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Decorin evokes rapid secretion of thrombospondin-1 in basal breast carcinoma cells via inhibition of RhoA/ROCK1

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Abstract

Pathological neovascularization relies on an imbalance between potent pro-angiogenic agents and equally effective anti-angiogenic cues. Collectively, these factors contribute to an angiogenic niche within the tumor microenvironment. Oncogenic events and hypoxia contribute to augmented levels of angiokines and thereby activate the so-called angiogenic switch to promote aggressive tumorigenic and metastatic growth. Soluble decorin functions as a paracrine pan-inhibitor of receptor tyrosine kinases (RTK), such as Met and EGFR, and thus is capable of suppressing angiogenesis under normoxia. This leads to a non-canonical repression of HIF1- α and VEGFA and a concurrent induction of thrombospondin-1. The substantial induction of endogenous tumor cell-derived thrombospondin-1, a potent anti-angiogenic effector, led us to the discovery of an unexpected secretory phenotype occurring very rapidly (within 5 min) after decorin treatment of the triple-negative basal breast carcinoma cell line, MDA-MB-231. Surprisingly, the effect was not mediated by Met receptor antagonism, as initially hypothesized, but required EGFR signaling to achieve swift and robust thrombospondin-1 release. Furthermore, this effect was ultimately dependent on the prompt degradation of RhoA, via the 26S proteasome, leading to direct inactivation of ROCK1. The latter led to a derepression of thrombospondin-1 secretion. Collectively, these data provide a novel mechanistic role for the ROCK1 kinase in addition of providing the first conclusive evidence of decorin exclusively targeting a RTK to achieve a specific effect. The overall effects of soluble decorin on the tumor microenvironment would cause an immediately-early as well as sustained anti-angiogenic response *in vivo*.

Keywords

Proteoglycan; angiogenesis; epidermal growth factor receptor; Met; receptor tyrosine kinase

Introduction

An intricate balance exists between pro- and anti-angiogenic factors that either promote or impede, respectively, pathological angiogenesis within the tumor microenvironment [1]. When this balance is altered as a consequence of oncogenesis to be in favor of pro-angiokines, an angiocompetent milieu is formed via the so-called angiogenic switch [2–6]. This pathobiological imbalance within the tumor microenvironment allows for

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neovascularization of the tumorigenic tissue, leading to enhanced aggressive behavior, increased metastasis, and poorer prognosis [1]. This balance of angiocrine factors is intimately orchestrated by the plethora of functions conveyed by the surrounding extracellular matrix constituents that are able to differentially, and often are the critical determinants, that regulate angiogenesis [7].

Proteoglycan-mediated control of tumor angiogenesis exemplifies this principle [8–10]. Of particular interest is the inherently powerful anti-angiogenic properties exerted by the small leucine rich proteoglycan (SLRP) decorin [11,12], a paradigmatic member of the SLRP superfamily. Decorin, originally identified as a regulator of type I collagen fibrillogenesis and [13–20] and as highly-induced gene in the stroma of human colon carcinomas [21,22], is encoded by a large and highly-conserved gene with a complex promoter structure [23–25]. Soluble, monomeric decorin [26] certainly functions in a paracrine manner to bind, with high affinity, multiple receptor tyrosine kinases (RTKs) including members of the ErbB family, such as the EGFR and ErbB4 [27–29], and has been identified as the only antagonistic ligand of Met, the hepatocyte growth factor receptor [30,31]. In addition, decorin binds to the insulin-like growth factor receptor I [32–34] and attenuates its activity in bladder cancer cells [35]. These diverse bioactivities convey highly effective anti-tumorigenic [31,36–39] and anti-metastatic [40,41] properties onto decorin. Decorin is also involved in innate immunity and hypersensitivity reactions [42–44], and in bone pathophysiology [45]. Genetic ablation of the decorin gene favor lymphomagenesis in a p53-null background [46], induces intestinal tumor formation in a C57Bl/6 background [47,48] and plays a role in wound healing [49]. Concurrent with these activities is the induction of cyclin-dependent kinase inhibitors such as p21^{WAF1} [50,51] coincident with the degradation, in a non-canonical fashion, of potent oncoproteins such as β -catenin and Myc [52]. Therefore, decorin acts as a paracrine tumor repressor by acting as a pan-RTK inhibitor at the cell surface of tumor cells [10,53].

Decorin represses pro-angiogenic factors (HIF-1 α and VEGFA) concurrent with simultaneous transcriptional induction of anti-angiogenic molecules such tissue inhibitor of metalloprotease 3 (TIMP3) and thrombospondin-1 (TSP-1) under normoxia via suppression of pro-angiogenic HGF/Met signaling [11,54–56]. Previous work [56] has demonstrated an acute transcriptional response for *THBS1* expression that correlated with increased TSP-1 protein in the triple-negative basal breast carcinoma cell lines, MDA-MB-231, as well as in tumor xenograft models composed of the same cell type. TSP-1 is an archetypical matricellular component of a gene family that encodes five large, modular, calcium-binding, secreted glycoproteins [57]. TSP-1 is a long, filamentous protein capable of binding several cell surface receptors enabling diverse regulation of cellular function among many different cell types [57]. Although originally identified as a secreted monomeric glycoprotein of ~140 kDa, TSP-1 functions primarily as a trimer and is derived from thrombin-stimulated platelets and platelet α -granules, accounting for ~3% and ~25% of total protein content, respectively [58]. It is now well established that TSP-1 is expressed by a wide variety of cell types, including predominant expression from vascular smooth muscle cells and endothelial cells [59]. Functionally, TSP-1 inhibits wound healing, inactivates MMP-9 and VEGFA liberation, triggers endothelial cell apoptosis via engagement of CD36 and signaling via Jun N-terminal kinase and p38 stress activated protein kinases and modulates adhesion [58–60]. Additional functions of TSP-1 include regulation of NO/cGMP signaling via engagement and ligation of CD47 with VEGFR2 within the cardiovascular system [61], regulation of synaptogenesis in the central nervous system [62], and modulation of TGF- β activation and fibrosis [63] and wound healing [64]. Moreover, TSP-1 inhibits angiogenesis via a direct effect on endothelial cell migration and survival, and by affecting VEGFA availability and VEGFR2 activity [65,66]. Notably, TSP-1 deficient mice display a lordotic curvature of the spine, increases in the number of circulating monocytes and eosinophils, and pulmonary

