Article

Decorin is an autophagy-inducible proteoglycan and is required for proper *in vivo* autophagy

Maria A. Gubbiotti [a], Thomas Neill [a], Helena Frey [b], Liliana Schaefer [b] and Renato V. Iozzo [a]

***a* - Department of Pathology,** *Anatomy and Cell Biology, and the Cancer Cell Biology and Signaling Program, Sidney Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107, USA*

***b* - Pharmazentrum Frankfurt/ZAFES,** *Institut für Allgemeine Pharmakologie und Toxikologie, Klinikum der Goethe-Universität Frankfurt am Main, Frankfurt am Main, Germany*

Correspondence to Renato V. Iozzo: *Department of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, 1020 Locust Street, Suite 336 JAH, Philadelphia, PA 19107, USA.* *renato.iozzo@jefferson.edu*

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Abstract

We have recently discovered that soluble extracellular matrix constituents regulate autophagy *via* an outside-in signaling pathway. Decorin, a secreted proteoglycan, evokes autophagy in endothelial cells and mitophagy in breast carcinoma cells. However, it is not known whether decorin expression can be regulated by autophagic stimuli such as mTOR inhibition or nutrient deprivation. Thus, we tested whether pro-autophagic stimuli could affect decorin expression in mouse cardiac tissue and whether the absence of decorin could disrupt the *in vivo* autophagic response. We found that nutrient deprivation induced decorin at the mRNA and protein level *in vivo* and *in vitro*, a process regulated at the transcriptional level by inhibiting the canonical mTOR pathway. Moreover, *Dcn*−/− mice displayed an aberrant response to fasting compared to wild-type mice. Our study establishes a new role for an extracellular matrix proteoglycan and provides a mechanistic role for soluble decorin in regulating a fundamental intracellular catabolic process.

Introduction

Along with playing an important role in maintaining the structural integrity of the cell, small leucine-rich proteoglycans (SLRPs) regulate important cellular functions such as proliferation, adhesion, and migration [1]. Decorin, a member of the class I family of SLRPs, demonstrates the versatility of these important matrix constituents. It embraces numerous functions [2] including: regulation of collagen fibrillogenesis [3–6], hepatic carcinogenesis [7,8], fetal membrane and calcium homeostasis [9,10], keratinocyte function [11], and suppression of angiogenesis due to both direct inhibition of vascular endothelial cell growth factor receptor 2 (VEGFR2) and insulin-like growth factor receptor I (IGF-IR) [12–17]. Moreover, soluble decorin represses tumorigenesis by antagonizing the epidermal growth factor receptor (EGFR) [18,19] and Met [20]. Most recently, soluble decorin has been shown to induce autophagy in endothelial cells and mitophagy in breast carcinoma cells [21–25]. These newly-found roles of decorin provide the first evidence that an extracellular matrix proteoglycan is implicated in the autophagic pathway.

Autophagy is a sophisticated process involving the degradation of superfluous cellular components *via* lysosomes [26,27]. Whether autophagy leads to cell survival or cell death is context-dependent. Under stressful conditions, autophagy may allow for the recycling of cellular products to maintain energy levels, promoting cell survival. On the other hand, autophagic cell death may be used as an alternative to apoptosis when apoptotic pathways are ineffective [28]. As autophagy plays a balancing act between cell

life and death, there exists a complicated relationship between autophagy and cancer. Traditionally, autophagy has been viewed as a pro-survival pathway in the setting of cancer [29]. However, recent changes in paradigm suggest that unrestrained autophagy may be able to slow the progression of tumorigenesis at certain stages of tumor growth by shielding non-malignant cells against metabolic stress caused by protein aggregates and other cellular debris [30,31]. Given this information, the ability of decorin to evoke autophagy in endothelial cells suggests that some of the anti-tumorigenic properties of decorin may involve its regulation of autophagy in the tumor microenvironment [32].

There are several well-established autophagic stimuli, one of which is nutrient deprivation. Following a depletion of nutrients, an imbalance in the AMP/ATP ratio results in the activation of adenosine monophosphate protein kinase (AMPK) [33] leading to the inhibition of the mammalian target of rapamycin (mTOR) [34] and subsequent de-inhibition of autophagy. We have recently demonstrated that decorin exerts its effects on autophagy in endothelial cells through this same pathway by activating AMPK through VEGFR2 signaling [22]. Though it has been established that decorin, like nutrient deprivation, is itself a pro-autophagic stimulus of canonical autophagy in select cell types, the question of whether autophagy can, in turn, regulate the expression of decorin remains unknown. In this study, we discovered that stimuli inducing autophagy concurrently evoke decorin expression at the mRNA and protein level both *in vivo* and *in vitro.* Moreover, *Dcn*−/− mice demonstrate an impaired autophagic response in the cardiac muscle. Mechanistically, we found that stimulation of decorin expression was transcriptionally mediated *via* a canonical inhibition of mTOR signaling. The results of this study provide new insight into the regulation of an extracellular matrix proteoglycan by autophagy and propose a new addition to the repertoire of SLRP functions. This may prove to be critical in understanding the role of decorin and other SLRPs in tumorigenesis and angiogenesis, and in other pathological conditions where autophagy is aberrant.

