Extracellular matrix-evoked angiostasis and autophagy within the tumor microenvironment represent two critical, but unconnected, functions of the small leucine-rich proteoglycan, decorin. Acting as a partial agonist of vascular endothelial growth factor 2 (VEGFR2), soluble decorin signals via the energy sensing protein, AMP-activated protein kinase (AMPK), in the autophagic degradation of intracellular vascular endothelial growth factor A (VEGFA). Here, we discovered that soluble decorin evokes intracellular catabolism of endothelial VEGFA that is mechanistically independent of mTOR, but requires an autophagic regulator, paternally expressed gene 3 (PEG3). We found that administration of autophagic inhibitors such as chloroquine or bafilomycin A1, or depletion of autophagy-related 5 (ATG5), results in accumulation of intracellular VEGFA, indicating that VEGFA is a basal autophagic substrate. Mechanistically, decorin increased the VEGFA clearance rate by augmenting autophagic flux, a process that required RAB24 member RAS oncogene family (RAB24), a small GTPase that facilitates the disposal of autophagic compartments. We validated these findings by demonstrating the physiological relevance of this process in vivo. Mice starved for 48 h exhibited a sharp decrease in overall cardiac and aortic VEGFA that could be blocked by systemic chloroquine treatment. Thus, our findings reveal a unified mechanism for the metabolic control of endothelial VEGFA for autophagic clearance in response to decorin and canonical pro-autophagic stimuli. We posit that the VEGFR2/AMPK/PEG3 axis integrates the anti-angiogenic and pro-autophagic bioactivities of decorin as the molecular basis for tumorigenic suppression. These results support future therapeutic use of decorin as a next-generation protein therapy to combat cancer.

Decorin, an archetypical member of the small leucine-rich proteoglycan gene family (1, 2), acts as a partial agonist for a diverse set of cell surface receptor-tyrosine kinases (RTKs) that directly regulate a surfeit of intracellular pathways via outside-in signaling (3–10). Classically, decorin evokes anti-angiogenic and angiostatic responses (11–13) in vitro (14, 15) and in vivo (16–18) via proteolytic degradation of oncogenes such as β-catenin, Myc (14), and HIF-1α (15), whereas concurrently inducing tumor suppressors, i.e. p21WAF1 (19). Decorin is unique among onco-suppressive agents (13) as it selectively and simultaneously binds RTKs expressed in the stroma such as VEGFR2 (20) and those expressed by the tumor parenchyma such as EGFR and/or Met (21, 22). Decorin-deficient mice are prone to tumorigenesis when either p53 is missing (23) or when subjected to a Western high-fat diet (24, 25).

Mechanistically, decorin reduces intracellular, cell-associated, and secreted vascular endothelial growth factor A (VEGFA) in a noncanonical manner and evokes the expression and rapid secretion of thrombospordin-1 downstream of VEGFR2 or Met (15, 26–29). The protracted suppression of potent pro-angiogenic factors constitutes a key hallmark of decorin as a soluble tumor repressor and emphasizes its etymology as a guardian from the matrix (2, 30–33). Decorin has been linked to metabolic reprogramming via regulation of cardiac O-β-N-acetylglucosylation (34, 35).

Using high-resolution transcriptomics to identify decorin-regulated genes in vivo, we discovered that paternally expressed gene 3 (Peg3) was specifically induced within the Mus musculus stroma in triple-negative breast carcinoma orthotopic tumor xenografts treated with systemic delivery of decorin (18). Peg3 is a genomically-imprinted Krüpple-like zinc finger transcription factor possessing inherent tumor suppressor properties (36, 37). Peg3 is commonly lost due to biallelic promoter hypermethylation (38, 39) or loss of heterozygosity (40). Querying the ONCOMINE database (18), we found that Peg3 expression is substantially and significantly reduced in invasive ductal breast carcinoma (41) and ductal breast carcinoma (42).

The abbreviations used are: RTK, receptor tyrosine kinase; AICAR, N’-[(β-D-ribofuranosyl)-5-aminimidazole-4-carboxamide; AMPKα, 5’-AMP-activated protein kinase catalytic subunit α; ATG, autophagy regulated genes; HAEc, human aortic endothelial cells; HIF-1α, hypoxia-inducible factor 1 subunit α; HUVEC, human umbilical vein endothelial cells; LC3, microtubule associated light chain 3; p62/Sequestosome 1; VEGFA, vascular endothelial growth factor-A; VEGFR2, vascular endothelial growth factor receptor 2; Peg3, paternally expressed gene 3; CQ, chloroquine; PAER2, porcine aortic endothelial cells overexpressing VEGFR2; Dic, differential interference contrast; EGFR, epidermal growth factor receptor; mTOR, mechanistic target of rapamycin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; STAT, signal transducers and activators of transcription; DAPI, 4′,6-diamidino-2-phenylindole; ANOVA, analysis of variance.
Due to the functional similarities of Peg3 for the noncanonical disruption of Wnt/β-catenin signaling in a glycogen synthase kinase 3β-independent manner (43), which is strikingly akin to the activity of decorin (14), Peg3 emerged as a prime candidate for further study.

We discovered that Peg3 co-localizes and physically interacts with the autophagic regulators LC3 and Beclin 1 (44) in response to decorin and canonical autophagic stimuli in endothelial cells (20). Peg3 is necessary and sufficient to maintain and evoke BECN1, MAP1LC3A, and TFEB expression (20, 28, 45, 46), and drives LC3-positive autophagosome formation and autophagic flux (28). Moreover, we found that Peg3 is required for thrombospondin-1 expression and secretion (28). Thus, Peg3 may act as a nexus to parse anti-angiogenic and pro-autophagic signals for the concerted regulation of decorin-evoked angiogenesis and autophagy.

