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Biglycan and decorin differentially regulate signaling in the fetal membranes

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Abstract

Preterm birth is the leading cause of newborn mortality in the United States and about one third of cases are caused by preterm premature rupture of fetal membranes, a complication that is frequently observed in patients with Ehlers-Danlos Syndrome. Notably, a subtype of Ehlers-Danlos Syndrome is caused by expression of abnormal biglycan and decorin proteoglycans. As compound deficiency of these two small leucine-rich proteoglycans is a model of preterm birth, we investigated the fetal membranes of *Bgn*$^{-/-}$;*Dcn*$^{-/-}$ double-null and single-null mice. Our results showed that biglycan signaling supported fetal membrane remodeling during early gestation in the absence of concomitant changes in TGFβ levels. In late gestation, biglycan signaling acted in a TGFβ–dependent manner to aid in membrane stabilization. In contrast, decorin signaling supported fetal membrane remodeling at early stages of gestation in a TGFβ–dependent manner, and fetal membrane stabilization at later stages of gestation without changes in TGFβ levels. Furthermore, exogenous soluble decorin was capable of rescuing the TGFβ signaling pathway in fetal membrane mesenchymal cells. Collectively, these findings provide novel targets for manipulation of fetal membrane extracellular matrix stability and could represent novel targets for research on preventive strategies for preterm premature rupture of fetal membranes.

Keywords

Small leucine-rich proteoglycan; Smad; MMP; TGF-β; TIMP; fetal membranes

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1. Introduction

As the leading cause of newborn morbidity and mortality in the United States (Murphy et al., 2012), preterm birth represents an enormous economic, clinical and psychological burden to society. Preterm premature rupture of fetal membranes (PPROM) accounts for 40% of preterm births (Steer, 2005). Causes of PPROM include environmental factors such as infection as well as genetic susceptibility (Parry and Strauss, 1998). To date, no targets have yielded successful therapeutic interventions (Ananth and Vintzileos, 2006) (Miyazaki et al., 2012).

A genetic cause of PPROM that has received little attention is Ehlers-Danlos Syndrome (EDS). Infants with this disorder have a significantly elevated incidence of preterm birth from PPROM (Barabas, 1966; Yen et al., 2006) compared to their unaffected siblings. EDS is a heterogeneous group of rare inherited connective tissue disorders associated with a decrease in tensile strength and integrity of skin, joints, and other connective tissues.

A cohort of women with a history of multiple PPROM was found to have undiagnosed connective tissue anomalies similar to Ehlers-Danlos syndrome. These findings consist of thin collagen bundles with variable diameters, a twisted appearance and irregular interfibrillar spaces as well as abnormal shapes and distribution of elastic fibers in the skin (Hermanns-Le et al., 2005). This observation suggests that recurrent PPROM may be associated with underlying connective tissue anomalies. It is likely that a spectrum of clinical presentations exists between Ehlers-Danlos syndrome with its full scope of connective tissue abnormalities and otherwise healthy women with recurrent PPROM.

In patients afflicted with the progeroid variant of EDS, the molecular basis of the connective tissue anomaly is a mutation that leads to the abnormal secretion of biglycan and decorin (Quentin et al., 1990), two small leucine-rich proteoglycans (SLRPs) that are constituents of the extracellular matrix of most organs. Xylosylprotein-4β-galactosyl-transferase I is the enzyme affected by the mutation, which prevents posttranslational glycosylation of biglycan and decorin, resulting in the secretion of non-glycosylated abnormal biglycan and decorin protein cores (Kresse et al., 1987; Quentin et al., 1990).

Connective tissue mechanical instability can lead to life threatening pathologies beyond PPROM. Weakening of aortic connective tissue extracellular matrix can lead to aneurysms and subsequent aortic rupture. Patients with Ehlers-Danlos Syndrome (Barabas, 1972), *biglycan* knockout mice (Heegaard et al., 2007) and *biglycan/decorin* heterozygous knockout mice (unpublished observations) are at increased risk of developing aortic rupture. A role for TGF-β signaling as well as for decorin and biglycan has been reported in aortic rupture. TGF-β, Smad-2 and biglycan and decorin are involved in the development of aortic aneurysms; Smad-2 levels correlate with extracellular matrix elastic fiber destruction, biglycan displays decreased expression and decorin expression is increased (Gomez et al., 2009). The inhibition of decorin degradation leads to enhanced collagen remodeling and decreases the rate of aortic rupture in a mouse model (Ang et al., 2011). The mechanism of connective tissue weakening leading to rupture of these tissues is similar to the pathophysiologic process in preterm premature rupture of fetal membranes.

Similarly, biglycan and decorin are present in atherosclerotic plaques (Riessen et al., 1994), the life-threatening rupture of which is associated with MMPs (matrix metalloproteinases) (Shah et al., 1995). In a mouse model of these plaques, TIMP-1 (tissue inhibitor of metalloproteinases) decreases progression (de Vries et al., 2012). These findings suggest that deregulation of connective tissue extracellular matrix signaling can lead to mechanical instability and thus tissue rupture.

Decorin and biglycan are members of the small leucine-rich proteoglycan (SLRP) gene family (Iozzo, 1999; Iozzo, 2011; Iozzo and Murdoch, 1996) that are involved in a number of biological processes including cancer growth (Iozzo and Cohen, 1993; Reed et al., 2005; Sofeu Feugaing et al., 2013), collagen fibrillogenesis and mechanical properties of connective tissues (Chen et al., 2011; Reed and Iozzo, 2002; Zhang et al., 2009), myogenesis (Brandan and Gutierrez, 2013), osteoarthritis and osteoporosis (Ameye et al., 2002; Ameye and Young, 2002; Nikitovic et al., 2012), stem cell biology (Berendsen et al., 2011; Bi et al., 2005; Ichii et al., 2012), immunity (Babelova et al., 2009; Merline et al., 2011; Moreth et al., 2012), and tumor angiogenesis and fibrosis (Neill et al., 2013; Neill et al., 2012a; Neill et al., 2012b).

We have previously shown that mice deficient in both biglycan and decorin, an animal model of EDS, deliver their pups prematurely (Calmus et al., 2011). While these SLRPs are the most abundant proteoglycans expressed in human fetal membranes (Gogiel et al., 2003; Meinert et al., 2001; Valiyaveettil et al., 2004), the mechanism by which biglycan and decorin protect from preterm birth is not known. Beyond their structural roles, both biglycan and decorin have been implicated in a host of signaling pathways that may provide insight into their mechanisms of action in the maintenance of fetal membrane integrity.

