Decorin Induces Mitophagy in Breast Carcinoma Cells via Peroxisome Proliferator-activated Receptor γ Coactivator-1α (PGC-1α) and Mitostatin*§

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Background: Decorin functions as a soluble tumor repressor via binding receptor-tyrosine kinases, such as Met, to curb rampant tumor neovascularization.

Results: Decorin evokes tumor cell mitophagy through dynamic co-regulation of PGC-1α and mitostatin via physical interactions between PGC-1α and mitostatin.

Conclusion: Decorin requires mitostatin to evoke mitophagy as the underlying basis for angiogenic attenuation.

Significance: We have identified mitostatin as a novel mitophagic effector.

Tumor cell mitochondria are key biosynthetic hubs that provide macromolecules for cancer progression and angiogenesis. Soluble decorin protein core, hereafter referred to as decorin, potently attenuated mitochondrial respiratory complexes and mitochondrial DNA (mtDNA) in MDA-MB-231 breast carcinoma cells. We found a rapid and dynamic interplay between peroxisomal proliferator-activated receptor γ coactivator-1α (PGC-1α) and the decorin-induced tumor suppressor gene, mitostatin. This interaction stabilized mitostatin mRNA with concurrent accumulation of mitostatin protein. In contrast, siRNA-mediated abrogation of PGC-1α-blocked decorin-evoked stabilization of mitostatin. Mechanistically, PGC-1α bound MITOSTATIN mRNA to achieve rapid stabilization. These processes were orchestrated by the decorin/Met axis, as blocking the Met-tyrosine kinase or knockdown of Met abrogated these responses. Furthermore, depletion of mitostatin blocked decorin- or rapamycin-evoked mitophagy, increased vascular endothelial growth factor A (VEGFA) production, and compromised decorin-evoked VEGFA suppression. Collectively, our findings underscore the complexity of PGC-1α-mediated mitochondrial homeostasis and establish mitostatin as a key regulator of tumor cell mitophagy and angiostasis.

Representing one of the eight hallmarks of cancer, tumor cell metabolism is commonly usurped to utilize aerobic glycolysis as the primary means to satisfy bioenergetic demands despite aerobic conditions (1) by way of enhanced glycolytic flux and an increased dependence on glucose. Altered cancer cell metabolism strictly relies on functional mitochondria, which are exploited to provide key biosynthetic precursors to aid in macromolecular synthesis via acetyl-CoA and derivatives thereof (2). Although the molecular underpinnings remain unclear, loss of tumor suppressor genes, alternate mRNA splicing of metabolic enzymes, conventional oncogenes, and receptor-tyrosine kinase signaling have all been shown to actively participate in mitochondrial reprogramming (2, 3). The latter contributors are particularly important as cancer cells display constitutively activated signaling pathways emanating from aberrant receptor-tyrosine kinases. The innate ability of transformed cells to be self-sufficient in the absence of instructional signaling cues promotes a switch from a quiescent, differentiated cell metabolism (predominantly catabolic for maximal ATP yield) to cellular anabolism (a consequence of direct mitochondrial reprogramming) for support of aggressive tumors and enhanced angiogenesis (1, 2, 4). Intriguingly, the Myc proto-oncogene, a target of decorin bioactivity, controls nuclear mitochondrial gene expression and, thus, mitochondrial biogenesis in concert with members of the PGC-1α (transcriptional co-activator family (5).

Decorin, the archetypical member of the small leucine-rich proteoglycan gene family (6, 7), is expressed in the stroma of various forms of cancer (8) and has been proposed to act as a

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“guardian from the matrix” (9). Decorin has been involved in the control of various biological processes encompassing collagen fibrillogenesis, wound healing, myogenesis, stem cell biology, and fibrosis (10–16). Initially identified as a cell growth inhibitor via blockade of transforming growth factor β (TGFβ) (17, 18), soluble decorin is emerging as a potent pan-receptor-tyrosine kinase inhibitor targeting EGF receptor, Met, IGF-I receptor, VEGF receptor 2, and PDGF receptor (19–29). Indeed, a decorin fusion protein linked to a wound-targeting peptide enhances wound healing and reduces scar formation via abrogation of TGFβ1/2 signaling (30). Interestingly, recent findings now suggest a much broader role for decorin insofar as modulating the biophysical properties of tendons and ligaments (14), orchestrating a critical signaling events during myogenesis (16), and regulating the innate immune receptors, Toll-like receptors 2/4, during inflammation (31).

Decorin suppresses cancer growth, migration, proliferation, and metastasis through concerted internalization of receptor-tyrosine kinases and consequent degradation of Myc and β-catenin (7, 9, 23, 32–39) with simultaneous induction of p21WAF1 (40). Importantly, decorin modulates the angiogenic network (9) via direct antagonism of the pro-angiogenic Met (41) and concurrent transcriptional suppression of HIF-1α (42), ultimately leading to a suppression of VEGFA (38) and retardation of angiogenesis (42). Decorin promotes an anti-angiogenic tumor microenvironment by acting as a partial EGF receptor agonist for rapid secretion of thrombospondin-1 (43), a central player in angiogenesis (44), fibrosis (45), and cardiovascular function (46). These studies have firmly established decorin as an endogenous tumoricidal agent (47) and is further supported by genetic evidence where loss of the decorin gene is permissive for tumorigenesis (48, 49). Moreover, mice with a compound germ-line deficiency in p53 and decorin die prematurely of aggressive T-cell lymphomas (50). Delivery of decorin via adenoviral-mediated gene transduction or systemic administration of recombinant human decorin or decorin protein core to several tumor xenograft models (breast and prostate carcinomas) suppresses cancer growth (34, 36, 51–54).

Consistent with the proclivity of decorin to induce tumor suppressor genes (PEG3, CDKN1A), decorin induces mitostatin, a mitochondrial protein with oncostatic activity (55). Upon induction, mitostatin displays several hallmarks of a classical tumor suppressor gene such as inhibiting tumor cell migration, growth, and proliferation and simultaneously triggering pro-apoptotic pathways (55, 56). Furthermore, mitostatin is absent in ~35% of human prostate carcinomas (56), whereas decreased expression is associated with advanced cancer stages (55). Thus, we hypothesized that decorin could compromise tumor mitochondria as the underlying mechanistic basis for suppressed tumor angiogenesis under normoxia. We discovered that decorin evoked mitostatin production via the Met receptor, thereby triggering a signaling cascade leading to a mitostatin-dependent mitophagy associated with a negative feedback control on VEGFA transcription, thus indirectly attenuating tumor angiogenesis. We further discovered a novel interaction between PGC-1α and mitostatin, and this interaction led to stabilization of mitostatin mRNA and concurrent accumulation of mitostatin protein.
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63 × 1.3 oil-immersion objective installed on a LEICA DM5500B microscope programmed with Leica Application Suite, Advanced Fluorescence v1.8 software from Leica Microsystems, Inc. (Frankfurt, Germany). Fluorescence imaging of MDA-MB-231 Su9-GFP cells was carried out as described above for immunofluorescence microscopy with identical parameters maintained for the treatment groups examined. Images were quantified with ImageJ (National Institutes of Health) software programmed with a special macro specifically designed to measure mitochondrial morphology variables as described previously (58). For live cell confocal microscopy, 3.5-cm inset dishes were coated with poly-l-lysine and 0.2% gelatin as before and seeded with ~5 × 10⁴ MDA-MB-231 Su9-GFP cells and visualized with a 63×1.3 oil-immersion objective using a Zeiss LSM-780 confocal laser scanning microscope and analyzed using the Zeiss LSM-780 software. All collected images were analyzed using ImageJ and Adobe Photoshop CS5.5 (Adobe Systems, Carlsbad, CA), and movies were processed and assembled with Adobe premiere Pro CS5.5 (Adobe Systems). Three-dimensional surface blots were created with ImageJ software as described (33).