Decorin actively signals via the VEGF2 tyrosine kinase to evoke autophagy (20, 47). Decorin differentially modulates AMPK and mTOR phosphorylation, critical rheostats for maintaining metabolic homeostasis (48–51), to favor pro-autophagic outcomes. Indeed, AMPK and mTOR subsume opposing roles in the governance of autophagic induction. AMPK is required for autophagic initiation viaULK1/2 phosphorylation (51–54) and mTOR for autophagic inhibition and termination (55, 56). We found prolonged phosphorylation of the AMPKα catalytic subunit at Thr172 denoting activation with a concurrent suppression of phosphorylated mTOR at Ser2448 and its downstream effectors (Akt and p70S6K) in endothelial cells for up to 48 h (48). Importantly, decorin-evoked endothelial cell autophagy occurs under nutrient-rich conditions, thereby designating decorin as a noncanonical stimulus for evoking excessive autophagy.

Our understanding of the molecular mechanisms of VEGFs regulating vascular homeostasis and angiogenesis has dramatically expanded in the past two decades (57, 58). Given that decorin suppresses VEGFA with the concurrent induction of autophagy via VEGFR2/AMPKα/Peg3 in endothelial cells (20, 28, 46, 48), we hypothesized these pathways could converge to catabolize intracellular VEGFA by autophagy as the mechanism for the onco-suppressive effects of decorin. In this study, we found that decorin evokes the protracted catabolism of intracellular VEGFA via endothelial cell autophagy in a VEGFR2/AMPK/Peg3-dependent manner as the functional apparatus to achieve angiostasis.

Results

Intracellular VEGFA protein levels are controlled by AMPK

We found via confocal imaging that decorin reduced intracellular VEGFA (Fig. 1A) in primary endothelial cells (HUVEC) and human aortic endothelial cells immortalized by stable expression of the human catalytic subunit of telomerase (TeloHAEC). VEGFA suppression proceeded in a VEGFR2-dependent manner as inhibition with the small molecule inhibitor, SU5416 (59–61) significantly abrogated the effect of decorin (Fig. 1A). We previously found that exogenous decorin evokes endothelial cell autophagy by activating the master energy sensor kinase, AMPK (52, 62, 63) by augmenting the phosphorylation of the α-catalytic subunit at Thr172 (48). We discovered that treatment with Compound C (Dorsomorphin), a reversible and selective AMPKα inhibitor (51), potently attenuated VEGFA suppression (Fig. 1A), underscoring a role for the VEGFR2/AMPK axis.

Next, we used AICAR, an analog of adenosine monophosphate that stimulates AMPK activity (64), to assess whether AMPK alone was capable of modulating VEGFA. We verified that AICAR potently activated AMPK as shown by increasing levels of P-Thr172 (Fig. 1B), that peaked at 4 h (Fig. 2C). This is in stark contrast to treating with vehicle only (DMSO), which has no effect on P-AMPKα or VEGFA levels (Fig. S1, A and B). We found that turning on AMPK via AICAR was sufficient to increase Peg3 (Fig. 1, B and D), a master autophagic regulator induced by decorin (20, 28, 46), and simultaneously decrease intracellular VEGFA with a t1⁄2 of 2.6 h (Fig. 1, B and E). Notably, immunofluorescence analysis showed that AICAR treatment caused a similar induction of Peg3 (Fig. 1F) and concurrent VEGFA suppression (Fig. 1G) with a comparable half-life (t1⁄2 = 3.2 h). We then assessed the effect of AMPK on PEG3 and VEGFA mRNA expression. AICAR dynamically increased Peg3 mRNA peaking at ~2.5 h and then declining to basal levels (Fig. 1H). Importantly, AICAR concomitantly decreased VEGFA mRNA (Fig. 1I). However, the half-life of VEGFA mRNA was nearly 5 h (t1⁄2 = 4.9 h), significantly longer (p = 0.0014) than VEGFA protein (cf. Fig. 1, B and E).

We sought to better define a role of the AMPK kinase for the differential regulation of Peg3 and VEGFA by treating HUVEC with AICAR, either individually or in combination with Compound C. We validated AICAR function by immunoblotting for P-Thr172 and found increased phosphorylation (Fig. 2, A and B). Treatment with or without Compound C did not alter basal or AICAR-induced P-Thr172 (Fig. 2, A and B). These data are congruent with the established mechanism of AMPKα activation via canonical phosphorylation by the upstream LKB1/MO25α/STRAD1 trimeric kinase complex (50). Importantly, Compound C significantly inhibited AICAR-mediated induction of Peg3 (p = 0.002, Fig. 2, A and C) and corresponding VEGFA suppression (p = 0.006, Fig. 2, A and D). We confirmed these biochemical findings using quantitative immunofluorescence under identical experimental conditions and found that Compound C markedly prevented AICAR-evoked induction of Peg3 (p = 0.0006, Fig. 2E) or reduction of VEGFA (p = 0.008, Fig. 2F).

Collectively, these data support a dynamic and reciprocal role of AMPK in inducing Peg3 and simultaneously decreasing VEGFA in endothelial cells. Moreover, activating AMPK alone with AICAR to mimic a low cellular energy state in an otherwise enveloping nutrient-rich environment appears equivalent to the decorin-dependent suppression of cellular VEGFA.

