Endorepellin-evoked Autophagy Contributes to Angiostasis*

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Endorepellin, the C-terminal domain of perlecan, is an angiostatic molecule that acts as a potent inducer of autophagy via its interaction with VEGFR2. In this study, we examined the effect of endorepellin on endothelial cells using atomic force microscopy. Soluble endorepellin caused morphological and biophysical changes such as an increase in cell surface roughness and cell height. Surprisingly, these changes were not accompanied by alterations in the endothelial cell elastic modulus. We discovered that endorepellin-induced autophagic flux led to colocalization of mammalian target of rapamycin with LC3-positive autophagosomes. Endorepellin functioned upstream of AMP-activated kinase α, as compound C, an inhibitor of AMP-activated kinase α, abrogated endorepellin-mediated activation and colocalization of Beclin 1 and LC3, thereby reducing autophagic progression. Functionally, we discovered that both endorepellin and Torin 1, a canonical autophagic inducer, blunted ex vivo angiogenesis. We conclude that autophagy is a novel mechanism by which endorepellin promotes angiostasis independent of nutrient deprivation.

Perlecan is a large modular proteoglycan that belongs to the pericellular and basement membrane class of proteoglycans (1–3). Its protein core alone is approximately 500 kDa and is comprised of five domains, with three heparan sulfate chains attached at the N terminus. It is encoded by an approximately 115-kb gene, HSPG2 (4, 5), and is expressed in a variety of tissues, both vascular and avascular (6, 7). The biological functions of perlecan span a range of processes, including cell adhesion (8, 9), endocytosis (10), bone and cartilage formation (11, 12), inflammation and wound healing (13, 14), thrombosis (15), lipid metabolism (16), autophagy (17), tumor angiogenesis and invasiveness (18–23), and cardiovascular development (24), where its angiogenic properties are among the most interesting. Perlecan transcription is also induced by TGF-β (25) and is rapidly repressed by interferon γ (26).

The network of heparan sulfate/growth factor interactions is a key regulator of angiogenesis (27). Perlecan acceptors VEGFA and FGFs via its N-terminal heparan sulfate side chains, which are then released by heparanases and subsequently bind to their cognate receptors, resulting in the induction of angiogenesis (9, 28, 29). In addition, there is a feedback loop in that VEGFA induces perlecan synthesis via the activation of VEGFR2, leading to increased angiogenesis (30, 31). Indeed, antisense targeting of perlecan inhibits in vivo tumor angiogenesis (32). During development, perlecan acts as a scaffold for blood vessel formation, and a restriction of Hspg2 expression in early embryogenesis results in cardiovascular defects (7, 33).

In contrast, the C-terminal domain V of perlecan, known as endorepellin, exhibits angiostatic properties (34). Endorepellin is found in vivo, where it is proteolytically processed from perlecan (14) via matrix metalloproteinases, a family of enzymes involved in a multitude of biological processes (35–39). Although perlecan mRNA can undergo alternative splicing, no evidence exists for endorepellin production in vivo via this mechanism (14). This domain of perlecan is an 85-kDa protein comprised of four EGF-like repeats and three laminin-like globular domains (LG1–3). Structurally, LG2/LG3 domains of endorepellin are separated by two EGF-like repeats that can be cleaved by BMP1/Tolloid-like proteases (40, 41) to release the LG3 domain (42).

As its name implies, endorepellin is an inhibitor of endothelial cell migration and capillary morphogenesis, thus preventing the formation of new blood vessels (34). These functional properties result from a “dual receptor antagonism” through its binding to VEGFR2 and α2β1 integrin (43): LG1/2 bind to the IgG3–5 repeats in the VEGFR2 ectodomain, whereas LG3 binds to α2β1 integrin (44). This biological interaction leads to rapid internalization of both receptors and, ultimately, attenuation of the PI3K/phosphoinositide-dependent kinase/Akt/mTOR6 and PKC/JNK/AP1 pathways and a decrease in expression of VEGFA and FGFs via its N-terminal heparan sulfate side chains, which are then released by heparanases and subsequently bind to their cognate receptors, resulting in the induction of angiogenesis (9, 28, 29). In addition, there is a feedback loop in that VEGFA induces perlecan synthesis via the activation of VEGFR2, leading to increased angiogenesis (30, 31). Indeed, antisense targeting of perlecan inhibits in vivo tumor angiogenesis (32). During development, perlecan acts as a scaffold for blood vessel formation, and a restriction of Hspg2 expression in early embryogenesis results in cardiovascular defects (7, 33).