Transient siRNA-mediated Knockdown—Transient knockdown of Met, PGC-1α, and mitostatin in MDA-MB-231 was achieved via transfection of three separate and validated siRNAs specific for either Met (sc-29397), PGC-1α (sc-38884), or mitostatin (sc-95954), all from Santa Cruz Biotechnology. Scrambled siRNA (siScr, sc-37007) served as a control for all siRNA experiments presented herein. The following protocol was used subsequent to protein (radioimmunoprecipitation extraction), RNA isolation (TRIZol reagent, Invitrogen), or mtDNA isolation via RNAmol B (from Molecular Research Center, Inc. (Cincinnati, OH)) from variably treated samples for further analysis. As such, 6-well plates were seeded with ~2 × 10⁵ MDA-MB-231 cells followed by overnight incubation at 37 °C/5% CO₂ until cultures were ~70% confluent. Targeting or scrambled siRNA duplex (80 and 20 pm, respectively) was added to diluted Lipofectamine 2000 RNAmol (Invitrogen) in 1% BCS-DMEM. The transfection mix was applied for 6 h at 37 °C, 5% CO₂ whereupon additional full serum (5% BCS-DMEM) media was added. The cells were then allowed to incubate overnight in the same culture conditions. The media were changed, and the transfection was carried out for an additional 48 h. Verification of RNAi-mediated knockdown of the target protein was determined via immunoblotting or quantitative real-time polymerase chain reaction (qPCR).

Quantitative Real-time PCR Analysis—Gene expression or mtDNA content analysis by qPCR was carried out on subconfluent 6-well plates seeded with ~2 × 10⁵ of MDA-MB-231 cells were treated variably depending on experimental parameters in full serum (5% BCS-DMEM) media. After incubation, cells were directly lysed in either 1 ml of TRIZol reagent (Invitrogen) or 1 ml of RNAmol B (Molecular Research Center) to extract total RNA or for later purification of mtDNA (see below). Subsequently, for gene expression analyses only, ~1 μg of total RNA was annealed with oligo(dT)₁₈–₂₀ primers, and cDNA was synthesized with SuperScript Reverse Transcriptase II (SSRT II, Invitrogen). PCR amplicons representing target genes and the endogenous housekeeping gene, ACTB, were amplified in quadruplicate, independent reactions with the Brilliant SYBR Green Master Mix II reagent (Agilent Technologies, Cedar Creek, TX). 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 independent reaction. -Fold change determinations were made utilizing the Comparative Ct method. ΔCt values represent normalized gene expression levels to ACTB. ΔΔCt values were then calculated and represent the experimental cDNA (for example, those samples were treated with 100 nM decorin) minus the corresponding gene levels (ΔCt values) of the calibrator sample (i.e. control samples). Last, -fold changes were calculated using the double ΔCt method 2⁻⁵ΔCT ± S.E. Data presented herein represent at least three independent trials run in quadruplicate for each gene of interest examined.

RNA Immunoprecipitation (RIP)—RIP followed by qPCR of precipitated RNA was employed to investigate the occupancy of PGC-1α binding directly to MITOSTATIN mRNA in the presence of decorin or in the presence of SU11274 and decorin in MDA-MB-231 cells. The RIP protocol was executed according to the manufacturer’s instructions enclosed with the Magna RIP kit (Millipore). Briefly, two confluent (~90%) 10-cm dishes of MDA-MB-231 per experimental condition (totaling ~16 × 10⁵ cells) were lysed in RIP lysis buffer on ice after washes in PBS and stored at −80 °C until further use. Magnetic beads were prepared by with initial PBS washes followed by incubation at room temperature for 30 min with primary antibody raised against PGC-1α (5 μg of total antibody used per immunoprecipitation). Extensive washes were performed before incubation of absorbed magnetic beads with previously collected cell lysates. Incubation of conjugated beads with lystate took place overnight at 4 °C with end-over-end rotation. The beads were thoroughly washed and digested with proteinase K (45 min at 55 °C) to disengage PGC-1α containing ribonucleo-protein complexes. RNA from immunopurified PGC-1α-positive ribonucleoproteins were harvested via a canonical phenol chloroform isooamyl extraction and further precipitated via ethanol. Immunoprecipitated RNA from PGC-1α (ribonucleoproteins) was then subjected to cDNA synthesis and qPCR analysis as described above.

mtDNA Isolation—Analysis of mtDNA was performed in MDA-MB-231 cells grown in a six-well plate. Isolation of mtDNA was done according to a modified protocol derived from Tom Getty (Michigan State University). Briefly, after treatment according to experimental conditions, confluent (~90%) MDA-MB-231 cells were lysed in 1 ml of RNAmol B and subjected to a chloroform extraction. A polycaryll carrier (Molecular Research Center) was utilized to facilitate precipitation of the DNA in conjunction with an ethanol extraction. After purification of DNA samples (containing both mtDNA and genomic DNA), 5 ng of purified DNA was used per qPCR reaction, and mtDNA content was measured using primers specific for NADH dehydrogenase subunit I (ND1) with Ct values normalized to the genomic marker, LPL (lipoprotein lipase). Reported -fold changes ± S.E. were calculated via the ΔΔCt method as described above.

Immunoblotting and Immunoprecipitation—After each treatment as described herein, MDA-MB-231 cells were lysed
in radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA/EGTA/sodium vanadate, 10 mM β-glycerophosphate, and various protease inhibitors including 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin/tosyl-phenylalanyl chloromethyl ketone/aprotinin each) for 20 min on ice and separated on SDS/PAGE. For immunoprecipitation experiments, protein A-Sepharose magnetic beads (GE Healthcare) were co-incubated with antibodies overnight at 4 °C. The next day the beads were washed extensively, and the lysates were added to the beads and incubated overnight at 4 °C with rotation. After extensive washing, the beads were boiled in reducing buffer, and supernatants were separated by SDS/PAGE. Proteins were then transferred to nitrocellulose membranes (Bio-Rad), immunoreacted with the indicated primary antibodies, subsequently developed with enhanced chemiluminescence (Thermo Scientific), and detected using an ImageQuant LAS-4000 (GE Healthcare).

Measuring Mitochondrial Membrane Potential—At least two individual assays were performed in MDA-MB-231 cells using the mitochondrial dye JC-1. JC-1 accumulates in actively respiring mitochondria forming “J-aggregates,” which emits a red/orange fluorescence at 590 nm. However, during times of low mitochondrial membrane potential (i.e. depolarization), JC-1 exists as a monomer and emits a green fluorescence at 525 nm. As such, MDA-MB-231 cells were grown in four-chambered glass slides coated with poly-L-lysine (50 μg/ml) and 0.2% gelatin for 24 h in 5% BCS-DMEM. Cells were then treated with nox-2 siRNA for 6 h. One chamber was treated with carboxylcyanide 4-trifluoromethoxy phenylhydrazone (FCCP) 10 min before staining. Each chamber was incubated with JC-1 (7.5 μM) for the last 10 or 20 min of the experiment. Cells were washed twice with PBS and imaged live using a Leica DM5500B microscope. All the images were procured using the same settings.

