Autophagic degradation of HAS2 in endothelial cells: A novel mechanism to regulate angiogenesis

Carolyn G. Chen⁹, Maria A. Gubbiotti⁹, Aastha Kapoor⁹, Xiaorui Han⁸, Yanglei Yu⁸, Robert J. Linhardt⁸ and Renato V. Iozzo⁹

⁹ - Department of Pathology, Anatomy and Cell Biology and the Cell Biology and Signaling Program, Kimmel Cancer Center, Sidney Kimmel Medical College at Thomas Jefferson University, Philadelphia, PA, USA
⁸ - Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, USA

Correspondence to Renato V. Iozzo: Renato.iozzo@jefferson.edu

https://doi.org/10.1016/j.matbio.2020.02.001

Abstract

Hyaluronan plays a key role in regulating inflammation and tumor angiogenesis. Of the three transmembrane hyaluronan synthases, HAS2 is the main pro-angiogenic enzyme responsible for excessive hyaluronan production. We discovered that HAS2 was degraded in vascular endothelial cells via autophagy evoked by nutrient deprivation, mTOR inhibition, or pro-autophagic proteoglycan fragments endorepellin and endostatin. Using live-cell and super-resolution confocal microscopy, we found that protracted autophagy evoked a dynamic interaction between HAS2 and ATG9A, a key transmembrane autophagic protein. This regulatory axis of HAS2 degradation occurred in various cell types and species and in vivo upon nutrient deprivation. Inhibiting in vivo autophagic flux via chloroquine showed increased levels of HAS2 in the heart and aorta. Functionally, autophagic induction via endorepellin or mTOR inhibition markedly suppressed extracellular hyaluronan production in vascular endothelial cells and inhibited ex vivo angiogenic sprouting. Thus, we propose autophagy as a novel catabolic mechanism regulating hyaluronan production in endothelial cells and demonstrate a new link between autophagy and angiogenesis that could lead to potential therapeutic modalities for angiogenesis.

© 2020 Elsevier B.V. All rights reserved.

Introduction

The extracellular matrix (ECM) encompasses a variety of different signaling effectors to regulate an abundance of dynamic roles ranging from tissue homeostasis to development [1–3]. Of note, extracellular proteoglycans masterfully synchronize a wide array of biological functions via outside-in cues, oscillating from maintaining tissue hydrodynamics and structural integrity to initiating cellular function and activating signal transduction via interaction with integrins and receptor tyrosine kinases (RTK) [4–13]. In particular, perlecan, a modular heparan sulfate proteoglycan (HSPG), supports a host of functions, including cell adhesion, lipid metabolism, angiogenesis, endocytosis, thrombosis, autophagy [14] and blood-brain barrier maintenance [15]. Perlecan is present not only in vascular basement membranes and pericellular spaces [16], but also in human sera as a major circulating protein. Structurally, it consists of a ~500 kDa protein core spanning 5 domains, which undergoes partial proteolysis via matrix metalloproteinases to cleave its C-terminal domain V, endorepellin [17]. Endorepellin consists of three laminin-like globular (LG) domains, allowing it to partake in partial antagonistic activity by dually signaling through the vascular endothelial growth factor receptor 2 (VEGFR2), the major vascular RTK [18] and α2β1 integrin to induce protracted autophagy and disassemble the actin cytoskeleton, respectively. These contemporaneous actions of endorepellin ultimately lead to a robust anti-migratory and anti-angiogenic phenotype [19–22]. Importantly, endorepellin-mediated signaling via VEGFR2 also results in phosphorylation of adenosine monophosphate kinase (AMPKα at Thr-172), leading to inhibition of mammalian target of rapamycin (mTOR) and induction of autophagy.
markers including Peg3, Beclin 1, LC3, and p62 [23–28]. Taken together, these actions of endorepellin position it as a key soluble pro-autophagic and anti-angiogenic molecule with promising therapeutic effects against tumor vasculature [17,19,29–31].

Hyaluronan (HA), another major component of the ECM, is a ubiquitous linear glycosaminoglycan composed of repeating disaccharide units of glucuronic acid and N-acetyl-glucosamine. HA is synthesized at the plasma membrane via multi-pass transmembrane enzymes of the HA synthase family (HAS1-3) [32,33]. Each of these enzymes polymerize varying chain lengths of HA, with both HAS1 and HAS2 producing high molecular weight HA (~2000 kDa) and HAS3 producing lower molecular weight HA (100–1000 kDa). Each HAS exhibits unique glycosyltransferase activity that incorporates UDP-sugars to the non-reducing end of the growing glycosaminoglycan at the cytosolic leaflet of the membrane while simultaneously extruding the reducing end from the cell [32]. Notably, HAS2 depends on key post-translational modifications that affect its trafficking and activity [34].

In contrast to most large extracellular matrix components, extracellular HA can be rapidly altered due to its impressive turnover rate. After synthesis via HASes, HA may be subjected to degradation via a variety of hyaluronidases (mainly HYAL1 and 2) [35]. Although simple in composition, HA regulates a variety of cellular functions including wound repair, inflammation, cell migration, and angiogenesis [36–40] and recently emerged as a key player in regulating the tumorigenic and inflammatory milieu [36,41–46]. Intriguingly, its physiological effects are size-dependent: full-length HA (~100–2000 kDa) is anti-inflammatory whereas its processed fragments (<36 kDa) are pro-inflammatory and pro-angiogenic.

In a variety of diseases including cancer, synthesis of HA via HASes and degradation via hyaluronidases, reactive oxygen species, and mechanical forces are globally increased, leading to an increased deposition of HA fragments [35]. From the synthesis end, HAS2 is one of the main drivers of increased extracellular deposition of lower molecular weight HA in a variety of inflammatory pathologies, creating a microenvironment favoring angiogenesis and inflammation that perpetuates disease progression [36,41,45,47]. In breast cancer, aberrant over-expression of HAS2 is involved in tumor growth and differentiation, axillary lymph node metastasis, and decreased patient survival [42,48–52]. Furthermore, knockdown of HAS2 inhibits breast cancer growth and attenuates HA expression. Likewise, in other solid tumors of the ovary, colorectum, and prostate, HAS2 is an important regulator of tumorigenicity and metastasis through excessive production of HA [42,53,54]. Notably, phosphorylation of HAS2 by AMPK and O-GlcNAcylation modulate its enzymatic activity and stability, respectively [55,56].

