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Interplay between collagenase and undescended testes in *Adamts16* knockout rats

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ABSTRACT

Background: The inguinoscrotal stage of testicular descent is characterized by an increase in cell density and collagen fibers as the gubernaculum undergoes cell division and increases Extracellular Matrix (ECM) activity. Rats that lack the enzyme *Adamts16*, a known ECM proteinase, develop cryptorchidism postnatally and are infertile. Therefore, this study aims to investigate the link between the Adamts16 enzyme and congenital undescended testes (UDT) in *Adamts16* knockout (KO) rats during postnatal development.
Methods: Formalin-fixed specimens of Wild-Type, *Adamts16* heterozygous and *Adamts16* homozygous KO rats post birth were sectioned and used for standard H&E histology and Masson's trichrome staining. A quantitative analysis on image J was performed to determine the intensity of collagen fibers within the inguinoscrotal fat pad (IFP) (n = 3 age/genotype).
Results: The migration of the gubernaculum within the *Adamts16* heterozygous and *Adamts16* KO rat was considerably disrupted. Furthermore, the Masson's trichrome staining demonstrated a significant increase in collagen fibers around the gubernaculum of rats that lacked *Adamts16* enzyme at day 8.
Conclusion: This study reports a failure of gubernacular migration leading to UDT in *Adamts16* KO rats during development, suggesting that the expression of *Adamts16* gene is critical for normal gubernacular migration through the breakdown of collagen fibers within the IFP.

Undescended testes (UDT) is a frequent urogenital irregularity either unilateral or bilateral seen in male children and can be separated into congenital UDT, where the stable scrotal position is never reached and acquired UDT, which is defined as a UDT where the testis was reported to be in the scrotum in early infancy [1]. The two major consequences of UDT are infertility and an increase risk of cancer of the germ line in adulthood, making it essential to provide treatment during the early stages of development [2, 3]. Testicular descent is very similar in rodents and humans, although, the second step occurs postnatally in rodents, whereas in humans the descent is normally completed before birth. Despite differences in timing, in both human and rodents the genito-inguinal ligament or gubernaculum, must migrate from the inguinal canal to the scrotum [4]. The migrating gubernaculum in a human fetus during inguinoscrotal descent is not attached to the adjacent mesenchyme, suggesting that enzymes are produced to dissolve the extracellular matrix (ECM) and to make a space for it [5]. During postnatal testicular decent in rodents, the ECM is also remodeled to clear a space for the migrating gubernaculum through the interaction of macromolecules such as collagens and glycosaminoglycans [4]. Furthermore, it has been suggested that the gubernaculum, which must migrate from the inguinal canal to the scrotum may produce metalloproteinases to remodel the ECM of the inguinoscrotal fat pad (IFP) to make space for the elongating gubernaculum [6].

The structure and function of the ECM is known to be under the control of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) enzymes, a family of proteases that exemplify procollagen-N-propeptidases in collagen fibril assembly and modification of ECM proteoglycans [7]. Following the first description of the prototype *Adamts1* gene and protein was identified, there are now 19 secreted proteinases known in humans with several common domains [8]. *Adamts16* gene has been shown to play an important role in

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hypertension, cryptorchidism, male infertility and aberrant renal development in rats [9–11]. *Adamts16* mRNA was identified in rat testes and ovaries at embryonic day 13 (E13) in both the spermatids and follicular granulosa cells of male and female rodents, respectively [9]. This study demonstrated a sex-specific gene expression of *Adamts16* mRNA and *Wilms tumor-1* gene (Wt1) in females and males during embryonic development. In addition, targeted knockout of *Adamts16* knockout (KO) in rats showed failed inguinoscrotal migration of the testes, UDT, and subsequent sterility, identifying an innovative role for *Adamts16* in testicular development [10].

This study examines the evidence from the *Adamts16* (KO) rat and interaction between the gubernaculum, collagen fibers and ECM during testicular descent. It highlights the structural organization of the gubernaculum and the relationship between collagen, ECM and *Adamts16* gene in rats during testicular descent.