In vivo studies have shown that endorepellin specifically targets the tumor vasculature and inhibits tumor angiogenesis (46). This bioactivity leads to inhibition of tumor growth without inducing apoptosis. Recently, we have discovered that sol-
Endorepellin induces autophagy in endothelial cells via the binding of its LG1/2 domains to VEGFR2 (47). This process occurs independently of the \( \alpha2\beta1 \) integrin and induces several autophagic markers (Beclin 1, LC3, and p62) under nutrient-enriched conditions (47). In this study, we examined in detail the physical properties of endothelial cells treated with endorepellin via atomic force microscopy (AFM) imaging and nanoindentation. We further elucidated the mechanism behind endorepellin-evoked autophagy. Specifically, we found that endorepellin evoked phosphorylation of AMPK at Thr172, a key residue necessary for autophagic progression. Moreover, endorepellin blunted vessel sprouting in \textit{ex vivo} angiogenesis assays, and this bioactivity was blocked by halting AMPK activation. Thus, we propose a new mechanism by which a fragment of an extracellular proteoglycan links angiostasis to autophagy.

**Results**

**Endorepellin and Torin 1 Evoke Nanoscale Molecular Bumps in Endothelial Cells**—To determine the nanoscale structural changes in porcine (PAER2) cells and human endothelial cells (HUVEC) evoked by endorepellin or Torin 1, a selective inhibitor of mTOR (48), we utilized tapping mode AFM imaging, which quantifies cell surface topography at nanoscale spatial resolution. We discovered that, although the vehicle-treated PAER2 cell surface was relatively smooth (Fig. 1A), that of endorepellin-treated cells (Fig. 1B) revealed increased surface roughness with the formation of discrete bumps. Identical bumps were detected in the cells treated with Torin 1 (Fig. 1C).

Next we determined the height of these bumps from the three-dimensional images (Fig. 1, D–F). Line scanning profiles of the three-dimensional bumps showed marked elevation in both endorepellin-treated (Fig. 1H) and Torin 1-treated cells (Fig. 1I), suggesting that these elevations may represent autophagosomes. In comparison, vehicle-treated cells exhibited a uniform height (Fig. 1G). We also used low-magnification AFM images (Fig. 1, I–K) to quantify the number of these bumps, which we interpret as autophagosomes. There was a significant increase in the number of autophagosome-like structures evoked by either endorepellin or Torin 1 \textit{vis à vis} vehicle-treated cells \( (p < 0.001, \text{Fig. 2L}). \)
To quantify the changes in cell morphology associated with autophagy, we analyzed cell surface roughness. The two surface roughness parameters, Ra (arithmetic mean height) and Rq (root mean square height), were determined in an area of \( 36 \mu m^2 \). We observed that, upon induction of autophagosome-like structures, the cell surface roughness (Ra) was markedly increased in both endorepellin-treated (\( p < 0.05 \), Fig. 2M) and Torin 1-treated HUVEC (\( p < 0.001 \), Fig. 2M). Rq values were similar to the Ra values (data not shown). We interpret these findings as representative of autophagosome generation within the cytoplasm of endothelial cells derived from both porcine and human large vessels.

Despite Cell Surface Nanostructural Changes, Endorepellin Does Not Alter the Elastic Modulus of Endothelial Cells—Following this observation of nanostructural changes in the endorepellin-treated endothelial cells, we hypothesized that autophagosome formation, induced both by endorepellin and nutrient deprivation, would result in changes in the cellular elastic modulus. To this end, we utilized a special type of AFM where nanoindentation was performed by a microspherical tip that directly indents the cell surface while the cells are alive and grown in appropriate culture medium (49, 50). This configuration provides information regarding the dynamic biomechanical properties of the target cells in response to different cellular milieus, allowing for an accurate representation of the changes in cellular elasticity under varying conditions. Although the focus of this study was on endorepellin-induced biomechanical changes, we also utilized Hanks’ balanced salt solution (HBSS), a nutrient deprivation method used to induce autophagy through inhibition of mTOR signaling (51). This acted as a positive control for autophagic induction to determine whether autophagy itself could alter the elastic modulus of endothelial cells.

