Compensatory fetal membrane mechanisms between biglycan and decorin in inflammation

Luciana Batalha de Miranda de Araujo[a], **Casie E. Horgan**[a], **Abraham Aron**[a], **Renato V. Iozzo**[b], and **Beatrice E. Lechner**[a,*]

[a]Department of Pediatrics, Women and Infants' Hospital of Rhode Island, The Warren Alpert Medical School of Brown University, 101 Dudley Street, Providence, RI 02905, USA

[b]Department of Pathology, Anatomy & Cell Biology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107, USA

Abstract

Preterm premature rupture of fetal membranes (PPROM) is associated with infection and is one of the most common causes of preterm birth. Abnormalities of biglycan and decorin, two extracellular matrix proteoglycans, lead to preterm birth and abnormal fetal membrane morphology and abnormal signaling in the mouse model as well as being associated with inflammatory cascades. In humans, decorin dysregulation is associated with inflammation in PPROM. We investigated biglycan and decorin's role in inflammation in fetal membranes using mouse models of intraperitoneal *E. coli* injections superimposed on biglycan and decorin deficiency. We assessed outcomes *in vivo* as well as *in vitro* using quantitative qPCR, Western blotting and ELISA techniques. Our results suggest that biglycan and decorin compensate for each other in the fetal membranes but lose the ability to do so in the setting of inflammation, leading to decreased latency to preterm birth. Furthermore, our findings suggest that biglycan and decorin play discrete roles in fetal membrane signaling pathways in inflammation, leading to changes in expression of MMP-8 and collagen α1VI, two components of the fetal membrane extracellular matrix that play a role in the pathophysiology of PPROM. In summary, these findings underline the importance of biglycan and decorin in fetal membranes as targets for the manipulation of fetal membrane extracellular matrix stability in the setting of inflammation.

Keywords

Preterm birth; MMP; collagen VI; PPROM; extracellular matrix

1. Introduction

Preterm birth is the leading cause of newborn morbidity and mortality in the United States (Murphy et al., 2012). About a third of preterm births are caused by preterm premature rupture of fetal membranes (PPROM) (Steer 2005). While no therapies for PPROM currently exist (Ananth and Vintzileos 2006) (Miyazaki et al. 2012) and the pathophysiology

*Corresponding author: Tel.: 401-274-1122; fax: 401-453-7571; blechner@wihri.org.

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is unclear, infection as well as genetic susceptibility are thought to play a role (Parry and Strauss 1998; Shen et al. 2008).

We have shown that mice deficient in biglycan and decorin, two highly homologous extracellular matrix proteoglycans, deliver their pups prematurely and display fetal membrane abnormalities in morphology and signaling (Calmus et al. 2011; Wu et al. 2014). Biglycan and decorin are members of the small leucine-rich proteoglycan (SLRP) family (Iozzo 1999; Iozzo 2011; Iozzo and Murdoch 1996) and are the most abundant proteoglycans expressed in human fetal membranes (Gogiel et al. 2003; Meinert et al. 2001; Valiyaveettil et al. 2004). Biglycan and decorin are involved in a number of biological processes including tumor angiogenesis and fibrosis (Neill et al. 2013; Neill et al. 2012a; Neill et al. 2012b), cancer growth (Iozzo and Cohen 1993; Reed et al. 2005; Sofeu Feugaing et al. 2013), stem cell biology (Berendsen et al. 2011; Bi et al. 2005; Ichii et al. 2012), myogenesis (Brandan and Gutierrez 2013) and osteoarthritis and osteoporosis (Ameye et al. 2002; Ameye and Young 2002; Nikitovic et al. 2012). Furthermore, biglycan and decorin are involved in collagen fibrillogenesis and contribute to the mechanical properties of connective tissues (Chen et al. 2011; Reed and Iozzo 2002; Zhang et al. 2009). The absence of these proteoglycans leads to a decrease in connective tissue tensile strength (Corsi et al. 2002). Humans with abnormal post-translational processing of biglycan and decorin display a subtype of Ehlers-Danlos syndrome, a syndrome associated with a decrease in tensile strength of connective tissues as well as an increased risk of PPROM (Barabas 1966; Quentin et al. 1990). Furthermore, we have shown that decorin is dysregulated in human fetal membranes with PPROM (Horgan et al. 2014).