Results and discussion

Decorin is induced *in vivo* in response to fasting

We serendipitously discovered that decorin mRNA was increased upon fasting in wild type (WT) C57BL/6 mouse hearts, known to express high levels of decorin [35]. This observation prompted us to test whether autophagic stimuli would induce synthesis and secretion of decorin proteoglycan and the effects of decorin deficiency. Following 25-h fasting, cardiac decorin transcript levels significantly increased vis-à-vis fed animals (Fig. 1A). Next, we determined whether the expression of biglycan, the SLRP member closest to decorin [36] and actively involved in regulating inflammation and angiogenesis [37–39], was also induced by fasting. Notably, biglycan mRNA was not induced upon fasting, suggesting that this response to nutrient deprivation may be unique to decorin (Fig. 1A). To further validate our findings, we determined the cardiac levels of *Atf4*, *Cdkn1a* and *Map1lc3a*, three genes previously identified as positively regulated by fasting [40]. We found induction of all these genes, thus validating our strategy (Fig. 1A). Next, we examined the transcript levels of two other matrix constituents, *Col18a1*, whose protein product produces the anti-angiogenic C-terminal fragment endostatin [41] and *Lama2*, whose protein product, laminin α2, is a major component of basement membranes [42]. These genes were specifically investigated as both endostatin and laminin α2 have been shown to be involved in the regulation of autophagy, where endostatin has the ability to evoke autophagy through its interaction with α5β1 integrin [43,44], and laminin α2 acts as an autophagic inhibitor [45]. However, like *Bgn*, neither *Col18a1* nor *Lama2* were induced upon fasting (Fig. 1A), further signifying the specificity of this response to fasting for decorin.

To validate the above findings at the protein level, we analyzed by immunoblotting whole tissue extracts from hearts. We found that both decorin proteoglycan and protein core were increased upon fasting (Fig. 1B). Moreover, decorin from both control and fasted hearts and kidneys was isolated, treated with Chondroitinase ABC to remove its single glycosaminoglycan chain, and analyzed. The data showed ~3-fold increase in decorin proteoglycan and protein core expression in the hearts and ~2.5-fold increase in decorin protein expression in the kidneys, suggesting that this autophagic induction of decorin occurs across multiple tissues (Fig. 1C to E).

Next, we determined the localization of decorin following fasting by performing immunohistochemistry on heart samples that had been derived from either fed or fasted animals. We found a significant increase in immunostaining in the fasted hearts (Fig. 1G to I), especially prominent in the epicardial and perivascular regions including nerves (Fig. 1I).

As decorin is a secreted proteoglycan found in the circulation [46], we examined decorin levels in the blood. To this end, we collected blood from WT mice, both fed and fasted (25 h), and isolated the plasma fraction for analysis *via* ELISA. We found that plasma decorin levels in fed mice were