As endorepellin evokes autophagy via AMPK [27,57], an enzyme critical for autophagic progression [58], and as AMPK regulates HAS2 activity [56], we hypothesized that HAS2 protein levels could be directly regulated via autophagy. Here, we leveraged three independent activators of autophagy to investigate the modulation of protracted autophagy on HAS2: endorepellin, the proteolytic fragment of perlecan [59], Torin 1, an ATP-competitive second generation mTOR inhibitor [60], and endostatin, the C-terminal fragment of collagen XVIII [57,61]. We showed that all three activators evoked downregulation and autophagic flux of HAS2 protein, with the former two resulting in a selective and marked decrease in extracellular HA. Upon autophagic activation, we found that HAS2 dynamically interacted with ATG9A, the only multi-pass transmembrane protein in the core autophagic complex. Finally, autophagic breakdown of HAS2 profoundly suppressed extracellular HA and angiogenic sprouting. Our findings provide a general physiological mechanism whereby an intracellular catabolic program can ultimately regulate the levels of a powerful pro-angiogenic and pro-tumorigenic polysaccharide. As such, leveraging autophagy could be of potential beneficial use against various forms of solid tumors where angiogenesis is prominent.

## Results

### Endorepellin downregulates HAS2 in vascular endothelial cells

To investigate the intracellular catabolism of HAS2, we first utilized endorepellin, the potent anti-angiogenic [19] and pro-autophagic [27,59] C-terminus of perlecan, one of the major heparan sulfate proteoglycans of basement membranes and cell surfaces [6]. Increasing concentrations of endorepellin activated AMPKζ via phosphorylation at Thr172 in human umbilical vein endothelial cells (HUVEC) (Fig. 1A and B). Remarkably, we found that endorepellin downregulated HAS2 levels in a dose-dependent fashion (IC50~270 nM, Fig. 1C). Time-course experiments revealed that endorepellin evoked sustained activation of AMPKζ at Thr172 (Fig. 1D and E), known to induce endothelial cell autophagy [26], while concurrently downregulating HAS2 protein levels (T1/2~7.5 h, Fig. 1F). Validating our published findings that endorepellin evokes autophagy by modulating autophagic marker LC3-II in vascular endothelial cells [27], we show significant increase in LC3-II levels upon time-dependent and dose-dependent treatments with endorepellin (Fig. S1A and B). Endorepellin also dose-
Fig. 1. Endorepellin downregulates levels of HAS2 protein through VEGFR2 signaling while concurrently inducing P-AMPKα T172. (A) Representative Western blot (WB) showing induction of P-AMPKα T172 and downregulation of HAS2 in HUVEC whole-cell lysates treated with increasing concentrations of endorepellin for 6 h. GAPDH served as the loading control. Quantification of P-AMPKα T172 (B) and HAS2 (C) from (A). (D) Representative WB of HUVEC time-course experiments with endorepellin (200 nM). Quantification of P-AMPKα T172 (E) and HAS2 (F) from (D). Data normalized to GAPDH; mean ± SEM. n = 3 biological replicates. (G) Representative WB of HUVEC whole-cell lysates pretreated with scrambled siRNA (siScr) or siRNA targeting VEGFR2 (siVEGFR2) ± endorepellin (200 nM) for 6 h. Quantification of VEGFR2 (H) and HAS2 (I) from (G). Statistical analyses were calculated via One-way ANOVA. (J) Representative WB of HUVEC treated with vehicle (DMSO) or AICAR (500 μM) for 6 h. (K) Quantification of HAS2, P-AMPKα T172/AMPKα, and P-ACC S79/ACC from (J). Statistical analyses were calculated via two-tailed unpaired Student's t-test. n = 3 biological replicates. Data normalized to GAPDH; horizontal bars = mean. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
dependently downregulated HAS2 protein levels in two other vascular endothelial cell types, porcine aortic endothelial cell overexpressing VEGFR2 (PAER2) and human aortic endothelial cells immortalized by stably expressing human telomerase catalytic subunit hTERT (TeloHAEC) (Fig. S2A and B). Modulation of HAS2 occurred post-transcriptionally, as mRNA profiles of HAS2 remained unchanged in HUVEC treated with endorepellin for 6 and 24 h vis-à-vis vehicle (Fig. S3A). Moreover, HAS3 mRNA levels showed no significant changes in the presence of endorepellin (Fig. S3B), whereas HAS1 mRNA levels were barely detectable (Fig. S3C).

Next, as endorepellin activity in endothelial cells is mediated by a direct interaction with the ectodomain of VEGFR2 [31] and to investigate key signaling players in this pathway, we transiently knocked down VEGFR2 via siRNA. Suppression of VEGFR2 expression prevented downregulation of HAS2 by endorepellin (Fig. 1G–I), confirming that VEGFR2 is critical for endorepellin-evoked decrease of HAS2. To verify whether AMPK activation was mechanistically involved in the downstream catabolism of HAS2, we treated HUVEC with AICAR, an AMP-mimetic that pharmacologically induces direct phosphorylation and activation of AMPK and downstream targets such as acetyl-CoA carboxylase (ACC). Concurrent with increased phosphorylation of both AMPK and ACC, treatment with AICAR for 6 h was sufficient to cause a two-fold reduction in HAS2 levels (Fig. 1J and K). Thus, signaling through both VEGFR2 and AMPK are mechanistically involved in this catabolic pathway of HAS2, and the pro-autophagic perlecan fragment endorepellin specifically and dynamically regulates HAS2 levels in

**Fig. 2.** Nutrient deprivation, mTOR inhibition, and endostatin downregulate HAS2 protein levels concurrent with LC3-II induction. (A) Representative WB of HUVEC time-course experiments with nutrient deprivation (EBSS). (B) Quantification of HAS2 and LC3-II from (A). (C) Representative WB of PAER2 time-course experiments with nutrient deprivation. (D) Quantification of HAS2 and LC3-II from (C). (E) Representative WB of HUVEC experiments treated with increasing concentrations of Torin 1 for 6 h. (F) Quantification of HAS2, p62 and LC3-II from (E). (G) Representative WB of HUVEC time-course experiments with Torin 1 (20 nM). (H) Quantification of HAS2, p62 and LC3-II from (G). (I) Representative WB of HUVEC whole-cell lysates treated with increasing concentrations of INK128 for 6 h. (J) Quantification of HAS2 and LC3-II from (G). (K) Representative WB of HUVEC treated with increasing concentrations of endostatin for 6 h. (L) Quantification of HAS2 and LC3-II from (K). Data normalized to GAPDH; mean ± SEM. n = 3 biological replicates. Statistical analyses were calculated via One-way ANOVA (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
endothelial cells by evoking autophagic catabolism of this important enzyme linked to cancer progression and angiogenesis.

**Nutrient deprivation and mTOR inhibition down-regulate HAS2 in multiple cell types and species**

To validate that HAS2 downregulation occurs a general autophagic mechanism, we induced autophagy globally via three mechanistic fronts: nutrient deprivation using EBSS (Earl’s Balanced Salt Solution) [62], mTOR inhibition using Torin 1 [63] and INK128 [64], and endostatin, the C-terminal fragment of collagen XVIII shown to signal through VEGFR2 [65] and evoke autophagy in endothelial cells [61]. First, we evaluated HAS2 levels after nutrient deprivation in human and porcine endothelial cells. Both cell types showed HAS2 downregulation and LC3-II modulation upon nutrient deprivation (Fig. 2A–D).

