The essential autophagy gene ATG7 modulates organ fibrosis via regulation of endothelial-to-mesenchymal transition*

Krishna K. Singh1,2,5,7, Fina Lovren1,5, Yi Pan1,5, Adrian Quan1,5, Azza Ramadan1,5, Pratiek N. Matkar3,6, Mehroz Ehsan1,5, Paul Sandhu1,5, Laura E. Mantella1,5, Nandini Gupta1,5, Hwee Teoh1,4,5,6, Matteo Parotto9, Arata Tabuchi5, Wolfgang M. Kuebler5,7,8,10, Mohammed Al-Omran2,5,7, Toren Finkel11, Subodh Verma1,5,7,†

From the Divisions of 1Cardiac Surgery, 2Vascular Surgery, 3Cardiology, 4Endocrinology & Metabolism, and Departments of 5Surgery and 6Medicine, Keenan Research Centre for Biomedical Science and Li Ka Shing Knowledge Institute of St. Michael’s Hospital, Toronto, ON, M5B 1W8, Canada; Departments of 7Surgery and 8Physiology, and 9Interdepartmental Division of Critical Care Medicine, University of Toronto, Toronto, ON, M5S 2J7, Canada; 10Institute of Physiology, Charité-Universitätsmedizin Berlin, 10117 Berlin Germany; 11Center for Molecular Medicine, National Heart, Lung and Blood Institute, NIH Bethesda, MD 20892, USA

Running title: ATG7, Endothelial-Mesenchymal Transition and Fibrosis

†To whom correspondence should be addressed: Subodh Verma, Division of Cardiac Surgery, St. Michael's Hospital, 8th Floor, Bond Wing, 30 Bond Street, Toronto, ON, M5B 1W8, Canada, Tel.: 1-416-864-5997; Fax: 1-416-864-5881; E-mail: vermasu@smh.ca

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Background: Endothelial-to-mesenchymal transition (EndMT) is implicated in the development of organ fibrosis.

Results: Loss of the autophagy gene ATG7 promotes EndMT and upregulates TGFβ signaling and associated pro-fibrotic genes. Endothelial-specific ATG7 knockout mice exhibit increased bleomycin-induced pulmonary fibrosis.

Conclusion: ATG7 is a novel regulator of EndMT-induced organ fibrosis.

Significance: Intact endothelial autophagy prevents aberrant EndMT and represents a potential target to limit organ fibrosis.

ABSTRACT

Pulmonary fibrosis is a progressive disease characterized by fibroblast proliferation and excess deposition of collagen and other extracellular matrix components. Although the origin of fibroblasts is multifactorial, recent data implicate endothelial-to-mesenchymal transition (EndMT) as an important source of fibroblasts. We report herein that loss of the essential autophagy gene ATG7 in endothelial cells (ECs) leads to impaired autophagic flux accompanied by marked changes in EC architecture, loss of endothelial and gain of mesenchymal markers consistent with EndMT. Loss of ATG7 also upregulates TGFβ signaling and key pro-fibrotic genes in vitro. In vivo, EC-specific ATG7-knockout mice exhibit a basal reduction in endothelial-specific markers, and demonstrate an increased susceptibility to bleomycin-induced pulmonary fibrosis and collagen
accumulation. Our findings help define the role of endothelial autophagy as a potential therapeutic target to limit organ fibrosis, a condition for which presently there are no effective available treatments.

The endothelium is composed of a monolayer of endothelial cells (ECs) that line the lumen of all blood vessels. It provides a protective barrier between all tissues and the circulating blood, and functions as a selective sieve to facilitate bidirectional passage of macromolecules and gases that facilitates vascular homeostasis (1,2). ECs, however, serve additional roles amongst which is the transition of ECs into mesenchymal cells (3). This form of EC plasticity is called EndMT (3). EndMT is characterized by acquisition of the mesenchymal or myofibroblastic phenotype which can be detected by positive staining using various mesenchymal markers including alpha smooth muscle actin (αSMA), neural (N)-Cadherin and types I/III collagen with complementary loss of endothelial markers such as CD31 and vascular-endothelial (VE)-Cadherin (4). In addition to the adoption of an activated, pro-fibrogenic phenotype, ECs undergoing EndMT also lose their cell-cell junctions and gain migratory and invasive capacity (4). EndMT is recognized as a crucial component of normal heart development, endocardial cushion formation (5,6) and wound healing (7). It has of late also been implicated in a wide variety of pathological conditions including cancer and organ fibrosis (8,9). The etiologic factors that initiate fibrotic disorders are quite diverse and in most cases remain obscure. Accumulation of activated myofibroblasts in the affected tissues remains a critical element in the evolution of such diseases as well as a potential therapeutic target (4). Fibroblasts have myriad origins, although recent data suggests that EndMT, similar to epithelial-to-mesenchymal transformation, may be a quantitatively important element of this process (10). Microscopy and EC-lineage analyses conducted in animal models of tissue fibrosis support EndMT as an important source of tissue myofibroblasts in various organs (11-14). For instance, Hashimoto et al. reported that 16% of the lung fibroblasts from bleomycin (BLM)-treated mice (a surrogate model for pulmonary fibrosis) were of EC origin vs. 3% of those from saline-treated mice (13). Mechanistically, it is generally accepted that TGFβ signaling plays a crucial role in tissue fibrosis (15-17) as well as in the generation of myofibroblasts through EndMT (3,12,18,19).