inflammation [67]. Interestingly, the TSP-1 null mouse was not embryonic lethal, perhaps due to redundancy among the other TSP gene members [58].

In the context of cancer, oncogenic Ras signaling [68] and altered Myc activity, downstream of Ras [69] combinatorially repress TSP-1 expression. The transcriptional inhibitor Id1 was recently shown to repress *THBS1* expression as Id1 deficiency is associated with increased TSP-1 levels [70]. As decorin is capable of unconventionally downregulating Myc [52], in conjunction with the concept that Myc drives Id1 induction [71], we sought to further characterize the mechanism for decorin-mediated induction of TSP-1 in the MDA-MB-231 cell line, presumably downstream of Met [56]. Unexpectedly, we found a prompt and robust secretory phenotype mediated by decorin that requires EGFR signaling, independently of Met, and orchestrated through the concerted degradation of RhoA and subsequent inactivation of ROCK1 to allow for rapid secretion of TSP-1 from MDA-MB-231 cells. Collectively, our results provide a novel secretory-inducing role for decorin and offer new perspective and understanding on the ability of this SLRP to attenuate the pro-angiogenic niche of the surrounding tumor microenvironment.

Results

Decorin evokes a rapid and biphasic release of TSP-1 in MDA-MB-231 breast carcinoma cells

We have previously described a function for exogenous soluble decorin to induce expression of *THBS1* mRNA and protein in MDA-MB-231 cells following engagement of Met as part of a potent angiostatic program [56]. Therefore, in the current study, we aimed to further characterize the mechanism of TSP-1 regulation and subsequent secretion while under the influence of decorin. Analysis of tumor cell-conditioned medium revealed a rapid mobilization of TSP-1 in as little as 5 min (~1.7-fold) following decorin exposure (Fig. 1A,B). It should be noted that decorin protein core was utilized throughout the study (hereafter referred to as decorin) unless otherwise indicated. Interestingly, the levels of TSP-1 spiked at 10min (~2.2-fold) then returned to near baseline levels at 20 min post treatment (Fig. 1B). However, TSP-1 began to increase a second time at 30 min and plateaued after 1 h (Fig. 1A,B). We believe that this biphasic release of TSP-1 reflects an initial burst of TSP-1 release most likely from pre-formed TSP-1 cytoplasmic storage followed by enhanced expression of *THBS1* mRNA for the second phase in a manner analogous to platelet derived growth factor (PDGF)-mediated induction of *THBS1* [72].

To further corroborate these finding, we performed confocal microscopy wherein MDA-MB-231 cells were exposed to decorin for 40 min and probed for TSP-1. The distribution of TSP-1 in control cells (Fig. 1C) was primarily arranged in a uniformly granular pattern. The appearance of TSP-1 staining insofar as both the number of granules and signal intensity of TSP-1 deposits in the decorin treated cells after 40 min were significantly diminished (Fig. 1D), in accordance with the above data detailing rapid release of TSP-1 from the cell.

In contrast to the rapid release evoked by decorin of secreted TSP-1, cellular TSP-1 at early time points (5–20 min) revealed no substantial changes in the intracellular stores of TSP-1 (Fig. 1E,F). However, at 30 min, there was a significant accumulation (~1.8-fold) of cellular TSP-1. These intracellular levels of TSP-1 remained high at 1 and 2 h post treatment (not shown).

Collectively, our results show an unexpected and novel role for decorin in evoking a rapid, biphasic, and time-dependent release of TSP-1 from quiescent (serum starved) MDA-MB-231 cells.

Decorin requires EGFR signaling, but not Met, to evoke rapid TSP-1

Next, we sought to identify the mechanism of this biological activity, i.e. we tested which of the known decorin RTK was responsible for coordinating this swift release of TSP-1 in response to soluble decorin. Our main hypothesis was that Met would be the primary receptor responsible for this activity, as Met has been previously implicated in coordinating the other aspects of decorin mediated angiostasis [56]. Therefore, we performed similar experiments in the presence or absence of the Met tyrosine kinase inhibitor SU11274, a highly selective inhibitor of the ATP binding domain for various Met mutants [73]. To this end, we pre-treated MDA-MB-231 cells for 30 min with SU11274 followed by an incubation with decorin for 10 min. Consistently, decorin facilitated a rapid burst of TSP-1 into the media; surprisingly, this effect was not blocked by SU11274 (Fig. 2A) as decorin still evoked TSP-1 secretion (~2-fold). Quantification of TSP-1 secretion in SU11274 pre-treated cells as well decorin alone revealed a marked induction of TSP-1 levels in both cases ($P < 0.001$, Fig. 2C), but no significant differences between the two experimental conditions ($P = 0.21$). Next, in an attempt to link these effects to Met, we pre-treated MDA-MB-231 cells with hepatocyte growth factor (HGF) for 30 min [74]. Similarly, decorin was able to induce secretion while HGF pre-treatment failed to block decorin mediated secretion of TSP-1 (Fig. 2B). Quantification of secreted TSP-1 revealed a significant induction of TSP-1 secretion in the presence or absence of HGF ($P < 0.001$, Fig. 2D) and no significant differences between the two experimental conditions ($P = 0.35$). Collectively, these results indicate that decorin engagement of Met is not involved in the secretion of TSP-1 by MDA-MB-231 cells.