Endothelial VEGFA suppression is mTOR independent

We have previously shown that decorin turns off mTOR signaling in a protracted fashion by attenuating phosphorylation at Ser2448 downstream of VEGFR2 and Akt, resulting in decreased activation of its effectors such as p70S6K (48).

Thus, we tested whether VEGFA suppression depended on mTOR inhibition. To this end, we used Torin 1, a highly-spe-
cific ATP competitive inhibitor of mTORC1 (65, 66). We assessed either p62/SQSTM1 (p62) or LC3 protein levels, two established autophagic markers regulated by mTOR inhibition (67). We found that Torin 1 dynamically modulated p62 in a dose-dependent manner in HUVEC (Fig. 3, A and B) and PAER2 (Fig. 3, C and D). Intriguingly, the responses were different between both models, with a maximal induction of p62 in HUVEC occurring with as little as 1 nM Torin 1, then decreasing at subsequently higher doses (Fig. 3, A and B). In PAER2, which have been immortalized by the stable integration and expression of VEGFR2, progressively increasing the amount of Torin 1 significantly decreased p62/SQSTM1 (Fig. 3, C and D). This was not a result of increased cell death as the internal loading control (GAPDH) remained equal (Fig. 3, C and D) and total cell numbers did not waver at the highest dose (500 nM) administered (data not shown). However, despite positive modulation of p62 as a surrogate for autophagy, we found no change of VEGFA with increasing amounts of Torin 1 in HUVEC (Fig. 3, A and B) or PAER2 (Fig. 3, C and D).

We next investigated the temporal effect of Torin 1 on VEGFA. We used 20 nM Torin 1 as we previously found this to be most effective dosage for autophagic induction in our system (68) and LC3 as a positive control for mTOR inhibition because p62 levels are only subtly modulated under this concentration of Torin 1 (Fig. 3, E and F). Similar time-dependent administration of Torin 1 induced dynamic LC3-II formation, the bioactive form of LC3, in HUVEC that peaked at 1 h (Fig. 3, E and F). In PAER2, LC3-II levels declined with Torin 1 treatment over time (Fig. 3, G and H). However, similar to the dose-response, VEGFA was not responsive to Torin 1 over time despite induction of autophagy in either HUVEC (Fig. 3, E and F) or PAER2 (Fig. 3, G and H).

We complemented Torin 1 inhibition with INK128 (Sapanisertib), which more potently attenuates mTORC1/2 and has a longer half-life than Torin 1 (69, 70). Under comparable experimental conditions, we found a similar trend in HUVEC where p62 exhibited a dynamic induction, peaking at 0.1 nM followed by a decline to baseline at higher concentrations (Fig. S2, A and C). In PAER2, INK128 caused a progressive decline in p62 levels.
from baseline, akin to the effect of Torin 1, as INK128 concentrations increased (Fig. S2, B and D). Importantly, using this more potent inhibitor of the mTOR complexes, VEGFA levels were unperturbed in both endothelial cell types (Fig. S2, A–D).

Collectively, these data demonstrate an mTOR-independent role of VEGFA modulation in endothelial cells utilizing pharmacological agents that mimic low-energy conditions, despite lively regulation of established autophagic markers. Thus, bioenergetics-mediated regulation of intracellular VEGFA proceeds in an AMPK-dependent and mTOR-independent mechanism in endothelial cells.

**VEGFA is sensitive to nutrient deprivation**

As VEGFA is untouched by mTORC1/2 inhibition as a mimic of the nutrient replete state, we performed nutrient deprivation in PAER2 cells using Earle’s balanced salt solution (71) and evaluated VEGFA over time. After confirming starvation-induced autophagy by increased P-AMPKα at Thr172 (data not shown), we showed a concurrent decrease in p62 (Fig. S2, E and F). Importantly, we found a significant decrease in VEGFA, nearly mirroring the profile of p62 over this same time frame (Fig. S2, E and F).

To evaluate the generality of the above results, we assessed VEGFA regulation by pro-autophagic stimuli (AICAR, Torin 1, and HBSS) in two other cell types, namely, human primary fibroblasts and NIH-3T3 immortalized mouse fibroblasts. Surprisingly, in response to AICAR, p62 levels did not appreciably change in either cell type (Fig. S3, A, C, B, and E). In contrast, AICAR significantly reduced VEGFA in human and mouse fibroblasts (Fig. S3, A, D, B, and F), similar to endothelial cells. Treatment with Torin 1 reduced p62 (Fig. S3, A, C, B, and E) without a concomitant decrease in VEGFA levels (Fig. S3, A, D, B, and F), akin to HUVEC and PAER2. Nutrient deprivation via HBSS suppressed p62 levels (Fig. S3, A, C, B, and E), whereas concurrently decreasing VEGFA (Fig. S3, A, D, B, and F). Collectively, our findings highlight a general sensitivity of cellular VEGFA to pro-autophagic agents that is not restricted to endothelia, further reinforcing the independence of mTOR in this pathway.

**Pro-autophagic stimuli evokes co-localization of VEGFA with LC3 positive autophagosomes**

We evaluated whether VEGFA would localize to autophagosomes using confocal imaging. We discovered that even basal levels of VEGFA co-localized with LC3-positive autophagosomes (Fig. 4, A and B). However, 6 h treatment with decorin, AICAR, or nutrient deprivation caused a marked increase in the number and size of the VEGFA/LC3 dually-positive autophagosomes (Fig. 4, C, E, and G) as did the signal intensity ascertained by semi-quantitative line scanning profiles for each stimulus (Fig. 4, D, F, and H).

