Ovarian Transplants in Wild Type and Biglycan/Decorin Double Knockout Mice

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Abstract

Preterm premature rupture of membranes (PPROM) is responsible for 40% of premature births. In humans, the progeroid variant of Ehlers-Danlos Syndrome (EDS) is associated with PPROM which results from the abnormal secretion of the two small leucine rich proteoglycans biglycan and decorin. Both biglycan and decorin are expressed in the uterus and fetal membranes during gestation; however the role of the maternal genotype versus the fetal genotype is not understood. Since biglycan/decorin double knockout mice display phenotypic changes similar to EDS, we use this model to determine the role of maternal genotype (uterus) in comparison to fetal genotype (fetal membranes) during gestation and labor. Three arms were developed to study the interactions between the uterus and fetal membranes, and each is predicted to have different parturition outcomes. Wildtype mothers will carry biglycan/decorin double knockout pups by using ovarian transplants. Biglycan/decorin double knockout females will carry wildtype pups by utilizing nonsurgical embryo transfers. Wildtype mice will also act as controls for the procedures by transplanting wildtype ovaries into wildtype mice and transferring wildtype embryos into wildtype mice. The first arm of this model was performed with ovarian transplants which were then evaluated with video recording system for duration of gestation, duration of labor, duration of delivery, and litter size. Wildtype ovary recipients had results similar to those of wildtype mice that did not undergo the procedure. Biglycan/decorin double knockout ovary recipients did not have premature birth as predicted and produced progeny with wildtype alleles rather than biglycan/decorin double knockouts. These unexpected genotypes are not uncommon in ovarian transplants, and this arm of the study needs to be redone with a different method to ensure the correct genotypes of the pups. Conclusions cannot be drawn from these results until the second arm is completed and analyzed.

Introduction

Despite major medical innovations in prenatal care, preterm birth remains the leading cause of newborn morbidity and mortality in the United States (Murphy et al., 2015). Preterm birth is defined as the delivery of an infant before the thirty seventh week of gestation. Since the baby is not yet fully developed when born, complications such as developmental delay, growth restriction, patent ductus arteriosus, and respiratory distress are common (Ward and Beachy, 2003). Preterm birth represents an economic, clinical and psychological burden to society but the incidence of prematurity has not improved over the last twenty years despite development of new interventions (Ananth and Vintzileos, 2006). The various risk factors for preterm birth include adverse sociodemographic status, ethnicity, infection, stress, trauma, and a history of preterm birth. About forty percent of premature births are attributed to preterm premature rupture of fetal membranes (PPROM) (Steer, 2005). While the pathophysiology of PPROM is not understood, proposed mechanisms include physical stress, biochemical alterations, and apoptosis (Arikat et al., 2006; El Khwad et al., 2006; Menon and Fortunato, 2004; Moore et al., 2006). Risk factors for PPROM include environmental factors, such as infection, and genetic susceptibility (Parry and Strauss, 1998).

Ehlers-Danlos Syndrome (EDS) is a genetic cause of PPROM. Affected children have an increased risk of preterm birth due to PPROM in comparison with their unaffected siblings (Barabas, 1972; Yen et al., 2006). EDS is a group of rare inherited connective tissue disorders which present with a decrease in tensile strength and integrity of skin, joints, and other connective tissues. It is caused by destabilization of the extracellular matrix (ECM). The major criteria for diagnosis include skin hyperextensibility, widened atrophic scarring, abnormal wound healing, joint hypermobility, and a positive family history of EDS (Malfait et al., 2010). Many

times patients have more minor symptoms including smooth velvety skin, easy bruising, and subcutaneous spheroids. Cardiac and aortic abnormalities have also been reported including mitral valve prolapse and aortic dissection/rupture. A diagnosis can be confirmed with specific biochemical or genetic defect testing (Yen et al., 2006). EDS is classified into 10 different clinical forms with varying degrees of symptom presentation.

In addition to typical symptoms, the progeroid variant is characterized by wrinkled faces, curly and fine hair, scanty eyebrows and eye lashes, and periodontitis (Malfait et al., 2010). This autosomal recessive disorder is caused by a mutation in the β4GALT7 gene, which codes for xylosylprotein-4beta-galactosyl-transferase I, an enzyme responsible for posttranslational glycosylation of proteoglycans decorin and biglycan (Schaefer and Iozzo, 2008). As a result, biglycan and decorin core protein lacking glycosaminoglycan side chains are secreted (E Quentin, 1990; Kresse et al., 1987). Decorin and biglycan are the most abundant proteoglycans in the fetal membranes. Mice deficient in both of these small leucine-rich proteoglycans (SLRPs) display connective tissue abnormalities resembling EDS, and these mice also display preterm birth and fetal membrane anomalies (Calmus et al., 2011; Danielson et al., 1997; Wu et al., 2012; Xu et al., 1998).