1. Materials and methods

1.1. Specimens

Formalin-fixed specimens of *Adamts16* homozygous KO rats, along with heterozygous (HET) and Wild-type (WT) littermates, were provided from the University of Toledo College of Medicine and Life Sciences, Ohio, USA [12]. All animals and animal protocols used in this study were approved by the University of Toledo IACUC (protocol number 104573). The Adamts16 knockout rat model was generated, as previously described, by deleting 17 base pairs of the first exon of the Adamts16 gene from the genome of the Dahl salt-sensitive (S) rat using the zinc-finger nuclease technology [12]. Both the homozygous KO and the heterozygous rat had significantly low systolic and diastolic blood pressures compared with the salt-sensitive Dahl-S rat, while the homozygote Adamts16 KO rat also had intra-abdominal UDT [10]. This is consistent with loss of the inguinoscrotal phase of testicular descent in the Adamts16 KO rat.

These animals were used to determine the cause of UDT by serially sectioning of the whole pelvis for histological analysis at birth (Day 0), D2, D4 and D8, (n = 3/ age group), which corresponds to the timing of normal migration of the gubernaculum to the scrotum in the rat [4]. Sagittal sections of the pelves were cut into 5 μm thickness, placed in a water bath for a few minutes at 40–45 °C and adhered to the slides. Sections were then dried overnight in an incubator at 37 °C, de-waxed and rehydrated for standard Myers hematoxylin and Eosin (H&E) histology and Masson's trichrome staining. As the gubernaculum migrates inferomedially from the inguinal canal to the scrotum, sagittal sections do not show both the inguinal canal and scrotum together on every slide. Therefore, we used the relationship of the gubernaculum to the pubic bone as an estimate of the migration.

1.2. Histochemical staining

Paraffin sections were all de-waxed and rehydrated prior to staining by immersing slides into 100% ethanol for 5 min twice, 95% ethanol for 5 min twice and 5 min in 70% ethanol twice after which sections are immersed in tap water; followed by Myers hematoxylin solution for 3 min, and then rinsed with running tap water. The sections were then immersed in 95% ethanol for 1 min, followed by acidic Eosin Y working solution for 2 min. The slides were dehydrated with 100% ethanol for 3 min three times, followed by 5 min of xylene and mounted with DPX (Sigma-Aldrich, Cat**:** 44581). Sections stained for Masson's trichrome were performed at The Royal Children's Hospital Anatomical Pathology Department as per manufacturer's protocol.

1.3. Quantification of Masson trichrome

The quantitative method of analyzing intensity of collagen fibers in a Masson's trichrome section was used as previously described using Image J (Fiji version 1.52i USA) [14]. The plugin 'color deconvolution' developed by [15], which separates a three-channel image into three colors (Red, Green and Blue) was installed. The inguinal fat pad was labeled as the region of interest (ROI) in each genotype during post-natal day 0, 2, 4 and 8 (n = 3 per genotype/age). Threshold value was set and kept consistent during each age between the genotypes and the mean of collagen fibers where calculated using the intensity (without background) over the ROI.

1.4. Immunohistochemical and immunofluorescence staining of ADAMTS16

Paraffin embedded, sagittal sections of the pelves from the wild-type, heterozygous and homozygous Adamts16 rats were de-waxed and rehydrated prior to staining (Section 1.2.2). Sections were heated in the microwave (5 min) in 0.01 M citric acid buffer (pH 7.0) for antigen retrieval, then incubated for 2 hours in blocking buffer (10% bovine albumin (BSA) and 10% horse serum in phosphate-buffered saline, PBS) at room temperature (RT). Slides were then stained as shown below;

For immunohistochemical staining, the sections were then incubated with unconjugated ADAMTS16 antibody (Cat # orb523568, Biorbyt) at 1:50 dilution (5%BSA, 5% horse serum in PBS), with secondary anti-rabbit IgG biotinylated antibody (Cat# E0432, DAKO) at 1:300 dilution (in PBS). Appropriately diluted peroxidase-conjugated streptavidin was added for 30 min. After three PBS washes, the brown color was developed with diaminobenzidine (DAB), and a hematoxylin counterstain was applied. Slides there then de-hydrated with ethanol and xylene before mounting with DPX. The corresponding negative controls were stained omitting the anti-ADAMTS16 antibody.

For immunofluorescence staining, sections were incubated overnight at 4 °C with primary ADAMT16 antibody in 1:50 dilution (10% horse serum, 5% bovine serum albumin and PBS 0.1% triton) following the 2 hour blocking buffer. Slides were then rinsed with PBS and secondary Alexa-488 anti-rabbit (Cat#A11008, Invitrogen) were applied for 2 h at RT. After PBS washes, cell nuclei were counterstained with DAPI (4,6-diamidino-2-phenylindole). After a final PBS wash, slides were mounted with Mowiol, coverslipped and stored at 4 °C in the dark until confocal imaging section 1.2.6.