Similar to endorepellin and nutrient deprivation, Torin 1 evoked a dose-dependent suppression of HAS2 protein (IC_{50}~188 nM) while modulating autophagic proteins, p62 and LC3-II, well-characterized components and substrates of the autophagosomal machinery [66,67] (Fig. 2E and F). Time-course experiments with Torin 1 (20 nM), showed a nearly identical HAS2 and p62 suppression (T1/2=5.2 h, Fig. 2G and H), and concurrent induction of LC3-II. Furthermore, INK128 produced a similar downregulation of HAS2 protein (Fig. 2I and J), confirming mTOR inhibition as a catalyst for HAS2 downregulation and linking this modulation to autophagic activation. As with endorepellin treatment, modulation of HAS2 via direct mTOR inhibition was also independent of transcriptional regulation, with no significant changes in HAS2 or HAS3 mRNA levels in HUVEC treated with Torin 1 for 6 and 24 h vis-à-vis vehicle (Fig. S3D and E). Lastly, we tested recombinant human endostatin, which, like endorepellin, signals through VEGFR2. We discovered that endostatin downregulated HAS2 in a dose-dependent manner while upregulating LC3-II (Fig. 2K and L), further highlighting VEGFR2 as a specific endothelial cell receptor that regulates HAS2. Collectively, these data implicate protracted autophagy as a general mechanism that effectively downregulates HAS2 in various cell types across species.

To test whether autophagic regulation of HAS2 occurs beyond endothelial cells, we measured HAS2 levels in nutrient-deprived NIH3T3 mouse fibroblasts, human embryonic kidney, Chinese hamster ovary and Mardin-Darby canine kidney cells. In all cases, there was a marked reduction of HAS2 protein levels upon nutrient deprivation (Fig. 3A–H), further generalizing the autophagic degradation of HAS2 as being present and conserved across a variety of species and cell types. Moreover, endorepellin and endostatin failed to modulate HAS2 in these cells lacking VEGFR2 (Fig. 3A–H). Tumor cell lines lacking VEGFR2 such as A431 and E0771 also did not modulate HAS2 in response to endorepellin (Fig. 3I to L), highlighting endorepellin’s ability to target HAS2 expression in endothelial cells specifically.

Next, we investigated HAS2 regulation via autophagy in vivo by starving wild-type mice for 48 h and measured HAS2 protein in heart and aorta whole-tissue extracts. Fasted mice possessed significantly reduced levels of HAS2 concurrent with elevated LC3-II in both the heart and aorta tissue, implicating that autophagic regulation of HAS2 occurs at the tissue level in vivo (Fig. 3M to P). Notably, we found no significant evidence of HAS2 degradation via autophagy in the liver, kidney or spleen after 48 h starvation (data not shown), suggesting that this biological process is organ-specific.

We further induced autophagic flux globally by injecting chloroquine (50 mg/kg) i. p. 24 h before sacrifice. Chloroquine is a well-established inducer of in vivo autophagic flux as it inhibits fusion of autophagosomes and lysosomes, thereby causing a build-up autophagosomes and their substrates [68]. We found that blocking autophagic degradation via chloroquine significantly increased HAS2 and LC3-II levels in both hearts and aortae vis-à-vis vehicle (Fig. 3Q to T). Thus, we provide robust in vivo evidence demonstrating HAS2 as an autophagic substrate that is degraded in vivo upon nutrient deprivation.

**Endorepellin or Torin 1 evokes autophagic flux of HAS2 and its intracytoplasmic co-localization with LC3**

To verify that HAS2 levels were modulated via autophagy, we performed autophagic flux assays with Bafilomycin A1, a pharmacological inhibitor of autophagosome-lysosome fusion [69]. This approach measures the build-up of autophagic substrates following provocation of this catabolic process by an autophagy inducer. Western blot analyses of HUVEC lysates treated with endorepellin or Torin 1 ±Bafilomycin A1 showed significantly increased HAS2 levels with Bafilomycin A1 vis-à-vis vehicle, with an even greater build-up of HAS2 in the combined treatments with either endorepellin or Torin 1 (Fig. 4A and B). Further, this accumulation of HAS2 upon inhibiting autophagic catabolism mirrored that of p62 and LC3-II (Fig. 4A and B). Quantification of immunofluorescence intensity of HAS2 also confirmed the same autophagic modulation via endorepellin (Fig. S4A).

Next, we corroborated these biochemical findings using confocal imaging. We found minimal co-localization of HAS2 and LC3-II under basal conditions (Fig. 4C). However, treatment with either
Fig. 3. Endorepellin has no effect on HAS2 levels in cells lacking VEGFR2 while nutrient deprivation downregulates HAS2 in HEK293, NIH3T3, and CHO cells. (A) Representative WB of NIH3T3 treated with endorepellin (200 nM), endostatin (200 nM), or nutrient deprivation (EBSS) for 6 h and (B) quantification of HAS2 protein. (C) Representative WB of whole-cell lysates of MDCK (Madin-Darby Canine Kidney cells) treated with endorepellin, endostatin, or nutrient deprivation for 6 h and (D) quantification of HAS2 protein. (E) Representative WB of whole-cell lysates of CHO treated with endorepellin, endostatin, or nutrient deprivation for 6 h and (F) quantification of HAS2 protein. (G) Representative WB of whole-cell lysates of HEK293 treated with endorepellin, endostatin, or nutrient deprivation for 6 h and (H) quantification of HAS2 protein. Data normalized to GAPDH. Horizontal bar = mean; n = 3. Statistical analyses were calculated via two-tailed unpaired Student's t-test (*, p < 0.05). (I) Representative WB of E0771 whole-cell lysates treated with increasing concentrations of endorepellin for 6 h. (J) Quantification of HAS2 protein. (K) Representative WB of A431 whole-cell lysates treated with increasing concentrations of endorepellin for 6 h. (L) Quantification of HAS2 protein; data normalized to GAPDH. Mean ± SEM. n = 3 biological replicates. Statistical analyses were calculated via One-way ANOVA. (M) Representative WB of heart tissue from mice fasted for 48 h. (N) Quantification of LC3-II and HAS2 protein from (M). (O) Representative WB of aorta tissue from mice fasted for 48 h. (P) Quantification of LC3-II and HAS2 protein from (O). (Q)
endorepellin, Torin 1 or Bafilomycin A1 caused enhanced co-localization of HAS2 and LC3 into autophagosomal vacuoles (Fig. 4D–F; Fig. S4C to E), an effect that was even more strongly increased in the combined treatments (Fig. 4G and H; Fig. S4F). Relative fluorescence intensity profiles along the cyan lines showed co-localization of HAS2 and LC3 (Fig. 4C–H, insets). Pearson’s coefficient of co-localization for overlapped pixels [70–72] of HAS2 and LC3 was significantly increased in all treatments vis-à-vis vehicle (Fig. S4B). Lastly, confocal imaging indicated an increased co-localization of HAS2 with autophagic substrate p62, with an increased Pearson’s coefficient of co-localization in endorepellin- or Torin 1-treated cells (Fig. S5A and B). However, immunoprecipitation using anti-p62 antibodies did not reveal direct interaction between HAS2 and p62 (Fig. SSC), a key player in the selective autophagy of ubiquitinylated substrates [67]. Collectively, our results established HAS2 as an autophagic substrate and that its intracellular catabolism was markedly enhanced upon stimulating flux through the autophagic pathway.