The original identification of decorin mediated antagonism towards RTKs began with EGFR and upon decorin binding there is receptor dimerization, and rapid trans-autophosphorylation reminiscent of the effect on Met within 10 min [27]. We therefore interrogated the role of EGFR signaling on TSP-1 secretion, as MDA-MB-231 do express EGFR, but not HER2/Neu. Utilizing immunofluorescence to probe TSP-1 in response to decorin, pre-treatment with the EGFR small tyrosine kinase inhibitor AG1478 [75], abrogated the ability of decorin to deplete TSP-1 (compare Fig. 2F with Fig. 2G), making control (Fig. 2E) and AG1478 pretreated cells look virtually indistinguishable. Further, application of the EGFR-specific monoclonal blocking antibody mAb425 [76] recapitulated the blockade of decorin-mediated secretion of TSP-1 and mimicked control conditions as determined by immunofluorescence. Application of either AG1478 or mAb425 prior to decorin treatment almost completely restored the punctate pattern of TSP-1 to control signatures and significantly obviated the ability of decorin to induce TSP-1 release as determined by the average TSP-1 fluorescence signal (Fig. 2I).

Taken together, these data advocate for the ability of decorin to differentially signal and integrate specific aspects of the angiostatic program across different RTKs expressed by the tumor cell. As such, rapid secretion of TSP-1 is almost exclusively dependent on EGFR, but not Met, in these basal breast carcinoma cells.

Rapid secretion of TSP-1 depends on ROCK1 inhibition

The role of p160-ROCK1 (Rho-associated coiled-coil containing protein kinases) in the control of TSP-1 has been evaluated and assigned an inhibitory role as a downstream effector of the Ras/PI3K/RhoA signaling pathway to achieve *THBS1* transcriptional repression [69]. Indeed, treatment with the ROCK1 inhibitor Y27632 derepresses TSP-1 repression following an 8-h treatment [69]. Suppression of ROCK1 by a variety of mechanisms reduces cancer metastasis and invasion in lung adenocarcinoma [60] and gastric cancers via miRNA-148a [77]. However, the role of ROCK1 in regulating secretion has been limited and surprisingly has focused on the effects of ROCK1 inhibition functionally

linked to hyperinsulinemia [78]. Therefore, we hypothesized that Y27632, a specific small molecule inhibitor of ROCK1, would be able to recapitulate the rapid pattern of decorin-evoked TSP-1 release from MDA-MB-231 cells. Incubation of MDA-MB-231 cells with Y27632 alone evoked a rapid and robust secretion of TSP-1 starting as early as 5 min, peaking at 10 min (~2.7-fold), with a plateau (~2.5-fold) that was sustainable for up to 2 h post-treatment with Y27632 (Fig. 3 A,B). These results recapitulate the decorin-evoked secretion of TSP-1 with comparable kinetics (*cfr.* Fig. 1A, B). Interestingly, no significant modulation of ROCK1 protein levels was seen with decorin up to 30 min (Fig. 3C,D) as well as up to 2 h (data not shown). Therefore, the activity of ROCK1, potentially via RhoA, must be curtailed to allow for rapid TSP-1 secretion and thus independent of ROCK1 protein level.

Next, we established that ROCK1 inhibition was the primary effector for decorin mediated TSP-1 release insofar as that treatment with either decorin or Y27632 alone achieved maximal TSP-1 secretion after 10 min (Fig. 3E,F). Further, pre-treatment with Y27632 followed by decorin application did not induce further liberation of TSP-1, indicating that ROCK1 inhibition is the primary target for decorin to evoke rapid TSP-1 mobilization (Fig. 3E,F).

The role of ROCK1 was functionally tied to the control of TSP-1 secretion through siRNA-mediated knockdown of ROCK1 (denoted as siROCK1). We achieved significant depletion of ROCK1 as detected by immunoblots (>85% of control levels, Fig. 3G) when compared to siScramble (siScram) transfected controls. Secreted TSP-1 in the presence of siROCK1 alone was significantly increased relative to siScram controls, indicating a role for ROCK1 to suppress basal TSP-1 secretion (Fig. 3H, I), and consistent with the above results that inhibition with Y27632 increased secreted TSP-1. Finally, decorin was unable to induce a further increase of secreted TSP-1 when in the presence of siROCK1 (Fig. 3H,I). These results indicate and further confirm that ROCK1 is epistatic to the bioactivity of decorin mediated secretion of TSP-1 and that upon inhibition or depletion of ROCK1, secreted TSP-1 is rapidly released.

Collectively, these data ascertain a novel role for ROCK1 in controlling basal levels of a highly anti-angiogenic molecule. Furthermore, ROCK1 serves as the main downstream effector in the mechanism of decorin-evoked TSP-1 release that emanates from positive EGFR signaling.