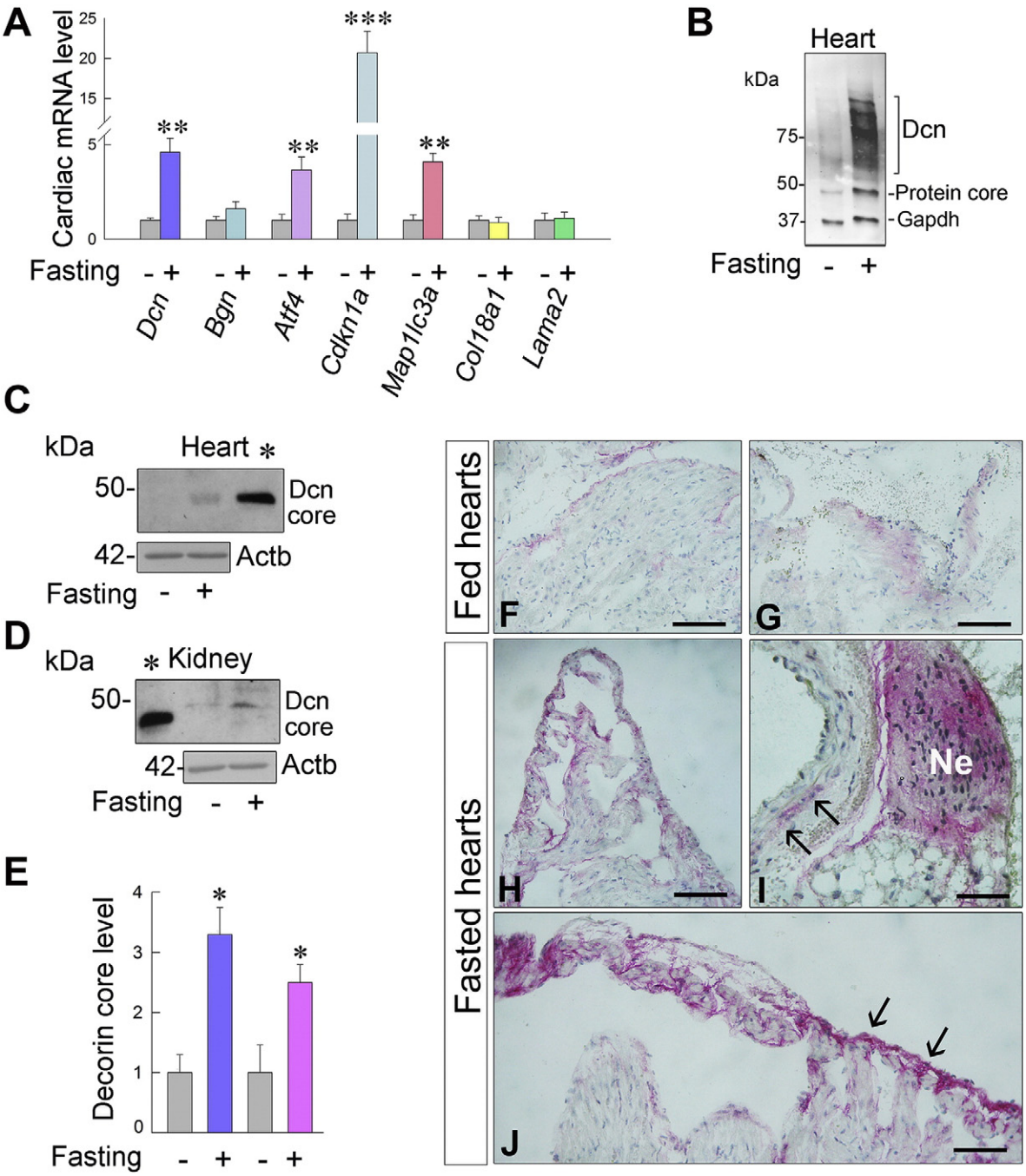

Fig. 1. Fasting induces decorin expression *in vivo*. (A) Gene expression analysis *via* qPCR of fed and fasted WT hearts. (B) Analysis of crude protein extracts for decorin proteoglycan and protein core in the heart tissue of fed and fasted WT mice. Gapdh was used as a loading control. (C, D) Representative western blots of decorin isolated from WT heart and kidney samples *via* DEAE-affinity chromatography and treated with Chondroitinase ABC to remove glycosaminoglycan chains. About 50 ng of decorin was used as a standard (asterisk). Actb was used as a loading control. (E), Quantification of decorin from (C) and (D) using densitometric analysis. (F–J) Immunohistochemistry of decorin in fed and fasted WT heart samples. Ne = Nerve. Arrows in panels I and J point to perivascular and subepicardial Dcn immunoreactivity, respectively. Bars = 100 μm. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars indicate SEM.

approximately 40 ng/ml. Analogous to our earlier data, we found that the fasted mice had levels twice (~80 ng/ml) that of the fed mice (Fig. 2A). Collectively, these results conclusively demonstrate that decorin is induced by nutrient deprivation *in vivo*, both in parenchymal tissues as well as in the

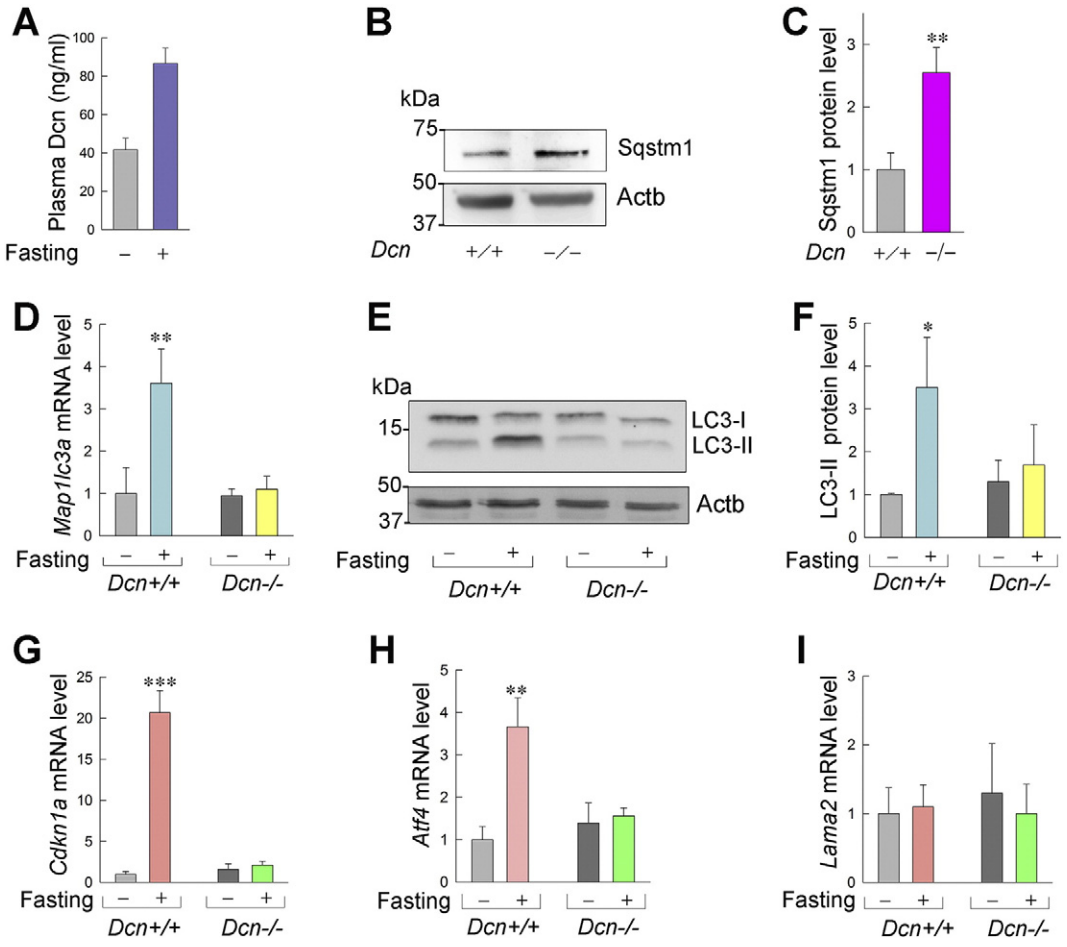

Fig. 2. The absence of decorin impairs the autophagic response. (A) ELISA results from plasma collected from fed and fasted WT mice depicting relative decorin levels. (B) Representative immunoblot of the autophagic marker, Sqstm1/p62, demonstrating accumulation in *Dcn*–/– heart tissue in comparison with WT samples. Actb was used as a loading control. (C) Quantification of Sqstm1/p62 protein from six independent hearts, either WT or *Dcn*–/–. (D) qPCR analysis of the *Map1lc3a* gene in WT and *Dcn*–/– hearts following fasting for 25 h. (E,F) Evaluation of LC3 protein levels in fed and fasted WT and *Dcn*–/– hearts depicted in a representative western blot. (F), Quantification of LC3 protein as seen in (D). (G, H, I) Gene expression analysis in fed and fasted WT and *Dcn*–/– hearts of *Cdkn1a*, *Atf4*, and *Lama2* respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars indicate SEM.

circulation, thereby identifying decorin as a secreted, autophagy-inducible proteoglycan.