Decorin and biglycan are important stabilizing components of the ECM. Biglycan is composed of a protein core with two chondroitin or dermatan sulfate chains. It binds to collagen VI, transforming growth factors α and β, chordin, and bone morphogenetic proteins (BMPs)(Chen et al., 2007). Decorin contains a single glycosaminoglycan chain of chondroitin or dermatan sulfate and demonstrates 55% homology with biglycan (Iozzo and Murdoch, 1996). Decorin also interacts with a number of ECM constituents and growth factors. Both decorin and biglycan are involved in many biological processes including: tumor angiogenesis and fibrosis

(Neill et al., 2012), 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 (Nikitovic et al., 2012). They are also important factors in collagen fibrillogenesis and contribute to mechanical properties of connective tissues. The absence of these proteoglycans results in reduced connective tissue tensile strength (Corsi et al., 2002).

Biglycan and decorin are involved with various receptors that regulate growth, motility, and the immune response. More specifically, they act as endogenous ligands of the toll like receptor (TLR) which are important in recognizing damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs). Decorin and biglycan can act as DAMPs, and under normally physiological conditions are sequestered to prevent an immune activation (Frey et al., 2013). Once released into their soluble form, these SLRPs can activate an immune response that contributes to inflammation. Biglycan and decorin are ligands of the same TLRs but the downstream signaling may differ due to crosstalk with other receptors, growth factors, or ECM components (Frey et al., 2013).

When binding to TLR2 or TLR4, biglycan requires both its protein core and side chains. It is incapable of activating these receptors in its matrix form but proteolysis of the ECM releases it into soluble form. If biglycan is degraded, some pieces will not be capable of activating the necessary immune pathway and therefore macrophages will compensate by releasing more biglycan. Downstream in the pathway biglycan leads to the activation of p38, Erk, and NFκB in a MyD88 dependent manner (Babelova et al., 2009; Moreth et al., 2010; Song et al., 2012). It also triggers synthesis of pro-IL-1β (Yamasaki et al., 2009). Mice deficient in biglycan show improved outcomes in experimental models of pathogen mediated and sterile inflammatory

disease. These mice are unable to activate an immune response via biglycan and therefore do not produce an inflammatory response in these circumstances.

On the other hand, decorin has multifaceted effects as a regulator of inflammation. This SLRP is able to bind to TLR2 and TLR4 with a comparable affinity to pathogen derived ligands. It also acts as a transcriptional inducer of tumor suppressor programmed cell death. A proinflammatory environment is created by stimulating PDCD4, TNF-a, and IL-12 and inhibiting TGF-B1 and anti-inflammatory IL-10 (Merline et al., 2011). Finally, decorin inhibits the adhesion of polymorphonuclear leukocytes, such as neutrophils, to the endothelial layer of blood vessels by downregulating the expression of ICAM-1 and syndecan-1 (Seidler et al., 2011). Without this adhesion, leukocytes are not able to migrate out of blood vessels to areas releasing signals for an inflammatory response. Organisms deficient in decorin would have difficulty mounting an immune response as well.

Decorin and biglycan are additionally involved in the transforming growth factor beta (TGF-β) signaling pathway (Kinsella et al., 2004). Both biglycan and decorin are regulated by TGF-β but decorin also acts a modulator for TGF-β through a negative feedback mechanism. Decorin and biglycan act as inhibitors by binding to TGF-β, and in the absence of both proteoglycans the pathway is dysregulated. In this pathway, TGF-β binds to its membrane receptor which leads to the phosphorylation of the Smad family of transcription factors. Phosphorylated Smad-2 and -3 travel to the nucleus and alter downstream gene expression of various collagens, matrix metalloproteinases (MMPs), and tissue inhibitors of metalloproteinases (Horgan et al., 2014). All of these proteins play a central role in the extracellular matrix stability of the fetal membranes and play a critical role in the pathogenesis of PPROM. Increased expression of matrix metalloproteinases leads to PPROM via degradation of extracellular matrix,

while mutations in TIMPs and collagens increase risk of PPROM (Menon and Fortunato, 2004; Parry and Strauss, 1998; Romero et al., 2010).

Throughout gestation, the fetal membrane ECM connecting the amnion to the chorion is continuously remodeled in order to accommodate the increasing volume and tension. Matrix metalloproteinases (MMPs) are enzymes that control this process by targeting and degrading specific substrates such as collagen and matrix proteoglycans. The activity of MMPs is modulated at the transcriptional level, during post translational modification, and through the activity of tissue inhibitors of metalloproteinases (TIMPs). There is balanced activity between MMPs and TIMPs during normal remodeling of the ECM. In dysregulated remodeling, like that which is present in biglycan/decorin double knockout mice, MMP-2, -8, -9 and -13 have been found to be involved in the pathogenesis of PPROM (Fortunato et al., 2003; Fujimoto et al., 2002; Maymon et al., 2000). In addition, TIMP-2 and TIMP-3 have a role in PPROM (El Khwad et al., 2006; Romero et al., 2010) and TIMP-1 is a target of the TGFβ-Smad-3 signaling pathway (Verrecchia et al., 2001). An imbalance between MMPs and TIMPs results in uncontrolled activity of MMPs which in turn causes more degradation of the ECM and eventual PPROM.