In eukaryotic cells, autophagy is a highly conserved and tightly regulated cellular process involved in the turnover of cytoplasmic organelles and proteins through a lysosome-dependent degradation process (20-23). Initially described as an alternative cell death pathway, accumulating evidence suggests that autophagy is an adaptive response aimed at promoting cell survival and limiting cell death following exposure to stressful stimuli (20-24). The molecular signaling pathways regulating autophagy are complex and the readers are directed to several comprehensive articles on the topic (20-23,25,26). Briefly, autophagosome formation, a critical step in autophagy, is dependent on a catalytic step mediated by ATG7 which exhibits homology to the E1 ubiquitin activating enzymes (27). ATG7 whole body knockout mice die within 24 hours of birth (28) and conditional deletion of ATG7 in an array of tissues leads to marked alterations in tissue hemostasis, with accumulation of damaged proteins and organelles (29-31). Emerging data supports autophagic induction as a critical response of normal and cancer cells to environmental changes. In cancer cells, autophagy also
regulates epithelial-to-mesenchymal transformation and cell invasion in a TGFβ-dependent manner (32).

In the present study, we hypothesized that preserved autophagic flux is important for the prevention of aberrant EndMT and organ fibrosis. We demonstrate that knockdown of ATG7 promotes EndMT in vitro, and upregulates key genes involved in TGFβ signaling and fibrosis. In mice, EC-specific knockout of ATG7 led to an increase in BLM-induced pulmonary fibrosis. These data suggest that autophagy may be an important and novel pathway linking EndMT to organ fibrosis.

EXPERIMENTAL PROCEDURES

Cell Culture, RNA Interference and Actinomycin D Treatment — Human umbilical vein ECs (HUVECs, Lonza) and human pulmonary aortic ECs (HPAECs, Lonza) were grown in EC growth medium-2 (EGM™-2 BulletKit™; Lonza) containing growth factors, serum and antibiotics. siRNA-mediated ATG7, ATG5 gene knockdown studies were performed with siATG7, siATG5 or scrambled control (Ambion) and the Dharmafect-4 transfection reagent (Dharmacon) in accordance with the manufacturer’s guidelines. For analysis of mRNA stability, HUVECs were transfected with siATG7 or scrambled control for 24 h followed by addition of 5 μM Actinomycin D (Sigma) and the cells were harvested at the indicated time-points.

Quantitative Real Time PCR and PCR array — Total RNA was extracted with Trizol® reagent (Invitrogen) and precipitated with isopropanol. Complementary DNA (cDNA) was synthesized with the Quantitect kit (Qiagen) and subjected to quantitative RNA polymerase chain reaction (qPCR) with the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Briefly, SYBR® Select Master Mix or TaqMan® Gene Expression Assays (both Applied Biosystems) were mixed with forward and reverse primers for ATG7, CD31, VE-Cadherin, Tie2, αSMA, N-Cadherin, fibroblast-specific protein-1 (FSP1), Slug, TGFβ1, TGFBRI, TGFBRII, collagen I, connective tissue growth factor (CTGF), β-actin and GAPDH (Table 1) according to the manufacturer’s instructions. Quantitative real-time PCR analysis of 84 TGFβ-related genes was performed with the human TGFβ/BMP signaling pathway RT² profiler PCR array (PAHS-035, QIAGEN). Data were analyzed with the manufacturer’s integrated web-based software package.

Immunoblot and Immunofluorescence — Lysates from ECs were prepared in RIPA buffer (Sigma) 24, 48 and 72 hours post-transfection with either siATG7 or its scrambled control. Total protein was isolated from mouse lungs in RIPA buffer. Equal amounts of protein were loaded on SDS polyacrylamide gels and processed for immunoblot analysis with antibodies specifically directed at the following proteins: ATG7 (Cell Signaling #2631), P62 (Cell Signaling #5114), LC3 (Cell Signaling #4108), CD31 (Cell Signaling #3528), VE-Cadherin (Santa Cruz Biotechnology #6458), Tie2 (Santa Cruz Biotechnology #324), N-Cadherin (abcam #ab76057), FSP1 (Abnova #H0006275-M01), αSMA (abcam #ab5694), α-actinin (Cell Signaling #3134), TGFβ1 (abcam #ab9758), TGFBRI (Cell Signaling #3102), SMAD2 (Cell Signaling #3122), pSMAD2 (Cell Signaling #3101), SMAD3 (abcam # ab28379), pSMAD3 (abcam # ab51451), SMAD2/3 (Cell signaling # 3102), pSMAD2/3 (Cell signaling # 8828), CTGF (abcam #ab6992) and GAPDH (Millipore #MAB374). Following incubation with the appropriate horseradish peroxidase-associated secondary
antibodies (Santa Cruz Biotechnology), bands were visualized with an enhanced chemiluminescence detection system (Amersham/GE Healthcare Biosciences), and their intensities quantified by densitometry using the ImageJ software. Immunofluorescence signals from ATG7, CD31, Tie2, αSMA and α-actinin staining were visualized with standard protocols 72 h post-transfection.

**Generation of Endothelial Cell-specific ATG7 Knockout Mice** — Floxed ATG7 mice (ATG7^floxed/floxed^ mice) were crossed with VE-Cadherin Cre transgenic mice to generate EC-specific ATG7 knockout (EC-ATG7−/−) mice (33). The characterization of these mice have been previously described (33). All animal-related procedures described herein were reviewed and approved by the St. Michael’s Hospital Animal Care Committee.

**Bleomycin Instillation** — A single dose of BLM (0.0015 U/g body weight, Sigma) was instilled endotracheally on day 0 to 20 weeks old mice (34-37).

**Immunohistochemistry** — Lungs were perfused, harvested, and cleared of extraneous tissue before being fixed in O.C.T. compound (Tissue-Tek®) for cryosectioning. Sections (4 μm) were stained with CD31 (abcam #ab28364), hematoxylin and eosin (H&E) or Masson’s trichrome.

**Statistical Analysis** — Data are expressed as mean±SE unless otherwise indicated. The Student’s t-test was applied when the means of two groups were compared. Differences between multiple means were evaluated by ANOVA and, when overall differences were detected, individual means were compared post-hoc with the Bonferroni’s test. A p value <0.05 was considered to indicate statistical significance.