Next, we investigated the formation of VEGFA/LC3 dually-positive autophagosomes using differential interference contrast (DIC) microscopy on HUVEC challenged with decorin, AICAR, or HBSS. We immunostained the cells with antibodies against either LC3/VEGFA (Fig. 5, A–D) or Beclin 1/VEGFA (Fig. 5, E–H) and found that all three stimuli significantly promoted the formation of dually-positive VEGFA/LC3 (Fig. 5, B–D; Fig. S4A) or VEGFA/Beclin-1 (Fig. 5, F–H; Fig. S4B) autophagosomes over basal conditions (Fig. 5, A and B; Fig. S4, A and B). Moreover, we found discrete VEGFA/LC3 puncta within large autophagosomes following decorin, AICAR, or HBSS (Fig. 5, B–D, white arrows) that were absent under basal conditions. Next, we evaluated VEGFA/p62 autophagosomal positivity and found that decorin significantly increases the number of VEGFA/p62 dually-positive autophagosomes as well (Fig. S4, C and D). Mechanistically, we found that the formation of VEGFA/LC3-positive autophagosomes in HUVEC was suppressed by treatment with the VEGFR2 kinase inhibitor SU5416 (p < 0.001, Fig. 5, F and J). We validated the immunofluorescence by omitting the primary or secondary antibodies in the presence or absence of BafA1 (Fig. S5). BafA1 served as a positive control for LC3. Treating with BafA1 recapitulated the augmented co-localization of VEGFA with LC3 (cf. Figs. 4 and 5; Fig. S5, A and B) over basal conditions. Omitting either the primary (Fig. S5, C and D) or secondary (Fig. S5, E and F) anti-
bodies annulled the immunofluorescence signal, even in the presence of BafA1. These data reinforce the robustness of our immunofluorescence data.

Collectively, these imaging data posit VEGFA as an autophagic substrate that is present in LC3- or p62-positive autophagosomes under basal conditions. The incorporation of VEGFA into autophagosomes was substantially augmented in response to decorin/VEGFR2 signaling, as well as conventional pro-autophagic cues.

Peg3 drives VEGFA clearance via autophagy

As AMPK activation up-regulates Peg3 (cf. Fig. 1, B and C), and Peg3 drives autophagosome formation (28), we postulated that Peg3 could be directly involved in regulating VEGFA clearance via autophagy. Structurally, Peg3 is composed of an N-terminal SCAN domain required for protein–protein interactions and an elongated C terminus of 12 C_{2}H_{2} Krüpple-like zinc fingers interspersed with proline-rich regions potentially used for DNA binding and transcriptional regulation (Fig. 6A) (36, 72–74). We transiently transfected PAER2 cells with empty vector (pcDNA3.1), HA-PEG3, or HA-SCAN (Fig. 6A) and verified their correct expression using anti-HA antibodies (Fig. 6B). Importantly, and in contrast to either empty vector or SCAN-domain, overexpression of full-length Peg3 drove the accumulation of VEGFA into LC3-positive autophagosomes (Fig. 6C and D).

Next, we performed loss-of-function experiments to determine the role of Peg3 in promoting VEGFA-positive autophagosomes in response to decorin. We verified Peg3 knockdown via RNAi and found a significant \((p = 0.002)\) depletion (Fig. S4E). Corroborating the gain-of-function experiments above, we discovered that transient depletion of Peg3 abrogated the ability of decorin to drive VEGFA into LC3-positive autophagosomes in HUVEC (Fig. 6F) vis-à-vis vehicles. Quantitation of the Pearson’s coefficient of colocalization as a readout for dual VEGFA/LC3 positivity supported (Fig. 6F) our confocal imag-
**Autophagic clearance of intracellular VEGFA**

We observed that VEGFA co-localized under basal conditions with LC3 (cf. Figs. 4 and 5), Beclin 1 (cf. Fig. 5), and p62 (cf. Fig. S4), suggesting that VEGFA could be directly degraded via autophagy. We pharmacologically blocked autophagic flux using bafilomycin A1 (BafA1 hereafter), a universally established method to assay autophagic processes (75–77). Mechanistically, BafA1 inhibits the vacuolar H^+-ATPase to preclude mature autophagosomes from fusing with lysosomes, thereby giving a metric on the substrates undergoing autophagic degradation. Using PAE2 cells, we performed a BafA1 dose response. Our positive control, proteolytic conversion of LC3-I to LC3-II, which amasses following autophagic inhibition (76, 78, 79), increased in a concentration-dependent manner with as little as 1 nM BafA1 (Fig. 7, A and B). Concurrent with LC3-II induction, we found that VEGFA increased proportionally (Fig. 7, A and B). Notably, we found nearly identical results in HUVEC (Fig. 7, C and D).

Next, we used a different autophagic inhibitor, chloroquine, to assay the sensitivity of VEGFA to basal autophagy. Chloroquine (CQ), a lysosomotropic agent, has previously been thought to prevent lysosomal acidification as a mechanism of action (80). However, recently it has been shown that CQ inhibits autophagic flux by decreasing autophagosome-lysosome fusion (81). Evaluating HUVEC with CQ, we found that LC3-II increased (Fig. 7, E and F), validating CQ function, concomitantly with an accumulation of VEGFA (Fig. 7, E and F). Treating PAE2 cells with CQ resulted in a similar pattern wherein p62 and VEGFA both increased (Fig. S6, A and B) in response to autophagic inhibition.