Decorin depends on EGFR to rapidly degrade RhoA and to evoke TSP-1 secretion

The cellular role of ROCK1 is activated and thus orchestrated by a direct and putative binding of RhoA [79], a small GTPase implicated in motility, invasion, and proliferation [80] as well as promoting oncogenic transformation and a more aggressive tumor phenotype [81]. Further, it has been found that increased RhoA and RhoC expression ultimately correlates with poorer prognoses in breast cancers [82]. Thus, as inhibition of ROCK1 with Y27632 even in the presence of decorin did not synergistically or additively increase total secreted TSP-1 together with the lack of ROCK1 modulation at the protein levels as assayed for up to 2 h, we hypothesized that ROCK1 would be unable to be activated via antagonism of RhoA, stipulated by decorin engaging EGFR. Immunoblotting revealed a stark reduction (< 50%) of RhoA in MDA-MB-231 within just 10 min of decorin treatment (Fig. 4 A). This observation supports the hypothesis that ROCK1 fails to become activated due to a pronounced reduction in RhoA levels. This effect was sensitive to EGFR kinase inhibition insofar as pre-treatment with AG1478 followed by decorin (10 min) failed to reduce total RhoA levels as compared to decorin treated samples (Fig. 4 B). This effect was further verified following quantification revealing a decorin dependent decrease (Fig. 4B) in RhoA (by over 60%) that was wholly abrogated by pretreating with AG1478 (Fig. 4B). Moreover,

pretreatment with the EGFR monoclonal blocking antibody, mAb425 elicited identical effects by abolishing suppression of RhoA when compared to decorin alone (Fig. 4C). This rapid decrease (within 10 min of decorin application) of RhoA is the result of active proteolysis via the 26S proteasome since lactacystin, a powerful proteasome inhibitor, was able to completely attenuate the decorin-evoked degradation of RhoA (Fig. 4D).

Taken together, these data advocate for a prompt and vigorous decorin-mediated response to degrade RhoA via the 26S proteasome dependent on both EGFR signaling and the caveat that decorin binds EGFR, and not Met.

Decorin depends on classical secretory pathways for rapid TSP-1 secretion

Exosomes constitute an emerging class of secreted, membrane bound vesicles that allow for intercellular communication by delivering a host of bioactive molecules ranging from proteins, lipids, RNA, and DNA that seem to play an immunosuppressive role [83]. These nanovesicular bodies have been shown to play a functionally important role in both the initiation and progression of pathobiological states, including cancer. Interestingly enough, tumorigenic cells secrete increased amount of exosomes relative to non-tumorigenic counterparts and display a distinct proteomic signature representative of the host cell [84]. We, therefore, hypothesized that exosomes might be responsible for the rapid *en masse* secretion of TSP-1 from MDA-MB-231 cells. To this end, we subjected tumor cell conditioned media following incubation with decorin for 6 h to differential ultracentrifugation to isolate exosomal fractions followed by immunoblotting for the detection of exosomal TSP-1. It is important to note that the decorin treatment time was significantly increased to allow for the maximal collection of exosomes from the media. Thus, using caveolin-1 (Cav-1) as an exosomal marker [85], we found no significant change between control and decorin exosomal isolates (Fig. 5A). Quantification of three independent experiments normalized to the fetal bovine serum detected in the media showed no significant changes in TSP-1 levels (Fig. 5B). Paradoxically, exosomal Cav-1 was increased relative to control (Fig. 5A) suggesting decorin triggers an increase in exosomal secretion, but this does not underlie the secretory mechanism for TSP-1.

Classical secretion from the cell depends on the movement of vesicles via the microtubular network followed by fusion with the plasma membrane and secretion of the contents to the extracellular space. Thus, pre-incubating MDA-MB-231 cells with nocodazole, which interferes with microtubule polymerization, was able to block decorin mediated release of TSP-1 (Fig. 5F) when compared to decorin alone (Fig. 5D) and was similar to control (Fig. 5C). There was a small, but significant reduction in TSP-1 levels following pretreatment with nocodazole and ensuing decorin incubation ($P = 0.041$, Fig. 5G), when compared to controls suggesting alternative release mechanisms. However, when compared to decorin alone, a significant block ($P = 0.009$, Fig. 5G) occurred indicating a requirement for microtubules as mediating the release of TSP-1 under the influence of decorin. Finally, RhoA is potently degraded by decorin via the 26S proteasome (*cf.* Fig. 4D), and this has functional consequences on the ability of decorin to trigger release of TSP-1. As shown (Fig. 5E), pre-treatment with lactacystin (which attenuates RhoA degradation) was able to hinder the decorin-mediated release of TSP-1, suggesting a requirement for RhoA degradation as part of the mechanism to allow for accelerated TSP-1 release. Indeed, quantification of TSP-1 fluorescence revealed an almost complete block in TSP-1 release when compared to decorin ($P = 0.001$, Fig. 5G) and virtually identical to control ($P = 0.29$, Fig. 5G).

Overall, we demonstrate that decorin utilizes the classical secretory pathway dependent on microtubules to elicit TSP-1 discharge. Further, this pathway is profoundly sensitive to the levels of RhoA, as preventing degradation of this cytoskeletal mediator completely blocked decorin triggered release of TSP-1.

Discussion

A fragile balance among the angiokine signaling factors as well as the tumor parenchyma and endothelial cells residing within the tumor microenvironment mediates the tumor angiogenic switch. Angiogenesis critically relies on matrix derived cues for coordination, particularly small leucine-rich proteoglycans [30]. From the perspective of the tumor proper, decorin is a potent inhibitor of tumor angiogenesis [55]. Recently, we found a potent transcriptional repression of numerous genes encoding soluble pro-angiogenic mediators responsible for initial sculpting and continued maintenance of the angiogenic response such as *VEGFA*, *FGF2*, and *ENG* with concurrent induction of soluble anti-angiogenic effectors including *THBS1* and *TIMP3* [56]. These data demonstrated, for the first time, a potent induction of soluble angiostatic gene products from MDA-MB-231 in the presence of decorin in both *in vitro* and *in vivo* models of triple negative breast carcinoma [56].