Quantification and Statistical Analysis—Immunoblots were quantified by densitometry using ImageJ software. Gene expression and mtDNA analysis were determined as described above and reported as -fold changes ± S.E. All experiments contained herein were carried out with a minimum of three independent trials performed in triplicate. 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 unpaired Student’s t test, and data were considered significant when p < 0.05. For quantification of immunofluorescence studies, fluorescence intensity and three-dimensional surface plots were quantified by measuring pixels with ImageJ software.

RESULTS

Decorin Requires Met for Suppression of Oxidative Phosphorylation—Decorin transcriptionally suppresses several critical oncosenes under normoxia including Myc and HIF-1α (42), which play instrumental roles in the reprogramming of metabolism, as it pertains to the dichotomy of aerobic glycolysis and mitochondrial respiration (4, 59 – 61). Thus, we determined the effect of soluble decorin protein core (62) on mitochondrial respiratory chain complexes (OXPHOS) in triple-negative breast carcinoma MDA-MB-231 cells. Utilizing a mixture of monoclonal antibodies targeting each OXPHOS subunit, we found a marked suppression of complexes II, III, IV, and V in response to decorin (Fig. 1A). Quantification of three independent experiments revealed a reduction of 40 – 60% relative to control (Fig. 1B) after 4 h, and this effect was long lasting (Fig. 1C).

Next, we investigated the involvement of the HGF/Met signaling axis in the regulation of OXPHOS. Exogenous HGF increased the amount of all complexes (Fig. 1D) by ~2-fold over basal state (p < 0.001; Fig. 1E), and Met was required, as depletion of Met via siRNA (Fig. 1F) abrogated this response for complex V (Fig. 1, F and G), complex III (Fig. 1, F and H), and complex IV (Fig. 1, F and J). Intriguingly, silencing of Met did not prevent suppression in response to decorin of complex II (Fig. 1, F and I). Therefore, it is possible that a differential pathway exists for complex II degradation (as complex II is also a key enzyme for the citric acid cycle) and/or decorin integrates signaling over multiple receptor-tyrosine kinases (e.g. EGF receptor). Thus, decorin suppresses the mitochondrial respiratory chain complexes via a Met-dependent pathway under normoxic and nutrient-rich conditions.

Decorin Evokes a Met-dependent Induction of Mitostatin—We hypothesized that decorin-mediated induction of mitostatin could suppress OXPHOS components by promoting mitochondrial turnover/degradation. Decorin induced mitostatin protein levels by ~2.5-fold (p < 0.001; Fig. 2A) with a concurrent relocation of mitostatin epitopes to large (2–3 μm) perinuclear vacuoles, reminiscent of autophagosomes (white arrows, Fig. 2B). Importantly, these experiments were performed in nutrient-rich conditions. Incubation with HGF depressed mitostatin protein levels (p < 0.01, Fig. 2C), implicating Met in the negative control of mitostatin. Preincubation with HGF followed by decorin resulted not only in a complete block of decorin-evoked induction of mitostatin (p < 0.01, Fig. 2D) but also in lowering mitostatin below control levels, analogous to HGF alone (Fig. 2D). Depletion of endogenous Met via siRNA (Fig. 2E) blocked the ability of decorin to evoke mitostatin as compared with either siMet alone or scrambled siRNA (siScr) (Fig. 2E). Thus, these novel findings indicate a dependence on Met for decorin to induce mitostatin in MDA-MB-231 cells, a process that is either blocked and/or subdue by the natural agonist, HGF.

PGC-1α Is Required for Decorin-evoked Mitostatin Induction—Next, we investigated whether decorin-mediated induction of mitostatin was due to suppression of mitochondrial biogenesis factors. It is well established that PGC-1α serves as the primary node in the transcriptional network for mitochondrial biogenesis (63). PGC-1α regulates loci encoding nuclear mitochondrial genes, TCA cycle enzymes, outer and inner membrane mitochondrial transporters, and TFAM (mitochondrial transcription factor A), the master regulator of mtDNA expression (63). Decorin significantly decreased PGC-1α mRNA (data not shown) and protein (p < 0.001, data not shown) in MDA-MB-231 and HeLa cells. In contrast, HGF substantially increased PGC-1α protein levels (data not shown). Exogenous decorin, after transient depletion of Met, resulted in no further suppression of PGC-1α relative to either decorin (p = 0.474) or siScr
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FIGURE 1. Decorin requires Met for suppression of OXPHOS in MDA-MB-231 cells. A, representative immunoblots of mitochondrial OXPHOS complexes II, III, IV, and V in the presence of decorin protein core (100 nM, 4 h) resolved by SDS/PAGE. Henceforth, decorin protein core will be referenced as “decorin” unless otherwise noted. B, quantification of OXPHOS immunoblots presented in A after normalization to a Coomassie-stained gel to demonstrate equivalent sample loading. C, quantification of normalized OXPHOS components after a 24-h exposure to decorin (100 nM). D and E, OXPHOS evaluation of complexes II, III, IV, and V after HGF treatment (50 ng/ml, 4 h) and corresponding quantification (F) normalized on total protein. F, immunoblot of OXPHOS complexes after transient depletion of the Met receptor via targeting siRNA (siMet, 80 ps) in the presence of decorin protein core (100 nM, 4 h) relative to scrambled (siScr, 20 ps) controls. G–J, quantification of representative immunoblots presented in F for individual OXPHOS components (II, III, IV, and V) normalized to total protein. We note that complex I subunit was below the levels of detection in these cells. Values are representative of at least three independent trials performed in duplicate and are expressed as -fold changes ± S.E.; **, p < 0.01; ***, p < 0.001.

(data not shown). Knockdown of Met alone decreased basal levels of PGC-1α (p < 0.01, data not shown), implicating Met as a necessary receptor-tyrosine kinase for steady state PGC-1α.

Next, we tested whether a functional relationship existed between PGC-1α suppression and mitostatin induction as a mechanism for decorin-evoked suppression of OXPHOS. Unexpectedly, depletion of PGC-1α resulted in a concomitant loss of basal mitostatin relative to siScr and abrogated decorin-mediated induction of mitostatin (p < 0.001, Fig. 3A). Paradoxically, the addition of decorin in the presence of PGC-1α siRNA resulted in further suppression of mitostatin (p = 0.024, Fig. 3A), whereas loss of PGC-1α did not affect HGF-mediated reduction of mitostatin (Fig. 3A).

Following this observation, time-course experiments performed at steady state levels revealed a dynamic regulation of PGC-1α (Fig. 3B). As early as 15 min, decorin evoked a significant increase in PGC-1α levels (>3-fold) with maximal amounts occurring at 30 min; however, at later time points, PGC-1α returned to base line (Fig. 3B). Decorin evoked a steady increase of mitostatin that paralleled the co-regulation of PGC-1α up to 30 min (Fig. 3B). However, as PGC-1α returned to base line, mitostatin levels continued to increase and eventually plateaued at ~120 min (Fig. 3B). Immunofluorescence studies utilizing antibodies specific for PGC-1α corroborated the transient increase in PGC-1α at 15 and 30 min with subsequent decline at 120 min (Fig. 3C). Importantly, induction of PGC-1α at 30 min was dependent upon positive Met signaling, as preincubation with the small molecule inhibitor, SU11274, blocked PGC-1α induction (Fig. 3D). Furthermore, confocal microscopy revealed rapid nuclear accumulation of PGC-1α with sustained nuclear occupancy (>2-fold over control) and lasting up to 60 min (Fig. 3E).