We surmised that the presence of autophagosomes would increase the stiffness of the cells. To our great surprise, despite the presence of structural differences visible by differential interference contrast microscopy in both the endorepellin- and HBSS-treated cells vis à vis vehicle-treated cells (Fig. 3, A–D), the elastic modulus of these HUVEC was not significantly altered (Fig. 3M). Intriguingly, the positive control cells treated with HBSS also did not show any significant change in elastic modulus, suggesting that the formation of autophagosomes may not result in changes in stiffness as measured at the cell surface, at least in endothelial cells.

Endorepellin Evokes Autophagic Flux and Co-localization of mTOR and LC3 into Puncta—Following this biophysical analysis, we sought to delve deeper into the mechanism by which endorepellin contributes to the induction and/or progression of the autophagic process. First, we validated our cell system by performing autophagic flux experiments. To this end, HUVEC were treated with or without chloroquine, an inhibitor of the fusion of the lysosome with the autophagosome, which allows...
Endorepellin Promotes Autophagy via AMPK Activation—It is well established that AMPK plays a key function in regulating autophagy and mTOR activity following phosphorylation of its catalytic α subunit at Thr172 (52, 53). We hypothesized that endorepellin could induce autophagy through a canonical activation of AMPKα. Under nutrient-rich conditions, endorepellin increased the phosphorylation of AMPKα at Thr172 over time (Fig. 5A), reaching a peak at 4 h, with levels almost three times greater than those of untreated cells (Fig. 5B).

To further verify the role of AMPKα in endorepellin-induced autophagy, we treated HUVEC with either endorepellin and/or compound C, a potent inhibitor of AMPK (53). Upon addition of this inhibitor, the phosphorylation of AMPKα was no longer detected in endorepellin-treated cells (Fig. 5, C and D). Compound C also attenuated the effect of endorepellin on LC3-II and Beclin 1 levels (Fig. 5, C, E, and F). These results indicate that the activation of AMPKα is involved in the endorepellin-mediated increase in the autophagic markers Beclin 1 and LC3-II.

Next we investigated the role of AMPKα in endorepellin-mediated autophagy by imaging the intracellular movement of LC3 and Beclin 1 after treatment with compound C, endorepellin, or both. Endorepellin alone promoted the co-localization of Beclin 1 and LC3 into large autophagosomes compared with vehicle-treated cells (Fig. 6, A and B). In contrast, compound C significantly decreased the formation of endorepellin-induced Beclin 1/LC3-positive autophagosomes (Fig. 6D) relative to endorepellin alone (Fig. 6E).

VEGFR2 Is Required for Endorepellin-evoked Phosphorylation of AMPKα Thr172—Next we investigated the relationship between AMPKα and VEGFR2 following treatment of endothelial cells with endorepellin by genetically targeting this receptor via RNAi. Following verification of successful knockdown of VEGFR2 (Fig. 6, F and G), we found that an ~50% reduction in VEGFR2 protein levels prevented the endorepellin-evoked phosphorylation of AMPKα at Thr172 (Fig. 6F), and these data were significant vis à vis scrambled siRNA in three independent experiments (p < 0.01, Fig. 6H). We conclude that VEGFR2 is required for the proper activation of AMPKα following interaction at the cell surface with the LG1/2 domains of endorepellin (44), as its knockdown leads to a significant attenuation of endorepellin-induced autophagy.

Endorepellin and Torin 1 Inhibit ex Vivo Angiogenesis—Given its well established angiostatic activity (34, 43, 47), we hypothesized that endorepellin could evoke autophagy and inhibit angiogenesis via a common pathway. To this end, we utilized an ex vivo model, the aortic ring assay (54). This assay closely mimics the in vivo environment required for angiogenesis, as it includes both endothelial and supporting cells, which surround the aorta in vivo (55). In rings grown in a nutrient-rich environment, we observed a well structured microvessel network with clearly defined tubules and regular branching (Fig. 7, A and B). In contrast, the samples treated with endorepellin, using the same concentration (200 nM) as in the biochemical assays, showed a significant decrease in the number of microvessels growing from the rings (Fig. 7, C and D).