Following the original discovery of decorin as a TGF-β inhibitor (Yamaguchi et al., 1990), there has been mounting evidence for a role of decorin in controlling the activity of several receptor tyrosine kinases encompassing EGFR (Schaefer and Iozzo, 2012), Met (Goldoni et al., 2009), IGF-IR (Iozzo and Sanderson, 2011), VEGFR2 (Buraschi et al., 2013) and PDGFR (Baghy et al., 2013).

TGFβ signals via Smads (Guo and Wang, 2009; Liu et al., 1996), transcription factors that play a role in the modulation of the extracellular matrix. Smad-2 and -3 modulate downstream gene expression of collagens and tissue inhibitors of matrix metalloproteinases (TIMPs) (Verrecchia et al., 2001), proteins that modulate fetal membrane extracellular matrix mechanical stability. A compelling body of evidence links matrix metalloproteinases (MMPs) to the pathogenesis of PPROM through the degradation of the proteoglycans and collagens of the extracellular matrix of fetal membranes (Ferrand et al., 2002). MMP-2, -8, -9 and -13 are involved in the pathogenesis of PPROM (Fortunato et al., 2003; Fujimoto et al., 2002; Maymon et al., 2000). In addition, MMP-9 is involved in the TGFβ-Smad-2 pathway (Kim et al., 2005). TIMP-1 is a target of the TGFβ-Smad-3 signaling pathway (Verrecchia et al., 2001), while TIMP-2 and TIMP-3 play a role in the pathophysiology of rupture of fetal membranes (El Khwad et al., 2006; Romero et al., 2010). Collagens α2I and α3IV, as well as TIMP-2 display small nucleotide polymorphisms that are associated with an increased risk of PPROM (Romero et al., 2010), while collagen α2I and collagen α1VI

are both targets of the TGFβ-Smad-3 signaling pathway (Verrecchia et al., 2001). In addition, collagen VI binds both biglycan and decorin (Wiberg et al., 2001).

While the biglycan- and decorin-dependent TGFβ– Smad-2/-3 signaling pathway may modulate PPROM via interactions with MMPs, TIMPs and collagens, this pathway has not been characterized in fetal membranes. Identifying the key components of the biglycan- and decorin-related signaling pathways in fetal membranes is critically important to understanding the extracellular matrix mechanisms that stabilize the fetal membranes. Thus, we hypothesized that biglycan and decorin play a role in signaling within the fetal membranes and that dysregulation of biglycan and decorin dependent TGF-β signaling pathways may be part of undiagnosed predisposing genetic factors for PPROM.

2. Results

2.1. Fetal membrane morphology is altered in the absence of biglycan and decorin

Given that fetal membrane strength and thus function likely is dependent on membrane thickness and morphology, we first examined the fetal membranes of wild-type, single *Bgn*$^{-/-}$ and *Dcn*$^{-/-}$ knockout as well as *Bgn*$^{-/-}$*;Dcn*$^{-/-}$ double knockout mice at embryonic day 18 (E18). This embryonic stage was chosen because it represents late gestation when mechanical forces on the fetal membranes are the greatest. We found that *Bgn*$^{-/-}$*;Dcn*$^{-/-}$ fetal membranes displayed an abnormal morphology. Specifically, membrane thickness varied to a greater degree ($p<0.001$) and cell size was less uniform than its wild-type and two single knockout counterparts [Fig. 1]. This means that while the mean thickness was not different between the wild-type and single knockouts on one side and the double knockout fetal membranes on the other side, the standard deviation between the regularly thick wild-type and single knockouts and the irregularly varied thickness of the double knockout was significantly different.

2.2. Biglycan and decorin regulate TGF-β expression in fetal membranes in a discrete and gestational age dependent manner

Because TGF-β regulates fetal membrane stabilizing proteins such as MMPs, TIMPs and collagens (Overall et al., 1989) and because TGFβ expression is regulated in part by biglycan and decorin, we examined TGFβ expression in fetal membranes of wild-type and mice deficient in either biglycan or decorin at E12 and E18, representing the early and late developmental stages, respectively. Overall the results showed a dynamic and differential expression of TGFβ in fetal membranes during the course of gestation according to the genetic background. At E12, TGFβ levels increased in the absence of decorin ($p=0.028$), but declined to control levels at E18 [Fig. 2]. In contrast, at E12, TGFβ levels were unchanged in the absence of biglycan but decreased at E18 ($p=0.036$). While the Western blot in Figure 2a suggests a decrease in TGFβ levels at E12, this decrease is not statistically significant on repeat blots. TGFβ protein expression is unchanged at E15 in both knockouts (data not shown).

2.3. Smad-2 and -3 expression and phosphorylation are regulated by biglycan and decorin in a discrete and gestational age-dependent manner

Given that TGF-β regulates the expression of the transcription factors Smad-2 and Smad-3 (Derynck and Zhang, 2003), we next assessed the expression of these proteins as well as their phosphorylated active forms, phospho-Smad-2 and phospho-Smad-3, via Western blotting. Our studies found differential regulation of these factors depending on the specific genetic background. The role of decorin in Smad regulation was biphasic. At E12, Smad-3 and phospho-Smad-3 expression increased in the absence of decorin [p=0.0144 and 0.0140, Fig. 3A and C], while Smad-2 and phospho-Smad-2 expression are unchanged. By E18, Smad-2, phospho-Smad-2 and phospho-Smad-3 decreased in the absence of decorin [p=0.0037, 0.0486 and 0.0388, Fig. 3A and C]. Biglycan also demonstrated developmental regulation of Smad expression. At E12, the expression of Smad-2 and-3 as well as their phosphorylated counterparts was unchanged in the $Bgn^{-/-}$ *biglycan* knockout, while Smad-2 decreased by E18 [p=0.05, Fig. 3B and D]. At E15, Smad-2 protein expression is decreased in the *biglycan* knockout, while Smad-3 expression is unchanged and the expression of both transcriptional factors is unchanged in the *decorin* knockout (data not shown).

