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# Relationship between annexin A7 and integrin β4 in autophagy

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ABSTRACT

We recently found that both annexin A7 and integrin β4 were involved in autophagy of vascular endothelial cells. But, their relation is not clear. In this study, we addressed this question by using a small molecule ABO that promoted autophagy by targeting annexin A7. The results showed that knockdown of integrin β4 partly inhibited ABO-induced autophagy in vascular endothelial cells. Furthermore, in HEK293 cells that express integrin β4 too low to detect by western blot, ABO could not induce autophagy. If integrin β4 was overexpressed in HEK293 cells, ABO could evoke autophagy. On the other hand, knockdown of annexin A7 also blocked ABO-induced autophagy although the level of integrin β4 was elevated. Moreover, by co-immunoprecipitation, we identified the interaction of integrin β4 and annexin A7, and found that ABO could modulate the interaction, at the same time, the phosphorylation of Y-1494 in integrin β4 cytoplasmic domain was inhibited significantly *in vitro* and *in vivo*. Hence, by identifying the interaction between integrin β4 and annexin A7, we demonstrated that both annexin A7 and integrin β4 were essential for small molecule ABO-induced autophagy and targeting annexin A7 by ABO could modulate integrin β4 phosphorylation, while Y-1494 phosphorylation of integrin β4 may negatively regulate autophagy.


## 1. Introduction

Integrin β4, a laminin-5 receptor, mainly locates in the adhesion structure of hemidesmosome (HD). As a putative model for phosphorylation-induced hemidesmosome disassembly, the tyrosine phosphorylation of the integrin β4 signaling domain in the cytoplasmic domain contributes to the disruption of hemidesmosome (Dans et al., 2001). Distinct from other β subunits, integrin β4 subunit contains an exceptionally large cytoplasmic domain, which comprises five major tyrosine-phosphorylation sites, such as Y-1422, Y-1440, Y-1494, Y-1526 and Y-1642 (Giancotti, 2007). Among them, Y-1494 has been reported as a key phosphorylation site of integrin β4 in regulating multiple signaling pathways important for tumor development and progression (Dutta and Shaw, 2008). Recent studies by our group also have shown the importance of Y-1494 phosphorylation in the nuclear translocation of integrin β4 and ethyl1-(3-(4-chlorophenoxy)-2-hydroxypropyl)-3-(4-chlorophenyl)-1H-pyrazole-5-carboxylate (ECPC) induced apoptosis in vascular endothelial cells (VECs) (Ge et al., 2013). Beside signals that regulate hemidesmosome, integrin β4 can also cooperate with growth factor receptors and other surface molecules to amplify intracellular signaling pathways through its subunit cytoplasmic domain, while phosphorylation events also play a critical role in these functions of integrin β4 by regulating its interactions with intracellular cytoskeleton and signaling intermediates (Dans et al., 2001; Dutta and Shaw, 2008).

As the adhesion structure of HD plays a key role in regulating tumor cell migration, most of the previous researches focused on the role of integrin β4 in cancer cells. However, high expression of integrin β4 has been shown in endothelial cells and our previous researches indicated the implication of integrin β4 in multiple signaling events in VECs, including cell death, angiogenesis, senescence, and autophagy (Wang et al., 2012). Representatively, our previous research showed that integrin β4 level is increased in nutrient deprivation induced-VEC apoptosis. Low concentration cadmium could effectively counteract the apoptosis and evoke autophagy accompanied with depressed the protein level of

*Abbreviations:* VEC, vascular endothelial cell; ANXA7, Annexin A7; HD, hemidesmosome; ECPC, ethyl1-(3-(4-chlorophenoxy)-2-hydroxypropyl)-3-(4-chlorophenyl)-1H-pyrazole-5-carboxylate; ABO, 6-amino-2, 3-dihydro-3-hydroxymethyl-1, 4-benzoxazine; oxLDL, oxidized low-density lipoprotein; HUVEC, Human umbilical vein endothelial cell; ApoE$^{-/-}$ mice, apolipoprotein E-deficient mice; LC3, microtubule-associated protein 1 light chain 3.

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integrin β4 (Dong et al., 2009), suggesting that integrin β4 may participate in the crosstalking between apoptosis and autophagy in VECs.

In addition, a novel small molecule 6-amino-2,3-dihydro-3-hydroxymethyl-1,4-benzoxazine (ABO) was previously synthesized in our laboratory (Jiao et al., 2006). Subsequently, ABO was identified as a protector and an autophagy enhancer of VECs, as ABO evidently inhibits oxidized low-density lipoprotein (oxLDL)-induced VEC apoptosis through depressing the level of integrin β4, at least partially, and stimulates autophagy by acting on annexin A7 (ANXA7) (Liu et al., 2009; Wang et al., 2010). Collectively, our results indicated that both ANXA7 and integrin β4 participate in the regulation of VEC autophagy and are regulated by ABO, however, the relationship between them during the process is not known.

More recently, we found that ABO could directly bind to ANXA7 and inhibit the phosphorylation of ANXA7 and its binding partners (Li et al., 2013), which had a significant inhibitory effect on its GTPase activity (Caohuy and Pollard, 2002). As mentioned above, Y-1494 phosphorylation of integrin β4 is involved in VEC apoptosis (Ge et al., 2013). However, the roles of integrin β4 and its phosphorylation in autophagy are not clear. Whether there is an interaction between integrin β4 and ANXA7 and whether ABO affects the phosphorylation of integrin β4 need further investigation.