**RESULTS**

**Loss of ATG7 Evokes Marked Morphological and Ultrastructural Changes in HUVECs** — We used siRNA technology to knock down ATG7 expression in HUVECs. Although three groups of HUVECs were studied — non-transfected, scrambled control-transfected, and siATG7-transfected — results from the non-transfected group are not presented as they were indistinguishable from those of the scrambled control-transfected group. Quantitative PCR and immunoblot analysis confirmed markedly lower ATG7 expression in HUVECs following siATG7 transfection (Figure 1A, B). Autophagic flux is commonly evaluated by the concomitant assessment of P62 protein levels and the LC3-II to LC3-I ratio (38,39). The increase in P62 and reduction in LC3-II levels observed in ATG7-silenced cells is consistent with impaired autophagic flux (Figure 1B, C) (38,39). Notably, ATG7-silenced HUVECs, when viewed under light microscopy, exhibited a conspicuous transition from the distinctive cobblestone-like appearance to an enlarged spindle-shaped pattern that is consistent with fibroblast-like morphology (Figure 1D). These changes were accompanied by an elevation in α-actinin expression and mesenchymal cell-like cytoskeletal protein re-organization in ATG7-silenced HUVECs (Figure 1E) (40).

**Loss of ATG7 Promotes Endothelial-to-Mesenchymal Transition-like Phenotypic Switching in ECs** — The differential transcript and protein levels of EC markers CD31, VE-Cadherin and Tie2 and mesenchymal markers αSMA, N-Cadherin, FSP1 and Slug in siATG7- vs. scrambled control-transfected HUVECs is indicative
of the loss of endothelial and gain of mesenchymal markers and is consistent with EndMT (Figure 2A-C). To verify whether the loss of ATG7-associated phenotype observed is unique to HUVECs, we conducted parallel studies with HPAECs and confirmed via real-time PCR, western blot and microscopic analysis that ATG7-silenced HPAECs also displayed a marked reduction in ATG7 levels (Figure 3A, B) that was associated with an increase in P62 and a decrease in LC3-II expression i.e. impaired autophagic flux (Figure 3B). Furthermore, loss of ATG7 expression in HPAECs coincided with changes in marker expression, morphological alterations and TGFBR2 expression that are consistent with EndMT (Figure 3C-G).

**Loss of ATG7 Activates the TGFβ Signaling Pathway in HUVECs** — HUVECs lacking ATG7 had significantly higher TGFβ1 transcript and protein levels than those that had been transfected with the scrambled control (Figure 4A, B). TGFβ1 binds mainly to two serine threonine kinase receptors, transforming growth factor receptor type I (TGFBR1) and type II (TGFBR2) (41). TGFBR2 is constitutively active, and binds with and phosphorylates TGFBR1 following ligand binding (41,42). The resultant complex goes on to phosphorylate SMAD proteins and enters the cell nucleus where it acts as a transcription factor for various TGFβ-dependent pro-fibrotic genes, such as CTGF, plasminogen activator inhibitor-1 and multiple collagens (41,43-46). ATG7 loss in HUVECs corresponded with increased TGFBR1 and TGFBR2 transcript content and SMAD2/3 phosphorylation (Figure 4B-D), suggesting a molecular link between autophagy and the TGFβ network. TGFβ-responsive pro-fibrotic genes CTGF and Collagen I were also significantly upregulated in the ATG7-silenced HUVECs in comparison to the control HUVECs (Figure 4E-G).

To understand the relationship between loss of ATG7 and TGFβ signaling in endothelial cells, we performed mRNA stability/expression assay using the transcription inhibitor Actinomycin D (Act D). Our data demonstrates that loss of ATG7 in HUVECs significantly up-regulated TGFβ1, TGFBR1 and TGFBR2 at the mRNA and protein levels (Figure 4A-D). However, following Actinomycin D treatment, the RNA and protein expression levels of TGFβ1, TGFBR1 and TGFBR2 were diminished indicating reduced transcription and translation (Figure 5A-D). These results were in agreement with the data from scrambled control treated HUVECs (Figure 5A-D). Our findings demonstrate that loss of ATG7-associated increased TGFβ activity in HUVECs is most likely due to increased transcription of TGFβ ligand and its cognate receptors (Figure 5A-D).

We next tested whether other pharmacological or genetic inhibitors of autophagy had similar effect on EndMT. Incubation of HUVECs with Bafilomycin, a known inhibitor of autophagic-flux, resulted in marked up-regulation of LC3-II and P62 protein levels (Figure 6A). Pharmacological inhibition of endothelial autophagy following Bafilomycin treatment was also accompanied by significant up-regulation of the mesenchymal markers Slug, αSMA and N-Cadherin (Figure 6B). Similarly, successful knockdown of another essential autophagy gene, ATG5, also inhibited autophagy as demonstrated by increased LC3-II and P62 protein levels (Figure 6D). Knockdown of ATG5 significantly increased transcript levels of αSMA and N-Cadherin in HUVECs (Figure 6E). Additionally, loss of ATG5 or Bafilomycin treatment both upregulated TGFβ1, TGFBR1 and TGFBR2 transcript levels, similar to what was
observed in ATG7-silenced HUVECs (Figure 6C, F). In contrast to ATG7 knockdown of HUVECs, genetic inhibition by knockdown of ATG5 or pharmacological inhibition by Bafilomycin did not show a significant effect on expression of endothelial markers; CD31, Tie2 and VE-Cadherin (Figure 6B, E).

TGFβ-associated Slug induction is known to play an important role in TGFβ-induced EndMT (47). In order to investigate the role of Slug in loss of ATG7-associated TGFβ induction and EndMT, we successfully knocked down Slug using siRNA (Figure 6G, H). Loss of Slug was associated with significantly increased CD31, VE-Cadherin and Tie2 expression in siSlug-transfected HUVECs in comparison to the scrambled control-transfected HUVECs (Figure 6I). TGFβ-treatment of the siSlug- and scrambled control-transfected HUVECs significantly upregulated mesenchymal markers; αSMA, N-Cadherin and FSP1 but this up-regulation was accompanied with significantly increased endothelial markers; CD31, VE-Cadherin and Tie2 (Figure 6J), suggesting that Slug is required to induce a complete TGFβ-mediated EndMT in endothelial cells.

