Knockdown of Decorin and Biglycan at Time of Tendon Injury Alters Gene Expression and Fibril Morphology

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Introduction

Tendon healing follows a typical wound healing process, including inflammatory, proliferative, and remodeling phases, though outcomes following tendon injury remain poor. The small leucine-rich proteoglycans (SLRPs), decorin (Dcn) and biglycan (Bgn), are critical regulators of fibrillogenesis and matrix assembly, but their specific roles in tendon healing are not fully understood. We previously showed that knockdown of Bgn or both Dcn/Bgn resulted in increased tendon modulus 6-weeks post-injury, suggesting improved function due to Bgn knockdown.[1] However, the mechanisms driving these differences remain unknown. Therefore, the objective of this study was to define the biological and structural regulatory roles of Dcn and Bgn in tendon healing using conditional knockdown of Dcn, Bgn, and both Dcn/Bgn at the time of injury. We hypothesized that induced knockdown of Bgn and both Dcn/Bgn would improve healing resulting in increased tendon extracellular matrix gene expression, reduced scarring, and superior fibril structure compared to wild-type mice.

Methods

Study design

Female $Dcn^{+/+}/Bgn^{+/+}$ control (WT, n = 44), $Dcn^{flox/flox}$ (I-$Dcn^{-/-}$, n = 32), $Bgn^{flox/flox}$ (I-$Bgn^{-/-}$, n = 32), and compound $Dcn^{flox/flox}/Bgn^{flox/flox}$ (I-$Dcn^{-/-}/Bgn^{-/-}$, n = 32) mice with a tamoxifen inducible Cre (B6.129-Gt(ROSA)26Sortm1(cre/ERT2)Tyj/J, Jackson Labs) were used (IACUC approved).[2] At 120 days old, Cre excision was induced via two (injured) or three (uninjured) consecutive daily IP injections of tamoxifen. At time of induction, injured groups underwent bilateral patellar tendon (PT) injury surgery as described and were sacrificed 1-, 3- or 6-weeks later. Uninjured groups were sacrificed at 150 days old.[3]

Gene Expression

PTs (n = 4/group) were homogenized, and RNA was extracted. RNA was converted to cDNA, pre-amplified, and loaded into a Fluidigm 96.96 Dynamic Array. The 96 target genes included categories of collagens, non-collagenous matrix, matrix remodeling, cell-ECM proteins, and cell and inflammatory markers. ΔCt was calculated by subtracting the gene cycle threshold (Ct) from average Ct of the housekeeping genes (*Abl1*, *Rps17*).

Histology

Knee joints (n = 4/group) were fixed, decalcified, and paraffin sectioned in the transverse plane of the PT at 10μm. Sections were stained with toluidine blue, and scar tissue was measured in the wound site adjacent to the native tissue.

Transmission Electron Microscopy

PTs (n = 4/group) were isolated, fixed, and processed as described.[4] Sections were cut at 85nm, stained, and imaged at 60,000x in the wound area. Fibril diameter distributions were quantified.

Statistics

For gene expression and scar area percentage, one-way ANOVAs with Tukey post-hoc tests were conducted at each timepoint. Fibril diameter distributions were compared using Kolmogorov-Smirnov tests. Significance was set at $p \leq 0.05$ and trends at $p \leq 0.1$.

Results

Gene Expression

Dcn and *Bgn* expression demonstrated efficient knockdown at each healing timepoint. *Dcn* was significantly reduced (4-6 fold) in I-$Dcn^{-/-}$ and I-$Dcn^{-/-}/Bgn^{-/-}$ tendons compared to WT and I-$Bgn^{-/-}$ mice (Figure 1A). Similarly, *Bgn* expression was 4-6 fold lower in I-$Bgn^{-/-}$ and I-$Dcn^{-/-}/Bgn^{-/-}$ tendons (Figure 1B). Further evaluation of gene expression profiles revealed subtle changes during early tendon healing. At 1-wk post-injury, *Col12a1*, *Tnmd*, and *Igf1* (Figure 2A) expression were significantly reduced in I-$Dcn^{-/-}$ tendons compared to WT. By 3-wks, *Igf1* expression in the I-$Dcn^{-/-}$ group was significantly greater than WT tendons, contrasting the difference at 1-week. And by 6-wks, there was no difference in *Igf1* expression between