Beyond their structural roles, both biglycan and decorin have been implicated in a number of signaling pathways, including the TGF-β pathway. TGF-β signals via Smads (Liu et al. 1996), transcription factors that play a role in the modulation of the extracellular matrix. Both biglycan and decorin are regulated by TGF-β (Bassols and Massague 1988) and decorin modulates TGF-β activity via negative feedback mechanisms (Yamaguchi et al. 1990). Smad-2 and -3 regulate downstream gene expression of tissue inhibitors of matrix metalloproteinases (TIMPs) and collagens (Verrecchia et al. 2001), proteins that in turn modulate the mechanical stability of the fetal membrane extracellular matrix. Matrix metalloproteinases (MMPs) are linked to the pathogenesis of PPROM through the degradation of fetal membrane extracellular matrix proteoglycans and collagens (Ferrand et al. 2002). Furthermore, mutations in matrix metalloproteinase genes (Fujimoto et al. 2002) as well as collagen genes (Anum et al. 2009) have been identified as possible predisposing factors to PPROM. Additionally, proteoglycans interact with MMPs. For example, decorin induces the expression of MMP-1 (Huttenlocher et al. 1996). Importantly, we have shown that biglycan and decorin modulate the TGF-β-SMAD-MMP-TIMP-collagen pathway in fetal membranes in a gestational age dependent manner (Wu et al. 2014).

Substantial clinical data suggest that infection is a significant risk factor for PPROM (Torricelli et al. 2013). When exposed to an infectious insult, term human fetal membranes display increased MMP expression (Estrada-Gutierrez et al. 2010). We have shown a link between infection and decorin expression in human fetal membranes with PPROM (Horgan et al. 2014). Coupled with studies indicating that biglycan and decorin both play a role in

inflammatory cascades (Babelova et al. 2009; Merline et al. 2011), these observations suggest that infection may significantly impact the biglycan and decorin-dependent signaling pathways that modulate fetal membrane mechanical properties.

However, while we know that mutations leading to abnormal processing of biglycan and decorin (Barabas 1966; Quentin et al. 1990) as well as infection (Torricelli et al. 2013) play a role in the pathogenesis of PPROM, the mechanism by which infection modulates the function of biglycan and decorin and their dependent signaling pathways in the fetal membranes is not known.

2. Results

2.1. In the setting of inflammation, latency to preterm birth as well as live birth is decreased in the absence of biglycan and decorin

In order to assess the role that biglycan and decorin play in the maintenance of gestation to term in the setting of inflammation, we first examined the natural history of mouse gestation after the injection of *E. coli* intraperitoneally into pregnant mice at embryonic day 15. We found that the period between injection of live *E. coli* and preterm birth is significantly decreased in the $Bgn^{+/-};Dcn^{-/-}$ and $Bgn^{-/-};Dcn^{+/-}$ nulls compared to the wild-type *E. coli* injected mice as well as compared to the PBS injected $Bgn^{+/-};Dcn^{-/-}$ and $Bgn^{-/-};Dcn^{+/-}$ null mice (Figure 1A) (P=0.001). Furthermore, while the incidence of live pup birth is significantly decreased in the *E. coli* injected wild-type dams compared to the PBS injected wild-type dams, it decreased to 0 in the *E. coli* injected $Bgn^{+/-};Dcn^{-/-}$ and $Bgn^{-/-};Dcn^{+/-}$ mice (Figure 1B) (P=0.004). For comparison with the wild-type, we chose mice that have only one of four possible SLRP genes (two biglycan and two decorin). These mice are more affected than the biglycan or decorin single nulls but are less severely affected than the double nulls ($Bgn^{-/-};Dcn^{-/-}$). The $Bgn^{-/-};Dcn^{-/-}$ mice delivered their pups extremely prematurely even without the additional environmental insult of an *E. coli* injection, and are thus not suitable for this experimental design. While there was no statistically significant difference in the latency between the E. coli exposed and the saline exposed wild-type mice, the standard deviation was larger in the E. coli exposed mice, demonstrating a range of latencies compared to the saline exposed mice from decreased latency to increased latency with dystocia.

2.2. Biglycan and decorin compensate for each other in fetal membrane transcription in the absence of inflammation

Next, we investigated whether biglycan and decorin would exhibit compensatory upregulation of gene expression in fetal membranes depending on exposure to the environmental insult of inflammation. We observed upregulation of biglycan transcript in decorin null fetal membranes in the absence of inflammation (PBS injected mice) (P=0.05), and similarly, upregulation of decorin transcript in biglycan null fetal membranes in the absence of inflammation (P=0.025)(Figure 2A and 2B).

2.3. Biglycan and decorin compensatory transcription increase does not occur in inflammation, while decorin transcription is decreased in the *E. coli* injected mice compared to the PBS injected mice

Interestingly, the compensatory mechanisms between biglycan and decorin in the fetal membranes that are present in the absence of inflammation are modified by the presence of inflammation. In the *E. coli* injected fetal membranes, biglycan transcription remained unchanged in the decorin null samples compared to the wild-type samples, thus departing from the compensatory increase that it displayed in the absence of inflammation. Decorin transcription also displayed a lack of compensatory upregulation in the biglycan null in the setting of inflammation. Additionally, in the absence of biglycan, decorin transcription decreased in the *E. coli* injected mice compared to the PBS injected mice (P=0.01) (Figure 2A and 2B).