The autophagic response is attenuated in Dcn–/– mice

Our results so far suggest that nutrient deprivation is capable of inducing decorin expression at both the mRNA and protein levels *in vivo*. As these findings underscore the importance of decorin in autophagy, we hypothesized that mice deficient in decorin would have an abnormal autophagic response to nutrient deprivation. To this end, we examined both WT and *Dcn*–/– heart tissue following 25-h fasting. We examined two well-established, canonical autophagic markers, Sqstm1/p62 and LC3, as accumulation of Sqstm1 is indicative of impaired autophagic flux and LC3 conversion is an ideal biomarker for the progression of autophagy. When we investigated basal levels of Sqstm1 in heart tissues, we found that Sqstm1 was elevated in the *Dcn*–/– hearts (Fig. 2B and C), giving plausibility to our hypothesis that autophagy is anomalous in the absence of decorin.

To confirm an impairment of autophagy in the *Dcn*–/– animals, we next determined the expression of LC3 in fed and fasted hearts in the absence or presence of decorin. In WT hearts, *Map1lc3a* mRNA was induced ~3.5-fold after fasting compared to levels seen in fed hearts (Fig. 2D). In contrast, there was little or no induction of *Map1lc3a* mRNA in the decorin-deficient mice (Fig. 2D). Moreover, in WT hearts, LC3-II, the autophagosome membrane-associated lipid-conjugated form of LC3, was increased in

response to fasting (Fig. 2E and F). However, in *Dcn*–/– hearts under the same conditions, there was little change in LC3-II levels (Fig. 2E and F) following fasting, indicating that *Dcn*–/– mice have aberrant autophagy. Thus, secreted decorin is the first proteoglycan to be directly involved in regulating an intracellular catabolic process such as autophagy.

Next, we examined a select group of genes responsive to nutrient deprivation in the *Dcn*–/– samples. Of importance was the response to fasting of *Cdkn1a*. *Cdkn1a* encodes the protein $p21^{WAF1}$, also known as cyclin-dependent kinase (CDK) inhibitor $p21^{WAF1}$, which binds to several cyclin-CDK complexes where it inhibits their activities. These interactions allow $p21^{WAF1}$ to regulate the cell cycle by inhibiting progression at the G_1 and S phase leading to growth arrest [47]. Notably, $p21^{WAF1}$ transcript and protein are induced by soluble decorin [48,49]. Following fasting, *Cdkn1a* appreciably increased in WT hearts (Fig. 2G), analogous to observed increases in previous *in vitro* studies in response to decorin treatment. However, in *Dcn*–/– hearts, this response to fasting was totally lacking, indicating that the presence of decorin *in vivo* is necessary for *Cdkn1a* induction under nutrient-poor conditions (Fig. 2G). Furthermore, a similar phenomenon was observed for *Atf4*, where the absence of decorin impaired its upregulation in response to fasting (Fig. 2H). We also investigated whether *Lama2*, a negative regulator of autophagy and a gene we found to be unchanged in WT samples following fasting, was affected in *Dcn*–/– samples. Interestingly, *Lama2* was unaffected in both fed and fasted *Dcn*–/– hearts (Fig. 2I), thereby suggesting specificity to regulation by decorin, and correspondingly autophagy, for a certain subset of genes.

In addition, we wanted to corroborate our biochemical data of LC3 by using transgenic mice expressing GFP-tagged LC3. This transgenic mouse allows the visualization of LC3-positive puncta in heart sections following a fasting stimulus [50]. To this end, we generated for the first time GFP-LC3;*Dcn*–/– mice and compared our results with the GFP-LC3 mice, which share a common C57BL/6 genetic background with the *Dcn*–/– mice. Following a 25-h fasting, we observed a significant increase in decorin immunostaining in the GFP-LC3;*Dcn*+/+ mouse hearts compared to fed hearts (Fig. 3A, B) in agreement with the data presented above (*cfr.* Fig. 1H–J). Note that in both fed and fasted hearts, the immunostaining is not in the cardiomyocytes but rather in the epicardial and pericardial regions. No decorin expression was detected in the GFP-LC3;*Dcn*–/– mice before or after fasting (Fig. 3A, C, D). Following a 25-h fasting, we observed a significant increase in LC3 puncta in GFP-LC3;*Dcn*+/+ mouse hearts (Fig. 3F and I) compared to fed hearts (Fig. 3E and I**)**. In contrast, we found a significant attenuation of LC3 puncta formation in fasted GFP-LC3;*Dcn*–/– hearts (Fig. 3H and I), as compared to fed hearts of the same genetic background (Fig. 3G and I**)**. Collectively, these *in vivo* data corroborate our biochemical data and provide robust genetic evidence for a role of decorin in regulating muscle cell autophagy. Subsequently, we also isolated embryonic fibroblasts from both wild-type and *Dcn*–/– mice. These fibroblasts were subjected to autophagic flux utilizing Chloroquine, an alkaline compound that crosses the lysosomal membrane to increase its pH thereby preventing merging with the autophagosome and hence allowing for a build-up of proteins, like LC3-II, that are typically degraded *via* lysosome-autophagosome fusion. In alignment with our hypothesis, we found that the ratio of LC3-II protein in the presence of Chloroquine and serum deprivation compared to LC3-II protein in cells treated with Chloroquine alone was less in the Dcn–/– fibroblasts compared to the changes seen in wild-type cells thus confirming that the abrogated response to autophagy is in fact due to impaired autophagic flux (Fig. 3J). Moreover, our findings have not only identified a new role for decorin as a key extracellular matrix proteoglycan that can be regulated positively by autophagic induction, but have further determined the importance of decorin in maintaining normal autophagy *in vivo*. Coupling this conclusion with the ability of decorin to act as a driving force for autophagy in certain cell types offers further insight into the function of decorin as the "*guardian from the matrix*," particularly in cancer or other diseases.

Nutrient deprivation induces Dcn expression in mouse fibroblasts

Having established that decorin is upregulated in response to autophagy *in vivo*, we then investigated the mechanism by which this induction occurs. To accomplish this goal, we first needed to develop a solid *in vitro* model in which to study this process. Mouse embryonic NIH-3T3 fibroblasts were ideal cell candidates for this model as they endogenously express decorin. Serum deprivation was chosen as an autophagic stimulus to mimic our *in vivo* studies using fasting.