Next, we complemented the pharmacological approach with RNAi-mediated silencing of ATG5, an E1-like activating enzyme for autophagy. Deleting ATG5 potently abrogates autophagosomal maturation leading to a cessation of autophagy for flux analyses (83, 84). Loss of ATG5 results in a build-up of LC3-I, the unprocessed form of LC3 (Fig. 7). We verified our siRNA and found a significant knockdown of ATG5 in HUVEC (Fig. 7, G and H). ATG5 loss led to a marked increase in LC3-I (Fig. 7, G and H), thus functionally validating ATG5 silencing. Analogous to our findings, genetically silencing a critical enzyme for autophagosome maturation, we discovered a significant (p < 0.001) increase in intracellular VEGFA with respect to our nontargeting vehicle (Fig. 7, G, H).

To determine the generality of our findings, we applied a similar strategy to nonendothelial systems. We evaluated NIH3T3 mouse fibroblasts with either BafA1 (Fig. S6, C and D) or CQ (Fig. S6, E and F), with LC3-II conversion as a positive control, and recapitulated the results insofar as VEGFA is degraded by basal autophagic flux. Finally, we evaluated highly...
malignant MDA-MB-231 triple-negative breast carcinoma cells by treating with BafA1. We found increased LC3-II with a build-up of VEGFA (Fig. S6, G and H). Collectively, these pharmacological and genetic data indicate that VEGFA is degraded by basal autophagy in a diverse number of cellular systems and types.

Decorin evokes intracellular degradation of VEGFA via autophagy and RAB24

A hallmark of decorin bioactivity is the dual property of being anti-angiogenic (13, 15, 27, 32, 86, 87) and pro-autophagic (18, 20, 28, 46 – 48). Moreover, the Dcn gene itself is an autophagic-inducible gene (88). We postulated that decorin-mediated autophagy is the mechanism by which intracellular VEGFA is reduced.

We investigated this hypothesis by performing autophagic flux assays in PAER2 and found that decorin significantly suppressed VEGFA levels (Fig. 8, A and B). Treatment with BafA1 alone increased both LC3-II and VEGFA above basal conditions (Fig. 8, A and B), further reinforcing our above findings (cf. Fig. 7, A and B) that VEGFA is degraded by basal autophagy. Combinatorial treatment of decorin and BafA1 increased LC3-II levels beyond the levels expected of basal autophagy (Fig. 8, A and B), corroborating the concept that decorin evokes excessive autophagy by increasing autophagic flux. Critically, VEGFA was no longer suppressed by decorin following...
Autophagic inhibition with BafA1. Intracellular VEGFA levels appeared modestly increased above basal levels with the combinatorial treatment indicating that decorin enhances the rate of VEGFA catabolism (Fig. 8, A and B). These data indicate that decorin clears intracellular VEGFA by inducing endothelial cell autophagy.

Next, we investigated the small GTPase RAB24, a gene induced by decorin via NanoString analysis. RAB24 has recently been implicated in the autophagic pathway (89).

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Figure 7. Interfering with basal autophagic flux leads to an accumulation of intracellular VEGFA. A, representative immunoblots of PAER2 treated with increasing concentrations of bafilomycin A1 (BafA1) for 6 h. B, quantification of targets as in A. C and D, immunoblot of HUVEC ± BafA1 (500 nM) (C) and quantification (D). E and F, immunoblot of HUVEC ± chloroquine (30 μM) for 6 h (E) and quantification (F). G and H, immunoblot depicting HUVEC following the transient transfection of siScr or siATG5 (100 pM each) (G) and corresponding quantification of targets (H) as in G. GAPDH served as the loading control for immunoblots depicted in A, C, E, and G. Data are reflective of four independent biological replicates. Data are expressed as arbitrary units (A.U.) on a dot density plot. Statistics were calculated via two-tailed Student’s t test for D, F, and H.

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RAB24 facilitates autophagic clearance of components undergoing basal autophagy, but it does not aid in autophagosomal formation, as depletion of RAB24 results in a significant increase in late autophagic compartments (90). Therefore, as VEGFA is degraded by autophagy and decorin promotes increased autophagic flux of VEGFA and lysosomal biogenesis via TFEB (46), we evaluated the role of RAB24. We silenced RAB24 by RNAi and verified its depletion (Fig. 8, C and D). We noted that decorin induced RAB24 by 1.9-fold (p < 0.014) (Fig. 8, C and D). Moreover, we found that LC3-I was significantly increased following loss of RAB24 indicating a role for RAB24 in autophagosome maturation with no significant changes seen in p62 (Fig. 8, C and D). Decorin reduced VEGFA in PAER2 cells and this effect was abrogated following RAB24 silencing, recapitulating the findings from the flux assays (Fig. 8, C and D). Moreover, loss of RAB24 alone increased VEGFA levels, corroborating the role of RAB24 in basal autophagic regulation and strengthening the shuttling of VEGFA in the autophagic pathway for clearance (Fig. 8, C and D).

Taken together, these data illustrate that decorin promotes VEGFA catabolism by autophagic clearance via RAB24 in endothelial cells. This mechanism serves as a nexus by which decorin integrates and exerts its powerful anti-angiogenic and pro-autophagic properties.