2.4. MMP-8 decreases in the wild-type and biglycan null fetal membranes in the setting of inflammation, while it remains unchanged in the decorin null

Next, we assessed the expression of the matrix metalloproteinases that play a significant role in fetal membrane rupture and PPROM. We found that inflammation leads to a decrease in MMP-8 expression in both the wild-type (P=0.03) and the biglycan null (P=0.05), but not in the decorin null, in which it remained unchanged irrespective of the presence of inflammation (Figure 3). MMP-9 expression was unchanged (data not shown).

2.5. Collagen α1VI gene expression is unchanged in the setting of inflammation in the absence of biglycan or decorin, while collagen α1VI protein levels are increased

Given the role that collagens play in the maintenance of connective tissue mechanical strength and integrity, we next examined the biglycan- and decorin-dependent expression of collagen α1VI, a biglycan and decorin binding collagen, in inflammation. In the absence or presence of both biglycan and decorin, there was no change in collagen α1VI gene transcription levels (Figure 4A). However, in the absence of either biglycan (P=0.05) or decorin (P=0.03), collagen α1VI protein expression increased in the setting of inflammation compared to non-inflammation controls (Figure 4B and C).

In summary, these results demonstrate that inflammation exacerbates the gestational phenotype of the biglycan/decorin null mouse and leads to abnormalities of transcriptional compensation between biglycan and decorin as well as leading to translational and transcriptional changes in downstream targets that play a role in fetal membrane stability.

3. Discussion

Biglycan and decorin are necessary for the maintenance of gestation to full term in a gene-dose dependent and compensatory manner (Calmus et al. 2011), while biglycan/decorin null fetal membranes display abnormal morphology (Wu et al. 2014). In this study, we show that biglycan and decorin play a role in the maintenance of gestation in the setting of inflammation as well as in the fetal membrane response to inflammation.

Others have shown that inflammation leads to preterm birth in the mouse model (Hirsch and Wang 2005; Reznikov et al. 1999) and is associated with PPROM in humans (Bendon et al. 1999). Our study has demonstrated that the superimposition of an environmental insult, in this case inflammation, on a genetic defect, leads to an exacerbation of the preterm birth phenotype compared to the preterm birth phenotype in the setting of an environmental insult without a genetic mutation. Furthermore, our study of the role of fetal membrane biglycan and decorin in the context of inflammation has demonstrated important mechanistic insight into the role of these proteoglycans in the pathophysiology of PPROM.

Our data indicate that biglycan and decorin compensate for each other in the fetal membranes by increasing transcription in a compensatory manner in the absence of inflammation. This compensatory upregulation of biglycan and decorin is similar to observations in multiple tissues including cornea, kidney, skin, bone and muscle (Ameye and Young 2002; Corsi et al. 2002; Zhang et al. 2009) and to our observations in the placenta as well as functionally *in vivo* during gestation (Calmus et al. 2011). We have not observed similar upregulation of biglycan protein expression in decorin-null fetal membranes and vice versa (Calmus et al. 2011). This may be secondary to protein compensation not being present in the fetal membranes or it may be because compensatory changes are present but not readily apparent with the techniques we utilized given the abundance of both SLRPs in fetal membranes. Interestingly, this ability to compensate for each other is disrupted by the presence of inflammation. On the other hand, when inflammation occurs in a wild-type genetic background, inflammation fails to evoke biglycan or decorin expression. Thus, it is likely that the underlying etiology for the decreased latency to preterm birth in the $Bgn^{+/-};Dcn^{-/-}$ and $Bgn^{-/-};Dcn^{+/-}$ mice is a result of a two hit hypothesis, in which a genetic predisposition is aggravated by an environmental factor. Specifically, genetic susceptibility is compensated for, but the compensation is then tempered by an environmental hit that deranges the compensation. This observation mirrors clinical observations in patients with Ehlers-Danlos syndrome, who display a significantly elevated incidence of preterm birth from PPROM compared to their unaffected siblings (Barabas 1966; Yen et al. 2006). The progeroid variant of Ehlers-Danlos syndrome displays a mutation leading to the secretion of abnormal, non-glycosylated biglycan and decorin (Quentin et al. 1990). Further support is lent to the concept of PPROM via connective tissue genetic abnormalities exacerbated by inflammation by the fact that women with a history of multiple PPROM have undiagnosed connective tissue anomalies similar to Ehlers-Danlos syndrome (Hermanns-Le et al. 2005).