As a working model, we envision two distinct mechanisms of action accounting for this positive regulatory function of PGC-1α. The first possibility is a post-translational mechanism involving direct protein-protein interactions between PGC-1α and mitostatin leading to accumulation of mitostatin. The second possibility would contemplate a post-transcriptional control whereby PGC-1α would stabilize mitostatin by directly binding to its mRNA via the C-terminal RNA recognition motif (RRM) found in PGC-1α (63, 64). To test the first model, as discussed above, we preincubated the cells with cycloheximide to block new protein synthesis and then added decorin at various time points. We found a time-dependent increase in both PGC-1α and mitostatin, albeit with delayed kinetics due to the non-steady state context (Fig. 3F). The rate of PGC-1α induction was analogous to steady state conditions, with maximal PGC-1α levels occurring at 60 min (>22-fold) and remaining high for up to 90 min (p < 0.01, Fig. 3F). Although mitostatin displayed slower kinetics, it did increase concomitantly with
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Next, we evaluated the second possibility of post-transcriptional control via PGC-1α and its C-terminal RRM. Cells were preincubated with the transcription inhibitor actinomycin D (ActD) to determine stability of PPARGC1A and MITOSTATIN mRNAs. Both mRNAs increased in a dose-dependent manner with respect to decorin, reaching ~6 and ~2-fold at 100 nM, respectively (p < 0.001, Fig. 4A). Time-course experiments showed rapid stabilization of PPARGC1A and MITOSTATIN mRNAs starting as early as 5 min (p < 0.001, Fig. 4B). Importantly, PPARGC1A mRNA levels peaked dramatically (>7-fold) at 30 min and then declined to ~2-fold above control (p < 0.001, Fig. 4B). Concurrent with the sudden burst in PPARGC1A mRNA stabilization, MITOSTATIN mRNA was also stabilized at an increased rate at 30 min (>4-fold) and remained elevated up to 60 min (p < 0.001, Fig. 4B).

To gain insight into whether PGC-1α would directly contribute to stabilizing MITOSTATIN mRNA, we performed additional mRNA stability assays under various conditions. First, we reproduced the decorin-evoked stabilization of PPARGC1A and MITOSTATIN mRNA after 30 min with each species stabilizing to a similar extent relative to the ActD-only treated control (p < 0.001, Fig. 4D). We then proceeded to deplete PGC-1α with targeting siRNA. After verification of PPARGC1A knockdown (Fig. 4C), we discovered that the loss of PGC-1α completely abrogated the ability of decorin to stabilize MITOSTATIN mRNA (p < 0.001, Fig. 4D). Protein methylation of RRMs contained within RNA-binding proteins catalyzed by protein arginine methyltransferases (PRMTs) is required for RNA-substrate interaction (63, 65). As such, siRNA-mediated depletion of PRMT1, the isoform specifically responsible for arginine methylation of PGC-1α (64), phenocopied the effect after depletion of PGC-1α (p < 0.001, Fig. 4C) insofar as abrogating decorin-evoked stability of MITOSTATIN mRNA (Fig. 4D). Additionally, and consistent with previous reports (63) suggesting that PGC-1α binds and stabilizes its own mRNA, loss of PRMT1 also precluded stability of PPARGC1A (Fig. 4D). These data reinforce the concept that decorin promotes direct PGC-1α binding to MITOSTATIN mRNA as the molecular basis for rapid stabilization.

Consistent with the dependence of Met on decorin-mediated mitostatin induction, siRNA-mediated depletion of Met (Fig. 4C) resulted in a total block of PPARGC1A and MITOSTATIN mRNA stabilization (Fig. 4D). Surprisingly, this rapid stabilization for both mRNA species was entirely dependent on Met signaling as co-incubation with SU11274 completely inhibited stabilization of PPARGC1A and MITOSTATIN mRNA (Fig. 4D).

Parallel findings concerning the requirement of PGC-1α in mediating decorin-evoked MITOSTATIN mRNA stability were made in an independent breast cancer cell line, T47D. After verifying depletion of PPARGC1A mRNA under non-steady state conditions (Fig. 4E), we found that decorin stabilizes both PPARGC1A and MITOSTATIN transcripts (Fig. 4F). However, loss of mitostatin-prevented stabilization of MITOSTATIN mRNA, akin to the triple negative breast carcinoma cell model (cf. Fig. 4D).

Finally, we investigated the link between involvement of the C-terminal RRM within PGC-1α and the potential for direct binding to the MITOSTATIN mRNA via this protein module. To this end, cells were stably transfected with a PGC-1α construct harboring a truncated RNA recognition motif known as the increase in PGC-1α at 60 min with plateauing at 90 min (Fig. 3F). These data indicate a dynamic regulation of PGC-1α and mitostatin under steady state and non-steady state conditions.

Next, we tested whether there was any physical association between PGC-1α and mitostatin. Using co-immunoprecipitation with anti-PGC-1α and immunoblotting with anti-mitostatin, we found a constitutive association between PGC-1α and mitostatin, and this binding was increased by decorin (Fig. 3G, left panel). Reciprocal co-immunoprecipitation experiments yielded analogous results (Fig. 3G, right panel), in contrast with a negative rabbit IgG control. Furthermore, quantification of the immunoprecipitated material after decorin stimulation revealed a significant (p < 0.01) increase in bound proteins (Fig. 3G, bottom). Collectively, decorin enhanced already preexisting binding between PGC-1α and mitostatin that might mechanistically underlie the requirement of PGC-1α for basal and decorin-induced mitostatin.

PGC-1α Stabilizes MITOSTATIN mRNA for Rapid Decorin-evoked Induction—Next, we evaluated the second possibility of post-transcriptional control via PGC-1α and its C-terminal RRM. Cells were preincubated with the transcription inhibitor

**FIGURE 2. Decorin evokes a Met-dependent induction of mitostatin in MDA-MB-231 cells. A, immunoblot of mitostatin after SDS/PAGE in response to decorin (100 nM, 4 h) and the corresponding quantification normalized on total cellular protein (Coomassie). The molecular mass of standard proteins is indicated on the right. B, immunofluorescence microscopy of control and decorin-treated MDA-MB-231 cells (100 nM, 4 h) as indicated after immunostaining for mitostatin (green) and DAPI (blue). All images were captured with the same exposure, gain, and intensity. Scale bar ~ 10 μm. C, immunoblot of mitostatin in response to HGF (50 ng/ml, 4 h) and corresponding quantification to total cellular protein. D, mitostatin evaluation in combination with decorin alone (100 nM) or after a 30-min pretreatment with HGF (50 ng/ml) for 4 h. E, representative immunoblot after transient transfection with either scrambled siRNA or siRNA targeting Met and mitostatin. Using co-immunoprecipitation experiments presented as -fold changes ± S.E. NS, not significant (p > 0.05); **, p < 0.01; ***, p < 0.001.
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A

B

C

D

E

F

G
HA-PGC-1α-ΔRRM (64). We achieved high expression of the transgene (Fig. 4G) after 48 h relative to empty vector (pcDNA3.1). qPCR analysis after transfection of empty vector showed a decorin-evoked increase in MITOSTATIN mRNA (p < 0.001, Fig. 4H). This is in stark contrast to the effect conveyed by HA-PGC-1α-ΔRRM, as lack of the RRM domain precluded decorin from stabilizing MITOSTATIN mRNA (p < 0.001, Fig. 4H). Importantly, transfection of HA-PGC-1α-ΔRRM alone in the presence of ActD destabilized MITOSTATIN mRNA (p < 0.001, Fig. 4H). This suggests that HA-PGC-1α-ΔRRM acts as a dominant negative effector, potentially competing with endogenous PGC-1α binding to MITOSTATIN mRNA to maintain a basal threshold of mRNA stabilization.