**VEGFA is reduced in vivo by protracted fasting**

To determine whether our *in vitro* results could also occur *in vivo*, we fasted WT C57BL/6 mice for 48 h. We found that VEGFA levels in both heart and aorta of fasted mice were significantly reduced compared with those fed *ad libitum* (Fig. 9, A–D). As a positive control for starvation-induced autophagic induction, we tested LC3-II levels and found a marked increase in both cardiac and aortic tissues (Fig. 9, A–D). To provide definitive proof for the *in vivo* autophagic catabolism of VEGFA, we starved the mice for 48 h and treated them with CQ (50 mg/kg) for the last 4 h. We found a significant increase in VEGFA in cardiac and aortic tissues relative to starved mice alone (Fig. 9, E–G). As a negative control, we evaluated β-catenin, a critical regulator of cell behavior in response to Wnt signals (91) and canonical decorin target (14). We found no significant changes in β-catenin levels in either the heart (p = 0.456) or aorta (p = 0.433) following *in vivo* autophagy inhibition (Fig. 9, E and F). Collectively, these results demonstrate that VEGFA is regulated in a nutrient-dependent manner.
in parenchymal tissues in vivo. These findings greatly reinforce our in vitro data whereby VEGFA is governed by prevailing nutrient conditions and by in vivo autophagic flux.

Discussion

Autophagic modulation by soluble extracellular matrix components is rapidly emerging as a common, conserved, and fundamental cellular mechanism intricately intertwined in health and disease outcomes (9, 92, 93). In this study, we functionally unified the anti-angiogenic and pro-autophagic properties of decorin downstream of VEGFR2, AMPK, and Peg3 signaling in endothelial cells and demonstrated that intracellular VEGFA is catabolized via autophagy (Fig. 10).

Stimulation with the AMP-mimetic AICAR phenocopies the effect of decorin. Our data support a reciprocal role of AMPK that requires the inherent kinase activity of the catalytic α-subunit, to induce Peg3 and simultaneously decrease VEGFA in endothelial cells at the protein level. Activating AMPK alone is biologically equivalent to decorin-dependent suppression of intracellular VEGFA, indicating that decorin and AMPK are in the same signaling pathway, presumably downstream of decorin/VEGFR2 interactions (Fig. 10). The precise connection between VEGFR2 and phosphorylated AMPK remains elusive.

Figure 9. VEGFA is reduced in vivo by protracted fasting. A, representative immunoblot of cardiac tissue from WT C57BL/6 mice following 48 h starvation. B, quantification of immunoblot targets as in A. C, representative immunoblot of aortic tissue from WT C57BL/6 mice following 48 h starvation. D, quantification of immunoblot targets as in C. E and F, representation immunoblot of in vivo autophagic flux in cardiac (E) or aortic (F) tissue following a 48-h starvation period with concurrent systemic administration of chloroquine (50 mg/kg) for the last 4 h. G, quantification of VEGFA from the tissues as shown in E and F. GAPDH served as a loading control for immunoblots depicted in A, C, E, and F. Data are reflective of four independent biological replicates and expressed as arbitrary units (A.U.) on a dot density plot. Statistics were calculated via two-tailed Student’s t test.

replete nutrient conditions (48) and cytosolic Ca²⁺ mobilization by decorin (94), it is feasible that decorin is engaging the nucleotide-independent pathway to activate AMPK via CAMKK2 (63) downstream of VEGFR2.

Decorin differentially modulates signaling to favor autophagic induction by attenuating mTOR at P-Ser²⁴⁴⁸ and inducing AMPKa at P-Thr¹⁷². It is known that mTOR can evoke VEGFA expression via p70S6K1 activation and STAT3 nuclear translocation to the VEGFA promoter (95, 96). Here, we demonstrate an mTOR-independent role of VEGFA suppression in endothelial cells utilizing pharmacological agents that inhibit the mTOR kinase and mimic low-energy conditions (97), despite the lively regulation of LC3-I/-II and/or p62 (Fig. 10). Thus, bioenergetics-mediated regulation of intracellular VEGFA proceeds in an AMPK-dependent and mTOR-independent mechanism in endothelial cells and fibroblasts. It is possible that decorin initiates autophagy solely via AMPK and ULK1/2 (50, 51, 98) as decorin requires the class III phosphatidylinositol 3-kinase or Vps34 and Beclin 1-positive complexes (20, 99) that are heavily involved in the early stages of autophagic progression (100).

We found that conventional autophagic stimuli trigger VEGFA incorporation into LC3- and Beclin 1-positive auto-
Autophagic clearance of intracellular VEGFA

Figure 10. Schematic depiction of decorin-evoked catabolism of VEGFA via endothelial cell autophagy. Please refer to the text for additional details. Star-shaped symbols denote lysosomal acid hydrolases.

phagosomes to a similar magnitude and frequency to that of decorin. This process occurs downstream of VEGFR2 as tyrosine kinase inhibition prevents autophagosomal incorporation, highlighting an active role of this RTK for the autophagic degradation of VEGFA in response to anti-angiogenic signals originating from the extracellular matrix. Decorin transduces signals through VEGFR2 that engage and augment catabolic hubs for the degradation of powerful angiokines residing within the cytosol. Interestingly, the overall levels of VEGFA are sensitive to the ambient nutrient conditions as nutrient deprivation in multiple cellular models representing diverse tissues, such as endothelium, stromal, and triple negative breast cancer all resulted in a significant decrease of VEGFA.