2.4. Biglycan and decorin regulate transcription and protein expression of downstream fetal membrane extracellular matrix effector genes MMPs,TIMPs and collagens

As part of the TGF-β signaling pathway, Smad-2 and Smad-3 transcriptionally regulate various components of the extracellular matrix. To elucidate the precise role of biglycan and decorin in this pathway in fetal membranes, we examined gene transcription and protein expression of downstream gene targets. Of the proteins that play a major role in the maintenance of extracellular matrix mechanical integrity and matrix remodeling (MMPs, TIMPs and collagens), we chose to examine those that are regulated by these Smads, play a role in fetal membrane extracellular matrix remodeling and the pathogenesis of PPROM or bind biglycan or decorin. Again, our findings indicated that biglycan and decorin played discrete roles in the regulation of these extracellular matrix components at various embryonic stages. MMP-8 and MMP-9 protein expression decreased at E12 in the $Bgn^{-/-}$, but not at E18, while MMP-13 expression is decreased at E18 but unchanged at E12 [Fig. 4B]. The gene expression of collagen α1VI decreased in the $Bgn^{-/-}$ membranes at E18 [Fig. 4E]. Concurrently, TIMP-1 gene expression increased at E18, while TIMP-3 gene expression decreased and TIMP-4 gene and protein expression decreased [Fig. 4C and D].

In the $Dcn^{-/-}$ membranes, we again observed a biphasic regulation. Gene and protein expression of MMP-8 and MMP-9 increased at E18, while both MMP-8 and MMP-9 protein expression decreased at E12. Also, MMP-13 gene expression increased at E18 [Fig. 4A and B). Additionally, TIMP-2 protein expression increased at E12 and TIMP-1 and -2 gene expression increased [Fig. 4C and D]. At E18, on the other hand, gene expression of TIMP-1 and gene and protein expression of collagen α1VI increased [Fig.4C, D and E]. However, none of the MMPs tested in $Bgn^{-/-};Dcn^{-/-}$ fetal membranes at E12 (MMP-8, -9 or -13) or at E18 (MMP-2, -8, -9 or -13) showed differences in protein expression compared to wild-type (data not shown). None of the proteins assayed at E15 displayed changes in either genotype (data not shown). Table 1 includes a summary of the results 2.2-2.4 of the TGFβ-Smad signaling cascade.

2.5. Exogenous decorin rescues the abnormal regulation of TGFβ signaling in decorin knockout fetal membrane mesenchymal cell culture

Finally, to confirm that decorin directly regulates the TGFβ signaling cascade in fetal membranes, we performed cell culture experiments utilizing mesenchymal cells from fetal membranes and soluble recombinant decorin that has been previously shown to have full biological activity (Buraschi et al., 2013). Manipulation of fetal membrane mesenchymal cells in culture showed that decorin is necessary for the function of the TGFβ-Smad-2 pathway. In the absence of decorin, the ratio of phospho-Smad-2/Smad increased. The addition of recombinant decorin protein core rescued the phenotype, decreasing the phospho-Smad-2/Smad ratio to wild-type levels. However, when the TGFβ receptor was blocked, the phospho-Smad-2/Smad ratio decreased in the absence of decorin compared to the wild-type and was not rescued by the addition of recombinant decorin protein [Fig. 5] ($p=0.04$).

In summary, these results demonstrate that biglycan signaling supports fetal membrane remodeling during early gestation in the absence of concomitant changes in TGFβ levels while in late gestation, biglycan signaling acts in a TGF-β dependent manner to aid in membrane stabilization. In contrast, decorin signaling supports fetal membrane remodeling at early stages of gestation in a TGFβ dependent manner, and fetal membrane stabilization at later stages of gestation without changes in TGFβ levels. Also, exogenous soluble decorin is capable of rescuing the TGFβ signaling pathway in fetal membrane mesenchymal cells.

3. Discussion

Previously, we demonstrated that *Bgn*$^{-/-}$;*Dcn*$^{-/-}$ mice have an increased incidence of preterm birth (Calmus et al., 2011). MMPs and their inhibitors, TIMPs, as well as their targets, collagens, play a key role in the control of turnover of extracellular matrix in fetal membranes at term rupture as well as at preterm premature rupture of fetal membranes (Parry and Strauss, 1998). In this study, we show that decorin and biglycan play a role in fetal membrane remodeling by regulating the TGFβ signaling pathway and its downstream targets, MMPs, TIMPs and collagens at the transcriptional as well as translational levels. Exogenous decorin gain-of-function experiments confirm that decorin regulates components of this pathway.

PPROM is associated with increased expression of the proteases MMP-2, MMP-8, MMP-9 and MMP-13 in the fetal membranes (Vadillo-Ortega and Estrada-Gutierrez, 2005) as well as with decreased expression of their inhibitors, TIMPs (Romero et al., 2010; Vadillo-Ortega et al., 1996). These proteases and their inhibitors are involved in extracellular matrix remodeling in both physiological and pathological processes (Malemud, 2006) and play a key role in the pathogenesis of PPROM (Menon and Fortunato, 2004; Vadillo-Ortega and Estrada-Gutierrez, 2005). Biglycan and decorin bind collagens in the extracellular matrix (Brown and Vogel, 1989; Wiberg et al., 2001) and both biglycan and decorin (Meinert et al., 2007) and collagens (Parry and Strauss, 1998) are expressed in the extracellular matrix of fetal membranes. The substrate affinities of MMP-2, -8, -9 and -13 suggest that they target collagens and proteoglycans of the fetal membrane extracellular matrix (Vadillo-Ortega and Estrada-Gutierrez, 2005), leading to the changes in collagen structure and content that are

thought to play a role in the pathogenesis of PPROM (Parry and Strauss, 1998). Furthermore, decorin induces the synthesis of a number of MMPs, including MMP-1, -2 and -14 (Schonherr et al., 2001).

Our data indicate that the absence of biglycan and decorin leads to morphological abnormalities of the fetal membranes. Furthermore, they indicate that biglycan and decorin play differential roles in mouse fetal membrane TGFβ signaling, leading to differential expression of the aforementioned MMPs, TIMPs and collagens, and that these roles are developmentally regulated. At E12, TGFβ levels as well as the transcriptional regulators Smad-3 and phospho-Smad-3 increase in the *decorin* null compared to wild-type mice. Downstream, TIMP-1 gene expression and TIMP-2 gene and protein expression increase, while MMP-8 and -9 protein expression decreases. These results suggest that decorin expression leads to a decrease in TIMPs and a concomitant increase in MMPs via a signaling cascade consisting of TGFβ and phospho-Smad-3. Thus, during early gestation, decorin plays a role in the process of tissue remodeling that occurs as the fetal membranes grow rapidly to accommodate the growing fetus.