Collectively, we provide a novel model whereby decorin requires Met signaling to stabilize PPARGCA and MITOSTATIN mRNA. Depletion of PGC-1α, prevention of PGC-1α methylation, or utilization of a PGC-1α truncation mutant deficient in the RRM all resulted in total loss of decorin-evoked mRNA stability.

Stabilized PPARGCA and MITOSTATIN mRNAs Reflect Increased Mitostatin and Are Dependent on PGC-1α—We determined whether the stabilized PPARGCA and MITOSTATIN mRNAs yielded a corresponding increase at the protein level. Preincubation with ActD followed by stimulation with decorin revealed a significant increase of PGC-1α and mitostatin (p < 0.01, Fig. 5A). Analogous to the effect of PGC-1α depletion on mRNA stability, PGC-1α loss abrogated the ability of decorin to evoke stabilization of mitostatin protein (Fig. 5B). These data are in line with the requirement of PGC-1α for mitostatin induction during steady state. Next, we utilized the HA-PGC-1α-ΔRRM construct to recapitulate the necessity of the RRM domain in mRNA stabilization. Unlike empty vector, expression of HA-PGC-1α-ΔRRM completely blocked decorin-evoked induction of mitostatin (p < 0.01, Fig. 5C), in full agreement with the mRNA data. These findings indicate a biologically relevant output for stabilized PPARGCA and MITOSTATIN mRNAs and provide support for a key role of PGC-1α in this pathway and for RRM requirement to stabilize mitostatin mRNA.

PGC-1α Directly Binds MITOSTATIN mRNA via a Met-dependent Pathway—Having established a biological and functional link between PGC-1α and mitostatin, we evaluated the direct binding of PGC-1α to MITOSTATIN mRNA by performing RIP. Immunoprecipitation of PGC-1α-positive ribonucleoprotein complexes followed by qPCR analysis revealed that, after a 30-min decorin treatment, PGC-1α protein was enriched >90-fold on MITOSTATIN mRNA as compared with negative rabbit IgG2 control (p < 0.001, Fig. 5D). Importantly, preincubation with SU11274 completely blocked PGC-1α binding (p < 0.001, Fig. 5D) to MITOSTATIN mRNA. In contrast, decorin failed to promote PGC-1α binding the Parkinson protein 7 (PARK7) mRNA, which encodes DJ-1 and functions as a positive regulator of androgen receptor signaling (66) (Fig. 5E), confirming specificity of the RIP as well as PGC-1α target selectivity. We further validated our RIP experiments via semiquantitative PCR to confirm the identity of mitostatin from our input and immunoprecipitated RNA. The MITOSTATIN primers used for analysis gave a 120-bp amplification. PCR analysis of input and RIP samples revealed a specific 120-bp band representing the presence of the MITOSTATIN amplicon (Fig. 5F) and thereby further complementing the specificity of PGC-1α immunoprecipitation. Thus, we demonstrate a novel role for Met-dependent signaling to rapidly promote PGC-1α binding to MITOSTATIN mRNA under the influence of decorin. We have, therefore, discovered a novel regulatory function for PGC-1α to maintain a basal threshold of mitostatin as well as being required for decorin-mediated induction vis-à-vis the dynamic co-regulation between PGC-1α and mitostatin.

Decorin Disrupts Mitochondria Membrane Potential and Requires Mitostatin to Evoke Mitophagy—Based on decorin suppression of OXPHOS components and re-localization of mitostatin into large perinuclear vacuoles (cf. Fig. 2B), we tested whether decorin could evoke mitophagy in tumor cells via mitostatin. Early signaling events leading to a mitophagic response are initiated immediately after loss of mitochondrial membrane potential, Δψm (67, 68). Thus, we assessed the effects of decorin on Δψm using the voltage-sensitive dye JC-1. JC-1 is a lipophilic cationic dye that accumulates in the mitochondrial inner membrane in response to the Δψm. At low Δψm, JC-1 monomers show green fluorescence but at high levels of accumulation resulting from a high Δψm leads to formation of JC-1 aggregates that shifts the JC-1 emission spectrum to red fluorescence (69). Therefore, red to green fluorescence ratio has been used as a semiquantitative indicator of Δψm. After the addition of decorin for 6 h and utilizing live cell

**FIGURE 3.** PGC-1α is required for decorin-evoked mitostatin induction in MDA-MB-231 cells. A, representative immunoblot depicting the effects of siRNA targeting PGC-1α (80 pmol) on total mitostatin protein levels in combination with either decorin (100 nmol) or HGF (50 ng/ml) after 4 h relative to siRNA scrambled controls (siScr, 20 pmol) and corresponding quantification. B, analysis of PGC-1α and mitostatin protein levels via SDS/PAGE immunoblotting in MDA-MB-231 cells treated with decorin (100 nmol) at the indicated time points. C, MDA-MB-231 cells exposed to decorin (100 nmol) at the indicated time points and subjected to immunofluorescence microscopy. Cells were immunoreacted with PGC-1α (green) and stained with DAPI (blue) for nuclear visualization. D, representative immunofluorescence microscopy of PGC-1α (green) and MITOSTATIN (red) in MDA-MB-231 cells after treatment with PBS (control), decorin alone (100 nmol, 30 min), or after preincubation with the Met-tyrosine kinase inhibitor, SU1274 (1 µmol, 30 min pretreatment). All images were captured with the same exposure, gain, and intensity. Scale bar = 10 µm. E, confocal immunofluorescence microscopy of PGC-1α (green) and DAPI (blue) after treatment with decorin (100 nmol) at the indicated time points as co-localization appears as cyan with corresponding quantification of nuclear-localized PGC-1α puncta. All images were taken with the same exposure, gain, and intensity. Scale bar = 15 µm. F, immunoblot determining the effects of decorin (100 nmol) on PGC-1α and mitostatin after pretreatment with cycloheximide (20 µg/ml, 30 min) at the indicated time points with corresponding quantification. G, representative co-immunoprecipitation (IP) and reciprocal immunoblot (IB) of MDA-MB-231 cells treated with decorin (100 nmol) for 4 h. Cells were lysed in modified radioimmunoprecipitation and immunoprecipitated with an anti-PGC-1α antibody (5 µg); subsequently the input samples and immunoprecipitates were resolved via SDS/PAGE and detected with an anti-mitostatin antibody. Reciprocal (right panel) co-immunoprecipitation experiments where decorin (100 nmol, 4 h) stimulated MDA-MB-231 cell lysates were subjected to immunoprecipitation via an anti-mitostatin (5 µg) antibody and immunoblotted with an anti-PGC-1α antibody. Importantly, for both experiments, a negative rabbit IgG control antibody was employed as a negative control. Quantification of immunoprecipitated material is reported as a fold-change after normalization to the respective input protein. Data reflect at least two independent experiments in triplicate and are expressed as fold changes ± S.E.; *p < 0.05; **p < 0.01; ***p < 0.001.
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![Graphs and images](image)