Notably, serum deprivation for 24 h caused a marked increase in decorin mRNA concomitant with that of *Atf4* and *Cdkn1a* (Fig. 4A), two genes evoked by fasting [40]. We should point out that soluble decorin induces $p21^{WAF1}$ [48,49,51] as well as Peg3 [32], both of which are highly induced in skeletal muscle during starvation and wasting [40]. As in the case of the *in vivo* experiments, the mRNA levels of *Bgn*, *Col18a1*, and *Lama2* remained unchanged (Fig. 4A). Thus, it appears that decorin might be a part of a circuit to control autophagy from the outside in, presumably *via* RTKs, as in the case of VEGFR2 [21–23] and Met [24]. Next, we performed time-course studies to determine the kinetics of decorin and biglycan induction in response to serum deprivation. As predicted, decorin mRNA increased in a linear

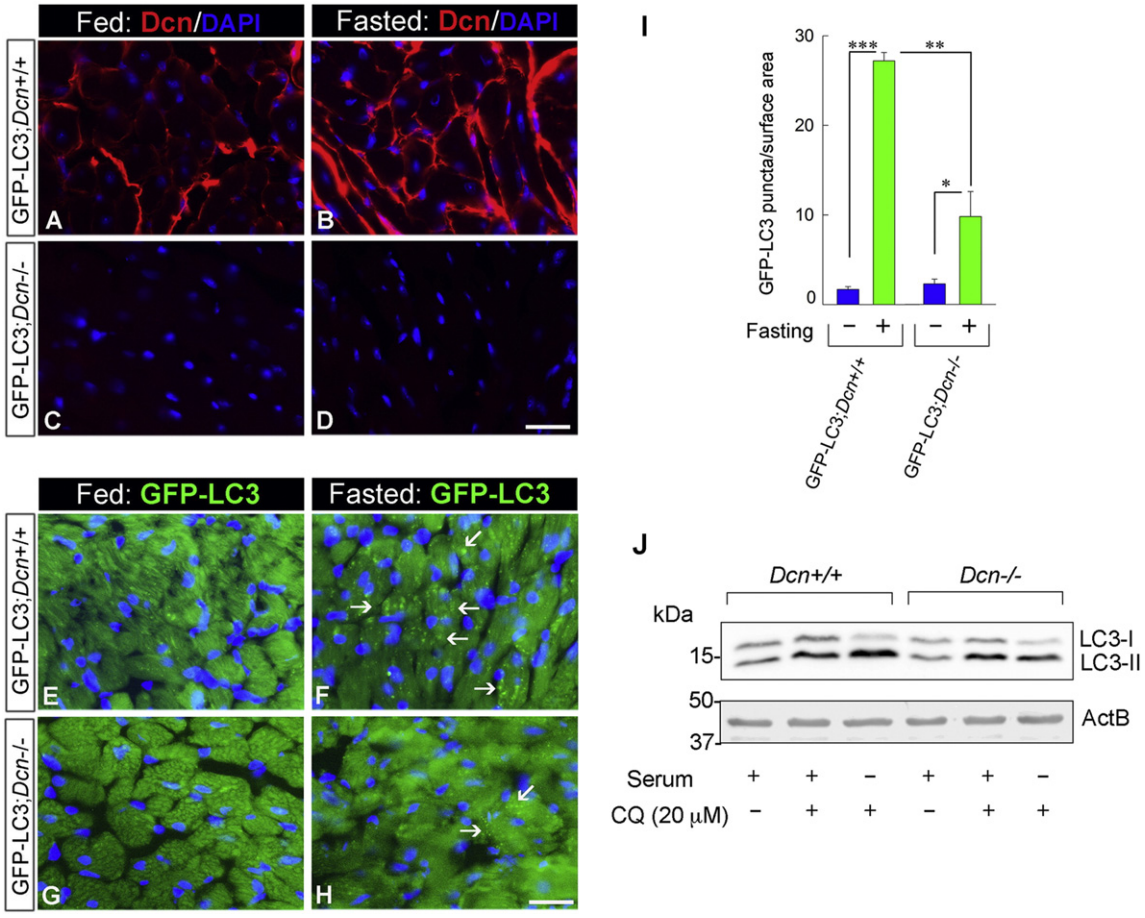

Fig. 3. Formation of GFP-LC3 puncta in response to fasting is diminished in GFP-LC3;*Dcn*–/– hearts. (A–D) Representative cryo-sections of GFP-LC3;*Dcn*+/+ hearts demonstrating the increase in decorin immunoreactivity in the fasted GFP-LC3;*Dcn*+/+ hearts (B) vis-a-vis fed (A). (C, D) Lack of decorin immunoreactivity in the GFP-LC3;*Dcn*–/– hearts. Bar ~50 μm. (E, F) GFP-LC3;*Dcn*+/+ heart sections showing abundant formation of puncta (white arrows in F) during starvation. (G, H) GFP-LC3;*Dcn*–/– heart sections depicting an attenuated puncta formation following fasting(white arrows in H). Bar ~50 μm. (I) Quantification of GFP-LC3 puncta in GFP-LC3;*Dcn*+/+ and GFP-LC3;*Dcn*–/– hearts with and without fasting. Quantification was determined using at least six different images from three separate mice for each category, using an NIH-ImageJ macro as described before [59,60]. (J) Autophagic flux of wild-type and *Dcn*–/– embryonic fibroblasts. Notice the reduced amount of LC3-II in the *Dcn*–/– embryonic fibroblasts after a 6 h pulse with Chloroquine (CQ). **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Error bars depict SEM.

fashion over the 24-h period, whereas biglycan mRNA did not appreciably vary during the time-course (Fig. 4B). These results validate our previous findings and suggest that the response to nutrient deprivation is not uniform across SLRP family members.