Collagen is the main structural component of the extracellular matrix. Collagen type I and III along with types V, VI, and VII provide the major tensile strength (Fortunato et al., 2003). Biglycan and decorin both bind to collage type VI (Wiberg et al., 2001), and in their absence some tensile strength is lost. Collagens α2I and α3IV have small nucleotide polymorphisms associated with an increased risk of PPROM (Romero et al., 2010). On the other hand collagen α2I and collagen α1VI are targets of the TGFβ-Smad-3 signaling pathway (Verrecchia et al., 2001). The TGFβ– Smad-2/-3 signaling pathway may be involved in the pathogenesis of PPROM through the above described interactions with MMPs, TIMPs, and collagens; however

this pathway has not been characterized in fetal membranes. Identifying the signaling pathways related to decorin and biglycan in the fetal membrane are critical to understand the mechanisms within the extracellular matrix that stabilize the fetal membranes (Wu et al., 2014).

Both biglycan and decorin are expressed in connective tissue of the pregnant mouse and their concentrations vary throughout gestation. Biglycan is found in the uterus while decorin is present in the placenta and myometrium during gestation (Hjelm et al., 2002; San Martin and Zorn, 2003; San Martin et al., 2003). Levels of decorin decrease during active labor while biglycan levels fluctuate minimally (Meinert et al., 2007). Upon further inspection it has been noted that decorin mutants demonstrate decidualized stroma of the uterus with large diameters and irregular contours of collagen fibrils (Sanches et al., 2010). Little is known about the role of these proteoglycans in the function of uterine muscle. It is hypothesized that biglycan and decorin could be protective of uterine smooth muscle dysfunction. Their absence would lead to labor dystocia (Wu et al., 2012) and adverse gestational outcomes. Additionally, the contributions of the maternal and fetal genotype to the PPROM phenotype are not understood. The placenta and uterus carry the mother's genotype while the fetal membranes reflect the fetal genotype. A biglycan/decorin double knockout mouse model could represent a novel and invaluable tool for evaluating these interactions as well as testing therapeutic approaches for human PPROM and dystocia prevention and treatment.

Methods

Mouse Model

Three experimental groups were developed to elucidate the contribution of maternal and fetal genotype to the PPROM and dystocia phenotypes. The uterine genotype is contributed by the mother while the fetal membranes and the placenta develop from the fetus. Biglycan/decorin homozygous double knockout ovaries were transplanted into wild-type dams. This model yields a wildtype uterus with biglycan/decorin double knockout fetal membranes. We predicted the mother will deliver early from PPROM without dystocia, or difficulty giving birth. We have chosen to perform biglycan/decorin double knockout ovarian transplants because these mice do not mate well. Since biglycan and decorin are absent from connective tissues, the mice are more fragile, and stress can cause weakened blood vessels to rupture easily. After being set up with males, the females are often found dead the next morning. Upon postmortem investigation, it is found that the aorta has ruptured and caused the moue to fatally hemorrhage. This is caused by the stress of copulation. Therefore, it is difficult to obtain biglycan/decorin double knockout embryos. Wildtype mice do not have these problems. An ovarian transplant allows us to produce biglycan/decorin double knockout embryos without risking the death of the mother. Our goal is to study the interaction between a wildtype uterus and biglycan/decorin double knockout fetal membranes, which the ovarian transplant will allow us to directly observe.

In the second model, wildtype embryos will be transferred into biglycan/decorin double knockout dams. These pregnancies should persist to term but dystocia is expected to be present during labor. We will use a nonsurgical embryo transfer (NSET) in which blastocysts are removed from pregnant wildtype females and then transferred transvaginally via cannula into the uterus of a pseudopregnant biglycan/decorin double knockout female.

As a control group for each procedure, wild-type ovaries were transplanted into wild-type dams and wild-type embryos will be transferred into wild-type dams. Figure 1 demonstrates a visual representation of our experimental design.

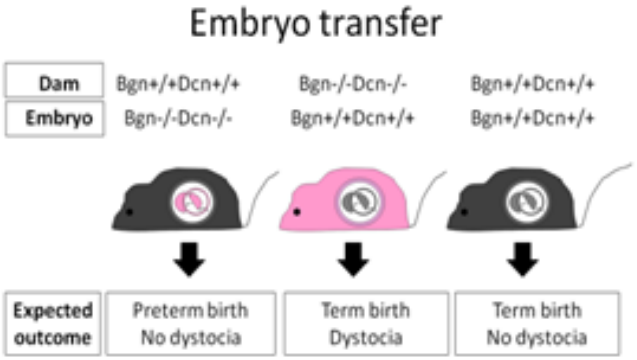

Figure 1: Experimental set up for maternal-fetal divergent genotype embryo transfer experiment. *Bgn-/-/Dcn-/- (knockout)* (pink) embryos will be implanted into *wildtype (WT)* (black) dams. *WT* (black) embryos in *Bgn-/-/Dcn-/- (knockout)* (pink) dams and *WT* (black) embryos in *WT* (black) dams will be controls**.**