1.5. Image processing

Paraffin Sections from Wild-type, heterozygous and homozygous *Adamts16* stained with Myers hematoxylin and Eosin, Mason's trichrome and immunohistochemically stained with anti-ADAMTS16 antibody were captured on Nikon DS-Ri1 (Nikon, Widefield) microscope at 4.2*x* magnifications. Each image was then stitched together using FIJI software (version 1.52i, USA) and scale bars were added (mm).

1.6. Confocal imaging

Sections of the Adamts16 rat pelves stained with fluorescent markers were imaged on the Dragonfly spinning disc confocal microscope and images acquired via the Fusion Software (version 2.0, Andor, Northern Ireland). Confocal images were captured at 10× and 60× magnification. Laser at 488 mm for ADAMT16 in green and 637 mm excited the DAP1 blue, respectively to create merged images, which were edited with Fiji Image J (version 1.50; LOC1, University of Wisconsin-Madison, Madison, Wisconsin) for color, brightness, contrast correction and scale-bar inserted in Fig. 4.

1.7. Statistics

The mean value of collagen within each genotype at varies age groups are presented as mean +/− SEM using Prism 6 (GraphPad Software) in the ROI. *P*-values were calculated with an unpaired Students' *t*-test using a non-parametric test (Mann–Whitney test).