Biglycan, on the other hand, plays a similar yet discrete role within the signaling pathway at E12. The absence of biglycan leads to a decrease in MMP-8 and -9 protein expression, suggesting that the presence of biglycan during the early growth phase of gestation leads to the increase in MMP activity that is typical of remodeling of the extracellular matrix. While biglycan and decorin regulate MMP-8 and -9 expression in a similar manner, the biglycan pathway is nonetheless discrete, since changes in biglycan expression do not lead to changes to TGFβ, Smad-3 or TIMPs.

By E18, a shift in decorin-related signaling occurs. TGFβ levels do not change dependent on the presence or absence of decorin, and Smad-2, phospho-Smad-2 and phospho-Smad-3 decrease in the *decorin* knockout fetal membranes. Downstream gene and protein expression also shifts. TIMP-1 and MMP-13 gene transcription increases, as well as MMP-8 and MMP-9 gene and protein expression. Gene and protein expression of collagen α1VI, which binds decorin (Wiberg et al., 2001), is also increased. This shift in expression towards matrix stabilizing proteins and away from matrix remodeling proteins suggests that decorin is necessary for the decrease in MMP activity and thus the stabilization of the fetal membranes in late gestation.

At E18, TGF-β and Smad-2 are both decreased in the *biglycan* knockout. Downstream, TIMP-1 gene expression is increased, while TIMP-3 gene expression, TIMP-4 gene and protein expression, MMP-13 protein expression as well as collagen α1VI gene expression are decreased. These findings suggest that biglycan plays a role in stabilizing the fetal membranes during late gestation by upregulating the TIMPs and collagens, fetal membrane extracellular matrix stabilizing proteins, via a TGFβ dependent pathway.

While decorin downregulates membrane destabilizing MMPs, biglycan upregulates membrane stabilizing TIMPs and collagen α1VI at E18. Thus, biglycan and decorin display pathways that are complementary to each other in stabilizing the fetal membranes during late gestation. At the same time, collagen α1VI is increased in the absence of decorin but

decreased in the absence of biglycan, suggesting functionally contrasting roles for these proteoglycans. These results suggest that biglycan and decorin also contribute to regulatory processes in which matrix remodeling is balanced with matrix stabilization. Similarly, decorin decreases prior to parturition as membrane tensile strength decreases, while biglycan increases (Meinert et al., 2007). Further suggesting a role for biglycan and decorin in regulatory processes is the observation that TIMP-1, which acts to stabilize the extracellular matrix, is increased in the absence of both biglycan and decorin, thus counteracting biglycan and decorin's predominant role of membrane stabilization. These observations suggest that the processes that determine the balance between membrane remodeling and membrane stabilization are fluid and complex. While these findings may seem contradictory, the balance between TIMPs and MMPs is an important and highly complex process in the pathophysiology of preterm birth (Fortunato et al., 1999; Tency et al., 2012).

Data from various tissues suggest that biglycan and decorin compensate for each other functionally. Examples include skin, bone, muscle, kidney and cornea (Ameye and Young, 2002; Corsi et al., 2002; Zhang et al., 2009) as well as our observations in the placenta (Calmus et al., 2011). Furthermore, fibromodulin and lumican, another pair of SLRPs, compensate for each other in tendon (Svensson et al., 1999). We have reported that in vivo, the preterm birth phenotype of the *biglycan/decorin* double knockout female is rescued in the *biglycan* or *decorin* single knockout, suggesting compensation between the two proteoglycans (Calmus et al., 2011). At the same time, while there is evidence of compensatory upregulation of biglycan in the *decorin* knockout and vice versa in the placenta, we did not observe similar upregulation of protein expression in the fetal membranes. This may be secondary to compensation not playing a role in the fetal membranes. Alternately, subtle compensatory changes may not be readily apparent with the techniques we utilized given the abundance of both SLRPs in fetal membranes. Thus, future experiments will need to be geared toward addressing the question of compensatory mechanisms between biglycan and decorin leading to the signaling changes we observed in fetal membranes.

Our cell culture experiments, which were undertaken to further characterize the mechanism of decorin action in fetal membrane TGFβ signaling, suggest that in a fetal membrane mesenchymal cell culture system, recombinant decorin rescues the phenotype. In the absence of decorin, the phospho-Smad-2/Smad ratio is increased, but the addition of recombinant decorin protein rescues the phenotype by decreasing the ratio back to wild-type levels. However, if the TGFβ receptor is blocked, then the phospho-Smad-2/Smad ratio decreases in the absence of decorin and is not rescued by recombinant decorin protein. This suggests the existence of an alternate TGFβ pathway that is independent of decorin in regulating transcription.

In summary, our findings suggest that biglycan signaling supports fetal membrane remodeling during early gestation in the absence of concomitant changes in TGFβ levels. In late gestation, biglycan signaling acts in a TGFβ–dependent manner to aid in membrane stabilization. Our findings suggest that decorin signaling, on the other hand, supports fetal membrane remodeling at early stages of gestation in a TGFβ–dependent manner, and fetal membrane stabilization at later stages of gestation without changes in TGF-β levels.

Furthermore, recombinant decorin rescues components of the TGFβ signaling pathway in fetal membrane mesenchymal cells. Collectively, our results suggest differential roles for biglycan and decorin in signaling pathways that lead to a switch from remodeling during early gestation to stabilization of the fetal membranes during late gestation. Thus, these findings present novel targets for manipulation of fetal membrane extracellular matrix stability and thus novel targets for research on preventive strategies in preterm premature rupture of fetal membranes.

4. Experimental procedures

4.1. Histochemistry

Wild-type, *Bgn*$^{-/-}$, *Dcn*$^{-/-}$ and *Bgn*$^{-/-}$;*Dcn*$^{-/-}$ fetal membranes were dissected at E18 in 0.1 mol l^{-1} phosphate-buffered saline, pH 7.4. The specimens were then flash frozen in isopentane and stored at −80°C. The frozen tissue was cryostat sectioned to 10 mm thickness, mounted on slides and stored at −20°C. Hematoxylin and eosin staining was performed according to standard protocols. Light microscopy was performed using an inverted stage Nikon Eclipse TE2000-E microscope equipped with epifluorescent filters and a Nikon Plan Apo 20 and 40 and a Plan Fluor 10 objective lens (Yokohama, Japan). Images were acquired and fetal membrane thickness and thickness variability were assessed using a Coolsnap HQ cooled CCD camera (Roper Scientific, Ottobrunn, Germany) and MetaVue software (Molecular Devices, Downingtown, PA).