To differentiate endothelial from non-endothelial cells, we immunostained the aortic ring explants with an antibody toward CD31/platelet endothelial cell adhesion molecule, a cell surface glycoprotein highly expressed by endothelial cells. The confocal images clearly showed that the sprouting vessel-like structures were indeed positive for CD31 (Fig. 7, E and F) and that endorepellin markedly reduced them (Fig. 7, G and H).
FIGURE 4. **Endorepellin induces autophagic flux and mobilization of mTOR into LC3-positive autophagosomes.** A, representative immunoblotting of HUVEC lysates following endorepellin treatment in the absence and presence of chloroquine. B, quantification of LC3-II using one technical replicate from each of six biological samples obtained from six independent experiments as seen in A, normalized to GAPDH. C–E, mTOR and LC3 staining in HUVEC following treatment with vehicle, endorepellin (200 nM, 6 h), or Torin 1 (20 nM, 2 h). Scale bar = 10 μm. The white arrows indicate autophagosomes. F, quantification of the number of autophagosomes (LC3- and mTOR-positive) per cell in HUVEC treated with vehicle (n = 76 cells from three biological replicates obtained from three separate experiments), endorepellin (n = 49 cells from three biological replicates obtained from three separate experiments), or Torin 1 (n = 60 cells from three biological replicates obtained from three separate experiments). *, *p < 0.05, Student’s t test.

FIGURE 5. **Endorepellin activates the autophagic pathway via AMPKα phosphorylation.** A, representative immunoblots of P-AMPKα (Thr172) in response to endorepellin (200 nM) at different time points. B, quantification of P-AMPKα:AMPKα as shown in A. One-way analysis of variance was used for statistical analysis. C, representative immunoblots of P-AMPKα, total AMPKα, Beclin 1, and LC3-II following incubation with endorepellin (200 nM, 6 h), compound C (30 μM, 30 min pretreatment + 6 h), or endorepellin and compound C. D, quantification of the P-AMPKα:AMPKα ratio shown in C. E and F, quantification of Beclin 1 and LC3-II as shown in C after normalization to GAPDH. Quantification was performed using one technical replicate each from three biological replicates obtained from three independent experiments. D–F, *, *p < 0.05, Student’s t test.
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Furthermore, treatment of aortic rings with Torin 1 revealed a pattern similar to that evoked by endorepellin (Fig. 7, I–L). Quantification of three to four independent experiments revealed a significant suppression of the number of sprouts per aortic ring by both endorepellin and Torin 1 vis à vis vehicle treatment (p < 0.01, Fig. 7M).

Autophagic Inhibition Reverses the Angiostatic Response Triggered by Endorepellin—Having established that both endorepellin and Torin 1 had comparable effects on angiogenesis, we sought to elucidate whether induction of autophagy was the impetus for the endorepellin-mediated angiostasis. To this end, we utilized compound C as a means to prevent endorepellin-mediated autophagy and angiostasis. In agreement with the findings presented above, endorepellin inhibited angiogenic sprouting from mouse aortic rings compared with those treated with vehicle (Fig. 8, A and B). Importantly, compound C blocked the inhibitory activity of endorepellin, as angiogenic sprouts grew to levels comparable with those seen in vehicle-treated rings (Fig. 8D). This outgrowth of vessels was statistically significant compared with endorepellin treatment alone but not significantly different from either vehicle-treated (Fig. 8A) or compound C-treated (Fig. 8C) rings (Fig. 8E). Thus, we can conclude that some of the anti-angiogenic effects of endorepellin are directly intertwined with its pro-autophagic capabilities, and, hence, we provide autophagy as a novel mechanism for the inhibition of neovascularization.