Endothelial Cell-specific Loss of ATG7 Exacerbates Bleomycin-induced Pulmonary Fibrosis in vivo — To evaluate the potential translational implications of our in vitro data and to circumvent the early lethality observed in systemic ATG7 knockout mice (28), we generated EC-ATG7-/− mice (48). These mice demonstrate loss of ATG7-associated impaired autophagic flux only in the ECs (33). EC-ATG7-/− mice are born in a Mendelian ratio and they do not present with any apparent basal phenotype (33). Histological assessments revealed significantly less CD31 staining in lung sections from EC-ATG7-/− mice vs. WT littermate controls (Figure 7A) and notably, these differences were most evident in the endothelial lining of the vessel walls (Figure 7A). Quantitative PCR performed on the RNA isolated from the lung tissue of the WT and EC-ATG7-/− mice demonstrated significantly reduced CD31, VE-Cadherin and Tie2 expression (Figure 7B). However, the basal transcript levels of mesenchymal markers; Slug, αSMA and N-Cadherin were similar between the groups (Figure 7B).

We observed profound alterations in the pulmonary microanatomy of BLM-treated EC-ATG7-/− when compared to similarly treated WT mice. These differences included increased alveolar septal thickening and infiltration of the parenchyma by inflammatory cells with a resultant increase in pulmonary fibrosis (Figure 7D) and collagen content (Figure 7D) 21 days after BLM instillation in the EC-ATG7-/− mice. Concordant with earlier reports (49,50), the collagen deposition and distribution pattern was primarily bronchocentric. Exacerbated pulmonary fibrosis in BLM-treated EC-ATG7-/− mice was further associated with increased TGFβ1 expression and activity, along with up-regulation of αSMA and TGFβ-responsive CTGF expression (Figure 7C).

DISCUSSION
The main observation made in this study is that loss of endothelial autophagic flux may lead to aberrant EndMT, and that organ fibrosis (as assessed in the lung), a known consequence of EndMT, was increased in vivo after conditional deletion of ATG7 in EC. These data suggest that endothelial autophagy may be a potentially novel and previously unrecognized target to limit organ fibrosis, a condition for which, at the present time, there remains no effective therapies.

The genetic and molecular basis of autophagy was initially characterized in yeast (51), and a number of autophagy-
related genes (ATG) were subsequently identified as being essential for the formation of the autophagosome, or for the subsequent fusion of the autophagosome with the lysosome (52). Since these autophagy genes are highly conserved throughout evolution, it is believed that autophagy plays a critical role in the survival of organisms. Current evidence suggests that autophagy maintains cellular homeostasis through the removal of damaged proteins and organelles, and also serves as an important pro-survival mechanism for cells during stress. Autophagic flux is precisely regulated by both extracellular and intracellular cues in an effort to promote pro-survival pathways while minimizing destruction of proteins and organelles that are required for cell survival (53). While nutrient/amino acid deprivation is recognized as the classic stress factor inducing autophagy, it has become increasingly evident that that multiple forms of stress can trigger a rise in autophagic flux. Within the cardiovascular field, most of the work on autophagy has centered on the role this process plays in the myocardium (54,55). Many are now evaluating how normal and abnormal autophagy also impacts vascular pathophysiology, and there is growing body of evidence that suggests that autophagic dysregulation may be a common pathway through which vascular aging and its associated pathologies develop (56).

Preliminary data suggest an important role for autophagic flux in the regulation of EC homeostasis and there is evidence that loss of autophagy may be a central mechanism through which risk factors cause endothelial dysfunction. Furthermore, autophagy may also be involved in the paracrine regulation and secretion of key endothelial effectors, such as nitric oxide and von Willebrand factor (vWF) (33,57). The biosynthetic pathway of vWF is complex and involves various post-translational modifications in the endoplasmic reticulum and golgi bodies, followed by assembly into discrete structures known as Weibel-Palade bodies. A reduction in vWF secretion was observed in mice lacking endothelial ATG5 or ATG7. Various vascular risk factors, including oxidized low-density lipoprotein, hyperglycemia, shear stress, β-amyloid, and angiotensin-II have been shown to impair EC function, in part through inhibiting autophagic flux (58-60). Although ECs are known to display a significant amount of plasticity, particularly under stressful and pathophysiologicaal conditions, there has been no report linking endothelial autophagy to maintenance of cellular architecture, phenotypic transformation and/or mesenchymal transition.

EndMT was first described during embryonic cardiogenesis whereby ECs give rise to mesenchymal cells that form the endocardial cushion, which in turn contribute to the development of the septa and cardiac valves. Evidence suggests that EndMT occurs in the post-natal state as well, and is involved in the development of organ fibrosis, pulmonary vein stenosis, aberrant vascular remodeling, calcification (via osteoblastic and chondrocytic differentiation), and more recently cerebral cavernous malformations (18,61-64). The hallmark of these phenomena is usually a loss of endothelial and gain of mesenchymal and stem-cell like markers. The TGFβ pathway is the major inducer of EndMT, which is mediated largely through phosphorylation of SMAD proteins, that in turn, regulate transcription of key target genes (12). Understanding the biological cues that regulate EndMT can therefore provide important insights into the pathophysiology and potential treatment of several specific diseases, in addition to the more general process of organ fibrosis.
Pulmonary fibrosis is a devastating disease for which there remains no cure. Since the accumulation of myofibroblasts is the pathologic feature of pulmonary fibrosis, research efforts to date have predominantly centered on identifying the source of fibroblasts. Although historically it was believed that fibroblasts arise from resident pulmonary mesenchymal cells, recent data suggest that a significant proportion of the fibroblast population in fact originate from epithelial-to-mesenchymal and EndMT, in addition to bone marrow progenitor cells that are recruited to the injured lung (61).