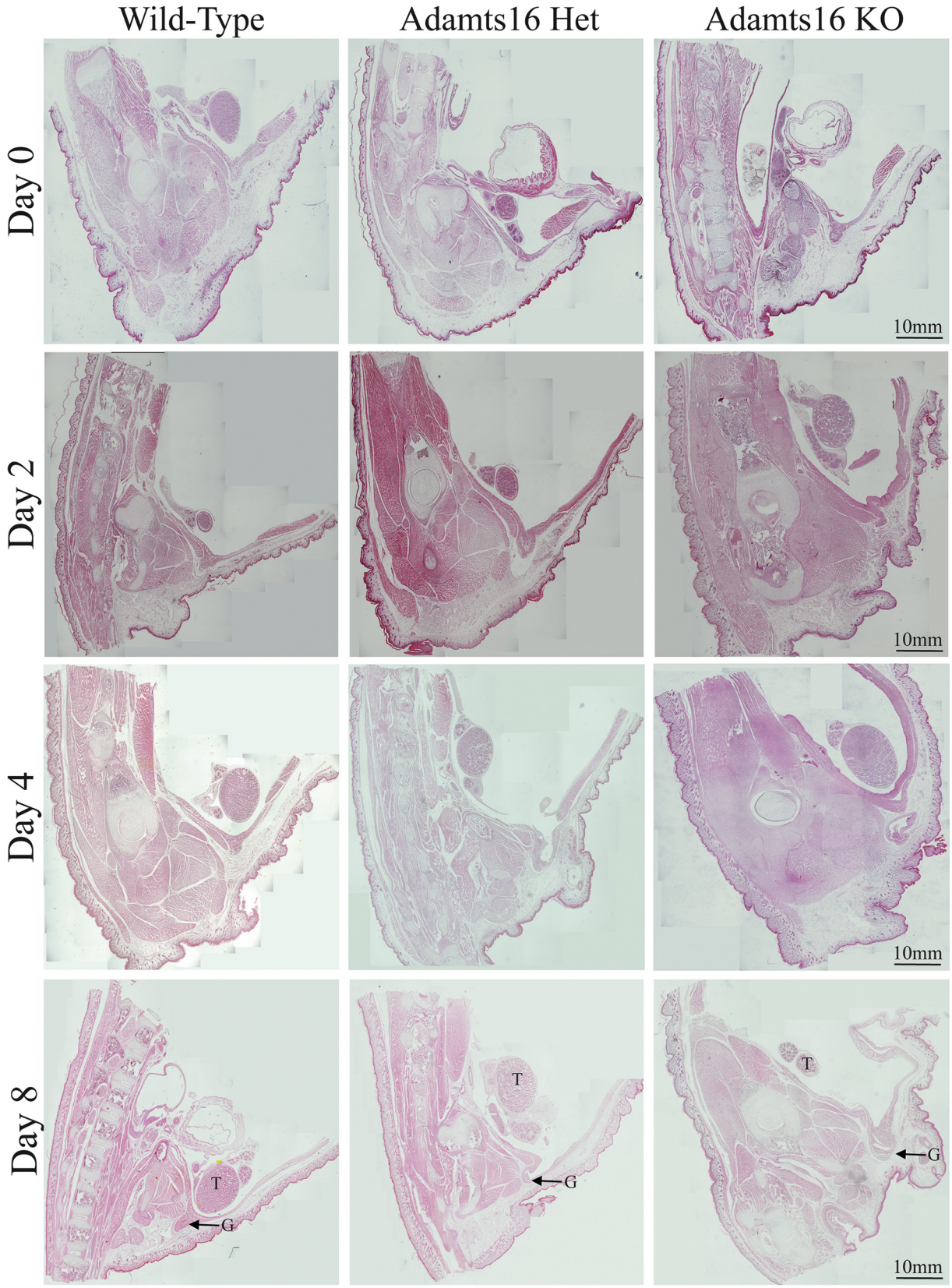

Fig. 1. *Adamts16* KO male rats develop undescended testes (UDT) during development. Haematoxylin and Eosin stain demonstrates the anatomical and morphological changes during testicular descent from day 0 to day 8 in WT, heterozygous and *Adamts16* KO rats. Rats that lack *Adamts16* gene developed UDT, as demonstrated with disrupted migration of the gubernaculum (G), testes (T). Magnification at ×4, images stitched together using image J, Scale bar 10 mm.