One caveat to these observations is that the mice used for the in vivo *E. coli* injection experiments are heterozygous/homozygous and homozygous/heterozygous biglycan/decorin nulls ($Bgn^{+/-};Dcn^{-/-}$ and $Bgn^{-/-};Dcn^{+/-}$). However, based on our prior data (Calmus et al. 2011), it is likely that our observations are a similar but tempered response compared to the homozygous/homozygous double nulls ($Bgn^{-/-};Dcn^{-/-}$), which have too severe a phenotype to lend themselves to these types of experiments. At the same time, these observations are likely an exaggerated response compared to the single nulls ($Bgn^{-/-}$ or $Dcn^{-/-}$), on which we have performed the in vitro component of this study.

When both biglycan and decorin are present in fetal membranes, inflammation leads to a decrease in MMP-8 protein expression, perhaps as a protective mechanism to counteract the membrane destructive/remodeling effects. Alternately, MMP-8 could be transcribed at the typical rate but secondary to inflammation there is higher turnover as it is being used to degrade the fetal membranes. In the absence of biglycan, MMP-8 protein expression is decreased in a manner similar to the wild-type. In the decorin null, on the other hand, MMP-8 protein expression is unchanged. Thus, the expected MMP-8 decrease in the presence of inflammation does not occur in the absence of decorin, but does occur in the absence of biglycan, indicating a role for decorin in the MMP-8 signaling pathway under inflammatory circumstances. Similarly, exogenous decorin gain-of-function experiments confirm that decorin regulates components of this pathway (Wu et al. 2014). These findings are similar to our previous findings, in which MMP-8 and MMP-9 gene and protein expression, as well as MMP-13 gene transcription increases in fetal membranes close to term (embryonic day 18) in the absence of decorin, but not in the absence of biglycan (Wu et al. 2014), suggesting that decorin is a membrane stabilizing factor. Furthermore, decorin suppresses the expression and activity of two MMPs associated with PPROM, MMP-2 and MMP-9 (Neill et al. 2012a). Also, decorin decreases prior to parturition as ozen in liquid nitrogen and stored at hile biglycan increases (Meinert et al. 2007). Interestingly, however, decorin induces the synthesis of a number of MMPs, including MMP-1, -2 and -14 (Schonherr et al. 2001) and during early gestation (embryonic day 12), the absence of decorin leads to a decrease in MMP-8 and -9 protein expression in fetal membranes. This suggests a role for decorin in extracellular matrix remodeling in early gestation as opposed to its membrane stabilizing role in late gestation and in inflammation.

Our data indicate that biglycan and decorin are both necessary for the maintenance of appropriate collagen α1VI levels in the setting of inflammation. Gene transcription of collagen α1VI is unchanged with inflammation or in the absence of biglycan or decorin. Protein expression, however, increases in the absence of biglycan or decorin in the setting of inflammation. One possible explanation for the increase in collagen α1VI in the absence of both biglycan and decorin is compensation for the structural role of the absent proteoglycans. In the setting of two hits, a genetic defect as well as inflammation, the increase in collagen α1VI expression may be a protective strategy to stabilize the fetal membranes. Decreased decorin in the kidney leads to increased accumulation of extracellular matrix with increased biglycan expression (Merline et al. 2009) and the absence of decorin leads to hepatic fibrosis (Baghy et al. 2011). Thus, alternately, the increase in collagen α1VI in the absence of these SLRPs may be a reflection of the anti-fibrotic role of these proteoglycans.

The diverse MMP-8 and collagen α1VI expression profiles in the absence of biglycan or decorin suggest that the two SLRPs signal via discrete pathways. Given that they compensate for each other, if they exerted their signaling via one common pathway, one would expect the MMP-8 and collagen α1VI levels to be unchanged between the wild-type and either null mouse. It is likely that compensation is not complete, coupled with some level of discrete functions. Biglycan and decorin play differential roles in mouse fetal membrane TGFβ signaling, leading to differential expression of the MMPs -8, -9 and -13, TIMPs -1, -2, -3 and -4, and collagen α1VI (Wu et al. 2014). Furthermore, the differences in

expression and function may be related to the mid-pregnancy gestational age studied here, since biglycan and decorin play distinct signaling roles in a gestational age dependent manner (Wu et al. 2014).

Biglycan plays a role in inflammatory signaling as an inflammatory cascade activator via its role as a ligand for Toll-like receptors 2 and 4 (Babelova et al. 2009; Moreth et al. 2014; Zeng-Brouwers et al. 2014). Furthermore, decorin expression is evoked by septic inflammation, and decorin boosts inflammatory response in both sepsis and cancer (Merline et al. 2011). Thus, the role of biglycan and decorin in the maintenance of fetal membrane integrity in the setting of inflammation is likely to reflect complex interactions between their roles as connective tissue stabilizing proteins and their roles as extracellular matrix proinflammatory signaling molecules.