Discussion

Autophagy is a catabolic process in which double or multiple membrane-delineated autophagosomes sequester cytoplasmic material and fuse with lysosomes, resulting in the degradation of their contents (56). This process allows for the recycling of long-lived proteins and damaged organelles, thus maintaining cellular homeostasis. In addition to its function in the maintenance of cellular housekeeping under normal conditions, autophagy is also highly up-regulated during nutrient deprivation, hypoxia, and other unfavorable conditions, where it promotes cell survival (57). Given both the cytoprotective and sometimes cytotoxic functions of autophagy, defects in this process can contribute to a number of human pathologies, including cancer and neurodegenerative diseases (51).

Despite recent progress, the functional role of autophagy remains unclear in many biological contexts, particularly in angiogenesis, where its role remains controversial (58, 59). Endorepellin, the C-terminal domain of perlecan, induces autophagy in endothelial cells (47). Here we investigated in depth the mechanism through which endorepellin evokes autophagy and angiostasis and discovered that protracted autophagy under nutrient-rich conditions can negatively regulate angiogenesis.

First, we analyzed the effect of endorepellin on the morphology and microphysical properties of endothelial cells using AFM. The latter enables high-resolution topographical imaging of a single cell surface with minimal sample preparation (60). Notably, nanomolar concentrations of soluble endorepellin caused morphological and biophysical changes in endothelial cells derived from either porcine or human macrovessels, including an increase in cell surface roughness, cell height, and number of autophagosome-like structures. Similar changes

![Image of aortic rings and beclin 1/3 staining](image)

**FIGURE 6.** AMPKα and VEGFR2 are required for endorepellin-mediated autophagy in HUVEC. A–D, immunofluorescence images depicting the co-localization of Beclin 1 (green) and LC3-II (red) shown upon treatment with vehicle (n = 31 cells from three biological replicates obtained from three independent experiments), 200 nM endorepellin for 6 h (n = 36 cells from three biological replicates obtained from three independent experiments), 30 µM compound C for 30-min pretreatment + 6 h (n = 28 cells from three biological replicates obtained from three independent experiments), or compound C + endorepellin (n = 28 cells from three biological replicates obtained from three independent experiments). E, immunoblot of Beclin 1 (± siRNA) and VEGFR2 (± siRNA) with respective quantification. F, immunoblot quantification of the number of autophagosomes per cell in HUVEC treated according to the conditions shown in A–D. G–H, quantification of VEGFR2 protein level (one technical replicate from each of three biological replicates obtained from three independent experiments) and P-AMPKα/AMPKα ratio (one technical replicate from each of three biological replicates obtained from three independent experiments) shown in F. *, p < 0.05; **, p < 0.01; ***, p < 0.001; Student’s t test.
were documented in cells treated with Torin 1, which induces autophagy through the inhibition of mTOR (48). Surprisingly, these changes were not accompanied by alterations in the endothelial cell elastic modulus. It is possible that endothelial cells do not incur any changes in elasticity following autophagic induction. However, it is also possible that, given the dynamic nature of autophagy, the lack of changes seen in the treated cells might be due to a high turnover rate of autophagosomes during this process. We note that there was slightly more variability in the modulus of endorepellin-treated cells compared with vehicle-treated samples, suggesting that there may be minor changes in modulus from transient formation of autophagosomes but that their rapid turnover makes any significant changes undetectable. Also, we must mention that, although the majority of cells undergo autophagy initiation following endorepellin treatment, it is possible that some cells do not. In these studies, the moduli were measured in cells chosen at random. Using AFM that can detect fluorescence-labeled autophagic markers could be a way to circumvent this limitation.

We also wanted to scrutinize more thoroughly the intracellular signaling events that accompanied these biophysical changes observed at the cell surface. We focused on AMPKα and mTOR, two opposing regulators of the autophagic pathway (61). When activated, AMPKα, the master nutrient-sensing enzyme, binds and phosphorylates ULK1, a key kinase in the initiation of this process (53, 62). Our working model (Fig. 9) delineates the necessity of phosphorylation of AMPKα at Thr172 for endorepellin-evoked autophagy. Interestingly, the kinetics of this phosphorylation are rather slow, peaking 4 h after endorepellin treatment, especially when compared with another extracellular matrix constituent, decorin (63–65). Decorin treatment also induces autophagy via AMPKα phosphorylation, which increases rapidly within 30 min of treatment and is maintained for up to 2 h (66, 67). However, the slow induction of AMPKα by endorepellin does correlate with increases in several autophagic proteins, including Beclin 1, LC3-II, Peg3, and p62 (47).