Mechanistically, the mobilization of VEGFA into LC3-positive autophagosomes requires the putative tumor suppressor gene, Peg3 (Fig. 10). Transient depletion or overexpression of full-length Peg3 is necessary and sufficient for the formation of VEGFA/LC3-positive structures, respectively, whereas genetically ablating the C-terminal zinc finger domains abrogates this process. Peg3 is capable of driving endothelial BECN1 gene expression, a critical effector necessary for autophagosomes biogenesis (101–103), and LC3-mediated autophagic flux (28). Truncating the zinc finger domains may result in a loss of BECN1 expression culminating in decreased autophagic flux, thereby manifesting as reduced VEGFA/LC3 autophagosomes. Alternately, loss of the zinc fingers may abrogate the scaffolding function of Peg3, as its incorporation into Beclin 1-positive protein complexes (20) may be impaired resulting in dampened autophagosome formation. The exact molecular partners that shuttle VEGFA into the autophagosome remain unidentified.

Bioinformatics analyses of the various VEGFA isoforms did not identify a canonical LC3-interacting region that would enable specific autophagosomal targeting; therefore, we cannot exclude the possibility that VEGFA is merely a constituent of the larger cytoplasmic milieu subject to bulk degradation by macroautophagy.

Intracellular VEGFA is subject to autophagic flux as pharmacological inhibition with BafA1 or CQ, lysosomotropic agents that block autophagosomal/lysosomal fusion, results in a considerable accumulation of VEGFA under basal conditions (Fig. 10). These findings were recapitulated by depleting ATG5, a critical protease for LC3 maturation (85). Importantly, decorin augments VEGFA flux, suggesting that this is a primary pathway for intracellular control of VEGFA. Mechanistically, decorin-evoked catabolism of VEGFA depends on RAB24, a small GTPase recently implicated in autophagic progression, particularly of substrates undergoing basal autophagy (89, 90). However, whether the control of RAB24 is under the auspices of the VEGFR2/AMPK/Peg3 signaling axis remains to be investigated (Fig. 10).

We validated our findings in vivo by demonstrating that starvation decreased VEGFA content in cardiac and aortic tissues, suggesting physiological relevance for the metabolic control over VEGFA. The starvation-mediated reduction of cardiac and aortic VEGFA was blocked by systemically administrating CQ, providing evidence for the in vivo role of autophagy in VEGFA catabolism. The precise reservoir of VEGFA that is being depleted, whether intracellular, cell-surface associated, extracellular matrix, and/or blood, is currently unknown.

Therapeutic targeting of VEGFA with biological agents, such as anti-VEGFA therapies using bevacizumab and ranibizumab, have revolutionized the treatment of metastatic colorectal cancer and severe ophthalmological disorders (57, 58). Mechanistically, these agents function by inhibiting VEGFA thereby preventing rampant neovascularization (58). Considering the effect of decorin in suppressing VEGFA via autophagic degradation, we recognize the potential clinical implications of decorin administered as an adjuvant that specifically targets the RTK-rich environment of the growing cancer (14). Moreover, our general finding of VEGFA sensitivity to starvation and subsequent accumulation with autophagic inhibitors transcends proteoglycan-driven endothelial cell autophagy. Starvation-evoked VEGFA clearance may augment the clinical benefits of caloric restriction as a potent modality to curb breast tumorigenesis by enhancing immunotherapies (105), radiation efficacy (106), and alleviating the metastatic burden (107, 108).

Collectively, we have integrated two critical properties of soluble decorin operating downstream of receptor binding for its putative tumor repressor properties. At the nexus of this network is Peg3, which acts as a molecular conduit for coordinated angiostasis and autophagic induction in response to decorin and perhaps other autophagic stimuli derived from extracellular matrix interactions (109). These data further reinforce the candidacy of decorin as a next-generation anti-angiogenic protein therapy (110) in the ongoing fight against cancer.
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Experimental procedures

Cells, chemicals, and general reagents

Primary HUVEC were obtained from Lifeline Cell Technology, grown in basal media supplemented with the VascuLife EnGS LifeFactors Kit, also from Lifeline Cell Technology, and used within the first five passages. Porcine aortic endothelial cells overexpressing VEGFR2 (PAER2) were described previously (111, 112). Human aortic endothelial cells immortalized by stable expression of the human catalytic subunit of telomerase (TeloHAEC), immortalized NIH-3T3 murine fibroblasts, and human MDA-MB-231 triple-negative breast carcinoma cells were procured from American Type Cell Culture. Cells were grown at 37 °C in a 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium containing 4.5 g/liter of glucose, 1-glutamine, and sodium pyruvate from Life Technologies and supplemented with 5% fetal bovine serum from ThermoFisher Scientific and 100 units/ml of penicillin/streptomycin from Life Technologies. Dimethyl sulfoxide (DMSO) and Hank’s balanced salt solution also came from ThermoFisher Scientific, whereas Earle’s balanced salt solution was from HyClone. Rabbit polyclonal antibodies against GAPDH, HA, AMPKα, and P-AMPKα were obtained from Cell Signaling Technologies. The rabbit polyclonal antibody against RAB24 was from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against p62 and LC3B were purchased from Sigma. Mouse monoclonal antibodies against VEGFA and ATG5 were purchased from Santa Cruz Biotechnology. A second mouse mAb against VEGFA was purchased from Abcam. The rabbit anti-Beclin 1 and horseradish peroxidase-conjugated goat anti-rabbit and donkey anti-mouse secondary antibodies were obtained from EMD Millipore. A custom rabbit polyclonal antibody against the N-terminal human SCAN domain of Peg3 (amino acids 164–177) was generated by GenScript. Compound C, AICAR, SU5416, bafilomycin A1, and chloroquine were purchased from Sigma. Torin 1 was purchased from Tocris Biosciences. INK128 was acquired from Cayman Chemicals. DMSO was used as vehicle where appropriate for all experiments. All primary antibodies were used at 1:1,000 diluted in 1% BSA/TBST except for GAPDH, which was used at 1:10,000 and VEGFA (from Santa Cruz Biotechnology) was used at 1:500. For immunofluorescence, primary antibodies were used at 1:200 in 1% BSA in PBS. Secondary antibodies for chemiluminescence were used at 1:5,000 in the same buffer as above. The SuperSignal West Pico Enhanced Chemiluminescence substrate was purchased from ThermoFisher Scientific (USA). Purification and validation of human recombinant decorin free of any co-purifying contaminants can be found elsewhere (18).