In the present report, we have extended our studies concerning the underlying mechanism of decorin-mediated angiostasis by further characterizing thrombospondin-1 regulation, as this matricellular component has a profound influence on the state of the angiogenic niche by compromising the viability of endothelial cells [86]. We discovered a novel mechanism for control of TSP-1 secretion (Fig. 6). In contrast to the protracted transcriptional response of *THBS1* and TSP-1 protein that occurs following an 8-h incubation with decorin [56], we found a very rapid secretion (within 5 min) of presumably preformed TSP-1 from MDA-MB-231 cells that appear to be biphasic in nature. The second phase of TSP-1 release might be mediated via the induction of *THBS1* mRNA expression or the consequent accumulation of the TSP-1 oligomeric form within the endoplasmic reticulum of cellular TSP-1 begins to increase, consistent with the ~30 min maturation time for TSP-1 accumulation within this compartment. These data outline an immediate-early transition from the tumor parenchyma to start reprogramming the otherwise pro-angiogenic niche.

We initially hypothesized that this effect would be coordinated by Met as decorin, which behaves as a partial agonist of Met, triggers rapid phosphorylation of the Met catalytic domain (at Tyr-1234/5) within five to ten min before returning to baseline [31]. Rapid TSP-1 release might be related to this spike in phosphorylation of the intracellular domains of Met immediately prior to receptor internalization. However, this was found to not be the case. Instead, decorin requires signaling via EGFR, as AG1478 and function blocking antibody mAb425 abrogated the immediate early release of TSP-1 (Fig. 6). This is consistent with the role of decorin in also triggering EGFR phosphorylation upon binding. This phosphorylation signature allows for the swift release of TSP-1 from MDA-MB-231 cells. This effect is intriguing insofar as this particular cell type expresses both Met and EGFR; however, decorin coordinates specific cellular events depending on the receptor it engages and thus integrates the signal over the repertoire of receptors known to bind decorin. This is thought to be mediated by differential binding affinities decorin has for EGFR and Met (K_d of ~80 nM and 2 nM, respectively). Despite the fact that decorin has a higher binding affinity for Met when compared to EGFR, we have clearly shown that Met does not play a role in TSP-1 release. Intrinsic differences that exist between EGFR and Met may influence decorin binding to EGFR, such as the topological requirements conferred by EGFR and/or the manifestation of fundamentally different phosphorylation patterns from that of Met to allow for TSP-1 release. Domain swapping experiments to precisely identify the bioactive determinants responsible for these differences between EGFR and Met will be of great importance for this phenomenon. Further, these data establish for the first time a role of decorin to not only bind, but also as a requirement to signal via an RTK to elicit downstream cellular events to achieve angiostasis.

Our results indicate that decorin-evoked TSP-1 release is not due to enhanced early transcriptional activity as TSP-1 levels peaked within 10 min of stimulation. Thus, we began to analyze downstream effectors of EGFR that could coordinate TSP-1 release. It has been established that Ras/RhoA/ROCK1 signaling is important in the control of Myc to repress *THBS1*, [69]. Increases in general protein secretion has been achieved through the inhibition of ROCK1 [87] and has been found to also be the case for decorin evoked TSP-1 secretion as no further secretion of TSP-1 was found to occur either upon small molecule inhibition or siRNA-mediated silencing of ROCK1. Furthermore, as no apparent changes in total ROCK1 levels occurred, we found a prominent degradation via the 26S proteasome of RhoA, the key small GTPase that activates ROCK1 to be required for this bioactivity (Fig. 6). Moreover, blocking degradation of RhoA prevented decorin-dependent release of TSP-1. Consistent with the finding of EGFR-dependent signaling, blocking with AG1478 also prevented RhoA degradation. These data identify not only a further anti-metastatic role for decorin through powerful suppression and degradation of RhoA, but also through derepression of potent angiostatic agent secretion in a non-canonical RhoA/ROCK1 dependent manner. Therefore, an immediate early loss of RhoA would presumably preclude activation of ROCK1 leading to the swift derepression of TSP-1 release and thus provide a mechanistic basis for this phenomenon downstream of EGFR signaling.

Finally we demonstrate the TSP-1 release does not involve secretion of TSP-1 from nanovesicles such as exosomes, but does depend on a stable microtubule network that is highly sensitive to the levels of RhoA and dependent on EGFR. This has several ramifications for the overall ability of decorin to impede tumorigenesis and metastatic capacity. Since decorin requires competent microtubule polymerization to drive secretion of TSP-1 (Fig. 6), stable microtubule growth is also a major detriment to cell motility and migration, as a decrease in microtubule stabilization leads to increased tumor cell invasion [88]. Interestingly, this is dependent on the activity of p27^{Kip1} [89], a cyclin-dependent kinase inhibitor inducible by decorin [90]. Therefore, rapid secretion of TSP-1 via RhoA/ROCK1 inhibition might be dependent on the activity of p27^{Kip1} thus leading to microtubule stabilization.

These data indicate an exceptionally early ability of decorin to begin to transform the surrounding tumor microenvironment by inducing secretion of soluble angiokine factors that inhibit tumor angiogenesis from the perspective of the tumor proper. Indeed, the profound effect decorin has on the tumor microenvironment is further exemplified in a recent study via systemic administration of decorin protein core to a triple negative orthotopic breast carcinoma xenograft model [91]. Decorin protein core was able to exclusively modulate stroma-specific (*Mus musculus* origin) genes without significant modulation of tumor xenograft (*Homo sapiens*) transcripts and thus resulting in a defined tumor microenvironment gene signature [91]. The mechanism of action remains quite unclear as to how soluble decorin, canonically viewed as a pan-RTK inhibitor acting on the tumor proper, is able to reprogram the surrounding tumor microenvironment through transcriptional induction of potent tumor suppressor genes. It is conceivable that decorin, acting on the tumor proper via RTK engagement, is able to prime the tumor microenvironment through the release of anti-angiogenic mediators to initiate this signature obtained from transcriptomic profiling. This might be of utmost importance for the contributions of endothelial cell-mediated angiogenesis following TSP-1 binding to CD36 and CD47.