Alongside the activation of the pro-autophagic pathway is the inhibition of the anti-autophagic mTOR pathway. mTOR

FIGURE 7. Endorepellin and Torin 1 inhibit angiogenesis ex vivo in mouse aortic ring assays. A–D, light microscopy images depicting the difference in microvessel growth between vehicle-treated and endorepellin-treated rings (200 nM). B and D, close-up views of the sprouts in A and C. E–H, confocal images of vehicle-treated and endorepellin-treated rings (200 nM) where nuclei were stained with DAPI (blue) and endothelial cells were stained with CD31 (red). F and H, close-up views of sprouts found in E and G. I–L, representative confocal images of rings treated with either vehicle or Torin 1 (40 nM). Sprouts were stained with BS-1 Lectin (red), and nuclei were stained with DAPI (blue). K and L, magnified views of I and J. M, quantification of the number of sprouts in the vehicle-, endorepellin-, and Torin 1-treated rings. There were 16 technical and four biological replicates from four independent experiments for the first vehicle group, 18 technical and four biological replicates from four independent experiments for the endorepellin group, 11 technical and three biological replicates from three independent experiments for the second vehicle group, and 12 technical and three biological replicates from three independent experiments for the Torin 1 group. Scale bars = 100 μm. **, p < 0.01; Student’s t test.
also regulates ULK1 at different phosphorylation sites from AMPK, which results in deactivating ULK1 and, thus, inhibiting the initiation of autophagy. We have established previously that endorepellin attenuates the mTOR pathway via dephosphorylation at Ser2448 (45). Here we found that, along with these biochemical changes, mTOR is taken up by autophagosomes upon endorepellin stimulation, in a fashion similar to that evoked by starvation in renal epithelial cells (68). We hypothesize that autophagosome-mediated degradation of mTOR caused by endorepellin/VEGFR2 signaling in endothelial cells would further enhance the pro-autophagic role of endorepellin. Proteoglycan receptors may thus play a role in regulating this important catabolic process (69).

Perhaps the most pivotal discovery of our study is the demonstration that autophagy can curtail neovascularization, as both endorepellin and Torin 1 reduce sprouting in ex vivo aortic ring assays. For the first time we were able to restore angiogenic sprouting following endorepellin treatment by blocking AMPKα-evoked autophagy using compound C. Interestingly, the synthesis of hyaluronan, a key component of the provisional angiogenic matrix under a complex metabolic control (70–72), is also inhibited by AMPK (73). This is due to the specific AMPK-evoked phosphorylation of hyaluronan synthase 2 at Thr110, a modification that blocks its enzymatic activity (73). Hence, along with the induction of autophagy, endorepellin may also simultaneously alter the cellular microenvironment to favor angiostasis by reducing the expression of this pro-angiogenic glycosaminoglycan. Given this information along with previous in vivo data depicting endorepellin as a powerful means to curtail tumor growth and angiogenesis (46), we believe that our findings from this study may yield unique therapeutic targets for novel drug design.

We should point out, however, that, although compound C typically crosses the plasma membrane to inhibit AMPKα, it also has affinity for VEGFR2. Because of this interaction, compound C can actually inhibit neovascularization through downregulation of VEGFR2 signaling in some models of angiogenesis (74). Indeed, we saw a non-significant reduction in vessel number in rings treated with compound C alone compared with vehicle. As endorepellin binds and signals through VEGFR2 (43, 45), it is possible that compound C may interfere with the endorepellin/VEGFR2 axis.

Remarkably, our findings are corroborated by other studies that depict a number of angiostatic matrix proteins and their domains, including endostatin (75) and Kringle V (76), which can concurrently induce autophagy. Notably, a recent study also illustrated that the natural compound capsicodendrin exhibits angiostatic activity and autophagy induction via VEGFR2 inactivation (77). Conversely, impairing autophagy in retinal epithelial cells leads to enhanced angiogenesis (78). Thus, our study contributes new information in support of a paradigm shift whereby autophagy may act primarily as an antiangiogenic mechanism.