To further establish that decorin was induced in our cell model, we isolated decorin proteoglycan from media of NIH-3T3 cells that had undergone serum deprivation for 24 h using DEAE chromatography. We found ~2.5 fold increase in the secreted decorin proteoglycan levels following serum deprivation as compared to controls, similar to our *in vivo* findings (Fig. 4C and D). Hence, we confirmed that nutrient deprivation is an impetus for the induction of both decorin mRNA and protein expression *in vitro*. We note that in previous experiments done by our laboratory [52] and others [53], decorin mRNA was found to be markedly increased by quiescence in both human dermal and lung fibroblasts. However, in both instances the quiescence was obtained by serum deprivation for 24–48 h. It is, thus, likely that some of the increase, if not all, was due to a cellular response to serum deprivation rather than quiescence.

To further reinforce the validity of our *in vitro* model, we performed autophagic flux experiments using Bafilomycin A1, an antibiotic that interferes with the late phase of autophagy by blocking the fusion of autophagosomes with lysosomes [54]. Thus, if autophagy is stimulated, there should be a greater accumulation of LC3-II protein, which cannot be further degraded in the acidic lysosomes, as

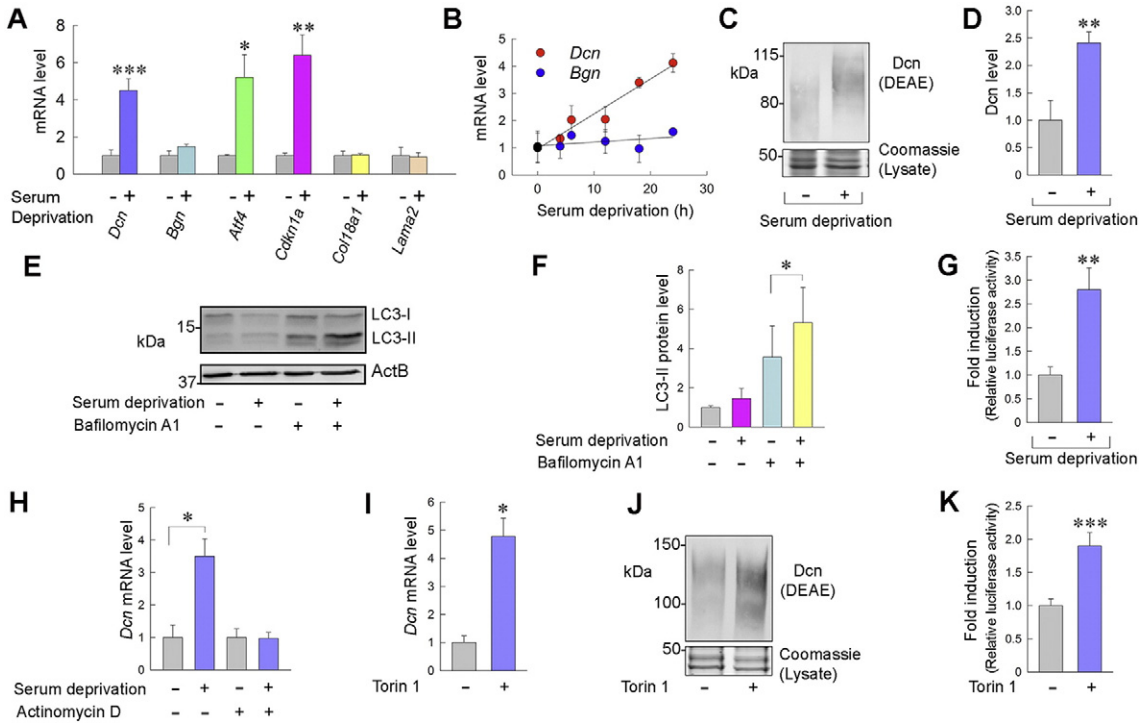

Fig. 4. Activation of autophagy induces decorin expression *via* a transcriptional mechanism utilizing the canonical mTOR pathway. (A) Validation of *in vivo* findings *via* qPCR analysis of NIH-3T3 fibroblasts serum-deprived for 24 h. (B) Time-course comparison of decorin and biglycan mRNA in 3T3 fibroblasts following serum deprivation. (C, D) Representative western blot for decorin proteoglycan following extraction from media of NIH-3T3 fibroblasts after 24 h of serum deprivation using a DEAE-affinity method. Proteoglycan levels were normalized to total protein from cellular lysates *via* Coomassie blue staining. (D) Quantification of decorin proteoglycan from (C). (E) Immunoblot demonstrating a representative autophagic flux experiment in 3T3 fibroblasts using Bafilomycin A1 (400 nM) in the presence or absence of serum. Actb was used as a loading control. (F) Quantification of LC3-II from (E). (G) Quantification of *Dcn* promoter-luciferase reporter assays before or after serum deprivation for 18 h. (H) qPCR analysis of decorin mRNA in 3T3 fibroblasts serum-deprived for 6-h in the presence or absence of Actinomycin D (1 mg/ml). (I) Gene expression of decorin in 3T3 fibroblasts following treatment with 400 nM Torin 1 for 6 h. (J) Immunoblot of DEAE-isolated decorin from media of 3T3 fibroblasts treated with 400 nM Torin 1 for 8 h. Proteoglycan levels were normalized to total protein from cellular lysates *via* Coomassie staining. (K) Quantitative analysis of luciferase activity following overnight treatment with 400 nM Torin 1 after transient transfection of *Dcn*-Luc. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars indicate SEM.

compared to nutrient-enriched conditions. Following a 6-h serum deprivation, we found that the autophagic flux in the NIH-3T3 fibroblasts was very efficient, whereby there was no change in LC3-II protein levels compared to vehicle-treated controls (Fig. 4E and F). However, upon Bafilomycin A1-evoked block of the flux, there was a significant increase in LC3-II protein following serum deprivation compared to LC3-II levels of cells grown in full serum (Fig. 4E and F).