HAS2 dynamically engages ATG9A in autophagosomal structures

We hypothesized that HAS2 could interact with ATG9A at the autophagosomal membrane. Notably, HAS2 and ATG9A are multi-pass transmembrane proteins, with the latter playing a key role in nucleating the pre-autophagosomal structure, cycling between the plasma membrane, trans-Golgi network and endosomes and contributing lipid bilayer to the developing autophagosome [73–76]. We found by co-immunoprecipitation complexes of ATG9A and HAS2 that were already detectable under basal conditions and markedly enhanced by autophagic induction via endorepellin or Torin 1 (Fig. 5A). Confocal microscopy also demonstrated an enhanced Pearson’s coefficient of co-localization for overlapped pixels of HAS2 and ATG9A evoked by either endorepellin or Torin 1 vis-à-vis vehicle (Fig. S4G-I; Fig. 5B). Super-resolution STED microscopy revealed minimal co-localization of HAS2 and ATG9A (Fig. 5C); however, endorepellin and Torin 1 evoked marked co-localization of the two proteins, especially evident along the membrane of large autophagosomal vacuoles (Fig. 5D and E).

To visualize dynamic interactions between HAS2 and ATG9A in real time, we performed live-cell confocal imaging of endorepellin-treated or nutrient-deprived PAER2 transfected with RFP-ATG9A [77] and GFP-HAS2 [78]. Upon autophagic stimulation with endorepellin, we observed a rapid (~10 min) translocation of GFP-HAS2 into RFP-ATG9A nucleating complexes, a process that was sustained for up to 14 min (Fig. 6A and B; movie S1). Nutrient deprivation showed faster kinetics, with interaction of HAS2 and ATG9A surrounding a dynamic intracellular vacuole forming within ~2 min of treatment (Fig. 6C and D; movie S2). Collectively, our results demonstrate an active interaction between two crucial multi-pass transmembrane proteins and highlight the possibility that, like ATG9A, HAS2 could play a functional role in the autophagic process.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.matbio.2020.02.001.

Autophagic degradation of HAS2 suppresses extracellular HA and inhibits ex vivo angiogenesis

Having established that induction of autophagy by various agents leads to suppression of HAS2, and given the fact that this enzyme is a major producer of HA [79], we measured the glycosaminoglycan composition of media conditioned by endothelial cells by quantitative disaccharide analyses with liquid chromatography-tandem mass spectrometry (LC-MS/MS) [80]. Relative to the vehicle, HA secreted from cells treated with endorepellin and Torin 1 had a notable 3- and 4.33-fold decrease, respectively (p < 0.01) (Fig. 7). In contrast, protracted autophagy by endorepellin or Torin 1 had no significant effects on extracellular heparan sulfate, chondroitin sulfate, or total glycosaminoglycan levels (Fig. 7). Thus, autophagic suppression of HAS2 by endorepellin or mTOR inhibition specifically depletes extracellular HA without appreciably affecting the levels of other glycosaminoglycans.

To directly test whether autophagic degradation of HAS2 would regulate angiogenesis, we performed ex vivo aortic ring assays [81]. Explanted aortic rings of wild-type C57BL/6 mice were treated with autophagic inducers endorepellin or INK128 over an extended period of 8–10 days. We utilized INK128 instead of Torin 1 due to its longer half-life and higher in vivo potency [64,82]. Analysis of the phase contrast images of rings treated with endorepellin or INK128 exhibited decreased vessel density vis-à-vis vehicle (Fig. 8A–D). To quantify
active sprouting emanating from the aortic rings, we measured the radial distance of the newly-formed vessels (Fig. 8E) and found a significant reduction in angiogenesis evoked by either endorepellin or mTOR inhibition ($p < 0.001$, Fig. 8F).

Next, we used confocal imaging to visualize aortic rings probed with HA binding protein (HABP) to label HA, isolectin B4 (IB4) to mark endothelial cells [83,84], or antibodies specific for HAS2. In all cases, autophagy induction by endorepellin or INK128 caused a prominent angiostatic effect (Fig. 8G and H; Fig. S6A and B). Quantitative analysis of confocal images showed a significant reduction in angiogenesis evoked by either endorepellin or INK128 ($p < 0.001$, Fig. 8I and J). Even after we normalized intensity of HABP to that of IB4, there was significant reduction of the relative fluorescence intensity of HABP/IB4 (1 and 0.28 averages of vehicle- and endorepellin-treated rings, respectively; $p = 0.000095$). Furthermore, conditioned media from aortic rings treated with endorepellin or INK128 showed markedly reduced levels of HA as quantified

---

**Fig. 4.** Endorepellin or Torin 1 induces autophagic flux and co-localization of HAS2 into LC3-positive autophagosomes. (A) Representative WB of HUVEC treated with endorepellin (200 nM, 6 h) or Torin 1 (20 nM, 6 h) ± Bafilomycin A1 (500 nM, 7 h). (B) Quantification of HAS2, p62, and LC3-II from (A). Data normalized to GAPDH; horizontal bars = mean; $n = 3$ biological replicates. Statistical analyses were calculated via One-way ANOVA (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

(C–F) Corresponding confocal images of HUVEC treated with (C) vehicle, (D) endorepellin, (E) Bafilomycin A1 (BafA1), or (F) endorepellin + BafA1 and labeled for HAS2 (red), LC3 (green) and DAPI (blue). In the right column, intensity profiles of HAS2 and LC3 along the cyan blue line. Scale bar, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
using LC-MS/MS analysis (Fig. S7). These results comprehensively implicate that the autophagic degradation of HAS2 suppresses extracellular HA both in functional in vitro and ex vivo assays leading to a profound and protracted angiostasis.