Mouse Husbandry

Institutional IACUC approval was obtained for the experiments. C3H wildtype mice were purchased from Jackson Laboratories (Bar Harbor, ME). A biglycan homozygous knockout mutant (bgn -/- dcn +/+) breeding pair (generated by Marian Young (Xu et al., 1998) was a gift from Justin Fallon. A C57BL6 decorin heterozygous knockout mutant (bgn +/+ dcn +/-) breeding pair were mated to produce homozygous pups (bgn +/+ dcn -/-). These offspring were bred to establish the decorin homozygous knockout mutant colony. A biglycan homozygous null mutant female was crossed with a decorin homozygous null mutant male to establish females heterozygous for both decorin and biglycan (bgn +/- dcn +/-) and males heterozygous for decorin but homozygous knockouts for biglycan (bgn -/0 dcn +/-). Males were homozygous for biglycan because it is an X chromosomal gene. These mice were mated to breed mixed genotype litters including biglycan/decorin double knockout pups (bgn -/- dcn -/-). Mice were housed under standard conditions. Pairs were set up for mating at 5-7 weeks of age.

To establish day 3.5 blastocysts outlined in the experimental design for timed pregnancies, breeding pairs were set up in evenings with plugs checked the following morning and all subsequent mornings. The first presence of a plug was defined as E0. Each morning cages were inspected for litters, and the day of birth was defined as the first morning that pups are observed.

The experiment began by crossing double heterozygotes (bgn +/- dcn +/-) together. Over the course of the experiment the breeding strategy was switched to three quarter knockout breeding pairs (bgn +/- dcn -/-; bgn -/- dcn +/-). This was done in hopes to increase the number of double knockouts produced.

Genotyping

For genotyping, a 2 mm tail biopsy specimen was obtained from each pup within a litter produced from a biglycan/decorin double heterozygous cross at weaning. Genomic DNA was extracted from the tail biopsy sample using a mixture of PBMD and proteinase K incubated in a 53°C hybridization oven. The samples were continuously rotated overnight, and proteinase K was inactivated by placing samples in heat blocks set to 95°C for 15 minutes. Polymerase chain reaction was performed to identify decorin and biglycan alleles using the PTC-200 thermal cycler. The PCR product was run on a 1.8% w/v agarose gel to visualize the following distinctive bands; the wildtype decorin allele PCR produced bands of 161 bp while the knockout allele produced 238 bp bands. The wildtype biglycan allele PCR produced bands of 212 bp and the knockout allele produced 310 bp bands.

Ovarian Transplants

Ovary Collection

We first weighed a female donor at 6-10 weeks of age. We then euthanized the mouse with isoflurane. The abdominal cavity was opened and the uterine horn and ovaries were located. Both ovaries were immediately removed and placed in sterile PBS at room temperature. The ovaries were cleaned and cut in half using sterile forceps. If the ovaries appeared smaller than usual, ovaries were not cut in half.

Recipient Transplants

We weighed 6-10 weeks old B3C3F1transplant candidates and chose a recipient with the same weight as the donor mouse. Ketamine and xylazine intraperitoneal (IP) injections were used to anesthetize recipients. Mouse toe reflexes were checked every 5 minutes to determine the onset of anesthesia. A small area of the mouse's back was shaved. Isopropyl and betadine were used to sterilize the shaved area. The mouse was placed dorsal side up on gauze and then moved to the sterilized surgical area.

Fine sterile scissors were used to make a small, paralumbar incision on the right side of the body. Next, an incision was made in the peritoneal membrane, and ovarian fat pad and ovary were located within the abdominal cavity. The ovary and upper part of the uterine horn were exposed, placed on a sterile gauze pad, and stabilized with a serrafine clamp (Figure 2). A small cut was made in the ovarian bursal sac and gently moved aside to expose the ovary. Forceps were used to maintain the position of both the ovary and the bursal sac. The ovary was removed using sterile scissors, taking great care to remove the entire ovary. The donor hemiovary was introduced into the opening of the recipient bursal sac and the bursa was placed back over the

ovary using forceps. Little movement was used to prevent movement of the ovary out of the bursal sac. The serrafine clamp was removed and the organs were carefully directed back into the peritoneal cavity. A suture was used to close the abdominal cavity incision.

An incision was then made on the left side of the body and the procedure repeated. After completed transplant of the second hemiovary, skin glue was used close the skin incision. Mice were given a subcutaneous (SC) injection of saline for hydration. Mice were given an injection of buprenorphine as an analgesic. The mouse was then monitored and after it was able to lift its head, it was placed in a cage with one end on top of a heating pad. The cage was placed back into its appropriate location once the mouse was able to fully walk around the cage on its own.