4.2. Western blotting

Fetal membranes from the various genotypes were dissected at E12, E15 and E18 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 and phosphatase 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 × *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 NuPAGE 4-12% Bis-Tris 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 Antibody Stripping Solution (Alpha Diagnostic Intl Inc, San Antonio, TX) 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, MD). Relative optical density (specific protein band to GAPDH) was normalized to the wild-type band. The experiment was repeated with three individual sets of samples.

4.3. Antibodies

The following antibodies were used: mouse monoclonal anti-TGF-β1,2,3 (MAB1835, dilution 1:1000, R&D Systems, Minneapolis, MN); rabbit monoclonal anti-phospho-Smad-2(Ser465/467)/Smad-3(Ser423/425) (8828, dilution 1:800), mouse monoclonal anti-Smad-2 (3103, dilution 1:1000), rabbit monoclonal anti-Smad-3 (9523, dilution 1:1000) and rabbit monoclonal anti-TIMP-3 (5673, dilution 1:1000) (Cell Signaling, Danvers, MA); rabbit polyclonal anti-TIMP-1 (sc-5538, dilution 1:500), rabbit polyclonal anti-TIMP-2 (sc-6835, dilution 1:400), goat polyclonal anti-TIMP-4 (sc-9375, dilution 1:800), 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) and anti-goat IgG horseradish peroxidase conjugate (Santa Cruz Biotech, Santa Cruz, CA). Mouse IgG and rabbit IgG (Vector Laboratories, Burlingame, CA), respectively, were used as controls.

4.4. ELISA

Wild-type, *Bgn*$^{-/-}$ and *Dcn*$^{-/-}$ dams as wells as dams heterozygous for both proteins were dissected at E12 and E18. Fetal membranes were collected and flash frozen in liquid nitrogen and stored at −80°C. Pups dissected from the double heterozygous dams were tail-snipped for genotyping. *Bgn*$^{-/-}$*;Dcn*$^{-/-}$ (n=8 at E12, n=11 at E18), wild-type (n=6 at E12, n=5 at E18), *Bgn*$^{-/-}$ (n=5 at E12, n=4 at E18), and *Dcn*$^{-/-}$ (n=5 at E12, n=4 at E18) fetal membranes were sectioned into halves (E12) or quarters (E18) on dry ice. Each fetal membrane sample was homogenized in 1mL of protein lysate buffer (2.5ml Triton × 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 and -13 from Anaspect; MMP-9 from Abnova) were run in triplicate according to the manufacturer's instructions. Plates were read on a Bio-Rad Laboratories Microplate Reader at 450nm.

4.5. Cell culture

The mesenchymal cell separation method is modified from (Sun and Myatt, 2003). E18 *Dcn*$^{-/-}$ and wild-type C57BL/6J mouse fetal membranes were dissected and washed with cold 1×PBS to remove blood. 15-20 fetal membranes were digested with 1 ml of 0.125% trypsin and 200 units/ml DNase I (Invitrogen) at 37 °C for 10 min, then spun at 800 rpm for 5 min. The supernatant was removed, and the digestion and spinning was repeated 4 times. The supernatant was discarded. The remainder was washed thoroughly with 1× PBS twice and transferred to a small dish. The membranes were dissected into small pieces with scissors and digested with 4ml of collagenase I and IA (5 mg collagenase 1 and 5 mg collagenase 1A in 10 ml of mixture buffer composed of 8.5 ml DMEM without phenol red, 0.5 ml 500mM HEPES, pH =7.4, 1ml heated inactivated FBS) and 200 units/ml DNase I (Invitrogen) at 37 °C for 1 hr. The digested mixture was filtered through a 100 μm membrane (BD Falcon) and the flow through was spun at 900 rpm (Allegra® 6R centrifuge, Beckman Coulter) for 10 min. The supernatant was discarded and the cells were re-suspended in 4 ml DMEM without phenol red and FBS. The suspended cell mixture was

loaded onto a discontinuous Percell column consisting of 60%, 40%, 20% and 5% gradients and spun at 2180 rpm (Allegra® 6R centrifuge, Beckman Coulter) for 25 minutes. The cell content located at the 20% gradient was collected and transferred to a 6-well cell culture plate with 20% FBS DMEM medium containing 100 units/ml of penicillin and streptomycin and incubated at 5% CO2, 37°C for 18 hours. Cells were washed in wells with sterile 1× PBS to remove Percell beads and dead cell debris, fresh 20% FBS DMEM medium containing 100 units/ml of penicillin and streptomycin was added and the cells were cultured another 2 days. The medium was changed to 10% FBS and 100 units/ml penicillin and streptomycin prior to experimental use. At 60-70% confluence they were changed to 10% FBS medium containing 100 units/ml penicillin and streptomycin, and 0.29 mg/ml of L-ascorbic acid 2-phosphate (Sigma Aldrich). Medium was refreshed every other day and cells were cultured for 6 days in 5% CO_2 at 37 °C. Wild-type and a subgroup of decorin knockout cells were cultured in medium without decorin, while another subgroup of decorin knockout cells were cultured in medium with 5 μg/ml decorin. On day 7, cells were washed with PBS three times. Two ml of 10% FBS medium containing 0, 5.0 and 10μM of the TGF-β type I receptor inhibitor SB431542 (Tocris Bioscience) was added to wild-type cells, decorin knockout cells and decorin knockout cells grown in 5 μg/ml recombinant decorin medium. The cells were incubated in 5%CO2 at 37°C for 5 hours. Cells were washed in cold 1×PBS, 200 μl of 1× cell lysis buffer was added (Fisher Scientific) containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche)(10 ml 1× cell lysis buffer + 1 tablet of protease inhibitor cocktail and 1 tablet of phosphatase cocktail). The plate was placed in an ice bath, all cell debris and fluid was harvested, transferred to a 1.5 ml microtube, sonicated for 3 × 5 s on ice bath, centrifuged at 10,000g and 4 °C for 8 min. The supernatant was collected and total protein concentration of samples was measured by BCA (Thermo Scientific). Samples were stored in small aliquots at −80°C until use. Mesenchymal cells from fetal membranes were stained using the mesenchymal cell marker anti-vimentin antibody as previously reported (Sun and Myatt, 2003).