Discussion

Unlocking the secrets of the tumor stroma has expanded our understanding of the intricate processes of cancer initiation and progression. Particularly, dynamic remodeling of the ECM is becoming increasingly acknowledged as an oncogenic and angiogenic force, as tumor cells and their vascularized stroma exert paracrine and angiocrine modes of regulation to stimulate angiogenesis, cell growth, and metastasis [85,86]. Over the years, our lab has provided robust evidence that the C-terminal module of perlecan, endorepellin, blocks VEGFR2 kinase activity, thereby evoking a strong pro-autophagic and anti-angiogenic response in vascular endothelial cells both ex vivo and in vivo [59]. We have also shown that systemic delivery of recombinant endorepellin inhibits tumor angiogenesis and growth and increases tumor hypoxia in squamous and Lewis lung carcinoma xenografts [29]. Since the cognate receptor for endorepellin, VEGFR2, is expressed

Fig. 5. Endorepellin and Torin 1 increase HAS2 interaction and co-localization with ATG9A. (A) Representative co-immunoprecipitation of HUVEC stimulated with endorepellin (200 nM) or Torin 1 (20 nM) for 3 h. The cells were lysed, immunoprecipitated (IP) with an anti-ATG9A antibody and subjected to WB with anti-ATG9A and anti-HAS2 antibodies as indicated. IgG heavy chain (Hc) as a loading control. (B) Pearson’s coefficient of co-localization quantifying signal overlap of HAS2 with ATG9A in confocal images of HUVEC treated with vehicle, endorepellin (200 nM), or Torin 1 (20 nM) for 3 h (Fig. S4, G to I). Co-localization of pixels within the entire cell was quantified; horizontal bars = mean; n = 3 biological replicates. Statistical analyses were calculated via One-way ANOVA (**, p < 0.01). Representative stimulated emission depletion (STED) super-resolution microscopy images of HUVEC treated with vehicle (C), endorepellin (D) or Torin 1 (E) for 3 h with intensity profiles of HAS2 (red) and ATG9A (green) along the white line. Nucleus (Nu) outlined in white dotted lines. Scale bar, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
primarily in endothelial cells and not in tumor cells, endorepellin’s anti-cancer activity is solely due to its ability to influence signaling in the tumorigenic milieu, thereby underscoring the importance and need for more in-depth study of the modulation of tumorigenesis via vascular signaling evoked by ECM effectors. Moreover, the most recent focus of endorepellin’s biological activity in endothelial cells involves its ability to induce protracted autophagy, a physiological housekeeping process that, in moderation, serves to eliminate damaged organelles, clear protein aggregates, and maintain cellular homeostasis [87]. However, a significant portion of the deranged signaling seen in malignant processes can be attributed to aberrations of autophagy both in the tumor parenchyma and supporting stromal cells [69,88]. Furthermore, suppression of the pro-oncogenic EGFR via RTK inhibition induces autophagy in non-small-cell lung carcinoma [89], whereas enhanced autophagy blocks HER2-mediated breast tumorigenesis [90]. However, despite these significant scientific contributions, no pharmacological

**Fig. 6.** Endorepellin evokes a dynamic physical interaction between HAS2 and ATG9A. (A) Time-course analysis of PAER2RFP/ATG9A transiently transfected with GFP-HAS2 and treated with endorepellin (200 nM) for up to 14 min. Representative image of 14 min of endorepellin treatment on left with the box outlining the frame area. Nucleus (Nu) outlined with dotted lines. (B) Time-lapsed imaging of white box in A performed at 1 frame per 30 s showing frames 9–14 min; white arrows point to area of convergence of the two differentially labeled proteins. (C) Time-course analysis of PAER2RFP/ATG9A transiently transfected with GFP-HAS2 and treated with nutrient deprivation for up to 13 min. Representative image of 5 min of endorepellin treatment with the box outlining the frame area. (D) Imaging was performed at 1 frame per 60 s showing frames from 1 to 13 min of nutrient deprivation. Nucleus (Nu) outlined with dotted lines; white arrows point to area of convergence of the two differentially labeled proteins.

**Fig. 7.** Endorepellin or Torin 1 suppresses extracellular HA in vitro. Quantification by liquid chromatography-mass spectrometry (LC-MS) of total secreted hyaluronan, heparan and chondroitin sulfate and total glycosaminoglycan (GAG) levels in HUVEC media conditioned for 24 h with endorepellin (200 nM) or Torin 1 (20 nM). WB of whole cell lysates of samples were run, and GAG levels in the media were normalized to the respective cell lysate GAPDH; horizontal bars = mean; n = 4 biological replicates. Statistical analyses were calculated via One-way ANOVA (**, p < 0.01; ***, p < 0.001).
An intervention specifically targeting autophagy is currently clinically available for human use to confront cancer [88,91]. Within the context of cancer, increased HA deposition in the matrix is a potent instigator of tumor angiogenesis and metastasis. Under physiological conditions, most of the extracellular HA is of relatively high molecular weight; however, during tumorigenesis and inflammation, HA polymers are significantly fragmented into oligosaccharides, evoking a pro-angiogenic and pro-inflammatory phenotype [51]. HAS1-3 isoforms comprise the family of HA synthases that polymerize HA in the intracellular face of the plasma membrane. Of all three hyaluronan synthases, hyaluronan synthase 2 (HAS2) is the main driver for HA deposition in breast cancer, highlighting the pro-angiogenic HAS2-HA system as a promising therapeutic target [9,52]. In the present work, we identify a novel mechanism to restrain angiogenesis that involves autophagic degradation of HAS2 driven by either suppression of VEGFR2 or canonical autophagic stimuli, thereby reducing the production and secretion of pro-angiogenic HA. A previous study showed modification of HAS2 with polyubiquitin chains linked at the Lys-48 residue, the linkage selectively targeted for degradation to proteasomes [92]. However, blocking proteasome-mediated degradation via MG132 only slightly increased the pool of polyubiquitinated HAS2 and did not significantly alter total HAS2 nor its capacity to synthesize HA [92]. We now provide robust evidence that HAS2 protein levels and extracellular HA are regulated by canonical autophagy in vitro and in vivo. This is a general mechanism found in four species (human, porcine, canine, and hamster) spanning five cell types (endothelial, ovarian, embryonic renal, mature renal, and fibroblastic cells). At the organ level in vivo, global physiological activation of autophagy via starvation downregulated HAS2 protein in whole-tissue extracts from heart and aorta in wild-type mice. We were able to successfully flux HAS2 in heart and aorta after inhibiting autophagic degradation via chloroquine treatment, further elevating the broad impact of this regulatory mechanism. Mechanistically, all five autophagic inducers including endorepellin, endostatin, Torin 1, INK128 and nutrient deprivation independently activated the canonical autophagic pathway by converging at mTOR inhibition. In support of our findings, an independent study has observed that activating mTOR in fibroblasts increased levels of HAS2 and secreted HA, and that this effect was partially reversed by rapamycin, an indirect inhibitor of mTOR [93]. However, this study did not explore autophagy as a mechanism of HAS2 regulation.
downstream of mTOR inhibition. Thus, our study provides the first evidence presenting an mTOR-dependent autophagic regulation of this enzyme that is ubiquitous among a variety of cell types and conserved among species.

Functionally, we show that autophagic clearance of HAS2 results in a marked depletion of secreted HA with no changes in other GAGs, specifically HS or CS. Since HA shares an overlapping pool of monosaccharide substrates with HS and CS, the selective reduction in HA is likely not due to depleted cytosolic stores of glucuronic acid or N-acetylglucosamine, the building blocks of HA [94], but rather the targeted depletion of HAS2. We would like to note out that an additional minor mechanism rather the targeted depletion of HAS2. We would like to note out that an additional minor mechanism caused by the phosphorylation of HAS2 by AMPK, which leads to decreased enzymatic activity [56], could also contribute to the suppression of HA levels. Interestingly, metformin, a known activator of AMPK, also decreases HA synthesis in vascular smooth muscle cells [95], further corroborating our findings that AMPK-induced autophagy downregulates HAS2.