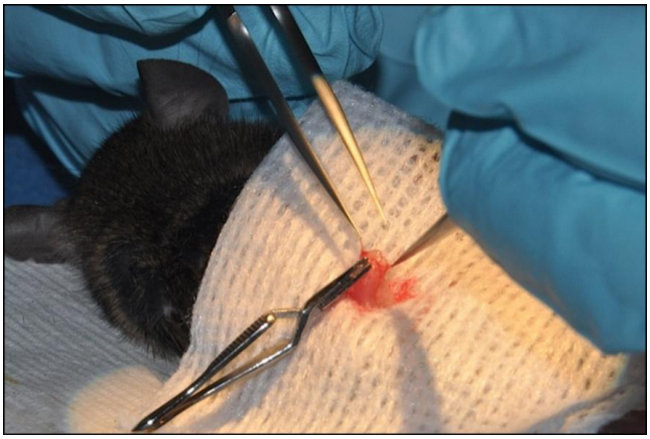

Figure 2: Ovaries of the recipient female are removed, and freshly harvested donor ovaries are inserted into the ovarian bursal sac, which physically holds the donor ovary in place.

Recipient Breeding

After a 2 week period of recovery, the transplanted females were bred with males to produce progeny. Wildtype ovary recipients were bred with wildtype males. Biglycan/decorin double knockout ovary recipients were bred with biglycan/decorin three quarter knockout males (bgn -/0 dcn +/-).

Video Monitoring

Pregnant females were placed in a single cage without nesting material on day 14.5 post coitus. A video recording system (Swan Communications, INC) was set up to capture continuous video (Figure 3) of the females during labor and birth. The mice were checked twice a day for presence of pups. Videos were continuously recorded until pups were seen during the morning or afternoon check. Videos were analyzed three times to determine labor onset behaviors and parturition parameters.

Figure 3: Video recording system set up to capture continuous image of the whole mouse cage.

Student Contribution

While this project involved all of the previously mentioned methods, I was not directly involved in every procedure. Dr. Underhill set up breeding pairs described in mouse husbandry and obtained tail snips from the offspring while I independently performed the genotyping on the tail snips. Dr. Underhill led the ovarian transplants while I assisted both in the preparation and the surgical procedure. I prepared all of the medications as well as set up the surgical equipment and weighed the mice. I then observed the surgery and performed any tasks Dr. Underhill required

assistance with such as anticipating needs of surgical tools as well as holding open the bursal sac while the hemiovary was being placed. Upon recovery Dr. Underhill then set up the breeding pairs and the video equipment to monitor the mice. She also evaluated the videos for the parturition parameter measurements.

Results

Genotyping

Over the course of 18 months, a total of 910 pups were genotyped over 9 generations of breeding. Of the pups genotyped, 29 were found to be biglycan/decorin double homozygous knockouts. The knockouts represented 3% of the cross offspring. Within this population, 17 males (bgn -/0 dcn -/-) were identified and 12 females (bgn -/- dcn -/-) were identified. The distribution of birth months of these mice is shown in Figure 4.

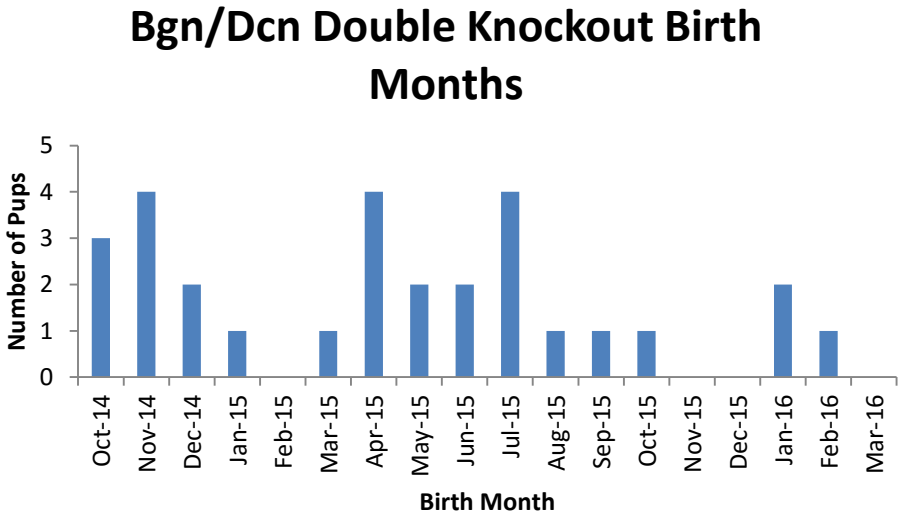

Figure 4: Distribution of biglycan/decorin double knockout pup births.

Video Recordings

Videos of labor and birth were evaluated to identify labor onset behaviors and measure parturition parameters. Labor onset behaviors were found to signify the onset of labor in the mice. Examples included reduced activity in which the mice tended to stay in one general area of the cage. Repeated back stretching and rhythmic back movements (contractions) were also

witnessed. The mother often tried to check her backside. Parturition parameters included length of gestation, length of labor, length of expulsion phase, and total number of pups in litter. Length of gestation was determined to be the time spanning from the presence of the plug to the onset of labor. The length of labor was determined to be the time starting when the mouse first began to display labor onset behaviors to the time of birth of the first pup. The expulsion phase included the time between the birth of the first and last pup.