Nutrient deprivation regulates decorin at the transcriptional level

The regulation of decorin in response to autophagic stimuli may occur *via* changes in transcription or increased mRNA stability. To differentiate between these two possibilities, we generated a 1.9 kb region of the mouse decorin promoter cloned into a luciferase construct, *Dcn-Luc.* Using transient transfection assays with this construct, we found that 18-h serum deprivation evoked ~3-fold increase in relative luciferase activity as compared to cells grown in full serum (Fig. 4G), suggesting that the increase in decorin expression might be likely regulated at the transcriptional level. To address this point, we used Actinomycin D, an antibiotic that suppresses transcription by forming a stable complex with double-stranded DNA, inhibiting DNA-primed RNA synthesis. We found that treatment of NIH-3T3 fibroblasts with Actinomycin D completely prevented the induction of decorin mRNA following 6 h of serum deprivation (Fig. 4H). These results corroborate the luciferase reporter assays and indicate that the regulation of decorin in response to autophagic stimuli primarily arises at the transcriptional level, rather than through an mRNA stabilizing mechanism.

Transcriptional regulation of decorin during autophagy occurs *via* inhibition of the canonical mTOR pathway

Next, we verified that decorin induction was directly linked to the activation of the autophagic program. As the canonical mTOR pathway is the primary mechanism that initiates autophagy following nutrient deprivation, we blocked this pathway using Torin 1, an ATP-competitive inhibitor of mTOR [55]. Torin 1 is a more potent autophagic inducer than rapamycin, as it inhibits both mTOR complexes 1 and 2 and also more completely inhibits mTOR complex 1. This is due to the fact that Torin 1 directly binds to the active site of mTOR rather than acting indirectly in an allosteric manner [55]. We found that treatment with Torin 1 for 6 and 8 h, respectively, was capable of replicating the induction of decorin mRNA and secreted proteoglycan as was observed following serum deprivation in NIH-3T3 cells (Fig. 4I and J). Moreover, we were able to establish functional relevance of these findings by verifying that 18-h treatment with Torin 1, following transient transfection with the *Dcn-Luc* vector, induced luciferase reporter activity by ~2-fold as compared to vehicle (Fig. 4K). Thus, our data suggest that the regulation of decorin is directly entwined with the activation of autophagy. The biological mechanism by which decorin is induced in response to pro-autophagic signals appears to be due to an inhibition of the canonical mTOR pathway.

Conclusions

The results of this study provide a new role for an extracellular matrix proteoglycan, decorin, in the process of autophagy. Though it has been previously shown that decorin is capable of inducing autophagy in endothelial cells, this study, to our knowledge, holds the first evidence that decorin itself can be regulated by autophagic stimuli. Our findings have far-reaching implications. First, as decorin is often downregulated or absent in tumors, this new evidence insinuates that there may be defective autophagy in cancer cells, which may contribute to their malignant phenotype. More importantly, the ability for decorin to be upregulated in the tumor stroma by treatment with specific mTOR inhibitors, like Torin 1, may provide a new avenue for therapeutic approaches for treating certain tumor types.

While the regulation of decorin was the focus of this paper, we briefly investigated changes in levels of biglycan expression in response to fasting. There was a stark contrast between the upregulation of decorin and the lack of transcriptional changes of biglycan despite both proteoglycans being members of the same family. More work needs to be done in this area as other SLRPs, such as lumican, may respond in a similar manner to decorin and may also play important roles in the autophagic process.

Also of note is the response to fasting of the *Dcn*−/− mice. Though they were exposed to the exact same conditions as the WT mice, they did not show significant elevation of LC3-II upon fasting nor were their GFP-LC3 puncta increased to the same extent. It is remarkable that the deficiency of one extracellular matrix proteoglycan can lead to impairment of a key autophagic event. As autophagy has been implicated in various diseases ranging from cancer to metabolic defects like diabetes, these data present innovative information which will provide a basis both for investigating disease mechanisms as well as for developing better treatment options for said diseases. Thus, the conclusions of this study further highlight how imperative decorin is for normal autophagy and emphasize the need for further inquiry regarding clinical relevance of these initial findings.

Experimental procedures

Chemicals and antibodies

Antibodies were purchased as follows: Decorin (R&D Biosystems, AF1060), LC3 (Sigma, L7543), Gapdh (Cell Signaling, 14C10), and Actb (Abcam, ab8227). All antibodies were used at a 1:1000 dilution except Actb, which was used at 1:10,000 and Gapdh, which was used at 1:4000. Actinomycin D, Bafilomycin A1, and Chloroquine were purchased from Sigma-Aldrich (A1410, B1793, and C-6628 respectively). Torin 1 was purchased from Tocris (4247). A portion of the mouse decorin promoter ranging from −2028 to −108 relative to the transcription start site was subcloned from genomic DNA extracted from mouse liver using the Wizard Genomic DNA purification kit (Promega, A1120). This promoter was then cloned into a pGL3-Basic luciferase plasmid (Promega, E1751) to make the *Dcn-Luc* construct. The pRLTK Renilla plasmid (Addgene, E2241) was used to determine transfection efficiency.

Animal experiments

C57BL/6 mice were purchased from Jackson Laboratories. *Dcn*−/− mice were generated as previously described [56]. GFP-LC3 transgenic mice were purchased from Riken. GFP-LC3;*Dcn*−/− mice were generated by crossing the GFP-LC3 mice with *Dcn*−/− mice until homozygosity was obtained, using genomic PCR according to a previously published protocol [56,57]. These mice were then crossed for at least five generations and their progeny was used for the experiments. Animal experiments were performed as per the Guide for Care and Use of Laboratory Animals and the Institutional Animal

Care and Use Committee of Thomas Jefferson University and in accordance with the German Animal Protection Law. Mice were of both male and female sex and of various ages ranging from 4 weeks to 6 months. Fasting experiments involved withholding food for 25 h, but water was allowed *ad libitum*. After animals were euthanized, organs were removed and immediately snap-frozen in liquid N_2. Embryos were isolated from 14-day pregnant wild-type and *Dcn*–/– mice and minced and placed in full serum media. They were allowed to grow to confluence before passaging to generate embryonic fibroblasts which were used for subsequent experiments.