In summary, our findings suggest that biglycan and decorin compensate for each other in the fetal membranes but lose their ability to do so in the setting of inflammation, leading to decreased latency to preterm birth, and thus preterm birth earlier than inflammation alone would have led to. Furthermore, our findings suggest that biglycan and decorin play discrete roles in fetal membrane signaling pathways leading to changes in expression of MMP-8 and collagen α1VI, two components of the fetal membrane extracellular matrix that play a role in the pathophysiology of PPROM.

4. Materials and Methods

4.1. Mouse husbandry and genotyping

Wild-type mice (C3H) were purchased from Jackson Laboratories (Bar Harbor, ME). A $Bgn^{-/-}$ breeding pair (C3H) (generated by Marian Young (Xu et al. 1998)) was a gift from Justin Fallon. A $Dcn^{+/-}$ breeding pair (C57BL/6) was mated to the birth of homozygous null pups, which were then bred to establish the $Dcn^{-/-}$ colony. A $Bgn^{-/-}$ female was crossed with a $Dcn^{-/-}$ male to establish double heterozygous breeding pairs ($Bgn^{+/-};Dcn^{+/-}$ and $Bgn^{-/0};Dcn^{+/-}$). These pairs were mated to breed $Bgn^{+/-};Dcn^{-/-}$ and $Bgn^{-/-};Dcn^{+/-}$ heterozygous-homozygous null pups. Breeding pairs were set up for mating at 5–7 weeks of age. Plugs were checked each morning, and the day of the plug was defined as embryonic day 0. Dams that were sacrificed at embryonic day 15 for tissue harvesting were sacrificed between 4 and 5 hours after *E. coli* or PBS injection. Approval was obtained from the Lifespan Institutional Animal Care and Use Committee. For genotyping, genomic DNA was extracted from each 3 mm tail biopsy sample using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). Polymerase chain reaction (PCR) was performed using the Taq DNA Polymerase kit (New England Biolabs, Ipswich, MA) and the PTC-200 thermal cycler. The PCR product was run on a 1.8% w/v agarose gel to visualize the following diagnostic bands. The decorin PCR produced bands of 161 bp for the wild-type allele and 238 bp for the null allele. The biglycan PCR produced bands of 212 bp for the wild-type allele and 310 bp for the null allele.

4.2. Bacteria preparation

Previously frozen *E. coli* was inoculated on an agar plate overnight. The next morning one colony from the plate was incubated overnight in 10mL in Luria-Bertani (LB) broth (MP

Biomedicals, Santa Ana, CA). The next morning, the culture was diluted 1:50 in fresh LB broth and incubated until an optical density of 0.5–0.8 was detected at 600nm, indicating the bacteria was in log phase growth. A more accurate bacterial concentration was determined post hoc by the plating of 10^6 serial dilutions in triplicate. Colony counts were performed on these plates the next morning. Colony forming units ranged from 3×10^6 to 1.4×10^7 CFU/ml.

4.3. Injection preparation

300mcl of the previously mentioned log phase bacterial broth was concentrated by centrifugation with the addition of 700mcl clean LB broth. The resulting pellet was washed with 1ml of sterile, room temperature phosphate buffered saline (PBS) and was concentrated again via centrifugation. The PBS was decanted and the pellet was re-suspended in 0.5ml of sterile, room temperature PBS. The resulting suspensions were drawn into 1ml tuberculin syringes and injected intraperitoneally into pregnant embryonic day 15 mice of the following genotypes: $Bgn^{-/-};Dcn^{+/-}$, $Bgn^{+/-};Dcn^{-/-}$ and wild-type ($Bgn^{+/+};Dcn^{+/+}$). Vehicle controls of 0.5ml sterile, room temperature PBS were also used. In order to assess preterm delivery rates, the mice were observed twice daily while health status and preterm deliveries were recorded. Health status was determined using an adaptation of the Mouse Sepsis Index scale (Biswas et al. 2002). The scale ranges from 5 for a normal healthy clinical status to 0 for death. Four is defined as slight illness with ruffled fur and lethargy, 3 is moderate illness with severe lethargy, ruffled fur and hunched back, 2 is severe illness with all signs of 3 plus exudative accumulation around the eyes, and 1 is a moribund state. Mice of both null genotypes injected with *E. coli* did not differ significantly from wild-type *E. coli* injected mice in their illness index. Nulls of both genotypes injected with *E. coli* had a mean score of 3.75 (SD 0.58) and wild-type mice injected with *E. coli* a mean score of 3.87 (SD 0.73) (P= 0.7).

Mice that were designated for tissue harvest were sacrificed 4 to 5 hours after *E. coli* or PBS injection on embryonic day 15.