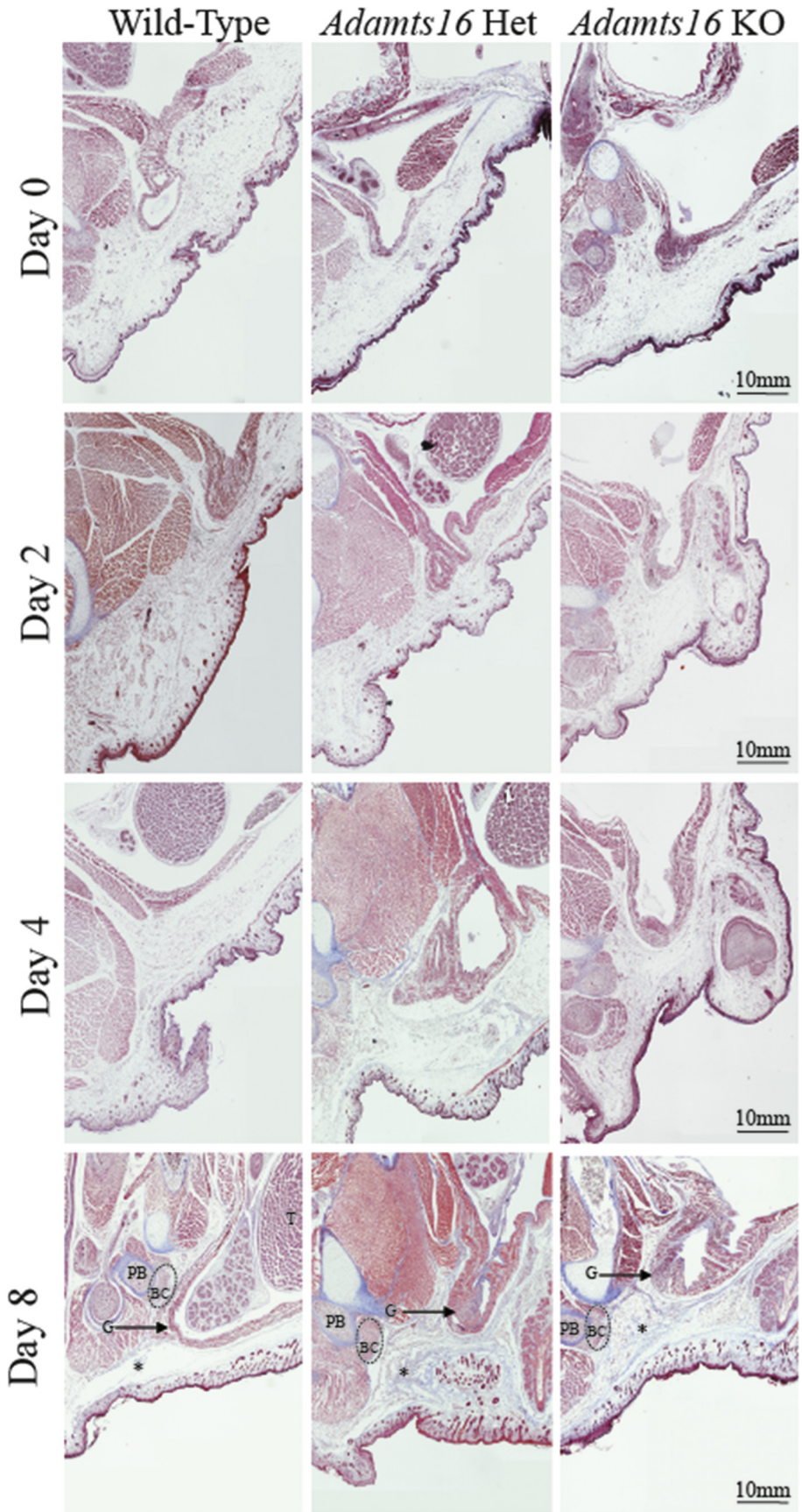

Fig. 2. Collagen fibers was considerably increased in the inguinal fat pad (IFP) (shown with *) of the heterozygous and *Adamts16* KO rats at day 8 when compared to the Wild-Type rat. Masson's trichrome stain shows an increase in collagen deposition (in blue) posterior of the gubernacular bulb within the IFP when compared to Wild-Type control. Key labels; Gubernaculum (G), Testes (T), Pubic bone (PB) and bulbocavernosus (BC). Magnification at 4×, images stitched together using image J, Scale bar 10 mm. Representative images of n = 3 rats per genotype/age were analyzed.

2. Results

H&E sections at 0, 2, 4 and 8 days of age showed that in the Wild-Type rat the end of the gubernaculum at day 0 was at the level of the pubic bone, but by 8 days old its distal end was in the scrotum. By contrast, in the heterozygous and *Adamts16* KO rat migration of the gubernaculum was disrupted (Fig. 1). Masson's trichrome staining showed that the collagen-rich ECM around the gubernaculum was deficient in the Wild-Type but dense in both the heterozygous and *Adamts16* KO rat (Fig. 2). The Wild-Type gubernaculum was loose under the skin, with a large clear space, which was absent in the heterozygous and homozygous animals at 8 days old. The quantitative analysis of the inguinal fat pad after Masson's trichrome staining showed a significant increase in the integrated density of collagen fibers in the heterozygous and *Adamts16* KO when compared to their Wild-Type counterpart at 8 days of age ($p < 0.05$) (Fig. 3). The immuno-stains showed that the collagen-rich ECM around the gubernaculum was deficient in the Wild-Type with increase ADAMTS16 protein, but dense in both the heterozygous and *Adamts16* KO rat along with reduced ADAMTS16 protein expression at day 8 (Fig. 4 A-B).

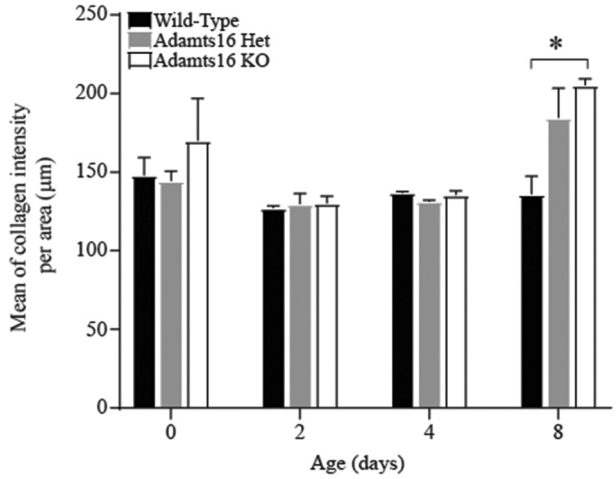

Fig. 3. Mean intensity of collagen fibers were significantly increased in heterozygous and *Adamts16* KO rat at post-natal day 8. Quantitative analysis showed an increase in the mean collagen fibers within the inguinal fat pad (IFP) (area in µm) of the heterozygous and *Adamts16* KO animals at day 8 post birth. Threshold of collagen and area was calculated using image J using color devolution plugin, following previously established method [15]. Statistical analysis using unpaired student's *t*-test was performed on graphpad prism. Standard error of mean was used and p-value of <0.05 (*) was considered significant n = 3 per genotype/age.