**FIGURE 4.** PGC-1α stabilizes MITOSTATIN mRNA for rapid decorin-evoked induction. A, dose-response curves for PPARC1A and MITOSTATIN mRNA after a 30-min incubation with decorin at the indicated concentrations. B, relative mRNA levels of PPARC1A and MITOSTATIN determined by qPCR in the presence of decorin (100 nM) at the stipulated time points. C, qPCR verification of PPARC1A, MET, or PRMT1 depletion in the presence of siScr or siRNAs targeting the respective genes. Verification was performed in the presence of ActD (20 μg/ml) with or without decorin (100 nM). D, qPCR evaluation of PPARC1A or MITOSTATIN mRNA stability as performed in the presence of ActD (20 μg/ml) and decorin (100 μM, 30 min) alone and/or in combination with decorin and siRNA targeting PGC-1α, the Met receptor, or PRMT1 or with pretreatment (30 min) with the Met-tyrosine kinase inhibitor, SU1274 (1 μM). E, verification of PPARC1A depletion in T47D cells. Confirmation was performed in the presence of ActD (20 μg/ml) with or without decorin (100 nM). F, qPCR-directed evaluation of PPARC1A and MITOSTATIN stability in the presence of ActD (20 μg/ml) and decorin (100 nM, 30 min) alone and/or in combination with siRNA targeting PGC-1α in T47D cells. G, qPCR-based verification of HA-PGC-1α-∆RRM expression after transient transfection (4 μg) of empty plasmid (pcDNA3.1) or HA-PGC-1α-∆RRM. H, microscopy, we observed a significant loss of Δψm (Fig. 6A) relative to control cells, similar to what was observed when depolarization was induced by the addition of the uncoupler FCCP (Fig. 6A). Quantification of two independent experiments revealed a significant decorin-mediated loss of Δψm comparable to that of FCCP (p < 0.001, Fig. 6B).

To further investigate decorin-evoked tumor cell mitophagy, we stably transfected MDA-MB-231 cells with GFP fused to the mitochondrial matrix protein, Su9, a subunit of the Fo-ATPase (70). Decorin evoked mitochondrial fragmentation and aggregation into large autophagosomes vis à vis control (inserts, Fig. 6C). Importantly, the decorin-evoked changes in mitochondrial morphology were identical to those evoked by rapamycin, an inhibitor of the mTORC1 nutrient sensor signaling complex and established autophagic inducer (71) (Fig. 6C). Depletion of mitostatin rendered the cells unresponsive to both decorin and rapamycin. Indeed, loss of mitostatin completely abrogated tumor cell mitophagy, as shown by the preservation and persistence of the mitochondrial network and characteristic tubular morphology while under either decorin or rapamycin (inserts, Fig. 6C). Unlike control cells (supplemental Movie 1), decorin-treated cells revealed progressive fragmentation of the mitochondrial network culminating into collapse and aggregation in autophagosomes as visualized in real time via live cell confocal microscopy (supplemental Movie 2). Similar changes to mitochondrial structure were achieved with rapamycin (supplemental Movie 3), indicating that decorin causes effects similar to blocking the PI3K/Akt/mTOR pathway.

Quantification of mitochondrial morphology (58) revealed a significant increase in total mitochondrial number evoked by both decorin and rapamycin (Fig. 6D). Moreover, relative mitochondrial surface area and length were significantly decreased by both decorin and rapamycin (p < 0.001, Fig. 6, E and F, respectively), consistent with mitochondrial turnover and fragmentation. We also determined mitochondrial form factor, a measurement of “roundness” achieved by calculating the reciprocal of mitochondrial circularity; the latter becomes closer to 1 as the mitochondrial become more circular and serves as a valid prognosticator of mitophagy (58). Measurement of this parameter revealed that both decorin and rapamycin had form factor values closer to 1 vis à vis control mitochondria (p < 0.001, Fig. 6G). No significant difference existed between decorin- and rapamycin-treated cohorts (p = 0.611), indicating that decorin evokes mitophagy similarly to rapamycin. Su9-GFP cells depleted of mitostatin via siRNA knockdown were resistant to both decorin and rapamycin treatments insofar as the mitochondrial network persisted as quantified for the analyzed parameters of mitochondrial number (Fig. 6D), occupied area (Fig. 6E), and mitochondrial length (Fig. 6F) were similar to controls. Furthermore, mitochondrial form factor returned to
near-basal levels for both decorin and rapamycin (Fig. 6G). Thus, our findings support a role for decorin in evoking tumor cell mitophagy in a mitostatin-dependent manner.

Decorin Requires Mitostatin to Suppress OXPHOS Components and mtDNA—Utilizing a siRNA targeting mitostatin, we found that its loss completely abrogated decorin-mediated suppression of OXPHOS complexes II, III, and V (Fig. 7A). Interestingly, complex IV was sensitive to mitostatin loss alone (Fig. 7A). As a functional output for decorin-evoked mitophagy, we found a significant suppression of Su9-GFP ($p < 0.001$, Fig. 7B), which was blocked upon depletion of mitostatin. Similarly, the VDAC, a protein located on the outer mitochondrial membrane, was markedly suppressed by decorin ($p < 0.01$, Fig. 7C), and this effect was blunted by mitostatin knockdown.

Next, we evaluated mtDNA content in the presence of decorin and found a highly significant suppression of mtDNA ($p < 0.001$, Fig. 7E). Furthermore, validation of mitostatin suppression via targeting siRNA (Fig. 7D) mimicked the effects of mitostatin loss for Su9-GFP and VDAC insofar as mitostatin knockdown prevented decorin-mediated suppression of mtDNA ($p < 0.001$, Fig. 7E). Moreover, decorin suppressed both steady state and non-steady state mRNA levels of the TFAM, a major regulatory component of the mtDNA genetic program (Fig. 7F) ($p < 0.001$, Fig. 7G). Depression of TFAM mRNA at the non-steady state was blocked by SU11274 (Fig. 7F). Importantly, we recapitulated our findings concerning the requirement of mitostatin in decorin-evoked mitophagy after validated knockdown of MITOSTATIN (Fig. 7G) in T47D cells prevented decorin-mediated mtDNA suppression (Fig. 7H) when compared with decorin stimulation alone (Fig. 7H).