4.4. Western blotting

Fetal membranes from each genotype and condition were dissected at embryonic day 15 between 4 and 5 hours after *E. coli* or PBS injection and frozen at −80°C Tissue samples were cut into small pieces and placed in 1.0 ml T-PER tissue protein extraction buffer (Pierce, Rockford, IL) with one tablet of proteinase inhibitor cocktail per 10 ml buffer (Roche, Basel, Switzerland). Tissue samples were then homogenized for 3×10s in an ice bath and kept on ice for 30 min. The homogenate was centrifuged at $10,000 \times g$ at 4 °C for 8 min. The supernatant was collected and stored at −80°C until use. Total protein content was determined using the BCA assay (Pierce, Rockford, IL). 30 μg of total protein was loaded on duplicate 10% SDS-polyacrylamide gels and subsequently blotted to a polyvinylidene difluoride membrane. Following blocking in 5% milk in PBS-T buffer (PBS with 0.2% Tween 20) for 30 min, each blot was incubated overnight at 4°C with primary antibody. The blot was then washed with PBS-T buffer briefly and incubated with secondary antibody for 40 min. The Super-Signal West Pico chemiluminescent substrate kit (Pierce, Rockford, IL) was used prior to development of the blot membrane. The bands were compared with protein markers of known molecular size run in parallel on the same SDS-polyacrylamide

gel (Bio-Rad, Hercules, CA). The membrane was stripped using Restore Western Blot stripping buffer (Thermo Scientific, Waltham, MA) for 10 min and blocked with 5% milk PBS-T for 20 min, then re-probed with anti-GAPDH antibody at a 1:800 dilution (Santa Cruz Biotech, Santa Cruz, CA) as an internal standard. Immunoblots were digitally scanned and densitometrically analyzed using Gel-Pro Analyzer (Media Cybernetics, Bethesda, MDRelative optical density (specific protein band to GAPDH) was normalized to the wild-type with PBS injection band. The experiment was repeated with three individual sets of samples.

4.5. Antibodies

The following antibodies were used: rabbit polyclonal anti-COLα1VI (sc-20649, dilution 1:800) and mouse monoclonal anti-GAPDH (sc-137179, dilution 1:1000) (Santa Cruz Biotech, Santa Cruz, CA). Secondary antibody labeling was performed with anti-mouse and anti-rabbit IgG horseradish peroxidase conjugate (Cell Signaling, Danvers, MA).

4.6. ELISA

Wild-type, $Bgn^{-/-}$ and $Dcn^{-/-}$ dams injected with PBS vehicle as wells as dams of each genotype injected with *E. coli* were dissected at embryonic day 15 between 4 and 5 hours after *E. coli* or PBS injection. Fetal membranes were collected and flash frozen in liquid nitrogen and isopentane and stored at −80°C. Four whole fetal membranes from each condition were used. Each fetal membrane sample was homogenized in 1mL of protein lysate buffer (2.5ml Triton X and one tablet of Roche Complete EDTA-free Protease Inhibitor filled to 50ml volume), flash frozen in liquid nitrogen and stored at −80°C until used. Commercially available ELISA kits (MMP-8 from Anaspec, Fremont, CA; MMP-9 from Abnova, Taipei City, Taiwan) were run in triplicate according to the manufacturer's instructions. Plates were read on a Bio-Rad Laboratories Microplate Reader at 450nm.

4.7. RNA, cDNA preparation and quantitative PCR (qPCR)

Fetal membranes from wild-type, $Bgn^{-/-}$ and $Dcn^{-/-}$ dams injected with PBS vehicle as wells as dams of each genotype injected with *E. coli* were dissected at embryonic day 15 between 4 and 5 hours after *E. coli* or PBS injection in 0.1mol l^{-1} phosphate-buffered saline, pH 7.4, snap-frozen in liquid nitrogen and stored at −80°C. RNA extraction, genomic DNA removal and conversion to cDNA was performed as described previously (Wu et al. 2014). qPCR reactions were performed on the 7500 Fast Real-Time PCR System thermocycler (Applied Biosystems, Foster City, CA) using the SYBR-Green method (Invitrogen, Carlsbad, CA). Primers were designed using Primer-Blast primer design software (National Library of Medicine, Bethesda, MD). GAPDH was used as a normalizer. Melting point analysis of the product was performed to ensure the absence of alternative products or primer dimers. Data analysis was performed via the comparative Ct method with a validation experiment. A standard sample of RNA pooled from samples of wild-type as well as biglycan and decorin null fetal membranes was used in each qPCR experiment as a calibrator whose relative transcript level was defined as 1. qPCR analysis was performed in triplicate. n=3–4 dams per genotype and condition.