Cell lines and cell culture conditions

NIH 3T3 fibroblasts were maintained in DMEM (Corning, 10–013) supplemented with 10% fetal bovine serum (SAFC Biosciences, 12103C) and 100 µg/ml penicillin/streptomycin (Corning, 30–002-CI). Cells were co-transfected with 4 µg of *Dcn-Luc* plasmid and 500 ng of pRLTK (Addgene, E2241) Renilla plasmid, using Lipofectamine LTX (Life Technologies, 15338100) following the manufacturer's protocol. The *Dcn-Luc* contained 1920 bp mouse decorin promoter driving *Firefly luciferase.* Autophagic flux experiments were performed using a final concentration of 20 µM Chloroquine for 6 h in the absence or presence of serum.

DEAE isolation of decorin

Secreted decorin proteoglycan was isolated from media as described previously [58]. Briefly, media were incubated overnight with 50 µl of 50% (w/v) of diethylaminoethyl (DEAE) Sephacel (Sigma-Aldrich, 17–0500–01) in 20 mM Tris–HCl (pH 7.5), 0.1% (w/v) Triton X-100, and 0.15 M NaCl at 4 °C. Pellets were washed twice with 1 ml of the same buffer and twice with buffer containing 0.2 M NaCl. Sample buffer was used to dissolve the pellets.

ELISA and western blot analysis

For ELISA, blood was collected from fed and fasted mice and placed in an Eppendorf tube coated with EDTA to prevent coagulation. The whole blood was centrifuged for 5 min at 10,000 rpm and the plasma fraction was collected for analysis. Plasma decorin levels were determined using a standard mouse decorin ELISA kit following the manufacturer's protocol (Abcam, ab155454).

For western blot analysis, cells were lysed following treatment in radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na_3VO_4, 10 µg/ml aprotinin, and 10 µg/ml leupeptin) for 20 min on ice. Secreted decorin was isolated from media using DEAE extraction. Tissue samples were lysed in T-Per Reagent with EDTA and protease inhibitor (Life Technologies, 78510). In some samples, decorin was purified and treated with Chondroitinase ABC to remove glycosaminoglycan chains. Samples were separated by SDS-PAGE and transferred to nitrocellulose where specific antibodies were used to visualize the proteins.

Immunofluorescence microscopy

Hearts removed from either GFP-LC3;*Dcn*+/+ or GFP-LC3;*Dcn*–/– mice were cross-sectioned and fixed in 4% paraformaldehyde (w/v PBS) (Fisher, T353-500) for 4 h. The cross sections were then incubated in 15% sucrose (Fisher, S6-500) for 4 h and 30% sucrose overnight after which they were placed in OCT (Tissue Tek, 4583). 5–8 µm sections were used for imaging. Sections were stained with a mouse-specific decorin antibody (R&D Biosystems, AF1060) at 1:200 dilution and, after processing, were mounted with Vectashield (Vector Laboratories Inc., H-1200). Images were acquired with Leica Application Suite, Advanced Fluorescence 1.8 software (Leica Microsystems, Inc.). Quantification of GFP-LC3 puncta was performed using a macro specific for ImageJ software that was designed by Ruben Dagda (University of Nevada School of Medicine) [59,60].

Luciferase assays and quantitative real time-PCR analysis

Luciferase assays were performed as per the manufacturer's instructions (Biotium, 30005). Fold change analysis was performed following normalization using a *Firefly*/*Renilla* ratio. Gene expression analysis by quantitative real time-polymerase chain reaction (qPCR) was performed as previously described [61] with minor modifications. Briefly, following treatment, 1×10^6 cells or 1 mm^3 of tissue were lysed in 750 µl of TRIzol® reagent (Life Technologies, 15596-026). RNA was isolated using a standard RNA isolation kit (Zymo Research, R2052). 1 µg of RNA was annealed with oligo(dT) primers (Life Technologies, 18418-012) and cDNA was synthesized using SuperScript Reverse Transcriptase II (Life Technologies, 18064-022) according to the manufacturer's directions. The target genes and housekeeping gene (*Actb*) were amplified in independent reactions using the Brilliant SYBR Green Master Mix II (Agilent Technologies, 600,828). Samples were run in triplicate on a LightCycler480-II (Roche Applied Science) and the cycle number (C_t) was obtained for each reaction. Fold-change determinations were made utilizing the comparative C_t method for gene expression analysis.

RNA stability and immunohistochemistry

Cells were pre-treated with Actinomycin D for 1 h at a concentration of 1 mg/ml and then treated with serum

deprivation and fresh Actinomycin D for 6 h. Following RNA extraction and cDNA synthesis, samples were assayed *via* qPCR to determine changes in gene expression. Immunohistochemistry of heart tissue for decorin was performed as previously described [46].

Statistical analysis

Experiments were repeated three or more times and all data are expressed as means ± SEM. Paired and unpaired two-tailed Student's *t*-tests were used to analyze significance using the SigmaStat program. $P < 0.05$ was considered statistically significant.

Competing financial interests

The authors declare no competing financial interests.

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Abbreviations used:
Actb, β-actin; AMPK, adenosine monophosphate protein kinase; Bgn, biglycan; CDK, cyclin-dependent kinase; Dcn, decorin; EGFR, epidermal growth factor receptor; GFP, green fluorescent protein; IGF-IR, insulin-like growth factor receptor I; LC3, microtubule-associated protein 1 light chain 3; mTOR, mammalian target of rapamycin; Peg3, paternally-expressed gene 3; qPCR, quantitative real time-polymerase chain reaction; SLRP, small leucine-rich proteoglycan; SQSTM1, sequestosome 1/p62; VEGFR2, vascular endothelial cell growth factor receptor 2; WT, wild type.

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