Next, we determined if mitophagic induction vis à vis mtDNA reduction was dependent on Beclin 1 as decorin did not modulate Beclin 1 levels in MDA-MB-231 cells (Fig. 7I), as recently shown to occur for other transformed cell lines ($p < 0.001$, Fig. 7J). Treatment with rapamycin or Hanks’ balanced salt solution (HBSS) to simulate nutrient deprivation reduced mtDNA to levels comparable to decorin alone ($p < 0.001$, Fig. 7J). Loss of mitostatin also abrogated the ability of both rapamycin and HBSS to induce mtDNA degradation (Fig. 7J). Collectively, these results corroborate the imaging studies using Su9-GFP-expressing cells where tumor cell mitophagy induced by
Decorin or canonical agents such as rapamycin or HBSS depends strongly on mitostatin and is Beclin 1-independent. Decorin Requires Mitostatin for VEGFA Suppression—Transcriptional repression of HIF-1α and suppression of VEGFA mRNA are hallmarks of soluble decorin bioactivity (42, 43). However, the underlying molecular mechanism remains unknown. Therefore, we postulated that decorin-evoked mitophagy via mitostatin could represent the underlying mechanism for VEGFA suppression. Mitostatin knockdown caused an increase in both cellular (Fig. 8A) and secreted (Fig. 8B) VEGFA levels (p < 0.001, Fig. 8C). Importantly, mitostatin depletion prevented decorin-mediated suppression of cellular (Fig. 8A) and secreted (Fig. 8B) VEGFA at either 1 or 2 h of decorin treatment (Fig. 8C). In the presence of either PGC-1α or mitostatin knockdown (Fig. 8D), decorin failed to suppress VEGFA mRNA levels vis à vis decorin alone (Fig. 8E). Immunofluorescence studies corroborated the mRNA and protein results as loss of mitostatin augmented VEGFA above siScr-transfected controls (Fig. 8F). Furthermore, siRNA depletion of mitostatin abrogated decorin-evoked suppression of VEGFA relative to decorin alone (Fig. 8F). Our results forge a novel pathway whereby decorin-evoked mitophagy is dependent on mitostatin and concomitantly necessary for VEGFA suppression. Therefore, induction of tumor cell mitophagy is detrimental for overall tumorigenic growth as decorin-evoked mitophagy attenuates VEGFA signaling under normoxic and nutrient-rich conditions.

Decorin Concurrently Induces Mitostatin and Suppresses mtDNA in Vivo—To translate our in vitro findings into a physiologically relevant setting, we established MDA-MB-231(GFP) triple-negative orthotopic breast carcinoma xeno-grafts. As such, introduction of 3 x 10^6 MDA-MB-231(GFP+) cells into the mammary fat pads of SCID mice permitted, once the tumors became palpable, daily systemic administration of

**FIGURE 6.** *Decorin evokes tumor cell mitophagy in a mitostatin-dependent manner.* A, representative fluorescence micrographs depicting live cell imaging of MDA-MB-231 cells after incubation with decorin (100 nM, 6 h) or with the protonophore FCCP serving as a positive control for the last 10 min. Tumor cells were cultured in nutrient-rich conditions and incubated with JC-1 (7.5 μM) at 37 °C to examine mitochondrial membrane potential for the last 10 min of the time point. B, quantification of two independent experiments utilizing ImageJ to determine JC-1 aggregates ± S.E. per cell. The number of cells quantitated per condition is denoted within the respective bars. C, gallery of fluorescence images of FACS-sorted MDA-MB-231 cells stably expressing the Su9-GFP transgene (MDA-MB-231 Su9GFP) treated with either decorin (100 nM, 6 h) or rapamycin (40 nM, 2 h) in the presence of scrambled siRNA (siScramble, 20 pm) or RNAi targeting mitostatin (siMitostatin, 80 pm). Inset images represent a correspondingly enlarged area of that outlined by the white box. All fluorescence images were taken with the same exposure, gain, and intensity. Scale bar = 10 μM. D–G, quantification of mitochondrial morphological parameters using ImageJ programmed with a macro enabling quantitation of mitochondrial values (58) including total mitochondrial number (D), area (E), length (F), and form factor (G) in the presence of decorin or rapamycin in conjunction with scramble siRNA or siMitostatin. Data represent at least two independent trials in A and B, whereas data presented in D–G reflect analysis with ImageJ, utilizing at least n = 50 MDA-MB-231 Su9GFP cells. ***, p < 0.001.
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Decorin protein core (10 mg/kg) via intraperitoneal injections. End point analysis of the breast carcinoma xenografts revealed a potent induction of MITOSTATIN mRNA (Fig. 9A) and protein, as confirmed by immunohistochemical staining (Fig. 9B) after systemic delivery of human recombinant decorin protein core (the same batch used for the current in vitro studies). Importantly, decorin suppressed mtDNA in the tumor xenografts (Fig. 9C). Collectively, these data support a role for decorin-mediated mitophagy within an established breast carcinoma xenograft model.

FIGURE 7. Decorin requires mitostatin to suppress OXPHOS components and mtDNA. A, representative immunoblots demonstrate the effects of siMito (80 pm) on the various mitochondrial respiratory complexes (II, III, IV, and V) in comparison to siScr controls (20 pm) under the influence of decorin (100 nm) at the indicated time points. B, the immunoblot reacted with a rabbit-anti-GFP primary antibody from SDS/PAGE-resolved lysates of MDA-MB-231 Su9-GFP in the presence of 100 nm decorin (4 h) with or without siMito (80 pm) relative to siScr transfected controls (20 pm) and accompanying quantification. C, representative immunoblot probed for VDAC in parental MDA-MB-231 cells under identical conditions as in B. Quantification was performed after normalization to GAPDH.

D, qPCR verification for RNAi-mediated depletion of MITOSTATIN (Mito, 80 pm) and BECN1 (Becn1, 80 pm) mRNA relative to siScr-treated controls (20 pm) with or without decorin (100 nm, 4 h). E, mtDNA analysis was performed in the presence of decorin (100 nm, 4 h) in the context of mitostatin depletion via siMito (80 pm) relative to siScr controls (20 pm). F, qPCR analysis of TFAM under the influence of decorin (100 nm, 4 h) in the absence or presence of either ActD alone (20 μg/ml) or in combination with SU11274 (1 μM, 30 min). G, RNAi-mediated knockdown verification of MITOSTATIN (siMito, 80 pm) in comparison with siScr controls (20 pm) in conjunction with decorin (100 nm, 4 h) in T47D cells. H, analysis if mtDNA in T47D cells after stimulation with decorin (100 nm, 4 h) in combination with MITOSTATIN depletion. I, immunoblot analysis of Beclin 1 in the presence of decorin (100 nm, 4 h) with a Coomassie stained gel to ensure equal sample loading. J, mtDNA analysis performed in the presence of either transient mitostatin or Beclin 1 depletion (siMito or siBecn1, respectively) and challenged with rapamycin (40 nm, 2 h), HBSS to simulate nutrient deprivation (4 h), or decorin (100 nm, 4 h) relative to siScr-treated control. Data represent at least three independent trials and are expressed as -fold changes ± S.E. For mtDNA analysis, data reflect three independent trials run in quadruplicate and normalized to the genomic DNA marker, LPL. ΔΔCt analyses were performed and are reported as -fold changes ± S.E.; **, p < 0.01; ***, p < 0.001. ND1, NADH dehydrogenase subunit 1. LPL, lipoprotein lipase.
DISCUSSION

Mitophagy is a conserved homeostatic process by which unnecessary or compromised mitochondria are subjected to selective turnover and lysosomal-mediated degradation (74, 75). Mitochondria, via oxidative phosphorylation, supply large amounts of ATP in quiescent, terminally differentiated cells. As molecular oxygen serves as the ultimate electron acceptor and due to the intrinsic nature of the OXPHOS process, mitochondria are the primary source of highly reactive oxygen species. Stringent safeguards that monitor mitochondrial integrity exist to circumvent the mutagenizing effects reactive oxygen species exert on subcellular structures, proteins, lipids, and nucleic acids. In the context of tumorigenesis, mitophagy is oncosuppressive to ensure mitochondrial quality control and preclude the propagation of damaged mitochondrial and genomic DNA (74). Decorin suppresses oncogenic signaling and curtails tumorigenic growth and angiogenesis (7, 26, 76) by triggering proteolysis of cyclin D1 and Myc (33) and inducing tumor suppressor genes such as p21, Peg3, and mitostatin (54). Furthermore, decorin antagonizes HIF-1 expression leading to an attenuation of the angiogenic network under normoxia (42). As Myc and HIF-1 control opposing effects for metabolic reprogramming (1), recent research indicates a complex regulatory cooperation controlling mitochondrial biogenesis (59) that is inherently advantageous for neoplastic growth.