4.8 Primer sequences

qPCR—GAPDH forward: CTCACAATTTCCATCCCAGAC, reverse: TTTTTGGGTGCAGCGAAC; collagen α1VI forward: CTGGTGAAGGAGAACTATGCAG, reverse: GTCTAGCAGGATGGTGATGTC; biglycan forward: ATTGCCCTACCCAGAACTTGAC, reverse: GCAGAGTATGAACCCTTTCCTG; decorin forward: TTCCTACTCGGCTGTGAGTC, reverse: AAGTTGAATGGCAGAACGC.

Genotyping—Biglycan wild-type allele forward TGATGAGGAGGCTTCAGGTT, reverse GCAGTGTGGTGTCAGGTGAG; biglycan null allele forward TGTGGCTACTCACCTTGCTG, reverse GCCAGAGGCCACTTGTGTAG; decorin allele forward CCTTCTGGCACAAGTCTCTTGG, decorin wild-type allele reverse TCGAAGATGACACTGGCATCGG; decorin null allele reverse TGGATGTGGAATGTGTGCGAG.

All primers were purchased from Invitrogen (Carlsbad, CA).

4.9 Statistical analysis

Analysis of differences in levels of gene and protein expression between genotypes was performed using the Student's t-test or, in the case of non-normally distributed data, the nonparametric Mann-Whitney Rank Sum test. ANOVA and χ^2 analysis were performed for the *in vivo* experiments. Where appropriate, Bonferroni correction was performed for multiple comparisons using the Student's t-test. Analysis was performed using SigmaPlot 11.0 (San Jose, CA).

Acknowledgments

Abbreviations

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PPROM	preterm premature rupture of fetal membranes
SLRP	small leucine-rich proteoglycan
Bgn$^{-/-}$	biglycan null
Dcn$^{-/-}$	decorin null
TGFβ	transforming growth factor β
MMP	matrix metalloproteinase
TIMP	tissue inhibitor of matrix metalloproteases