In summary, these studies have shown that both human and porcine endothelial cells undergo morphological and biochemical changes following treatment with endorepellin. In both cell types, we observed autophagosome formation in response to endorepellin treatment, accompanied by changes in cell surface roughness and height. Mechanistically, endorepellin-induced autophagy is dependent on AMPKα activation downstream of...
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**Experimental Procedures**

**Cells and Materials**—Human umbilical vein endothelial cells (HUVEC) were grown in basal medium supplemented with the VascuLife EnGS Life Factors Kit (LifeLine Cell Technology, Frederick, MD), with cells being utilized within the first five passages. Porcine aortic endothelial cells expressing VEGFR2 (PAER2) were described previously (43, 45). The rabbit polyclonal LC3B antibody (L7543) was obtained from Sigma-Aldrich (St. Louis MO). The goat polyclonal LC3 antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, sc-16756). The rabbit primary antibodies against Beclin 1 (3738S), VEGFR2 (55B11), mTOR (7C10), AMPKα (2532), phospho-AMPKα at residue Thr172 (40H9), and GAPDH (14C10) were procured from Cell Signaling Technology (Danvers, MA). The rabbit CD31 (Ab28364) primary antibody was purchased from Abcam (Cambridge, MA). For immunoblots, all primary antibodies were used at a 1:1000 dilution, except GAPDH, which was used at 1:5000. Secondary antibodies were used at 1:4000 dilutions. For immunofluorescence studies, primary antibodies were used at a concentration of 1:200, and secondary antibodies were used at a 1:400 dilution. The goat anti-rabbit secondary antibody conjugated with HRP (12–348) was purchased from Millipore, Inc. (Billerica, MA). Donkey anti-rabbit Alexa Fluor 488 (A21206), donkey anti-rabbit Alexa Fluor 594 (A11012), and donkey anti-goat Alexa Fluor 594 (A11058) antibodies were purchased from Invitrogen. Compound C and gelatin were obtained from Sigma-Aldrich. Vascular West Pico chemiluminescence substrate was purchased from Thermo Fisher Scientific (Philadelphia, PA). RNAi targeting human VEGFR2 and corresponding control siRNA (siScr) were purchased from Santa Cruz Biotechnology. Lipofectamine RNAiMAX was acquired from Invitrogen. Human recombinant endorepellin was expressed and purified as described previously (43, 45, 47). Type I collagen was purchased from BD Biosciences. BS-1 Lectin was purchased from Thermo Fisher Scientific and Sigma-Aldrich. DAPI was purchased from Invitrogen.

Scanning Cell Morphology and Ultrastructure by Atomic Force Microscopy—A Dimension Icon atomic force microscope was used for nanostructural studies (BrukerNano, Santa Barbara, CA). A confluent monolayer of HUVEC or PAER2 cells was grown on 0.2% gelatin-coated four-chamber slide (Thermo Fisher Scientific). After treatment, cells were washed in ice-cold PBS before fixing on ice for 2 h in glutaraldehyde diluted to 2% in HBSS. The fixed cells were washed again in PBS in ice-cold PBS before fixing on ice for 2 h in glutaraldehyde diluted to 2% in HBSS. The fixed cells were washed again in PBS before drying in a desiccation chamber. Images were acquired in tapping mode using a nanosized silicon tip (NCHV-A; BrukerNano; tip radius, ~10 nm; spring constant, ~42 N/m). To determine the elastic modulus of individual cells, cells were grown in either basal medium with growth factors ± 200 nM endorepellin (6 h) or HBSS (4 h) and tested without fixing. Assisted by an optical microscope to locate individual cells, nanoindentation was performed at 7 μm/s indentation depth rate using a microspherical tip ($R = 2.5 \mu m$, $k = 0.1 N/m$) and the Dimension Icon AFM in the same medium. A Hertz model with finite cell height correction (79) was applied to each force–depth loading curve to calculate the effective elastic indentation modulus. The Poisson’s ratio of the cells was assumed to be 0.5 (80).

Immunoblotting—HUVEC were treated as necessary for the given analyses and lysed in radioimmune precipitation assay 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/tosylphenylalanil chloride methyl ketone/aprotinin each)) for 20–25 min on ice. Resolved proteins were then transferred to nitrocellulose membranes (Bio-Rad), probed with the indicated antibodies, and devel-
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oped with the enhanced chemiluminescence technique. Resulting chemiluminescent signatures were detected via an ImageQuant LAS-4000 (GE Healthcare) visualization platform as described previously (81).