Recent studies have implicated the physiologically active forms of HAS as homo- or heterodimers [55,92], with HAS2 playing a critical role in the formation of enzymatically active HAS2 homodimers and HAS2/HAS3 heterodimers. Thus, downregulating HAS2 may affect overall HA synthesis by disrupting the cooperative dimerization of these synthases. These data present an autocrine and paracrine mode of regulation in vascular endothelial cells where an intracellular degradative pathway alters a distinct component of the secretome that, in turn, suppresses pro-angiogenic signals to the cell, leading ultimately to an anti-angiogenic phenotype. This was robustly confirmed in ex vivo murine aortic ring explants showing marked suppression of intracellular HAS2, extracellular HA, and vessel growth upon autophagic activation via endorepellin or mTOR inhibition. Thus, we propose that selectively targeting HA by HAS2 modulation via the induction of autophagy is a promising avenue of investigation for chemotherapeutics as HA is a major proponent of cancer cell angiogenesis, proliferation, and metastasis [96].

Mechanistically, we present novel evidence that autophagic activation induces a dynamic interaction between two multi-pass transmembrane proteins, HAS2 and ATG9A, involved in HA synthesis and contributing to the pre-autophagosomal structure, respectively. ATG9A is crucial for providing contact sites between the endoplasmic reticulum and the phagophore [37], thereby promoting formation of the autophagosomal membrane. Other studies report that the trans-Golgi network and plasma membrane can also contribute to the autophagosomal membrane [73,98]. Notably, unlike other proteins that undergo selective autophagy, HAS2 does not have a LC3-interacting region (LIR). As an integral membrane protein shuttling to and from the plasma membrane, HAS2 could play a role in forming the pre-autophagosomal structure, perhaps by shuttling lipid components to the growing autophagosome via complexing with ATG9A upon autophagic activation. This may be a function of HAS2 independent of its enzymatic activity, a notion requiring further investigation. As HAS2 is the main HAS in vascular endothelial cells and adult tissue, we have not investigated the effect of autophagy on the interaction of HAS3 (HAS1 is not expressed in endothelial cells) with ATG9A.

Taken together, these results shift the current archetype of autophagy from a mere quality control process to one that precisely regulates the expression of key ECM constituents affecting cancer progression and invasion. We believe that these findings will forge new paths to build an arsenal of powerful and effective cancer treatments that augment autophagy and regulate its downstream targets, especially in the tumor microenvironment, resulting in medical advances to improve cancer patient treatment.

Materials and methods

Cells and materials

Human umbilical vein endothelial cells (HUVEC) were grown and maintained in basal media supplemented with VascuLife EnGS LifeFactors Kit (Lifeline Cell Technology, Oceanside, CA). Cells were plated on 0.2% gelatin-coated cell culture plates (Thermo Fisher Scientific, Waltham, MA) and utilized within the first five passages. Antibodies were purchased from the following sources and utilized at the designated dilutions: GAPDH (14C10, 1:10,000 dilution; Cell Signaling, Danvers, MA), VEGFR2 (5B11, 1:1000 dilution; Cell Signaling), P-VEGFR2 (19A10, 1:750 dilution; Cell Signaling), ALK (4H9, 1:1000 dilution; Cell Signaling), ATG9A [ab108338, used for Western blot (WB) at 1:1000 dilution, immunofluorescence (IF) at 1:200, and immunoprecipitation (IP) at 4 μg; Abcam, Cambridge, MA], p62/SQSTM1 (ab91526, used for WB at 1:250 and 1:150, respectively; Santa Cruz Biotechnology, Dallas, TX), HAS2 (sc-34068 and sc-365263, used for WB at 1:250 and 1:150, respectively; Santa Cruz Biotechnology, Dallas, TX), and normal rabbit IgG (2729, used for IP at 4 μg; Cell Signaling, Danvers, MA), Alexa Fluor 555 goat anti-mouse IgG (A11034, 1:100; ThermoFisher), and Alexa Fluor 594 goat anti-rabbit IgG (A11036, 1:100; ThermoFisher). Secondary antibodies for STED imaging were purchased from the following sources and utilized at the designated dilutions: Alexa Fluor 559 donkey anti-goat H + L IgG (A11058, 1:400; ThermoFisher) and Alexa Fluor 488 goat anti-rabbit H + L IgG (A11008, 1:400; ThermoFisher).
H + L IgG (A-21422, ThermoFisher) and Oregon Green 488 goat anti-rabbit H + L IgG (O-11038, ThermoFisher). The following siRNA was purchased from Santa Cruz Biotechnology and used at the designated amounts: Fk1-1 siRNA (sc-9318, 100 pM) and scramble control siRNA-A (sc-37007, 100 pM). Earl’s Balanced Salt Solution (24010043, ThermoFisher) was used for nutrient deprivation. Torin 1 and Bafilomycin A1 were purchased from Sigma. Protein A Sepharose magnetic beads were purchased from GE Healthcare. Recombinant human endorepellin was produced and purified as described previously [17]. Murine Fc-Endostatin (1 mg/ml) was purchased from GE Healthcare. Recombinant human HAS1 were as follows: HAS1 forward, 5’-CTTGTCAGGCTTCTCCACTG-3’; HAS1 reverse, 5’-CGGTCATCCCTC A A A A G T A C A G -3’; HAS2 forward, 5’- ATGGTTGAGGTTGTTGGG-3’; HAS2 reverse, 5’- AGGTTCCACTAATGACTGAAC-3’; HAS3 forward, 5’- ATCATGCGAAGTGGAAGG-3’; HAS3 reverse, 5’- TCCAGGACTGCAAGATCTCT-3’; B2M forward, 5’- TCCATCGCACTTAAGTGGTGG-3’; B2M reverse, 5’- ACACGGCAGGCTAATCTACT-3’; HPRT1 forward, 5’- GCTATAAATTCTTTTGTGATGCTGCTG-3’; HPRT1 reverse, 5’-AATTCTTTATGTCCTAGTGTTGACTGG-3’; Master Mix II (Agilent Technologies). All samples were run on the Roche LightCycler 480-II Real Time PCR platform (Roche Applied Sciences), and cycle number (Ct) was recorded for each reaction. Fold changes were normalized to B2M or HPRT1 and calculated using the ΔΔCT method (2^ΔΔCT). Data were derived from three independent biological replicates, each carried out in quadruplicate for every gene of interest. The primers used in qPCR analyses were as follows: HAS1 forward, 5’-CTTGTCAGGCTTCTCCACTG-3’; HAS1 reverse, 5’- CGGTCATCCCTCAAAAGTACAG-3’; HAS2 forward, 5’- ATGGTTGAGGTTGTTGGG-3’; HAS2 reverse, 5’- AGGTTCCACTAATGACTGAAC-3’; HAS3 forward, 5’- ATCATGCGAAGTGGAAGG-3’; HAS3 reverse, 5’- TCCAGGACTGCAAGATCTCT-3’; B2M forward, 5’- TCCATCGCACTTAAGTGGTGG-3’; B2M reverse, 5’- ACACGGCAGGCTAATCTACT-3’; HPRT1 forward, 5’- GCTATAAATTCTTTTGTGATGCTGCTG-3’; HPRT1 reverse, 5’-AATTCTTTATGTCCTAGTGTTGACTGG-3’;