Quantitative real-time PCR and analysis

Briefly, quantitative PCR was carried out on subconfluent 6-well-plates seeded with ~2 × 105 of HUVEC cells, treated per experimental conditions, and lysed in 1 ml of TRIzol reagent (Life Technologies). RNA isolation was carried out with Direct-zol RNA Miniprep Kit as per the manufacturer’s protocol (Zymo Research). Total RNA (1 μg) was annealed with oligo(dT) primers, and cDNA was synthesized using SuperScript Reverse Transcriptase III (Life Technologies). Gene-specific primer sets for Homo sapiens PEG3 and VEGFA were designed and validated. Gene expression analysis was performed on a Roche LightCycler 480-II and calculated with the comparative ΔΔCt method. A full description can be found in Ref. 20.

Transient DNA expression and RNAi-mediated silencing

We transiently transfected PAER2 with empty vector (pcDNA3.1) or plasmids encoding HA-Peg3 or HA-SCAN using Lipofectamine 2000 (Life Technologies) in Opti-MEM reduced serum media (Gibco). Expression was verified by immunoblot using target-specific antibodies. A full description of the DNA transfection protocol has been described elsewhere (113). Cells were transiently transfected using Lipofectamine RNAiMAX (Life Technologies) mixed with siRNA against Peg3, ATG5, and RAB24 that were purchased from Santa Cruz Biotechnology. The siRNA used represents a validated mixture of 3–5 targeting oligonucleotides for each gene of interest. Scrambled siRNA (sc-37007, Santa Cruz Biotechnology) served as a control for all siRNA experiments presented herein. The full protocol is described in Ref. 45.

Immunofluorescence, confocal laser microscopy, line scanning, and Pearson’s coefficient of colocalization

Typically, ~5 × 105 HUVEC or PAER2 were plated on 0.2% gelatin-coated 4-well-chamber slides (Nunc, ThermoFischer Scientific) and grown to full confluence in their respective growth media at 37 °C. Cells were treated as per the experimental conditions contained herein. Slides were incubated with conjugated secondary antibodies such as: goat anti-rabbit IgG Alexa Fluor® 488 and goat anti-mouse IgG Alexa Fluor® 564 (Life Technologies). Nuclei were visualized with DAPI (Vector Laboratories). Immunofluorescence (104) and differential interference contract images were acquired with a ×63, 1.3 oil-immersion objective installed on a LEICA DM5500B microscope with the Leica Application suite, advanced fluorescence version 1.8 software from Leica Microsystems, Inc. Confocal analyses were carried out utilizing a ×63, 1.3 oil-immersion objective of a Zeiss LSM-780 confocal laser-scanning microscope with Zen Imaging Software. To determine colocalization of proteins, Z-stack series were acquired maintaining the same number of slices (n = 30). All images were then analyzed in ImageJ (NIH) and Photoshop CS6 (Adobe Systems). Line scanning plots were generated using SigmaPlot software (Systat Software). A full description can be found elsewhere (20).

Animal experiments

All animal experiments contained herein 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. C57BL/6 mice were purchased from Jackson Laboratories. Both male and female mice and of various ages ranging from 4 weeks to 6 months were used. Fasting experiments involved withholding food for 48 h, but water was allowed ad libitum. Chloroquine (50 mg/kg in sterile dH2O) was administered by intraperitoneal injection during the last 4 h of the starvation period. After animals were euthanized, organs were removed and immediately snap-frozen in liquid nitrogen. Cardiac and aortic tissues were homogenized, solubi-
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lized in T-PER Tissue Protein Extraction Buffer from ThermoFischer Scientific, and resolved by SDS-PAGE.

Statistical analysis

Immunoblots were quantified by scanning densitometry using Sigma Stat 3.10. Experiments with three or more comparison groups were subjected to one-way ANOVA followed by a Bonferroni post hoc test using the Systat Package of SigmaPlot 13.0. Differences were considered significant at two sided p < 0.05.

Data availability

All data presented here are contained within the manuscript.

Author contributions—T.N., C. G. C., and R. V. I. conceptualization; T. N., C. G. C., S. B., and R. V. I. data curation; T. N., C. G. C., S. B., and R. V. I. formal analysis; T. N. and R. V. I. supervision; T. N. and R. V. I. funding acquisition; T. N., C. G. C., S. B., and R. V. I. investigation; T. N., C. G. C., and R. V. I. writing-original draft; T. N. and R. V. I. project administration.

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