In conclusion, our data further establish decorin as a key inhibitor of tumorigenesis by contributing to the so-called “tumor secretome” and further ameliorating the competency of the tumor proper to initiate and sustain competent angiogenic processes from the stroma.

Materials and methods

Cells, inhibitors and antibodies

MDA-MB-231 triple negative breast carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 5% fetal bovine serum (FBS)(SAFC Biosciences, Lenexa, KS) and 100 μ g·mL⁻¹ penicillin/streptomycin (MediaTech, Manassas, VA). Primary antibodies against ROCK1 (H-85), used at a 1:1000 dilution, RhoA (26C4), used at a 1:500 dilution, and Caveolin-1 (1-C), also used at a 1:500 dilution, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal anti-thrombospondin-1 primary antibody (used at 1:500) came from Abcam (Boston, MA). Secondary antibodies, Goat- α -Rabbit IgG HRP (AP307P) and Donkey- α -mouse IgG HRP (AP192P) were from Millipore (Billerica, MA) and used at 1:2000 and 1:6000 dilutions, respectively. Hepatocyte growth factor (H9661), lactacystin (L6785), and nocodazole (M1404) were purchased from Sigma-Aldrich (St. Louis, MO). The Met kinase inhibitor SU11274 (448101), the ROCK1 inhibitor Y27632 (68800) and EGFR kinase inhibitor AG1478 (658552) were from Calbiochem (Darmstadt, Germany). The mouse monoclonal EGFR blocking antibody was a generous gift from Dr. Ulrich Rodeck (Kimmel Cancer Center, Thomas Jefferson University). The purification of decorin protein core has been described extensively elsewhere [41,91]. Concisely recombinant human decorin, existing as a poly-His₆ fusion protein, was expressed in 293-EBNA cells. The 293-EBNA cells were serum starved for maximal secretion of both decorin species: the glycanated and unglycanated forms of human decorin. This material was then passed on an Ni-NTA chelating column followed by exposure to increasing concentrations of imidazole (up to 250 mM) in 20 mM Tris-HCl, 500 mM NaCl, 0.2% CHAPS, pH 8.0 for elution. Finally, decorin was separated via anion-exchange chromatography on Q-Sepharose.

Immunoblotting

Following the end points of the specified treatments, MDA-MB-231 cells were briefly washed in ice-cold phosphate-buffered-saline and lysed in radioimmune precipitation assay (RIPA) buffer (1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na₃VO₄, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin) for 20 min on ice. Samples were resolved by SDS-PAGE on 8%, 10%, or 12% gels subsequent to transfer on nitrocellulose for immunoblotting against the desired protein target. Equivalent loading and normalization of the samples were confirmed following Coomassie Blue staining carried out on a non-transferred portion of the SDS-PAGE gel.

Slot blot assays for detection of secreted TSP-1

Slot blot analysis of secreted TSP-1 in MDA-MB-231 cells entailed treating with 200 nM decorin protein core at the indicated time points as required by the experimental condition, following a 2 h serum starvation. MDA-MD-231 cells were seeded at a density of approximately 3 $\times$ 10⁵ cells within 6-well dishes and allowed to grow to around 85% confluency (reflects about 1 $\times$ 10⁶ cells). Next, tumor conditioned media (TCM) was then collected, centrifuged, and passed through disposable 0.22 μ m syringe-driven filter unit (Millipore). Serial dilutions (maintaining a total volume of 400 μ L) of the conditioned media were applied to the sample acceptor of the slot blot apparatus with suction (60 mBar) for a minimum of 30 min to ensure sample adsorption to the nitrocellulose membrane. The resulting membrane was blocked overnight in 1% BSA and incubated with a 1:500 dilution of the mouse monoclonal anti-thrombospondin-1 primary antibody, followed by incubation with a 1:6000 dilution of the Donkey- α -mouse IgG HRP-conjugated secondary, with visualization via enhanced chemiluminescence on an ImageQuant LAS-4000 (GE Healthcare). We quantified, via densitometry on the ImageJ program (NIH, Bethesda, MD),

the top band (as reported above) which represents a 1:2 dilution (200 μ L of TCM) while the bottom band is a 1:4 dilution (100 μ L of TCM) within a constant volume of 400 μ L (DMEM was used as diluent) loaded into each slot and reacted with the anti-TSP-1 antibody. Quantification of secreted TSP-1 bands was normalized to total cell number over time. The 1:2 and 1:4 dilutions were averaged and reported. This method was applied for all subsequent slot blot analyses.