Ovarian Transplants

Recipient ID	Donor-Recipient Genotype	Duration of Gestation (days)	Duration of Labor (min)	Duration of Delivery (min)	Number of Pups
OT#1	WT-WT	20.5	61	N/A	1
OT#3	WT-WT	19.5	129	19	3
OT#4	WT-WT	19.5	73	27	4
OT#6	WT-WT	19.5	78	15	3
OT#7	WT-WT	19.5	120	25	5
OT#8	KO-WT	19.5	98	42	4
OT#9	KO-WT	20.5	229	9	2
OT#12	KO-WT	19.5	79	19	4
OT#14	KO-WT	19.5	113	13	2

Table 1: Video recordings of wildtype-wildtype and biglycan/decorin double knockout-wildtype ovarian transplants were evaluated for duration of labor, duration of delivery, and number of pups.

WT-WT Ovarian Transplants

Wildtype ovaries were transplanted into wildtype females to serve as a control for the procedure. A total of 6 procedures were performed. After recovery, 5 of the females were able to become pregnant. Parturition parameters of each pregnancy are shown in Table 1. Gestation lasted an average of 19.7 days (SD=0.44) with a labor of 92.2 minutes (SD=30.3). No dystocia was present during birth. The expulsion phase typically lasted 21.5 minutes (SD=5.5). The

average litter size was 3.2 pups (SD=1.4). The pups were not genotyped since it was clear that they would be wildtype.

KO-WT Ovarian Transplants

Biglycan/decorin double knockout ovaries were transplanted into wildtype females for the first arm of the study. A total of 7 procedures were performed which required the use of 5 biglycan/decorin double knockout females. Some of the donors had small ovaries which were not cut in half since they could easily fit into the ovarian bursa. Of these surgeries, 4 were successfully able to become pregnant. Parturition parameter measurements are shown in Table 1 for each pregnancy. Gestation lasted an average of 19.7 days (SD=0.5) with a labor of approximately 130 minutes (SD=67). Dystocia was not noted during delivery. Expulsion lasted for 20.7 minutes (SD=14.7) and produced litters of 3 pups (SD=1.2). Pups from each litter were genotyped and the results are shown in Table 2.

Table 2: Biglycan/decorin double knockout (bgn -/- dcn -/-) ovary recipients were mated with biglycan/decorin three quarter knockout males (bgn -/0 dcn +/-). Offspring of these crosses were genotyped and found to display genotypes containing wildtype alleles in both biglycan and decorin.

Recipient ID	Pup ID	Gender	Genotype
OT#8	04 05 06 06-2	F M M M	Bgn +/- Dcn +/+ Bgn +/0 Dcn +/- Bgn +/0 Dcn +/- Bgn +/0 Dcn +/-
OT#9	01	F	Bgn -/- Dcn +/-
OT#12	02 03 07 09	F F M M	Bgn +/- Dcn +/- Bgn +/+ Dcn +/- Bgn +/0 Dcn +/+ Bgn -/0 Dcn -/-
OT#14	11 12	F M	Bgn +/- Dcn +/- Bgn +/0 Dcn +/-

Discussion

Mouse Model

Biglycan and decorin double homozygous null mice were used as a model for a genetic cause of PPROM. Since the roles of biglycan and decorin in the uterus versus in the fetal membranes are not known, the mouse model of divergent maternal and fetal genotypes served to help elucidate this interaction. The first arm, which we were able to perform, is a wildtype mother carrying a biglycan/decorin double knockout fetus, representing a wildtype uterus and biglycan/decorin double knockout fetal membranes. We predicted this mother would deliver early without dystocia based on the idea that the weakened fetal membranes will not be able to sustain the strength needed to carry the offspring to term. We have chosen to perform biglycan/decorin double knockout ovarian transplants into wildtype females for this portion of the experiment. Due to the nature of the actions of biglycan and decorin in the ECM, the biglycan/decorin double knockout females are rarely able to become pregnant. After setting up biglycan/decorin double knockout females with males overnight, the females often do not survive; therefore it is not possible to obtain embryos from these mice. An ovarian transplant allows biglycan/decorin double knockout offspring to be generated from a mouse that is able to survive copulation and carry pups without risking major damage to connective tissue.

The second experimental arm requires a biglycan/decorin double knockout female to carry a wildtype fetus. This produces a biglycan/decorin double knockout uterus with wildtype fetal membranes. We predict this mother will deliver at term but present with dystocia during labor. In this case we hypothesize the deficiency of biglycan and decorin in the uterus will lead to nonproductive contractions. We have chosen to perform embryo transfers to inflict the least amount of stress on the fragile biglycan/decorin double knockouts. An ovarian transplant would

require the biglycan/decorin double knockout recipient to mate with a wildtype male which has led to complications in females. Since the connective tissues, including the skin, of the biglycan/decorin double knockout females is fragile and surgery can cause complications, a Nonsurgical Embryo Transfer (NSET) will be performed on pseudo-pregnant females. At this point in the experiment we do not have results to report on this arm of the experimental set up.