3. Discussion

The results of this study show that in the rat with disruption of the *Adamts16* gene, migration of the gubernaculum did not occur, with the distal end of the gubernaculum protruding just beyond the external inguinal ring, rather than reaching the scrotum, as in the Wild-Type littermate. In addition, Masson's trichrome staining identified persistence of collagen-rich ECM around the gubernaculum, which suggests that the *Adamts16* gene is a metalloproteinase required for dissolving the ECM in the inguinoscrotal fat pad (IFP) to allow elongation of the gubernaculum to the scrotum. When the enzyme function was interrupted, the ECM persisted and became a physical barrier for gubernacular migration.

Macroscopic and microscopic studies have been done of the gubernaculum, since it was first described by John Hunter in the 18th century, when he noted the lack of distal attachment of the gubernaculum immediately after descent [16]. Backhouse also observed a clear space around the gubernaculum in pigs [17], while recent studies have concentrated on testicular migration [18], or the gubernaculum itself [19, 20], with its developing cremaster muscle and processus vaginalis.

In both humans and rodents the inguinoscrotal phase of testicular descent requires the gubernaculum to elongate from the inguinal abdominal wall to the scrotum, and during this process there is no ECM attached to the outside of the gubernaculum [21], so that its distal end is completely free, and can be picked up by forceps during dissection [5]. In the rodent it also shows rhythmic contractility, which has been suggested may be important for orientating the tip of the gubernaculum so that it elongates towards the scrotum [4, 22].

The inguinoscrotal phase of decent is controlled by androgens, as it is completely missing in humans and animals models with androgen blockage [23]. How androgens act remains under debate, as these are no androgen receptors (AR) in the rodent gubernaculum during the critical window for action (E16–19) [24]. However, at this time there are AR in the IFP [25]. It has been suggested that the macroscopic clear space around the migration gubernaculum may be generated by the gubernaculum itself producing enzymes to dissolve the adjacent ECM [1]. Alternatively, it may be that androgen triggers release of metalloproteinase from the fibroblasts in the IFP, which have AR. Despite the fact that in this morphological study we were unable to determine the site of Adamts16 production, loss of this enzyme completely interrupted normal migration of the gubernaculum. One proposal to explain the action of androgen is that it triggers release of neurotrophins from the IFP which then act on the genitofemoral nerve (GFN) so that its sensory nerve endings in the IFP release calcitonin gene-related peptide (CGRP), which then provides a concentration gradient to guide gubernaculum migration [26]. It is important to acknowledge that heterozygous Adamts16 exhibited gubernaculum migration disruption and increase collagen fibers at 8 days of age, while previous studies demonstrated that only the homozygous Adamts16 rats had bilateral UDT and sterile [10]. As testicular descent in rats is normally completed between 18 and 21 days, we suggest that the decrease in Adamts16 mRNA within the heterozygous rat may have caused a delay in the early stages of descent and further investigation is necessary. We can, however, speculate that the delay in testicular descent in the Adamts16 heterozygous at day 8 may be compensated by Adamts10 which also interacts with Fibrillin-1 and stimulates its disposition in the ECM. Adamts16 mRNA expression is also activated cyclic AMP pathway on the differentiating luteinizing granulosa cells during sexual maturity at 50 days of age in rats [8, 27], this may influence changes in the cellular composition of the testis during spermatogenesis and ultimately affect fertility in the homozygous Adamts16 rat. Persistence of the collagen-rich ECM in animals with Adamts16 disruption and UDT suggests that remodeling of the ECM in the IFP is an essential part of the early process of inguinoscrotal descent of the testis, without which elongation of the gubernaculum is prevented.

In humans, it is recognized that the cause of UDT is likely to be multifactorial, but searches for genetic anomalies in the complex morphological processes required for the inguinoscrotal phase of descent are lacking. Disruption of inguinoscrotal descent of the testis in this rodent model suggests that abnormalities in gubernacular migration may be a fruitful place to find the common causes of UDT in children.