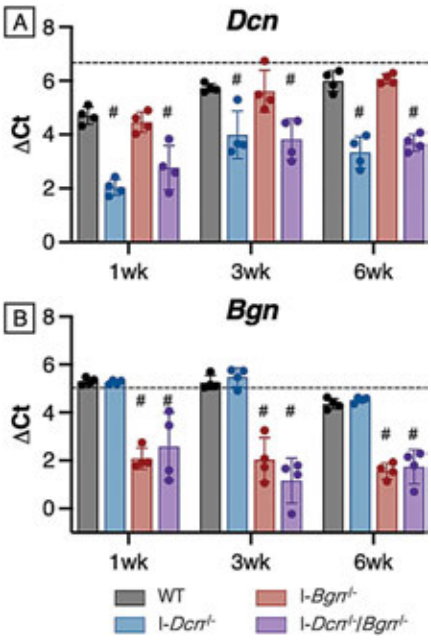


Figure 1. Induced knockdown of **(A)** *Dcp* and **(B)** *Bgn* expression resulted in a significant reduction in expression levels. (#: $p \leq 0.05$ from WT). Uninjured WT expression level is shown as a dashed line.

WT and I-*Dcn*$^{-/-}$ tendons, while expression was significantly higher in the I-*Dcn*$^{-/-}$/*Bgn*$^{-/-}$ group compared to WT tendons (Figure 2A). Contrasting the subtle changes at 1- and 3-wks post-injury, several significant gene changes during late tendon healing at 6-wks were observed in I-*Dcn*$^{-/-}$/*Bgn*$^{-/-}$ tendons. For example, there were no differences in *Fmod* at 1 or 3 wks, but *Fmod* was significantly increased at ± wks compared to WT (Figure 2B). Similar trends were observed across several target genes, and those exhibiting increased expression in the I-*Dcn*$^{-/-}$/*Bgn*$^{-/-}$ group compared to WT at 6-weeks are listed in Figure 2C.

Histology & Fibril Morphology

No differences in scar area percentage were observed at any healing timepoint (data not shown). However, fibril size distributions were significantly different between all groups at each timepoint with a shift towards smaller diameter fibrils in the I-*Dcn*$^{-/-}$/*Bgn*$^{-/-}$ at both 3- and 6-wks post-injury compared to WT and I-*Dcn*$^{-/-}$ (Figure 3A,B). Additionally, the fibril diameter distribution was narrower in I-*Bgn*$^{-/-}$ tendons compared to WT and I-*Dcn*$^{-/-}$ at 6-wks (Figure 3B).

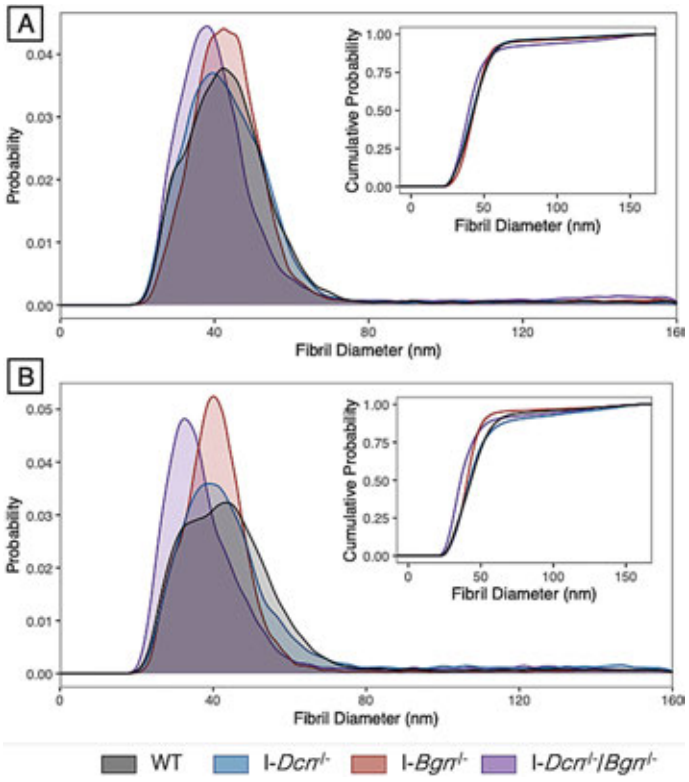
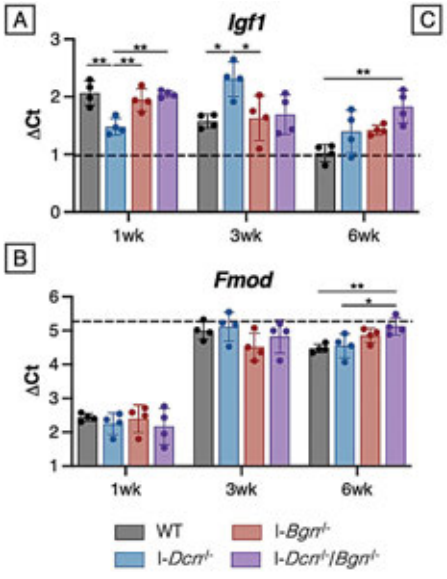