Finally, wildtype females will carry wildtype fetuses. Since both the uterus and fetal membranes have a normal composition of biglycan and decorin in the ECM, we expected to see a normal pregnancy (delivery at term with no dystocia). We performed both ovarian transplants and NSETs in this arm to ensure accuracy of both procedures. At this point we have completed and have results for wildtype-wildtype ovarian transplants.

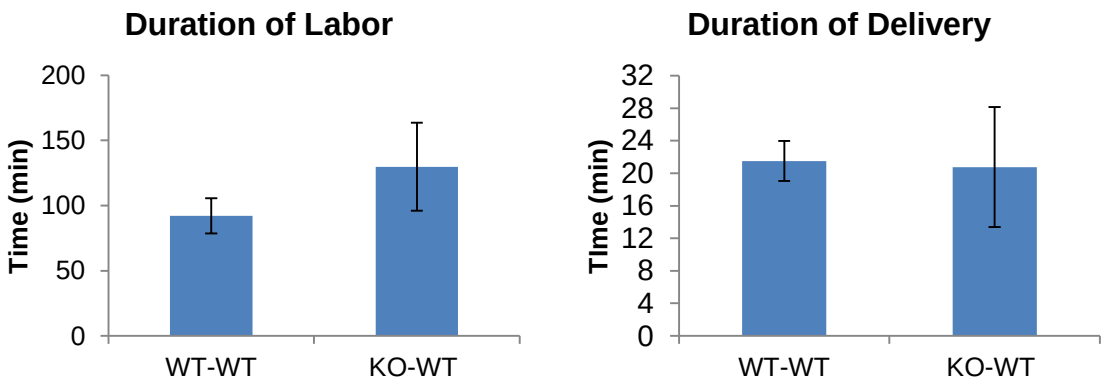

Figure 5: Average duration of labor and delivery for wildtype and biglycan/decorin double knockout recipient pregnancies.

Based on our model, we predicted different outcomes for the two arms we observed. The wildtype ovarian transplants were expected to have normal parturition outcomes. Based on our observations, this was achieved. The recipients had results similar to those of mice that did not undergo the procedure. The biglycan/decorin double knockout ovarian transplants were predicted

to have produced pregnancies of a shorter gestation without dystocia. There was not a difference between the gestation times of our wildtype/wildtype and wildtype/biglycan/decorin double knockout pregnancies. Differences between the wildtype and biglycan/decorin double knockout ovary recipients were seen in duration of labor (Figure 5) with a trend towards longer labor in the double knockout. While these differences are present, conclusions cannot be drawn until the second and third arms of the model are completed.

Upon genotyping the offspring, we found some pups had wildtype alleles, which, based on our breeding strategy, was unexpected. Only one pup born to a biglycan/decorin double knockout ovarian transplant recipient was a biglycan/decorin double knockout. Since we crossed a biglycan/decorin double knockout male and female (bgn -/- dcn -/-; bgn -/0 dcn -/-), we expected all of the offspring to be biglycan/decorin double knockout. Therefore none of the offspring from this cross should have contained a wildtype allele. The pups were genotyped again with new tail snips to ensure results remained the same. We also genotyped the donor ovaries to confirm that we had correctly identified the donors as biglycan/decorin double knockouts. We hypothesized the recipient's residual ovarian tissue had regenerated during the course of her recovery to give rise to these pups. With the genotype of the pups in mind, we concluded our interpretations of the parturition parameters would not necessarily be representative of our goal model.

Genotyping

Biglycan is an X-linked gene and decorin is an autosomal gene. Based on Mendelian genetics, we would expect a double heterozygous cross (bgn +/- dcn +/-, bgn -/0 dcn +/-) to produce biglycan/decorin double knockouts as 1/16, or 6.25%, of the population. Based on previous studies, only 5% are biglycan/decorin double knockouts due between day 18.5 of

gestation and birth (Calmus et al., 2011). Of this population, our experiments require more females than males. Therefore, from our breeding colonies we expected to be able to obtain 3.12% females for use in our study. From our crosses we found that only 3% were biglycan/decorin double knockouts and of these only 41% were female. Of the total population, only 1.3% were biglycan/decorin double knockout females and could be used for the first and second arms of our model (Calmus et al., 2011).

We also observed that over time fewer biglycan/decorin double knockout mice were identified. After a few generations we switched to using three quarter female knockouts (bgn +/- dcn -/-; bgn -/- dcn +/-) in hopes of increasing the biglycan/decorin double knockout yield. However, the opposite outcome was observed; fewer double knockouts were found. We hypothesize that over time either the pups were born less healthy to the point in which they did not survive or three quarter knockout nursing moms are less healthy. The biglycan/decorin double knockout mice are more fragile due to the nature of the actions of biglycan and decorin. The lower rate of biglycan/decorin double knockouts recovered is due to the poorer health of these individuals. Prior studies have shown that some die before birth (Calmus et al., 2011) and those that do survive may die before reaching the age for pregnancy. It was also found that once these mice do reach the mating age, the females die during mating.