Confocal microscopy, immunofluorescence imaging and quantification

Confocal microscopy and immunofluorescence studies were performed as described previously [52,92,93]. Briefly, approximately 5×10^4 MDA-MB-231 cells were seeded on 4-well glass chamber slides (BD Biosciences), which had been previously coated with 0.2% gelatin. MDA-MB-231 cells were serum starved (2 h) prior to incubation with decorin (200 nM). Cells were then washed with PBS and fixed using 4% paraformaldehyde on ice for 20 min, blocked in PBS 1% BSA overnight at 4°C, incubated with appropriate primary antibodies (1 h) at room temperature, washed in PBS, and then incubated in secondary antibody (goat anti-rabbit IgG Alexa-Fluor®488, Invitrogen) [94]. The nuclei were stained with DAPI (Vector Laboratories, Inc., Burlingame, CA). Images were obtained with a 63x, 1.3 oil-immersion objectives on a Zeiss LSM-780 confocal laser scanning microscope. The images were acquired with the ZEN 2010 software with the filters set at 488 nm for imaging and further analyzed with ImageJ and Adobe Photoshop CS5.1 (Adobe Systems, San Jose, CA). For the Immunofluorescence imaging studies, the slides were visualized using a Leica DM5500B microscope equipped with a Leica D-LUX3 camera in conjunction with the Advanced Fluorescence 1.8 software (Leica Microsystems, Inc.). Quantification of the relative fluorescence intensity of the TSP-1 signal was done via ImageJ [95]. Representative images were converted to black and white images, using the split channel option. The background was then subtracted and the threshold adjusted so that only fluorescent particles remained. The total amount of fluorescent particles was then quantified using the analyze particle option in ImageJ.

siRNA-mediated silencing of ROCK1

Transient transfection utilizing siRNA has been described elsewhere [56]. Briefly, ROCK1 was silenced through utilization of a cocktail comprised of three validated siRNAs specific for ROCK1 mRNA (ROCK1 siRNA sc-29473 Santa Cruz Biotechnology). Six-well plates of MDA-MB-231 cells (containing $\sim 2 \times 10^5$ cells) were transfected with either the siScramble control (sc-37007, Santa Cruz Biotechnology) at 20 pM or siROCK1 at 60 pM using diluted Lipofectamine 2000 (Invitrogen) in transfection medium (1% BCS-DMEM) at $\sim 70\%$ confluency. The transfection was carried out for a total of 48 h at 37°C followed by treatment with decorin (200 nM) at the indicated time point. Validation of ROCK1 depletion was verified by immunoblotting using ROCK1 specific primary antibodies prior to slot blot analysis of the tumor-conditioned media for TSP-1.

Exosomal Isolation and Purification

Exosome isolation and purification was carried as described elsewhere [96]. Briefly, conditioned media from four 10-cm dishes with $\sim 4 \times 10^7$ MDA-MB-231 cells was collected. Cells were treated for 6 h with 200 nM decorin in 1% BCS-DMEM whereupon media was pooled and centrifuged initially at $2000 \cdot g$ for 15 min at 4°C, then $4000 \cdot g$ for 15 min at 4°C in order to remove debris. Finally, exosomes were collected from the supernatant by ultracentrifugation at $105,000 \cdot g$ for 90 min at 4°C. The resulting pellet was resuspended in PBS and subjected to immunoblotting analysis.

Statistical Analysis

Each experiment presented herein was repeated three or more times with a comparable pattern of responses. All data were expressed as means $\pm$ SEM. Results were statistically analyzed with the Student's *t*-test or paired *t*-test using Sigma-Stat Software 11.0 (SPSS Inc). A probability value of $P < 0.05$ was considered statistically significant.

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Abbreviations

TSP-1	thrombospondin-1 protein
VEGFA	vascular endothelial growth factor A
RTK	receptor tyrosine kinase
EGFR	Epidermal Growth Factor Receptor
SLRP	small leucine rich proteoglycan
ROCK1	Rho-associated coiled-coil containing protein kinase 1
RhoA	Ras homolog gene family, member A
mAb	monoclonal antibody
CB	Coomassie Blue
HGF	hepatocyte growth factor
HIF-1α	hypoxia inducible factor 1-alpha

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Fig. 1.

Decorin evokes a rapid and biphasic release of Thrombospondin-1 (TSP-1) in MDA-MB-231 breast carcinoma cells. (A) Slot blot analysis of secreted TSP-1 in triple negative breast carcinoma MDA-MB-231 tumor conditioned media following exposure to 200 nM decorin at the indicated time points. Indicated on the left are the volumes of conditioned media in μL . Each sample was diluted with DMEM to reach a constant volume of 400 μL . (B) Quantification of secreted TSP-1 following normalization to total cell number over time. Values represent the mean $\pm$ SEM of three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (C,D) Representative confocal images of MDA-MB-231 cells of control (C) or decorin (200 nM, 40 min) treated (D) following immunostaining for TSP-1 (green) or stained with DAPI (blue) to visualize nuclei. Images were captured with the same exposure, gain, and intensity. Bar $\sim 10 \mu\text{m}$. (E) Immunoblot analysis via SDS-PAGE of cellular TSP-1 following treatment with decorin (200 nM) at the indicated time points. Equal loading of the cell lysates was determined by Coomassie Blue (CB) staining. (F) Immunoblot quantification of cellular TSP-1 at the time intervals reported. Normalization of signal intensities was achieved based on Coomassie blue staining. Slot blots and immunoblots were visualized via HRP-conjugated secondary antibodies. The data are representative of at least 3 independent experiments and reported as fold change $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as determined by the Student's t -test.

Fig. 2.