Several risk factors that contribute to pulmonary fibrosis appear to overlap and potentially modulate autophagic flux. For example, advanced age is one of the key risk factors for pulmonary fibrosis. It is also well recognized that autophagy diminishes with advanced age, and it has been suggested that the resultant accumulation of damaged proteins and organelles may incite a profound ER stress response leading to cellular senescence characteristic of pulmonary fibrosis (65). Indeed, impaired epithelial autophagy has been found in idiopathic pulmonary fibrosis; immunohistochemical evaluation of human IPF specimens revealed that epithelial cells showed increased cellular senescence, and both epithelial cells and fibroblasts in the fibroelastic foci expressed increased ubiquitinated proteins and P62 (66). Furthermore, in *in vitro* models, autophagy inhibition was sufficient to induce epithelial cell senescence and myofibroblast differentiation (67). These data argue that loss of autophagy may be a central mechanism through which pulmonary fibrosis occurs (65). Although both epithelial to mesenchymal and EndMT transition are important sources of fibroblasts, we focused our study specifically on the role of autophagy in EndMT-induced pulmonary fibrosis.

In both HUVECs and HPAECs, loss of endothelial autophagy, induced by silencing of the essential autophagy gene ATG7, promoted EndMT. Indeed, silencing of ATG7 was accompanied by marked morphological and ultra-structural changes changing cells from an initial “cobblestone-like EC morphology” to an enlarged spindle-shaped, smooth surfaced “fibroblast-like morphology” (Figure 1D, E and 3E, F). These morphological changes further corresponded to cytoskeletal protein re-organization, confirmed by α-Actinin staining (Figure 1E and 3F). The “fibroblast-like morphology” was further associated with reduced expression of the endothelial markers CD31, VE-Cadherin, and Tie2, and increased expression of the mesenchymal markers αSMA, N-Cadherin, and FSP1 (Figure 2A-C, 3C, D). VE-Cadherin is processed through the endosomal-lysosomal pathway, and autophagy/lysosomal inhibitors have been previously shown to prevent VE-Cadherin degradation resulting in accumulation of VE-Cadherin protein (68). In accord with these past observations, we observed a significant reduction in VE-Cadherin transcript content but accumulation of VE-Cadherin protein, arguably due to impaired processing of VE-Cadherin in ATG7-deficient HUVECs (Figure 2B). Loss of ATG7 associated accumulation of VE-Cadherin protein was not specific to HUVECs as similar accumulation was observed in ATG7-silenced HPAECs (Figure 3D).

Since TGFβ signaling is the main driver of EndMT, we evaluated this signaling pathway following ATG7 silencing. We found that in ATG7-silenced HUVECs, TGFβ signaling was upregulated, and specifically expression of TGFβ1 and its receptors TGFBR1 and TGFBR2 were increased. This upregulation of TGFβ1 and its receptors was further associated with increased SMAD2/3 phosphorylation, that
contributed to the observed enhanced transcription of pro-fibrotic genes (Figure 4A-G). A PCR array for human TGFβ and BMP signaling was performed to further assess the TGFβ signaling activation. As shown in Table 2, several TGFβ signaling-associated genes including NOG, BMPR1B, TGFβ1, ITGB5, PDGFB, FOS, FST, COL1A1, SOX4, CDC25A, DLX2 and BMP5 were significantly upregulated (Table 2).

We also tested whether other pharmacological or genetic inhibitors of autophagy had similar effect on EndMT. Incubation of HUVECs with Bafilomycin, a known inhibitor of autophagic flux resulted in marked up-regulation of LC3-II and P62 protein levels (Figure 6A). Inhibition of autophagy following Bafilomycin treatment was accompanied by significant up-regulation of the mesenchymal markers; Slug, αSMA and N-Cadherin (Figure 6B). Similarly, successful knockdown of other essential autophagy gene ATG5 also inhibited autophagy as shown by increased LC3-II and P62 protein levels (Figure 6D) and significantly upregulated the transcript levels of αSMA and N-Cadherin in HUVECs (Figure 6E). Similar to loss of ATG7 in HUVECs, pharmacological (by Bafilomycin) and genetic inhibition (by loss of ATG5) both upregulated TGFβ1, TGFBR1 and TGFBR2 expression and increased mesenchymal markers (Figure 6C, F). In contrast to ATG7 knockdown in HUVECs, genetic inhibition by knockdown of ATG5 or pharmacological inhibition by Bafilomycin did not show a significant effect on the expression level of endothelial markers CD31, Tie2 and VE-Cadherin (Figure 6B, E). The basis for these differences is not entirely clear at this point, although ATG7 does have autophagy-independent functions (69).

The BLM-induced model of pulmonary fibrosis is a widely employed experimental model of organ fibrosis. To directly link endothelial autophagy with the pathobiology of pulmonary fibrosis, we treated ATG7 EC-specific conditional knockout mice with BLM. Lungs from EC-ATG7-/- mice demonstrated a modest but significant basal reduction in the endothelial marker CD31, VE-cadherin and Tie2 (Figure 7A, B). Twenty-one days following BLM treatment, there was greater fibrosis noted in the lungs of EC-ATG7-/- mice compared to WT controls. Exacerbated pulmonary fibrosis in BLM-treated EC-ATG7-/- mice was further associated with increased TGFβ1 expression and activity, along with up-regulation of αSMA and TGFβ-responsive CTGF expression (Figure 7C). Collagen content is an established measure of murine pulmonary fibrosis (70) and increased collagen content were observed in lungs of EC-ATG7-/- mice compared to their WT littermate controls following BLM administration (Figure 7D). A putative model is presented in Figure 8 that summarizes the effect of EC-specific loss of ATG7 on EndMT and organ fibrosis.