Immunoblotting and immunoprecipitation

Treated HUVEC were rinsed once in ice-cold phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA/EGTA/sodium orthovanadate, 10 mM β-glycerophosphate, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin/tosylphenylalanyl chloromethyl ketone/aprotinin each)] for 15 min rocking on ice. Lysates were boiled for 3 min and resolved on SDS-PAGE as described before [99–101]. Proteins were then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA), probed with primary and secondary antibodies, and developed with SuperSignal West Pico Chemiluminescence substrate (Thermo Fisher Scientific). Signal detection was performed via an ImageQuant LAS-4000 (GE Healthcare) platform.

For Immunoprecipitation, protein A Sepharose magnetic beads (GE Healthcare) were incubated with antibodies for 4 h at room temperature (RT), and precleared cell lysates were added to the bead-antibody conjugate for 18 h at 4 °C. After extensive washing, beads were vortexed at high speed in reducing buffer for 30 min at RT (ATG9A IP) or boiled for 5 min (p62 IP), then separated using magnets. Visualizing ATG9A using the anti-ATG9A antibody was most clear in non-boiled lysates, as recommended by the manufacturer (Abcam). Remaining proteins in reducing buffer were analyzed via WB.

qPCR analysis

Transcriptional expression analysis by real-time quantitative PCR (qPCR) was carried out on confluent 12-well plates with -2 × 10^5 HUVEC treated with endorepellin (200 nM) or Torin 1 (20 nM) for 6 and 24 h in full serum Basal Endothelial Medium (Lifeline Cell Technology). Following incubation, cells were lysed in 500 μl TRIzol reagent (Invitrogen) to extract total RNA. Then, 1 μg of total RNA was annealed with oligo (dT<sub>18-20</sub>) primers, and SuperScript Reverse Transcriptase III (Invitrogen) was utilized to synthesize cDNA. Amplicons representing target genes and the endogenous housekeeping gene, β-2-microglobulin (B2M), were amplified in quadruple independent reactions using the Brilliant SYBR Green Master Mix II (Agilent Technologies). All samples were run on the Roche LightCycler 480-II Real Time PCR platform (Roche Applied Sciences), and cycle number (Ct) was recorded for each reaction. Fold changes were normalized to B2M or HPRT1 and calculated using the ΔΔCT method (2^ΔΔCT). Data were derived from three independent biological replicates, each carried out in quadruplicate for every gene of interest. The primers used in qPCR analyses were as follows: HAS1 forward, 5’-CTTGTCAGGCTTCTCCACTG-3’; HAS1 reverse, 5’-CGGTCATCCCTCAAAAGTACAG-3’; HAS2 forward, 5’-ATGGTTGAGGTTGTTGGG-3’; HAS2 reverse, 5’-AGGTTCCACTAATGACTGAAC-3’; HAS3 forward, 5’-ATCATGCGAAGTGGAAGG-3’; HAS3 reverse, 5’-TCCAGGACTGCAAGATCTCT-3’; B2M forward, 5’-TCCATCGCACTTAAGTGGTGG-3’; B2M reverse, 5’-ACACGGCAGGCTAATCTACT-3’; HPRT1 forward, 5’-GCTATAAATTCTTTTGTGATGCTGCTG-3’; HPRT1 reverse, 5’-AATTCTTTATGTCCTAGTGTTGACTGG-3’;

Immunofluorescence, confocal laser microscopy, and Pearson’s coefficient of co-localization

HUVEC were grown on four-chambered slides (Thermo Fisher Scientific). Cells were then washed briefly with PBS and fixed for 20 min rocking in 4% (w/v) paraformaldehyde on ice. Bovine serum albumin (1% in PBS) was utilized to block cells for 1 h, washed thrice in PBS, incubated for 1 h at RT with primary antibodies, washed thrice in PBS, and then incubated for 1 h at RT with secondary antibodies. After washing thrice in PBS, nuclei were stained with DAPI and mounted with a hard set mounting medium (Vectashield) and coverslips. Using a 63×, 1.3 oil-immersion objective in a Zeiss LSM-78 confocal laser-scanning microscope, fluorescence images were acquired at single optical sections of 1.0 μm, collected with the pinhole set to 1 Airy Unit for all three channels. Line scanning was performed as described previously [31,102]. All images were analyzed in ImageJ and Photoshop CS6 (Adobe Systems). Images were acquired in a single plane to determine co-localization. We calculated the weighted Pearson’s coefficient of co-localization using the co-localization function in the LSM-780 Zen software package as described previously [103].

Stimulated emission depletion (STED) super-resolution microscopy

Super-resolution imaging was performed using the 63×/1.4 oil-immersion objective in a Leica TCS SP8 STED 3× DMi8 inverted super-resolution microscope. HUVEC were grown and treated on gelatin-coated glass coverslips and fixed using freshly-prepared 4% paraformaldehyde on ice. After incubation for 1 h each at RT with primary and secondary antibodies and mounted on glass slides using ProLong Glass Antifade Mountant (P36980, ThermoFisher). Fluorescence images were acquired at single optical sections of 1.0 μm, collected with the pinhole set to 1 Airy Unit for all channels. Depletion lasers of 660 nm and 592 nm were utilized.
Transient siRNA-mediated knockdown

Transient knockdown of VEGFR2 (Fik-1) in HUVEC was accomplished via transfection of validated siRNA directed against human VEGFR2 (sc-9318; Santa Cruz Biotechnology). Scrambled siRNA (sc-37007; Santa Cruz Biotechnology) was used as control. Gelatinized six-well plates seeded with $2 \times 10^6$ HUVEC were grown at 37 °C and 5% CO$_2$ until 70% confluence. VEGFR2 or scrambled siRNA duplex (10 pM) were added to Lipofectamine 2000 (Invitrogen) in serum-free OptiMEM (Gibco). Cells were washed with PBS, incubated with the OptiMEM solution for 6 h at 37 °C and 5% CO$_2$, and incubated overnight with full HUVEC media in the same culture conditions. The media was then replenished, and treatment with vehicle or endorepellin began after 48 h of transient knockdown. Verification of siRNA-mediated knockdown was accomplished via immunoblotting.