Decorin requires EGFR signaling, but not Met, to evoke rapid TSP-1 release in MDA-MB-231 cells. (A,B) Slot blot analysis of secreted TSP-1 following treatment with either decorin alone (200 nM, 10 min) or in combination with a 30 min pre-treatment with the Met kinase inhibitor SU11274 (1 μ M) as in (A) or HGF (50 ng·mL⁻¹) as in (B) followed by a 10 min incubation with decorin (200 nM). (C,D) Quantification of secreted TSP-1 signal intensity following normalization to total cell number for either decorin alone or in combination with SU11274 (C) or HGF (D) pre-treatments. (E–H) Representative immunofluorescence images of TSP-1 (green) in control cells (E), cells treated with 200 nM decorin for 10 min (F), or in conjunction with a 30-min pretreatment with the EGFR kinase inhibitor AG1478 (1 μ M, G) or with the EGFR blocking antibody mAb425 (10 μ g·mL⁻¹, H) followed by a 10-min decorin treatment (200 nM). All images shown were taken with the same exposure, gain, and intensity. Nuclei appear blue after DAPI staining. Bar ~ 10 μ m (I) Average TSP-1 fluorescence intensity was quantified (n = 10/treatment). Data are representative of at least 2–3 independent experiments and reported as normalized fold changes $\pm$ SEM (C,D) or as average fluorescence intensity $\pm$ SEM (I). ** P < 0.01; *** P < 0.001; NS, not significant.

Fig. 3.

Rapid secretion of TSP-1 depends on ROCK1 inhibition. (A) Analysis of secreted TSP-1 in MDA-MB-231 tumor conditioned media via slot blot analysis in the presence of the ROCK1 inhibitor Y27632 (10 μ M) at the indicated time points. (B) Quantification of secreted TSP-1 probed as in (A) at the reported time points following averaging of both bands and normalization to total cell number. (C) Immunoblotting of ROCK1 via SDS-PAGE in MDA-MB-231 at the time intervals noted with 200 nM decorin. Coomassie blue (CB) stained portions of the gel served as equal loading controls for each lysate. (D) ROCK1 quantification following decorin (200 nM) over time as determined in (C) and normalized to the signal intensities given by Coomassie blue. (E) Slot blot determination of TSP-1 in MDA-MB-231 following individual treatment for 10 min with decorin (200 nM), Y27632 (10 μ M) or pre-treatment (30 min) with Y27632 (10 μ M) followed by decorin (200 nM) for 10 min. (F) Quantification of secreted TSP-1 in response to the applied treatments as described in (E). Secreted TSP-1 intensity was normalized to total cell number. (G) Immunoblot verification of ROCK1 depletion following transfection with siRNA (60 pM) specific for ROCK1 (siROCK1) relative to scramble siRNA (2 pM) notated as siScram in the absence or presence of 200 nM decorin for 10 min. Coomassie blue served as positive loading control for the siRNA-mediated knockdown of ROCK1 in MDA-MB-231 cells. (H) Effect of ROCK1 depletion on secreted TSP-1 assayed via slot blot analysis of the tumor-conditioned media harvested from the same samples as reported in (G) with corresponding quantification of secreted TSP-1 following normalization to total cell number in (I). Three independent experiments were performed for the above studies and reported as normalized fold changes $\pm$ SEM. *** $P < 0.001$; NS, not significant.

Fig. 4.

Decorin depends on EGFR to rapidly degrade RhoA and evoke rapid TSP-1 secretion. (A) Immunoblot analysis of RhoA in response to 200 nM decorin for 10 min in MDA-MB-231 cells (top panel) and quantification of RhoA levels (bottom panel). (B) Immunoblot analysis of RhoA after exposure to decorin alone (200 nM, 10 min) or following pretreatment (30 min) with the EGFR inhibitor, AG1478 (1 μ M) (top panel), with accompanying quantification of RhoA levels (bottom panel). (C) RhoA analysis following decorin treatment (200 nM, 10 min) or after pretreatment (30 min) with the EGFR blocking antibody, mAb425 (10 μ g·mL⁻¹) (top panel) and corresponding quantification (bottom panel). (D) RhoA exposed to decorin alone (200 nM, 10 min) or in conjunction with a 30 min pretreatment of the proteasome inhibitor, lactacystin (10 μ M) (top panel) and quantification (bottom panel). In all instances, the upper portion of the gel was Coomassie blue stained for equal loading and normalization of RhoA signal intensity. The data reported are representative of at least 3 independent experiments and expressed as the average fold change $\pm$ SEM. *** $P < 0.001$.

Fig. 5.

Decorin depends on classical secretory pathway for rapid TSP-1 secretion. (A) Immunoblot analysis of TSP-1 positive exosomes from MDA-MB-231 in response to 200 nM decorin (6 h) followed by ultracentrifugation ($100,000 \times g$, 90 min) to isolate the exosome-containing fraction. (B) Quantification of exosomal TSP-1 after normalization to Coomassie blue. (C–F) Representative immunofluorescence images of MDA-MB-231 cells probe for TSP-1 (green) for control (C), decorin-only treated (200 nM, 10 min) or after pre-treatment (30 min) with the proteasome inhibitor, lactacystin ($10 \mu\text{M}$) as in (E) or with the microtubule inhibitor nocodazole ($100 \text{ ng}\cdot\text{mL}^{-1}$) as in (F). The nuclei appear blue due to DAPI. All images were collected at the same exposure, gain, and intensity. (G) Quantification of average TSP-1 fluorescence intensity ($n=10/\text{treatment}$). Data are representative of at least three independent experiments and reported as normalized fold changes $\pm\text{SEM}$ (B), or as the average fluorescence intensity $\pm\text{SEM}$ for the reported immunofluorescence as in (G). * $P < 0.05$; *** $P < 0.001$; NS, not significant.

Fig. 6. Diagram depicting the role of decorin in inducing rapid release of thrombospondin-1 (TSP-1) from breast cancer cells via the EGFR. Soluble decorin functions as a partial agonist of EGFR to elicit RhoA degradation via the 26S proteasome. Loss of RhoA leads to deactivation of ROCK1 signaling and mobilization of TSP-1-enriched vesicles. This novel decorin-evoked bioactivity is mediated exclusively through the EGFR but not via the Met receptor. Please refer to text for additional details.