In conclusion, we provide novel evidence to suggest that loss of in vivo endothelial autophagy exacerbates the fibrotic response in mice. We propose that this occurs through a regulatory effect of autophagy on restraining TGFβ-dependent EndMT.
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VTG7 in Endothelial-Mesenchymal Transition and Fibrosis


FOOTNOTES

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†To whom correspondence should be addressed: Subodh Verma, Division of Cardiac Surgery, St. Michael's Hospital, 8th Floor, Bond Wing, 30 Bond Street, Toronto, ON, M5B 1W8, Canada, Tel.: 1-416-864-5997; Fax: 1-416-8645881; E-mail: vermasu@smh.ca
‡The abbreviations used are: EndMT, endothelial-to-mesenchymal transition; EC(s), endothelial cell(s); αSMA, alpha smooth muscle actin; BLM, bleomycin; CTGF, connective tissue growth factor; N-cadherin, neural cadherin; EC-ATG7−/−, endothelial-cell specific ATG7 knockout; FSP1, fibroblast-specific protein-1; H&E, hematoxylin and eosin; HPAECs, human pulmonary artery endothelial cells; HUVECs, human umbilical vein endothelial cells; pSMAD, phosphorylated SMAD; qPCR, quantitative RNA polymerase chain reaction; TGFB, transforming growth factor receptor type; VE-cadherin, vascular-endothelial-cadherin; vWF, von Willebrand factor

From the Divisions of ¹Cardiac Surgery, ²Vascular Surgery, ³Cardiology, ⁴Endocrinology & Metabolism, and Departments of ⁵Surgery and ⁶Medicine, Keenan Research Centre for Biomedical Science and Li Ka Shing Knowledge Institute of St. Michael’s Hospital, Toronto, ON, M5B 1W8, Canada; Departments of ⁷Surgery and ⁸Physiology, and ⁹Interdepartmental Division of Critical Care Medicine, University of Toronto, Toronto, ON, M5S 2J7, Canada; ¹⁰Institute of Physiology, Charité-Universitätsmedizin Berlin, 10117 Berlin Germany; ¹¹Center for Molecular Medicine, National Heart, Lung and Blood Institute, NIH Bethesda, MD 20892, USA

FIGURE LEGENDS

Figure 1. Loss of ATG7 impaired autophagic flux and induced marked morphological changes in HUVECs. (A) Cultured HUVECs were transfected with either siATG7 or scrambled control (5 nM) for up to 72 h. RT-PCR revealed successful silencing of ATG7 (~80% reduction) at 24 h. Representative image of an agarose gel showing the PCR products of ATG7 and GAPDH primers derived from equal amounts of cDNA. (B) ATG7 silencing was accompanied by a reduction in autophagic flux. Western blotting confirmed lower ATG7 and LC3-II levels, as well as higher P62 levels, in HUVECs lacking ATG7. GAPDH was used as a loading control. (C) Quantitative analysis indicated that siATG7 transfection significantly and time-dependently reduced the LC3-II/LC3-I ratio in HUVECs. (D) Scrambled control-transfected HUVECs, cultured on a two-dimensional plate, formed a confluent monolayer with the typical EC ‘cobblestone’ morphology (left panel). ATG7 silencing resulted in marked morphological changes whereby HUVECs took on an enlarged spindle-shaped appearance with smooth surfaces (right panel). Both micrographs were taken at the same magnification, scale bar = 200µm. (E) Immunofluorescent micrographs demonstrating cytoskeletal protein re-organization and ATG7 expression in HUVECs following ATG7 silencing. α-Actinin positivity is indicated in red; ATG7 positivity is indicated in green
and nuclei were stained with DAPI (blue); scale bar = 10µm. *p<0.05 and ***p<0.001 vs. corresponding scrambled control. n = 3-4 in triplicates.

**Figure 2.** Loss of ATG7 promotes EndMT in HUVECs. HUVECs were transfected with scrambled control or siATG7. Total RNA and protein were extracted at 24 h and 48 h, respectively. Differential (A) transcript (qPCR) data presented as a fold change to the scrambled control and (B) protein (western blotting) levels of key endothelial and mesenchymal markers as well as (C) CD31 (green), Tie2 (green), αSMA (green) and ATG7 (red) immunofluorescent staining in scramble control- and siATG7-transfected HUVECs indicate EndMT with ATG7 silencing. Nuclei were stained with DAPI (blue). Micrographs are representative images of HUVECs taken 72 h post-transfection; scale bar for scrambled = 10µm and scale bar for siATG7 = 20µm.

**Figure 3.** Loss of ATG7 promotes EndMT in HPAECs. (A) Cultured HPAECs were transfected with either siATG7 or scrambled control (5 nM) for up to 48 h. RT-PCR revealed successful silencing of ATG7 (~80% reduction) at 24 h. (B) ATG7 silencing was accompanied by a reduction in autophagic flux. Western blotting confirmed lower ATG7 and LC3-II levels, as well as higher P62 levels, in HPAECs lacking ATG7. GAPDH was used as a loading control. Differential (C) transcript (qPCR) data presented as a fold change to the scrambled control and (D) protein (western blotting) levels of key endothelial and mesenchymal markers. (E) ATG7 silencing resulted in marked morphological changes whereby HPAECs took on an enlarged spindle-shaped appearance with smooth surfaces. (F) Immunofluorescent micrographs demonstrating cytoskeletal protein re-organization and ATG7 expression in HPAECs following ATG7 silencing. α-Actinin positivity is indicated in red; ATG7 positivity is indicated in green and nuclei were stained with DAPI (blue); scale bar = 10µm. (G) Western blotting for TGFBR2 on HPAECs protein lysate following 48 h post-transfection. *p<0.05, **p<0.01, ***p<0.001 vs. corresponding scrambled control group. n = 3 in triplicates.