4.6. Expression and purification of recombinant decorin

Recombinant decorin was expressed as polyhistidine fusion protein in 293-EBNA cells using a Celligen Plus bioreactor (New Brunswick Scientific, Edison, NJ) as described previously (Goldoni et al., 2004; Mercado et al., 2006). In brief, cells were grown in DMEM containing 5% FBS to achieve the desired cell number. Media was then changed to serum-free and conditioned media collected every 48 hours. Initial purification of decorin was performed by passing 293 conditioned media over a nickel-chelating column followed by elution with a gradient of 0-250 mM imidazole in 20 mM Tris-HCl, 500 mM NaCl, 0.2 % CHAPS, pH 8.0.

4.7. Mouse husbandry and genotyping

C3H wild-type mice were purchased from Jackson Laboratories (Bar Harbor, ME). A *biglycan* homozygous knockout mutant breeding pair (generated by Marian Young (Xu et al., 1998)) was a gift from Justin Fallon. A *decorin* heterozygous knockout mutant breeding pair was mated to the birth of homozygous pups, which were then bred to establish the *decorin* homozygous knockout mutant colony. A homozygous $Bgn^{-/-}$ knockout female was crossed with a homozygous $Dcn^{-/-}$ male to establish breeding pairs in which the females

were heterozygous for both biglycan and decorin ($Bgn^{+/-};Dcn^{+/-}$) and the males were heterozygous for decorin but homozygous knockouts for biglycan ($Bgn^{-/0}Dcn^{+/-}$), given that biglycan is an X-chromosomal gene. These pairs were mated to breed $Bgn^{-/-};Dcn^{-/-}$ double knockout pups. Mice were housed under standard conditions. Breeding pairs were set up for mating at 5-7 weeks of age. Breeding pairs were set up in the evening. Plugs were checked the following morning and every morning thereafter. The day of the plug was defined as embryonic day 0. Dams were sacrificed at E12 and E18. IACUC approval was obtained. For genotyping, a 3-mm tail biopsy specimen was obtained for each pup within a *biglycan/decorin* double heterozygous litter at weaning. Genomic DNA was extracted from each tail biopsy sample using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). Polymerase chain reaction (PCR) was performed to identify the decorin and biglycan alleles 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 knockout allele. The biglycan PCR produced bands of 212 bp and 310 bp for the wild-type and knockout alleles, respectively.

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

Wild-type, *Bgn* homozygous knockout mutant and *Dcn*- homozygous knockout mutant mouse fetal membranes were dissected at E12, E15 and E18 in 0.1mol l^{-1} phosphate-buffered saline, pH 7.4, snap-frozen in liquid nitrogen and stored at −80°C. RNA extraction was performed using the Trizol method (Invitrogen, Carlsbad, CA). Genomic DNA was removed by incubating the RNA sample with DNase I (Invitrogen, Carlsbad, CA) for 30 min at 37°C with subsequent RNA re-extraction using the RNeasy Cleanup Column (Qiagen, Germantown, MD). The purified RNA was converted to cDNA using the Superscript III First-Strand Synthesis System Kit (Invitrogen, Carlsbad, CA). 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 using the comparative Ct method with a validation experiment. A standard sample of RNA pooled from E12, E15 and E18 samples of wild-type as well as *biglycan* and *decorin* knockout 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=4 dams per genotype.

4.9. Primer sequences

qPCR—GAPDH forward: CTCACAATTTCCATCCCAGAC, reverse: TTTTTGGGTGCAGCGAAC; TIMP-1 forward: CTCTGGCATCTGGCATCC, reverse: TGGTCTCGTTGATTTCTGGG; TIMP-2 forward: CAGGAAAGGCAGAAGGAGATG, reverse: GATCATGGGACAGCGAGTG; TIMP-3 forward: TGAAGGCAAGATGTACACAGG, reverse: GAGGTCACAAAACAAGGCAAG; TIMP-4 forward: GTTCGAGAAGGCCAAGGATATT, reverse: CTGCTTGTGACTGTTGGTTTC; MMP-8 forward: TCAACCAGGCCAAGGTATTG, reverse:

ATGAGCAGCCACGAGAAATAG; MMP-9 forward: GATCCCCAGAGCGTCATTC, reverse: CCACCTTGTTCACCTCATTTTG; MMP-13 forward: GATTATCCCCGCCTCATAGAAG, reverse: TCTCACAATGCGATTACTCCAG; Cola2I forward: AAGGATACAGTGGATTGCAGG, reverse: TCTACCATCTTTGCCAACGG; Cola3IV forward: TTTACACTCACCCAGCCATAC, reverse: CGCACCCTTTTCTGATCCATAG; Cola1VI forward: CTGGTGAAGGAGAACTATGCAG, reverse: GTCTAGCAGGATGGTGATGTC.

Genotyping—Biglycan wild-type allele forward TGATGAGGAGGCTTCAGGTT, reverse GCAGTGTGGTGTCAGGTGAG; biglycan knockout allele forward TGTGGCTACTCACCTTGCTG, reverse GCCAGAGGCCACTTGTGTAG; decorin allele forward CCTTCTGGCACAAGTCTCTTGG, decorin wild-type allele reverse TCGAAGATGACACTGGCATCGG; decorin knockout allele reverse TGGATGTGGAATGTGTGCGAG. All primers were provided by Invitrogen (Carlsbad, CA) except the MMP-8 and TIMP-4 primers, which were from Integrated DNA Technologies (Coralville, IA).

4.10. Statistical analysis

Analysis of differences in levels of gene and protein expression between genotypes was performed using the Student's t-test via Excel. SigmaPlot 11.0 and Matlab 2009a Student Version were used for standard deviation analysis. SigmaPlot 11.0 was used for ANOVA analysis.