FIGURE 8. Decorin requires mitostatin dependent mitophagy to reduce VEGFA. A, representative immunoblot resolved by SDS/PAGE and reacted with an anti-VEGFA antibody in the presence of targeting RNAi for mitostatin (siMito, 80 pM) or a scramble control (siScr, 20 pM) before challenge with decorin (100 nM) at the indicated time points. B, slot blot analysis of tumor-conditioned media evaluated for secreted VEGFA under the same conditions as in A. C, quantification of cellular VEGFA (cVEGFA, red) and secreted VEGFA (sVEGFA, blue) after normalization to either Coomassie or total cell number for cellular or secreted, respectively, VEGFA enumeration. D, verification of RNAi-mediated knockdown of PGC-1α (siPGC-1α, 80 pM) and mitostatin (siMito, 80 pM) relative to siScr control (20 pM) after a challenge with decorin (100 nM, 4 h). E, VEGFA expression analysis via qPCR under identical conditions as in D. F, immunofluorescence microscopy of MDA-MB-231 cells transfected with either siScr (20 pM) or siMito (80 pM), stimulated with decorin (100 nM, 4 h), and immunostained for VEGFA (green) and stained with DAPI (blue) to visualize nuclei. All images shown were taken with the same exposure, gain, and intensity. Scale bar = 10 μm. Data are representative of at least three independent experiments and are expressed as -fold change ± S.E. qPCR data presented in D and E were derived from three independent experiments run in quadruplicate and normalized to the endogenous housekeeping gene, ACTB. **, p < 0.01; ***, p < 0.001.

FIGURE 9. Systemically administered decorin induces mitostatin and suppresses mtDNA in vivo. A, qPCR expression analysis of MITOSTATIN harvested from MDA-MB-231(GFP+) orthotopic tumor xenografts treated with daily intraperitoneal injections of decorin (10 mg/kg). B, mitostatin detection via immunofluorescence of frozen sections of control tumor xenografts or decorin-treated tumors. Three-dimensional surface plots were created with ImageJ and directly represent mitostatin expression corresponding to the immunofluorescence signal. Accompanying scale bars (to the right) depict signal intensity. C, mtDNA analysis of MDA-MB-231(GFP+) tumors after systemic decorin treatments. For qPCR and mtDNA analyses, data represent three independent samples run in quadruplicate and normalized to either ACTB or lipoprotein lipase, respectively, and reported as -fold change ± S.E. (***, p < 0.001). ND1, NADH dehydrogenase subunit 1. LPL, lipoprotein lipase.
In this study we demonstrate for the first time that a soluble small leucine-rich proteoglycan member induces mitochondrial turnover and degradation in breast carcinoma cells as the underlying link for angiogenic suppression by relying on a novel mitochondrial localized tumor suppressor, mitostatin. We demonstrate that decorin evokes a protracted suppression of mitochondrial OXPHOS complexes, and this process is mediated by the decorin/Met axis. Notably, we were unable to detect complex I (NADH dehydrogenase) due to low abundance within our lysates. Complex I functions as an important regulator of the electron transport chain insofar as removing two electrons from reduced NADH via transfer to ubiquinone for shuttling. In concert with electron transfer from NADH, complex I simultaneously translocates four proton from the mitochondrial matrix into the intermembrane space. This begins the formation of a proton motive force and underlies the fundamental principle of oxidative phosphorylation for mass ATP production (74). Importantly, decorin significantly represses downstream complexes, although we cannot state conclusively the abrogation of this initial step. However, in the context of tumor mitochondrial physiology, suppression of downstream complexes is more consistent with inhibiting biosynthetic pathways necessary for tumorigenic growth. Interestingly, HGF/Met signaling augments glycolytic flux (77); however, we show a significant enhancement of OXPHOS components, potentially to support the cell with increased biosynthetic abilities consume with the pro-proliferative and pro-angiogenic nature of HGF/Met signaling. These data substantiate a novel anti-tumorigenic role for decorin to compromise mitochondrial chondrial turnover. (potentially function) through the concerted suppression of multiple key components of OXPHOS (Fig. 10). This function is critical as metabolic intermediates are shunted to support the shift to anabolic metabolism (1).

Consistent with reports utilizing urothelial and prostate carcinoma cells (55), decorin induces mitostatin in basal breast carcinoma cells where it rapidly accumulates, beginning at 30 min and remains elevated for up to 4 h. Importantly, an antagonistic relationship existed for regulation of mitostatin via the HGF/Met axis. Image analysis revealed a surprising and unexpected subcellular localization of mitostatin; that is, after decorin treatment; mitostatin localized to large (2–3 μm) perinuclear structures reminiscent of autophagosomes. This provides tantalizing possibilities consistent with mitostatin localization to mitochondria that decorin evokes mitophagy in a mitostatin-dependent fashion, as the basis for OXPHOS suppression.

Mitochondrial homeostasis proceeds via a PGC-1α-mediated transcriptional network (63). As decorin repressed PGC-1α mRNA and protein via Met, we hypothesized that an antagonistic relationship might exist between mitostatin and PGC-1α in controlling mitochondrial turnover. Unexpectedly, siRNA-mediated knockdown of PGC-1α did not lead to a derepression of mitostatin. In contrast, PGC-1α served as a positive regulator to maintain not only basal levels but also the decorin-evoked induction of mitostatin. Paradoxically, depletion of PGC-1α was permissive for further mitostatin loss. These data indicate that other factors are involved in the decorin-evoked induction of mitostatin that are no longer active (or present) due to PGC-1α depletion.

We surmised two possible roles for PGC-1α to function as a positive regulator of mitostatin, either via direct protein-protein interactions or through a post-transcriptional pathway involving PGC-1α binding to MITOSTATIN mRNA via the C-terminal RNA recognition motif. We further refined our analyses to unravel the mechanistic relationship between PGC-1α and mitostatin. Reciprocal co-immunoprecipitation experiments revealed a novel protein complex containing PGC-1α and mitostatin, with enhanced binding in the presence of decorin. Regardless of decorin involvement, this new association of PGC-1α in complex with mitostatin has important regulatory ramifications for mitochondrial homeostasis and cellular bioenergetics. We demonstrated that knockdown of PGC-1α resulted in a loss of basal levels of mitostatin. Functionally, this might result in loss of mitostatin protein stability.
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