**Figure 4.** Loss of ATG7 activates TGFβ signaling in HUVECs. HUVECs were transfected with scrambled control or siATG7. Total RNA and protein were extracted at 24, 48 and 72 h post-transfection. ATG7 knockdown elevated, in a time-dependent fashion, (A) TGFβ1 transcript and (B) TGFβ1, TGFBR1, pSMAD2/3 and SMAD2/3 protein expressions. These changes were accompanied by up-regulation of (C, D) TGFBR1 and TGFBR2 transcript levels, (E, F) CTGF and collagen I transcript level and (G) protein levels. *p<0.05, **p<0.01, ***p<0.0001 vs. corresponding scrambled control group. n = 3 in triplicates.

**Figure 5.** Loss of ATG7 up-regulates transcription of TGFβ and its receptors. HUVECs were transfected with siATG7 or scrambled control for 24 h followed by addition of 5μM Actinomycin D (Act D) and the cell lysates were then collected at indicated time-points. Quantitative PCR for TGFβ1, TGFBR1 and TGFBR2 show significant reduction at transcript (A, B, C) and protein (D) levels following Act D treatment. *p<0.05, **p<0.01, ***p<0.0001 vs. corresponding scrambled control group. n = 3 in triplicates.

**Figure 6.** Effect of pharmacologic and genetic inhibition of autophagy in HUVECs. Incubation of HUVECs with Bafilomycin (20 µM) resulted in marked up-regulation of LC3-II and P62 protein levels (A). Quantitative PCR data (presented as a fold change to the scrambled control)
on HUVECs for EndMT markers (B), TGFβ1, TGFBR1 and TGFBR2 24 h post-treatment (C). Transfection of HUVECs with siATG5 (5nM) resulted in reduced ATG5 and marked up-regulation of LC3-II and P62 protein levels (D). Quantitative PCR data on HUVECs for EndMT markers (E), TGFβ1, TGFBR1 and TGFBR2 24 h post-transfection (F). Transfection of HUVECs with siSlug (5nM) resulted in marked reduction in Slug at transcript (G) and protein levels (H). Quantitative PCR data on HUVECs for CD31, VE-Cadherin and Tie2 after 24 h post-silencing (I), and EndMT marker analysis of the Slug-silenced HUVECs 24 h post TGFβ1 (10 ng/mL) -treatment (J). All qPCR data is presented as a fold change to the scrambled control. *p<0.05, **p<0.01, corresponding control group. n = 3 in triplicates.

**Figure 7.** Endothelial-specific loss of ATG7 exacerbates BLM-induced pulmonary fibrosis in vivo. Lungs, harvested from 20-weeks old EC-ATG7−/− mice and their WT littermate controls, were used for RNA extraction and also immunostained for CD31. (A) Representative photomicrograph and quantification data confirm significantly lower CD31 expression in lungs from EC-ATG7−/− mice. Blue arrow points to the endothelial lining of the vessel wall exhibiting reduced CD31 (brown) expression. Scale bar = 20µm. V; vessel, B; bronchiole. (B) Quantitative PCR data for EndMT markers. (C) Western blot for CTGF, TGFβ1, pSMAD2/3, SMAD2/3, αSMA and GAPDH, and (D) representative images of H&E- and Masson's trichrome-stained lung sections revealing that 21 days post-BLM instillation, there was greater pulmonary fibrosis and significantly augmented collagen deposition (green) in EC-ATG7−/− vs. WT mice. Corresponding quantitative assessment of collagen content in lungs from BLM-treated mice. n = 3-4; *p<0.05, **p<0.01 vs. corresponding WT data.

**Figure 8.** A schematic representation of the complex interplay between ATG7 and the TGFβ-signaling pathway in the regulation of EndMT and fibrosis. EC-specific loss of ATG7 upregulates and activates TGFβ and its receptors culminating in phosphorylated SMAD-mediated Slug, CTGF and Col1 transcription. Slug in turn downregulates the expression of the endothelial markers VE-cadherin and CD31 which propel the EndMT process. EndMT along with increased expression of CTGF and Col1 exacerbate the fibrotic phenotype.
### Table 1
qPCR Primers

<table>
<thead>
<tr>
<th>Genes</th>
<th>Nucleotide Sequences</th>
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<tbody>
<tr>
<td>Hs-ATG7- Forward</td>
<td>5'-ATTGCTGCATCAAGAAACCC-3'</td>
</tr>
<tr>
<td>Hs-ATG7- Reverse</td>
<td>5'-GATGGAGAGCTCTCCTCAGCA-3'</td>
</tr>
<tr>
<td>Hs-CD31- Forward</td>
<td>5'-CCTTCTGCTCTGTCAAGCC-3'</td>
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<tr>
<td>Hs-CD31- Reverse</td>
<td>5'-GGGTCAGGTCTTTCCCATTT-3'</td>
</tr>
<tr>
<td>Hs-VE-Cadherin- Forward</td>
<td>5'-ACAGAGCTCCACTACGCTC-3'</td>
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<td>Hs-VE-Cadherin- Reverse</td>
<td>5'-CATGAGCTCTGCATCTTCC-3'</td>
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<tr>
<td>Hs-Slug- Forward</td>
<td>5'-TCGGACCAATACATCCTT-3'</td>
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<td>Hs-N-Cadherin- Reverse</td>
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<td>Hs-FSP-1- Reverse</td>
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<td>Mm-Tie2-Forward</td>
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</tr>
<tr>
<td>Mm-Tie2-Reverse</td>
<td>5'-TTTCGCGCATCAGACACAAGA-3'</td>
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List of primers used to perform qPCR for human (Hs; *Homo sapiens*) and mouse (Mm; *Mus musculus*) genes.
Table 2
TGFβ/BMP Signaling Pathway PCR Array