Live-cell imaging

Live-cell fluorescence imaging was performed using a 63×, 1.3 oil-immersion objective in a Zeiss LSM-78 confocal laser-scanning microscope. PAER2 stably expressing RFP-ATG9A were placed on a glass bottom confocal laser-scanning microscope. Live-cell imaging was performed using a Zeiss LSM780 NLO confocal/multiphoton microscope. Visualization was conducted via immunofluorescence staining using Alexa Fluor 488 goat anti-mouse H+L IgG (A11001, 1:400; Thermo Fisher) and Alexa Fluor 594 Isolectin GS-IB4 (1:200, ThermoFisher Scientific) for 3 h at RT. Visualization was conducted via Zeiss LSM780 NLO confocal/multiphoton microscope. Lastly, z-stack images of aortic rings were analyzed via ImageJ to measure the fluorescence intensity of Isolecitin B4 (IB4). Region containing the sprouts was outlined and the region containing the ring was eliminated. Lastly, mean fluorescence intensity was quantified. Using ImageJ and phase contrast images of the rings, we measured the live-cell fluorescence intensity was quantified. Using ImageJ and phase contrast images of the rings, we measured the

LC-MS/MS analysis of conditioned media

Media of HUVEC treated for 24 h with vehicle, endorepellin (200 nM), or Torin 1 (20 nM) were filtered (0.22 μm), flash frozen in liquid nitrogen, and stored at −80 °C. Media from sprouted aortic rings treated with endorepellin or INK128 for 8 days were flash frozen in liquid nitrogen, and stored at −80 °C. Unsaturated disaccharide standards of CS (0SCs-CS: ΔUA-GalNAc; 4SCs-CS: ΔUA-GalNAc4S; 6SCs-CS: ΔUA-GalNAc6S; 2SCs: ΔUA2S-GalNAc; 2S4s-CSs-B: ΔUA2S-GalNAc4S; 2S6s-CSs: ΔUA2S-GalNAc6S; 4S6s-CSs: ΔUA2S-GalNAc4S6S; TriScs: ΔUA2S-GalNAc4S6S), unsaturated disaccharide standards of HS (0SHs: ΔUA-GlcNAc; NSHs; ΔUA-GlcNAc; 6SHs: ΔUA-GlcNAc6S; 2SHs: ΔUA2S-GlcNAc; 2SNShs: ΔUA2S-GlcNAc6S; NS6shs: ΔUA-GlcNS6S; 2S6shs: ΔUA2S-GlcNS6S; TriShs: ΔUA2S-GlcNS6S), and unsaturated disaccharide standard of HA (0SHA: ΔUA-GlcNAc), where ΔUA is 4-deoxy-6-threo-hex-4-enopyranosyluronic acid (purchased from Iddron, UK). Chondroitin lyase ABC from Proteus vulgaris was expressed in the Linhardt laboratory. Recombinant Flavobacterial heparin lyases I, II, and III were expressed using Escherichia coli strains provided by Jian Liu (College of Pharmacy, University of North Carolina). AMAC and sodium cyanoborohydride (NaCNBH$_3$) were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of HPLC grade. Spectra/
radial distance of sprouting by subtracting background and highlighting sprouts using the threshold function. Then, circles were drawn around the ring to the edge of the sprouts and the radii were measured.

Animal fasting and in vivo autophagic flux

Animal experiments were performed as per the Guide for Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee of Thomas Jefferson University and in accordance with the German Animal Protection Law. C57BL/6 mice were obtained from The Jackson Laboratory and both male and female sex ranging from 5 to 6 months old were equally included in the study. Fasting protocol consisted of withholding food for 48 h with water accessible ad libitum. Autophagic flux experiments were performed with i. p. chloroquine injection of 50 mg/kg 24 h prior to sacrificing the animals. Following euthanasia, organs were isolated, snap-frozen in liquid nitrogen and stored at −80 °C. Mouse organs were submerged in liquid nitrogen and crushed using a mortar and pestle. Homogenates were lysed in T-PER (Tissue protein extraction reagent, Thermo Fisher) with 5 mM EDTA and protease inhibitor, centrifuged for 10 min at 400 × g at 4 °C. Sample buffer was added to the supernatants. Samples were then boiled for 2 min and run on 1.5 mm 10% acrylamide gel and transferred for Western blot analysis. To detect HAS2, anti-HAS2 antibody (sc-365,263, 1:150; Santa Cruz Biotechnology, Dallas, TX) was used for mouse tissue samples.

Quantification and statistical analysis

Immunoblots were analyzed via scanning densitometry (ImageJ). Significance in experiments with two groups were determined by two-tailed unpaired Student’s t-test, whereas statistical analyses for experiments with more than two groups were calculated via One-way ANOVA. Mean differences were considered statistically significant at p < 0.05.

Author contributions

R.V.I., C.G.C. and M.A.G. designed and directed the study. R.V.I., C.G.C. and M.A.G. wrote the manuscript. C.G.C. carried out the biochemical and cell biological experiments. R.J.L. directed and X.H. and Y.L. carried out the LC/MS-MS analyses of glycosaminoglycan content in conditioned media. R.V.I., A.K. and C.G.C. performed the aortic ring assay and A.K. performed microscopic analyses. All authors discussed the results and commented on the manuscript.

Conflicts of interest

The authors declare no competing interests.

Acknowledgements

We thank Dr. K. Rilla for the GFP-HAS2 plasmid and Drs. L. Claesson-Welsh, U. Naik, and J. Uitto for providing PAE-VEGFR2, CHO, and NIH3T3 cells, respectively. We also thank members of the Iozzo laboratory for helpful input and the Bioimaging Shared Resource of the Sidney Kimmel Cancer Center (NCI 5 P30 CA-56036), M. Covarrubias for STED imaging assistance and D. Weaver for help with the live cell confocal imaging. All grants supporting this work were issued by the National Institute of Health (NIH) in the USA: two R01 grants (CA39481 and CA47282) and two T32 training grants (AR052273 and AA07463).

Appendix A.Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.matbio.2020.02.001.

Received 6 January 2020; Received in revised form 5 February 2020; Accepted 5 February 2020
Available online 19 February 2020

Keywords:
ATG9A;
Endorepellin;
Extracellular matrix;
Hyaluronan;
mTOR

Abbreviations used:
AMPK,AMP-activated protein kinase; ATG9A,autophagy-related 9A; CQ,chloroquine; ECM,extracellular matrix; GAG,glycosaminoglycan; HA,hyaluronan; HUVEC,human umbilical vein endothelial cells; LC3,microtubule-associated protein light chain 3; PAER2,porcine aortic endothelial cells overexpressing VEGFR2; RTK,receptor tyrosine kinase; VEGFR2,vascular endothelial growth factor receptor 2.

References

Autophagic degradation of HAS2


Autophagic degradation of HAS2


[71] V. Zinchuk, Y. Wu, O. Grossenbacher-Zinchuk, E. Stefani, Quantifying spatial correlations of fluorescent markers using enhanced background reduction with protein proximity index and correlation coefficient estimations, Nat. Protoc. 6 (2011) 1554–1567.


[99] L. Rudnicka, J. Varga, A.M. Christiano, R.V. Iozzo, S.A. Jimenez, J. Uitto, Elevated expression of type VII collagen is associated with...