**Immunofluorescence Microscopy**—HUVEC, grown on 0.2% gelatin-coated four-chamber slides (Thermo Fisher Scientific), were treated for the respective analyses. Cells were subsequently washed with PBS and fixed on ice for 30 min in 4% paraformaldehyde at room temperature (82, 83). Cells were blocked in PBS + 5% bovine serum albumin, incubated with various antibodies for 1 h, washed in PBS, and incubated for 1 h with an appropriate secondary antibody. Nuclei were stained and visualized with DAPI (Vector Laboratories). Fluorescence images were acquired with a ×63, 1.3 oil immersion objective using a Leica DM5500B microscope programed with the Leica Application Suite, Advanced Fluorescence v1.8, from Leica Microsystems, Inc. All resulting immunofluorescence images were analyzed using ImageJ software (National Institutes of Health) and Adobe Photoshop CS5.1 (Adobe Systems).

**Transient siRNA-mediated Knockdown**—Transient knockdown of VEGFR2 in HUVEC was achieved via transfection with validated siRNAs specific for VEGFR2 (sc-29318) from Santa Cruz Biotechnology. Scrambled siRNA (siScr, sc-37007) served as a control for all siRNA experiments presented here. Six-well plates were seeded with 2 × 10^5 HUVEC, followed by incubation at 37 °C + 5% CO₂ until cultures were ~70% confluent. Targeting or scrambled siRNA duplex was mixed with transfection medium and Lipofectamine RNAiMAX. After incubation at an ambient temperature (~25 °C), the ribonucleic acid/cationic complexes were applied directly to the cells. Following a 48-h transfection, the cells were treated and lysed in radioimmunoprecipitation assay buffer. Verification of RNAi-mediated knockdown of the target protein was determined via immunoblotting.

**Aortic Ring Assays**—All animal protocols were performed according to the Guide for Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee of Thomas Jefferson University. Thoracic aortae from 5–7-week-old C57BL6 mice (The Jackson Laboratory) were surgically isolated, cleaned, and dissected into 0.5-mm rings. Rings were embedded in 1 mg/ml of type I collagen in a 96-well plate as described previously (84). When embedded, the rings were divided into respective groups: vehicle (PBS or DMSO), endorepellin (200 nM), Torin 1 (40 nM), compound C (1 μM), and the Institutional Animal Care and Use Committee of Thomas Jefferson University. Thoracic aortae from 5–7-week-old C57BL6 mice (The Jackson Laboratory) were surgically isolated, cleaned, and dissected into 0.5-mm rings. Rings were embedded in 1 mg/ml of type I collagen in a 96-well plate as described previously (84). When embedded, the rings were divided into respective groups: vehicle (PBS or DMSO), endorepellin (200 nM), Torin 1 (40 nM), compound C (1 μM), and compound C + endorepellin. Endothelial microvessels sprouts growing out from the rings were counted during the exponential growth phase to obtain angiogenic response data. Before the regression phase, rings were fixed for immunofluorescence staining of CD31 or BS-1 Lectin. Pictures were taken on day 12, and the total number of branches was counted using ImageJ.

**Quantification and Statistical Analysis**—Immunoblots were quantified by densitometry using ImageJ software. All experiments contained here were carried out with a minimum of three independent trials (85). Results are expressed as the mean ± S.E. Statistical analysis was performed with SigmaStat for Windows version 3.10 (Systat Software, Inc., Port Richmond, CA). Significance of differences was determined by paired and unpaired Student’s t test or one-way analysis of variance followed by Tukey-Kramer post-hoc multiple comparison. Data were considered significant with p < 0.05.

**Author Contributions**—R. V. I. conceived the study and coordinated the work. A. G., M. A. G., and R. V. I. performed experimental work and wrote the manuscript. D. R. C. performed the AFM measurements, data analysis, and interpretation. L. H. supervised the AFM work. All authors reviewed the results, contributed to data interpretation, and approved the final version of the manuscript.

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