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Abbreviations

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

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Highlights

- We have studied fetal membranes using a biglycan/decorin double-null mouse model of preterm birth
- Biglycan supports fetal membrane remodeling in early gestation without affecting TGFβ levels
- In late gestation, biglycan signaling promotes TGFβ-dependent membrane stabilization
- Decorin signaling supports fetal membrane remodeling during early gestation in a TGFβ-dependent manner
- At later gestation, decorin signaling promotes fetal membrane stabilization without TGFβ changes
- We provide genetic evidence for a crucial role of biglycan and decorin in fetal membrane homeostasis

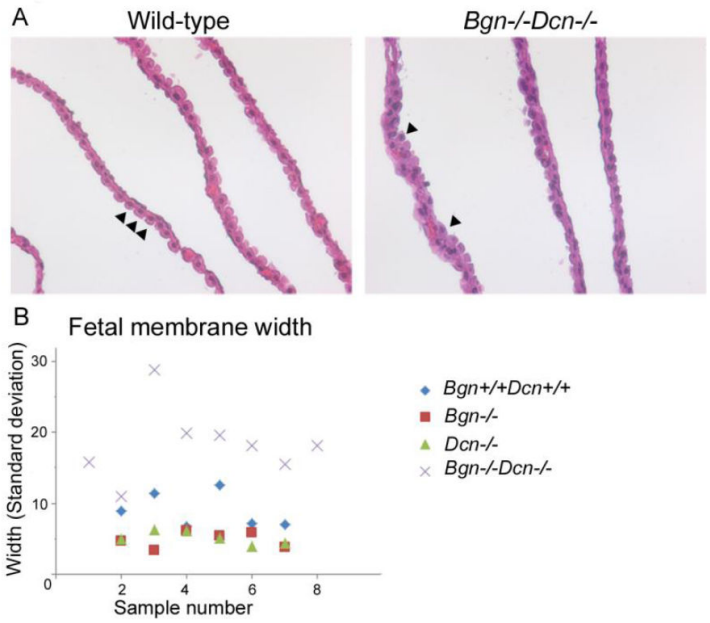

Figure 1.

[A] Fetal membrane morphology is altered in the $Bgn^{-/-};Dcn^{-/-}$ double knockout at embryonic day 18. The $Bgn^{-/-};Dcn^{-/-}$ double knockout displays strong variability in epithelial cell size and shape as well as membrane width compared to the wild-type. The arrows on the left show uniform size and shapes of epithelial cells. The arrows on the right show varying size and shapes of epithelial cells. H&E staining. 40× [B] Standard deviations of $Bgn^{-/-};Dcn^{-/-}$ double knockout fetal membranes are increased at embryonic day 18 compared to wild-type and single knockout ($p<0.001$). n=6 wild-type, 6 $Bgn^{-/-}$, 6 $Dcn^{-/-}$ and 8 $Bgn^{-/-};Dcn^{-/-}$ double knockout. Bgn=biglycan; Dcn=decorin.

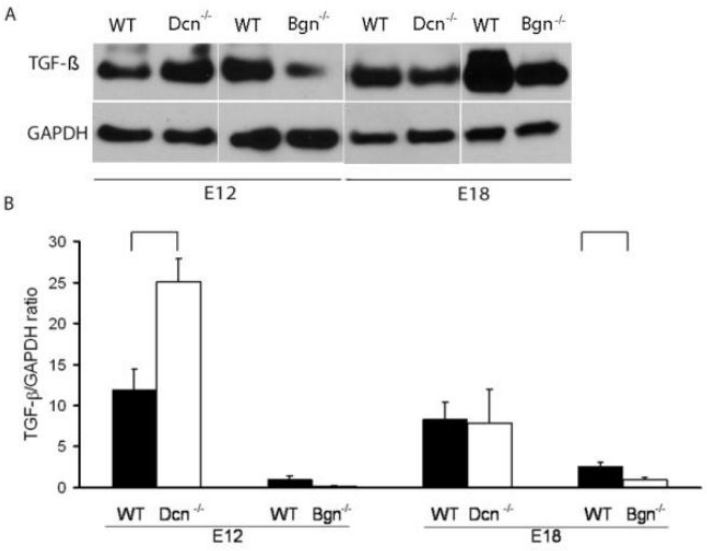

Figure 2.

TGF-β is increased in *decorin knockout* fetal membranes at E12 and decreased in *biglycan knockout* fetal membranes at E18 [A] Representative Western blots [B] Summary data. n=3 per genotype and gestational age (*p=0.028 and 0.036*). Error bars = SEM. E = embryonic day. Bgn=biglycan; Dcn=decorin; WT=wild-type. Student's T-test was performed for each set of data.

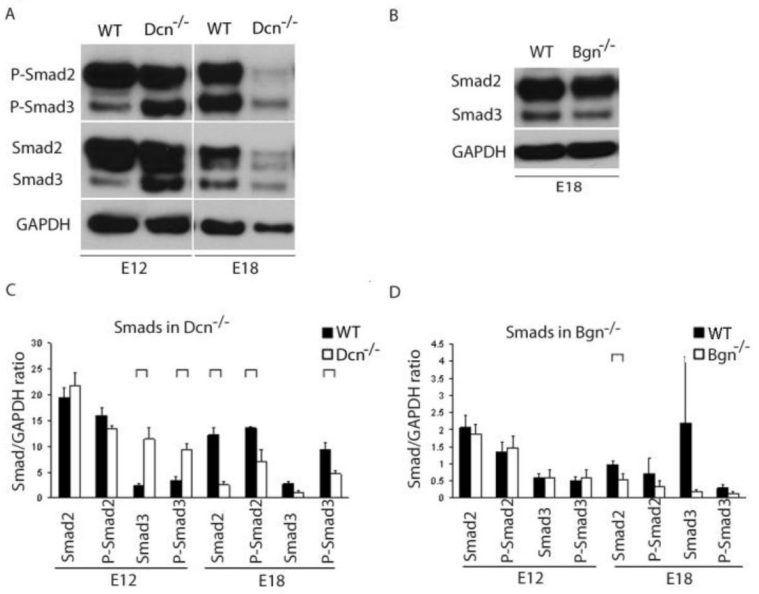

Figure 3.

