days. **A1-D1, A2-D2**: The representive high magnification images from the sites indicated by dotted squares in A-D respectively. **A3-D3**: The H&E stained transverse sections at the levels indicated by dotted lines in A1-D1 respectively. **E**: The bar chart showing

the comparison of blood vessel densities in CAM following the treatments with different concentrations of RAPA. **F**: The bar chart showing the comparison of blood vessel diameter in CAM following the treatments with different concentrations of RAPA. **G**: The RT-PCR data showing the expressions of VEGFR2, VEGFA, ANG1, ANG2 and HIF 2α in CAM following the treatments with different concentrations of RAPA. Scale bars = 1 cm in A-D; 400 μm in A1-D1, A2-D2 and 500 μm in A3-D3.

Supplementary Fig 4. The bar chart showing the comparisons of gene expressions in Fig 4G.

Supplementary Fig 5. The exposure of tunicamycin increases angiogenesis in chick CAM.

A-B: The representive images of vessel plexuses in CAM, which were treated with 0.1% DMSO (control, A) or 1μg/mL tunicamycin (B) for 2 days. **A1-B1**: The representive high magnification images from the sites indicated by dotted squares in A-B respectively. **C**: The bar chart showing the comparison of blood vessel densities in CAM following the treatments of tunicamycin. **D**: The RT-PCR data showing the expressions of Atg7, HIF 2α, VEGF and VEGFR2 in CAM following the treatments with tunicamycin. Scale bars = 1 cm in A-B and 4 mm in A1-B1.

Supplementary Fig 6. Proposed mechanism for autophagy-involved in regulating embryonic angiogenesis.

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