Based on this knowledge the model was therefore altered to ensure survival of biglycan/decorin double knockout females. As previously mentioned, the mice rarely become pregnant from copulation. If a mouse is able to survive the stress of copulation, we did not want to introduce another stress factor. Rather than performing surgical procedures that would require the mice to recover from skin incisions, we decided to perform nonsurgical embryo transfers in which embryos are introduced transvaginally into the uterus. This procedure does not require

opening the mouse's abdominal cavity and puts less stress on the mouse. Ovarian transplants also prevent the mouse from undergoing the stress of copulation. The ovary recipient, a wildtype mouse, is able to survive copulation and can become pregnant.

Ovarian Transplants

Laboratory mice are an important source for biomedical research. Their genome can be manipulated to simulate human diseases to allow for better understanding and development of treatments. Currently mouse lines can be transferred in the form of embryos, gametes or dissected mouse reproductive organs as well as shipping live mice for breeding. There are various methods for transfer of each tissue as well; some entail cryopreservation while others do not. Each method has its own advantages and disadvantages. If cryopreservation is going to be performed rather than immediate transplantation, it must be done as soon as possible to prevent accidental loss, genetic drift and to reduce cost (Du et al., 2010).

The first successful ovarian transplant in the mouse was performed in 1940 by Robertson (Robertson, 1940). In this procedure, both ovaries were removed from the recipient but only one whole ovary was transplanted. In order to determine that the transplanted ovary produced pregnancies, the donor was a yellow mouse while the recipient was agouti. The agouti recipient was then mated with a yellow male. Not all of the offspring were yellow as predicted by the cross. The procedure was used in 1945 to study variation in the prenatal maternal environment (Russell and Hurst, 1945). The same procedure developed by Robertson was used with the exception of the size of the ovary; only a half ovary was implanted rather than a whole ovary. Pregnancies from this procedure and cross produced progeny that demonstrated genotypes not predicted by the cross. This was attributed to regeneration of the host ovary, and thought to be

unavoidable even with careful surgical techniques. Although the ovaries did not produce the correct offspring, they remained functional.

The procedure was later modified by Stevens to increase the yield of offspring from transplanted ovaries (Stevens, 1957). Ovaries were cut in half and then one half replaced an ovary on each side of the recipient. Again the offspring from these procedures demonstrated signs of recipient ovary regeneration. Some litters were mixed with pups derived from both the transplanted and recipient ovary. The procedure was later updated to the version that is currently performed (Cunliffe-Beamer, 1983).

Ovaries have successfully been frozen by slow freezing or vitrification and transplanted into immunologically compatible mice to generate live pups (Parrot, 1960; Sztein et al., 1998; Tokieda et al., 2002). Frozen ovaries were successfully used to rescue a transgenic mouse line (Sztein 1999). While cryopreservation is a viable option when recipients are not prepared to receive the ovaries when they are collected, tissues may be damaged by cryoprotectant fluids that are necessary (Paris et al., 2009). Recipients with cryopreserved ovaries tend to produce fewer pups and fewer pregnancies than those with ovaries directly transplanted (Dawes et al., 2010).

Ovarian transplants have typically been successful in producing pregnancies; however, the genotypes of the pups do not match with those predicted by breeding patterns. Prior researchers ascribed this complication to ovary regeneration. In our biglycan/decorin double knockout ovary recipients, the pups contained wildtype alleles. Donated ovaries were removed from recipients and genotyped to confirm we had correctly identified the donors. Clearly this phenomenon is not novel with this procedure. Alongside regeneration of host ovaries, the transplanted ovary may not be sufficient to support pregnancies. Since the majority of oocytes

are located in the outer cortex of the ovary, it may have been damaged during the procedure. The fewer pregnancies may be due to the small size of the graft; perhaps it is not able to produce sufficient endocrine hormones.

The transplantation of ovaries is an important procedure for saving transgenic lines that are unable to mate or cannot sustain pregnancy. If the biglycan/decorin double knockout recipients had produced biglycan/decorin double knockout recipients, the results not only would have benefited our study but also would have been an important improvement within the field of reproductive surgery. We also would have had a more accurate depiction of biglycan/decorin double knockout fetal membranes in combination with a wildtype uterus. The pregnancy parameters may have been closer to our predictions which would have validated our hypothesis that the absence of biglycan and decorin in the fetal membrane causes PPROM. Since this procedure was unable to reliably produce biglycan/decorin double knockout progeny, our methods will have to be updated. This completed arm with unexpected results needs to be repeated with another method to ensure the appropriate genotypes. Before conclusions can be drawn from this study, the second arm (wildtype embryos implanted into biglycan/decorin double knockout recipients) needs to be completed and evaluated.

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