Figure 3. Probability density and cumulative distribution (inset) plots of fibril diameter demonstrated a moderate shift towards smaller fibril diameters in the I-*Dcn*$^{-/-}$/*Bgn*$^{-/-}$ group at **(A)** 3-weeks and **(B)** 6-weeks post-injury. I-*Bgn*$^{-/-}$ tendons also had a narrower distribution of fibril diameters at 6-weeks compared to I-*Dcn*$^{-/-}$ and WT tendons.

Discussion

Using our novel inducible models to minimize compensation typically present in traditional models, our findings support biological and structural regulatory roles of Dcn and Bgn



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Gene	ΔCt diff	p-value
Collagens		
Col1a2	0.67	0.006
Col6a1	0.55	0.008
Col11a1	0.58	0.02
Non-Collagenous Matrix		
Fbn2	1.97	0.005
Fmod	0.65	0.01
Hspg2	0.49	0.01
Thbs4	0.83	0.009
Tnc	0.48	0.05
Matrix Remodeling		
Adamts2	0.42	0.006
Mmp2	0.73	0.007
Mmp3	1.38	0.001
Mmp14	0.96	0.003
Cell-ECM		
Itga11	0.65	0.01
Ptk2	0.36	0.005

Gene	ΔCt diff	p-value
Signaling		
Bmp2	0.5	0.02
Igf1	0.81	0.003
Mtor	0.47	0.001
Pdgfa	0.55	0.009
Pdgfb	0.59	0.009
Pdgfrb	0.47	0.04
Tgfb1	0.51	0.006
Tgfb3	0.66	0.005
Vegfb	0.57	0.01
Cell Cycle/Proliferation		
Cdkn1a	0.61	0.02
H2afx	0.49	0.02
Inflammation		
Ccl4	1.61	0.02
Pge2	0.33	0.05

Figure 2. **(A)** I-*Dcn*$^{-/-}$ tendons exhibited an altered healing profile of *Igf1* expression with a significant decrease at 1-week and increase at 3-weeks post-injury compared to WT tendons. At 6-weeks post-injury *Igf1* and **(B)** *Fmod* expression were significantly increased in I-*Dcn*$^{-/-}$/*Bgn*$^{-/-}$ tendons compared to WT tendons. Uninjured WT expression level is shown as a dashed line. (*$p \leq 0.05$, **$p \leq 0.01$) **(C)** Target genes exhibiting increased expression in I-*Dcn*$^{-/-}$/*Bgn*$^{-/-}$ tendons compared to WT tendons at 6-weeks post-injury. The signaling category had the greatest number of differentially expressed genes. ΔCt diff represents the increase in mean ΔCt of I-*Dcn*$^{-/-}$/*Bgn*$^{-/-}$ compared to WT.

during tendon healing, as evidenced by alterations in gene expression profiles and fibril structure. In addition to their structural roles in fibrillogenesis and matrix assembly, Dcn and Bgn regulate inflammation and growth factor activity.[5] Though only moderate changes were observed in 1- and 3-weeks post-injury, increased expression of several growth factors and matrix proteins at 6-weeks post-injury suggest that Dcn and Bgn play more critical roles during the remodeling phase of healing.This may be due to the role of Dcn and Bgn in regulating signaling pathways such as Igf, Pdgf, and Tgfb, which results in downstream effects on matrix synthesis and remodeling.[6] While no compensatory changes in *Dcn* or *Bgn* expression were observed, the most pronounced effects in the I-$Dcn^{-/-}$/$Bgn^{-/-}$ group indicate overlapping functions of Dcn and Bgn and that functional compensation may occur in the single knockdown models.[7,8] Contrary to our hypothesis, induced knockdown of Bgn in both the single and double knockdown groups resulted in a narrower distribution of fibril diameters at 6-weeks post-injury, which deviates from an uninjured distribution. Therefore, increased modulus in the I-$Bgn^{-/-}$ and I-$Dcn^{-/-}/Bgn^{-/-}$ groups is likely not due to superior fibril structure and may instead be driven by alterations in the non-collagenous matrix.[1] Future work is necessary to elucidate the roles of decorin and biglycan in regulating growth factor activity and evaluate the composition of the healing matrix.

References

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