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Up-regulated genes</th>
<th>24 h (Fold change)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>NOG</td>
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<tr>
<td>2</td>
<td>BMPR1B</td>
<td>3.8406*</td>
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<tr>
<td>3</td>
<td>TGFβ1</td>
<td>1.8821*</td>
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<tr>
<td>4</td>
<td>ITGB5</td>
<td>1.9903*</td>
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<tr>
<td>5</td>
<td>PDGFβ</td>
<td>1.5168*</td>
</tr>
<tr>
<td>6</td>
<td>FOS</td>
<td>1.854*</td>
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<tr>
<td>7</td>
<td>FST</td>
<td>1.2684</td>
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<td>8</td>
<td>COL1A1</td>
<td>1.42 *</td>
</tr>
<tr>
<td>9</td>
<td>SOX4</td>
<td>1.6062*</td>
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<tr>
<td>10</td>
<td>CDC25A</td>
<td>1.2775*</td>
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<td>11</td>
<td>DLX2</td>
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<tr>
<td>12</td>
<td>BMP5</td>
<td>2.148*</td>
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</table>

Genes upregulated in ATG7-silenced vs. control ECs. *p<0.05, ***p<0.001 vs. control group.
FIGURE 1

A

ATG7/GAPDH mRNA levels

Scrambled Control
siATG7

ATG7
GAPDH

B

LC3-I
LC3-II
P62
GAPDH

Scrambled Control
siATG7 (24 h)
siATG7 (48 h)
siATG7 (72 h)

C

LC3-II/LC3-I Ratio

Scrambled Control
siATG7 (24 h)
siATG7 (48 h)
siATG7 (72 h)

D

Scrambled Control
siATG7

Microscopy

E

DAPI α-Actinin ATG7 Merged

Scrambled Control
siATG7

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FIGURE 2

A

mRNA levels (Fold change)

CD31  VE-Cadherin  Tie2  Slug  α-SMA  N-Cadherin  FSP1

-1  0  1  2  3  4

*  **  ***  ***

B

Scrambled Control

siATG7

CD31  VE-Cadherin  Tie2  GAPDH

α-SMA  N-Cadherin  FSP1  GAPDH

C

Scrambled Control

DAPI CD31 ATG7 Merged

DAPI Tie2 ATG7 Merged

DAPI α-SMA ATG7 Merged

siATG7

DAPI CD31 ATG7 Merged

DAPI Tie2 ATG7 Merged

DAPI α-SMA ATG7 Merged
ATG7/GAPDH mRNA levels

Scrambled Control  siATG7

FIGURE 3

ATG7
LC3-I
LC3-II
P62
GAPDH

Scrambled Control  siATG7 (24 h)
siATG7 (48 h)
siATG7 (72 h)

Scrambled Control  siATG7

CD31
Tie2
VE-Cadherin
αSMA
N-Cadherin
FSP1
GAPDH

Scrambled Control  siATG7

TGFBR2
GAPDH

Scrambled Control  siATG7

TGFBR2
GAPDH

Scrambled Control  siATG7

Microscopy

Scrambled Control  siATG7

GAPDH

Scrambled Control  siATG7

GAPDH
FIGURE 4

A

B

C

D

E

F

G

Scrambled Control
siATG7 (24 h)
siATG7 (48 h)
siATG7 (72 h)

Scrambled Control
siATG7 (24 h)
siATG7 (48 h)
siATG7 (72 h)

Scrambled Control
siATG7 (24 h)
siATG7 (48 h)
siATG7 (72 h)

Scrambled Control
siATG7 (24 h)
siATG7 (48 h)
siATG7 (72 h)

TGFβ1/GAPDH mRNA levels
(Fold change)

TGFβ1
TGFB1
TGFB1R1
pSMAD2
SMAD2
pSMAD3
SMAD3
GAPDH

CTGF/GAPDH mRNA levels
(Fold change)

CTGF
GAPDH

Collagen I/GAPDH mRNA levels
(Fold change)

Collagen I
CTGF
GAPDH

*  **  ***

0 1 2 3

0 1 2 3

0 1 2 3

0 1 2 3

TGFBR1/GAPDH mRNA levels
(Fold change)

TGFBR1
GAPDH

*  ***

0 1 2 3

*  ***

*  ***
FIGURE 5

A. TGFβ1/GAPDH mRNA levels (Percent)

B. TGFBR1/GAPDH mRNA levels (Percent)

C. TGFBR2/GAPDH mRNA levels (Percent)

D. Western Blot

- TGFβ1
- TGFBR1
- TGFBR2
- GAPDH

Scrambled Control
siATG7
Scrambled Control + Act D
siATG7 + Act D
FIGURE 7

A

CD31

WT

EC-ATG7−/−

B

mRNA levels (Fold change)

CD31

VE-Cadherin

Tie2

Slug

αSMA

N-Cadherin

C

WT (BLM) EC-ATG7−/− (BLM)

CTGF

TGFβ1

pSMAD2/3

SMAD2/3

αSMA

GAPDH

D

WT (BLM) EC-ATG7−/− (BLM)

H&E

Masson's Trichrome

Collagen Ratio in Lung (AU)
FIGURE 8

Loss of ATG7

- P53
- P21
- TGFβ1
- TGFB1 1/2
- SMAD2
- SLUG
- COL1
- CTGF
- VE-Cadherin CD31

Proliferation

EndMT

Fibrosis
The essential autophagy gene ATG7 modulates organ fibrosis via regulation of endothelial-to-mesenchymal transition

Krishna K. Singh, Fina Lovren, Yi Pan, Adrian Quan, Azza Ramadan, Pratiek N. Matkar, Mehroz Ehsan, Paul Sandhu, Laura E. Mantella, Nandini Gupta, Hwee Teoh, Matteo Parotto, Arata Tabuchi, Wolfgang M. Kuebler, Mohammed Al-Omran, Toren Finkel and Subodh Verma

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