Therefore, the aim of the present study is to determine the relationship between ANXA7 and integrin β4 in autophagy by utilizing ABO that directly targets ANXA7. We initially found that ABO could not induce autophagy in integrin β4-knockdown VECs and HEK293 cells, in which the level of integrin β4 is too low to detect by western blot, suggesting integrin β4 is essential for ABO-induced autophagy. Consistently, overexpression of integrin β4 in HEK293 cells restores the function of ABO in autophagy induction. Subsequently, we found that suppression ANXA7 expression by RNA interference in HEK293 cells dramatically increases the protein level of integrin β4. Under this scenario, ABO also could not induce autophagy in HEK293 cells. Finally, we identified the interaction between integrin β4 and ANXA7 by using co-immunoprecipitation. Meanwhile, ABO modulates the interaction and decreases the Y-1494 phosphorylation of integrin β4 *in vitro* and *in vivo*. In combination with our previous result that ABO could not induce autophagy in ANXA7 silencing VECs, we conclude that both integrin β4 and ANXA7 are essential for ABO induced-autophagy, and Y-1494 phosphorylation of integrin β4 may negatively regulate autophagy.

## 2. Materials and methods

### 2.1. Antibodies

Antibodies for ANXA7 (11389), integrin β4 (9090), β-actin (47778), GAPDH (365062), CD31/PECAM-1 (sc-18916) and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody for LC3 (2775) was purchased from Cell Signaling Technology. Antibody for phospho-Y1494 integrin β4 (29043) was purchased from Abcam. Secondary antibodies for immunofluorescence, donkey anti-rabbit IgG Alexa Fluor-488 (A21206) and goat anti-rat IgG Alexa Fluor-633 (A21094), were purchased from Invitrogen.

### 2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained as described by Jaffe et al. (1973) and cultured on gelatin-coated plastic dishes in M199 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, USA) and 2 ng/ml bFGF-2 at 37 °C in a humidified incubator with 5% $CO_2$. Passage number of HUVECs for experiments ranged from passages 5 to 10. HEK293 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured on plastic dishes in DMEM medium (Gibco, USA) supplemented with 10% heat-inactivated FBS at 37 °C in a humidified incubator with 5% $CO_2$.

### 2.3. Immunoprecipitation

HUVECs were washed with ice-cold PBS and lysed in IP buffer (Beyotime, China) containing 150 mM NaCl, 20 mM Tris–HCl (PH 7.5), 1% Triton X-100, and proteinase inhibitor mix. After centrifuging at 4 °C, the supernatant was collected and precleared with protein A/G agarose beads (Beyotime, China) for 1 h at 4 °C. The supernatant was collected after centrifuging and incubated with specific antibodies or normal IgG (as control), followed by incubation with Protein A/G beads overnight at 4 °C. The beads were washed three times with IP buffer and then eluted with 2× SDS loading buffer. The immunoprecipitated proteins were detected by western blot assay.

### 2.4. Western blot

Following separation by SDS-PAGE and transfering to PVDF membrane (Millipore, USA), proteins were probed with primary antibodies, then horseradish peroxidase-linked secondary antibodies, and detected with use of an enhanced chemiluminesence detection kit (Thermo). The relative quantity of proteins was analyzed by ImageJ software and normalized to loading controls.

### 2.5. Immunostaining of cells

Cells were fixed in 4% paraformaldehyde (w/v) for 15 min at room temperature and blocked in 1× PBS, 0.01% Triton X-100 (v/v) and 5% goat serum (v/v), then incubated with primary antibody overnight at 4 °C. Then cells were rinsed in 1× PBS 3 times and incubated with Alexa 488-labeled species-specific secondary antibody (1:200) for 1 h at 37 °C. For negative controls, cells were incubated with normal IgG. All samples were examined using a Leica TCS SP2 AOBS confocal laser scanning microscope. Different fields of view (>3 regions) were analyzed for each labeling condition, and representative results are shown.

### 2.6. RNA interference

Three specific siRNAs separately against integrin β4 and ANXA7 were designed and custom synthesized by Invitrogen, and scramble siRNA (37007) was purchased from Santa Cruz Biotechnology. When cell density reached 50–60% confluence, 40 nM siRNAs against integrin β4, ANXA7, and scramble siRNA (negative control) were transfected into cells with RNAiFect Transfection Reagent according to the manufacturer's protocol (QIAGEN, 1022076). We monitored the efficiency of gene silencing by western blot assay and chose the one with the best efficiency for subsequent experiments.

### 2.7. Plasmid and integrin β4 overexpression

Transient transfection was performed with lipo-fectamine TM 2000 transfection reagent according to the manufacturer's instructions. HEK293 cells at 60–70% confluence were transfected transiently with full-length integrin β4-cDNA constructs (pCMV-β4) or corresponding empty constructs for 4–6 h and normally cultured for 24 h. Then total proteins were extracted for western blot assay. The transfection efficiency of integrin β4 into HEK293



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