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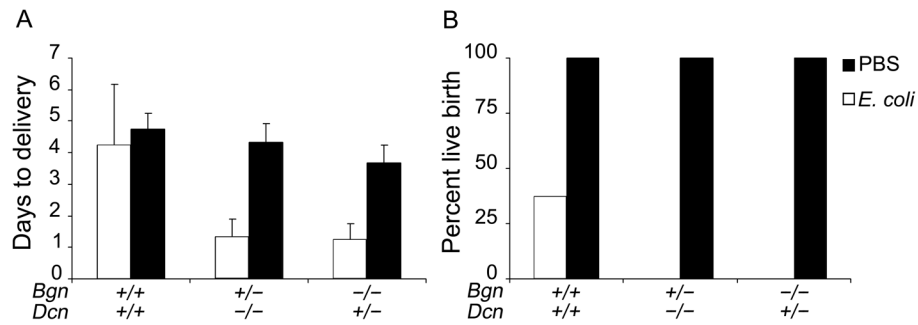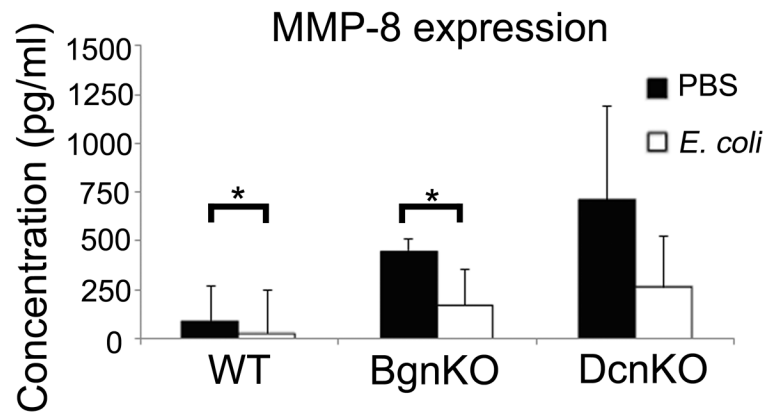
Figure 1.
[A] Days to delivery are decreased in the *Bgn$^{+/-}$;Dcn$^{-/-}$* and *Bgn$^{-/-}$;Dcn$^{+/-}$* dams injected with *E. coli* compared to both the same genotype injected with PBS and the wild-type regardless of injection category (p=0.001 via ANOVA). Error bars = SD. [B] While the percentage of live pup births is decreased in the wild-type injected with *E. coli* compared to PBS injected wild-type dams, it is decreased to zero in the *Bgn$^{+/-}$;Dcn$^{-/-}$* and *Bgn$^{-/-}$;Dcn$^{+/-}$* dams injected with *E. coli* (p=0.004 via χ^2). Gestational age embryonic day 15. n=3–8 per condition. *Bgn*=biglycan; *Dcn*=decorin.
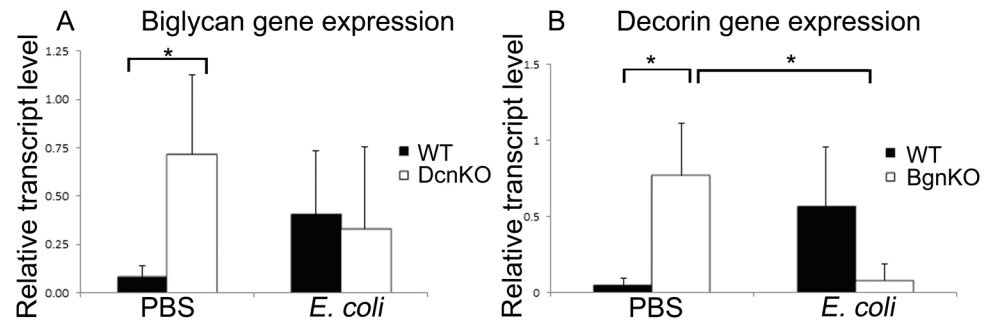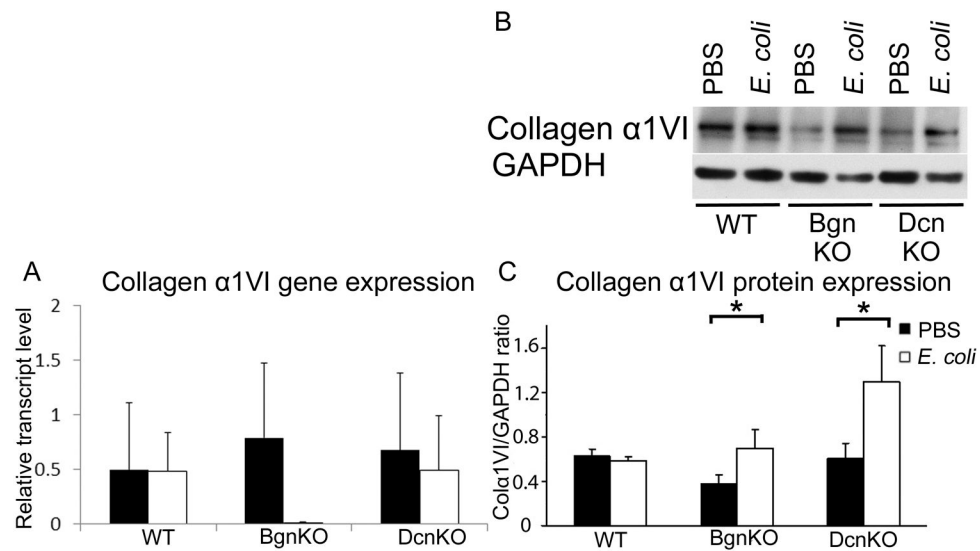
Figure 2.
Biglycan and decorin compensate for each other in fetal membranes in the absence of inflammation, but not in the presence of inflammation. [A] Biglycan gene expression is increased in the PBS injected decorin null fetal membranes compared to wild-type (p=0.02); this compensatory increase does not occur in the setting of inflammation [B] Decorin gene expression is increased in the PBS-injected biglycan null fetal membranes compared to wild-type (p=0.006); this compensatory increase does not occur in the setting of inflammation, but decreased decorin gene expression occurs in the *E. coli* injected biglycan null fetal membranes compared to the PBS injected biglycan null fetal membranes (p=0.009). n=4 per genotype and condition. Error bars = SD. *BgnKO*=biglycan null; *DcnKO*=decorin null; WT=wild-type; PBS=phosphate buffered saline. Student's t-test was performed for each set of data.

Figure 3.
MMP-8 expression is decreased in fetal membranes of *E. coli* injected mice in the wild-type as well as the biglycan null mouse but not in the decorin null mouse. n=4 per genotype and condition (p=0.03 and 0.028). Error bars = SD. *BgnKO*=biglycan null; *DcnKO*=decorin null; WT=wild-type. Student's t-test was performed for each set of data.

Figure 4.

In the setting of inflammation, collagen α1VI protein expression is increased in the biglycan null and decorin null fetal membranes but not in the wild-type, while gene expression is unchanged. [A] Collagen α1VI gene expression is unchanged in the biglycan null and decorin null with or without inflammation compared to the wild-type. [B] Representative Western blots and [C] Summary data demonstrating increased collagen α1VI protein expression in the biglycan null and decorin null fetal membranes but not in the wild-type in the setting of inflammation (P=0.05 and 0.03). n=4 per genotype and condition. Error bars = SD. *BgnKO*=biglycan null; *DcnKO*=decorin null; WT=wild-type. Student's t-test was performed for each set of data.