[A] and [C] Smad-3 and phospho-Smad-3 are increased in the *decorin knockout* fetal membranes at E12, while Smad-2, phospho-Smad-2 and phospho-Smad-3 are decreased at E18 (E12 p=0.0144 and 0.0140; E18 p=0.0037, 0.0486 and 0.0388). n=3 per genotype and gestational age. [B] and (D) Smad-2 is decreased in the *biglycan knockout* at E18 (*p=0.05)*. n=3-5 per genotype and gestational age. Error bars = SEM. E = embryonic day. Bgn=biglycan; Dcn=decorin; WT=wild-type. P-Smad-2=phospho-Smad-2; P-Smad-3=phospho-Smad-3. Student's T-test was performed for each set of data.

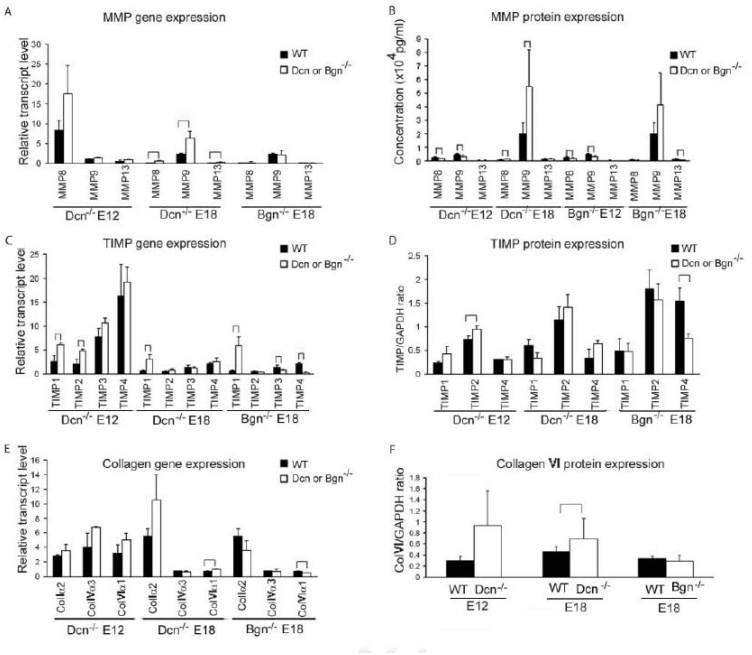

Figure 4.

Downstream extracellular matrix targets of the TGF-β-Smad2/3 pathway are altered in the absence of biglycan or decorin in a discrete and gestational age dependent manner. [A] Gene expression of MMP-8, MMP-9 and MMP-13 increased at E18 in $Dcn^{-/-}$ membranes (p=0.0175, 0.0468 and 0.0405, respectively). [B] MMP-8 and MMP-9 protein expression decreased at E12 (p=0.0223 and 0.0045) and MMP-13 decreased at E18 (p=0.0224) in $Bgn^{-/-}$ fetal membranes. MMP-8 and MMP-9 protein expression decreased at E12 (p=0.0136 and 0.0158) and increased at E18 in $Dcn^{-/-}$ membranes (p=0.0417 and 0.0298). [C] TIMP-1 gene expression increased at E18, while TIMP-3 and TIMP-4 gene expression decreased in $Bgn^{-/-}$ fetal membranes (p=0.05, 0.0152 and 0.0016, respectively). TIMP-1 and TIMP-2 gene expression increased at E12 (p=0.035 and 0.0272) in $Dcn^{-/-}$ membranes and at E18, TIMP-1 gene expression increased (p=0.0371) [D] TIMP-4 protein expression decreased in $Bgn^{-/-}$ fetal membranes (p=0.0085). TIMP-2 protein expression increased at E12 (p=0.0295) in $Dcn^{-/-}$ membranes. [E] Collagen α1VI gene expression decreased in $Bgn^{-/-}$ membranes at E18 (p=0.0339) and increased (p=0.0356) in $Dcn^{-/-}$ membranes. [F] At E18, protein expression of collagen α1VI increased (p= 0.0383) in $Dcn^{-/-}$ membranes. n=3-7 per genotype and gestational age. Error bars = SEM. Bgn=biglycan; Dcn=decorin; WT=wild-type. Student's T-test was performed for each set of data.

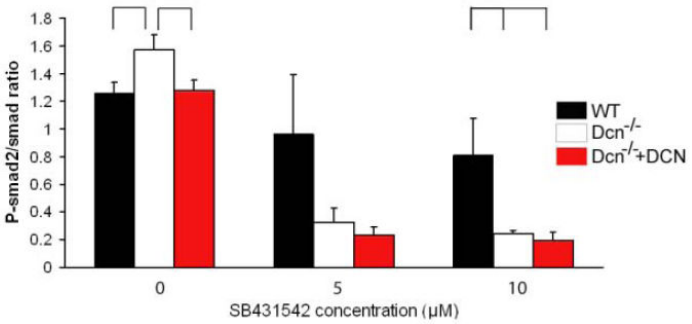

Figure 5.

Recombinant decorin phenotype rescue. The p-Smad-2/Smad ratio increased in the absence of decorin but is rescued back to the wild-type levels by the addition or recombinant decorin protein (p=0.04). Blocking the TGF-β receptor using the receptor blocker SB431542 leads to a decrease of the p-Smad-2/Smad ratio in the absence of decorin in a dose dependent manner (*p*=0.04), and the addition of recombinant decorin protein does not rescue the phenotype. Student's T-test was performed for each set of data. n=3 samples from 3 pregnant dams per condition. Error bars = SEM. $Dcn^{-/-}$=*decorin* knockout, WT=wild-type.

Table 1

Summary of fetal membrane expression patterns

	Dcn−/−				Bgn−/−		
	E12		E18		E12	E18	
	qPCR	Protein	qPCR	Protein	Protein	qPCR	Protein
TGF-β		↑		→	→		↓
smad2		→		↓	→		↑
P-smad2		→		↓	→		→
smad3		↑		→	→		→
P-smad3		↑		↓	→		→
TIMP1	↑	→	↑	→		↑	→
TIMP2	↑	↑	→	→	→	→	→
TIMP3	→	X	→			↓	→
TIMP4	→	→	→	→		↓	↓
MMP2				→			→
MMP8	→	↓	↑	↑	↓	→	→
MMp9	→	↓	↑	↑	↓	→	→
MMP13	→	→	↑	→	→	→	↓
Colα2I	→		→			→	
Colα3IV	→		→			→	
Colα1VI	→	→	↑	↑		↓	→

↓	knockout decreased compared to wild-type
↑	knockout increased compared to wild-type
→	no change in expression at E12
→	no change in expression at E18