4. Conclusion

This study demonstrates the interplay between the *Adamts16* gene and testicular migration by highlighting the structural organization of the gubernaculum and the relationship with collagen in the ECM in rats. The disruption and inability of the gubernaculum to migrate in *Adamts16* KO rats illustrates the importance of this enzyme in breakdown of collagen fibers within the IFP creating a pathway from the testes to reach the scrotum. It is not known whether the *Adamts16* gene has similar function in the human, but this would be worth investigating to see if it may be involved in facilitating the complex morphological process of testicular descent.

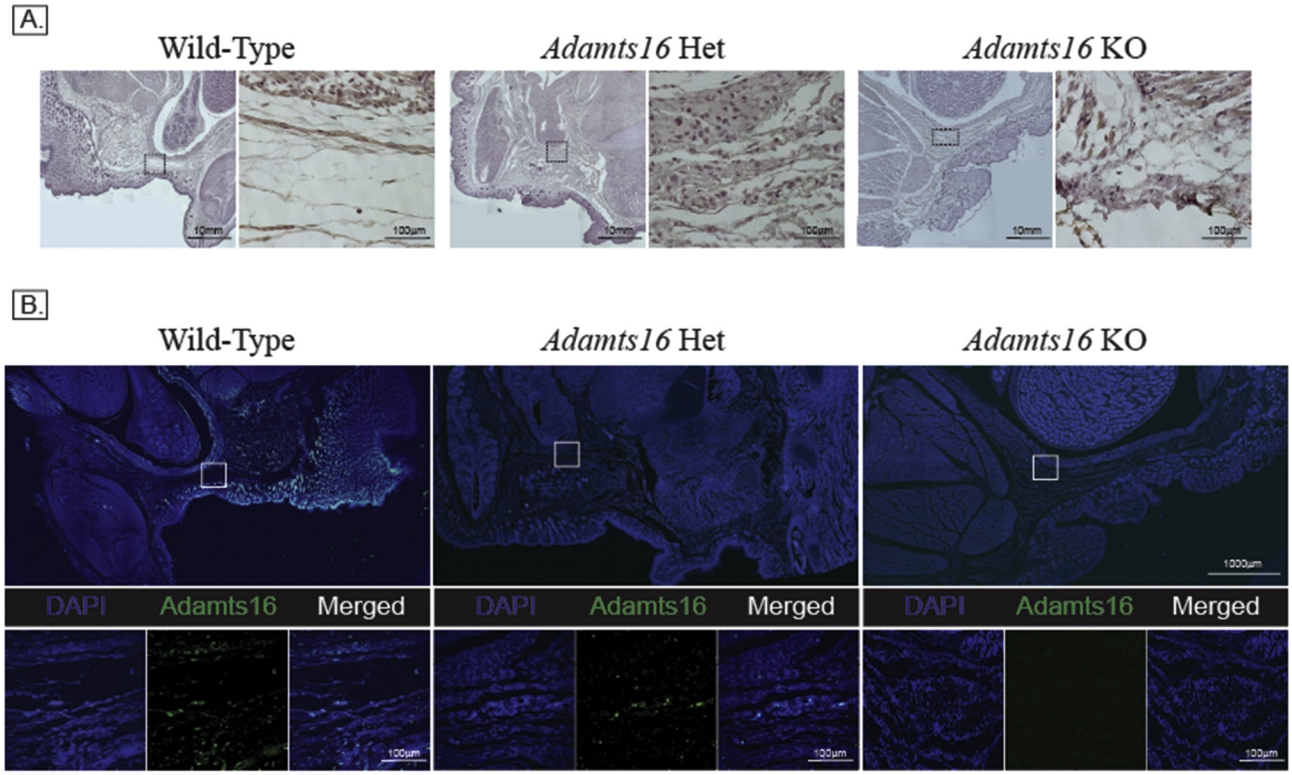

Fig. 4. ADAMTS16 protein is localized in the inguinal fat pad in rat and reduced in the Adamts16 KO animals at post-natal day 8. Wild-type rat shows localisation of the ADAMTS16 protein within the inguinal fat pad (IFP) near the gubernaculum, with a reduction observed in the homozygous KO rat using (A) Immunohistochemistry staining (ADAMTS16 protein shown in brown) and (B) Immunofluorescence staining (ADAMTS16 protein shown in green). White and black squares illustrate the IFP and the area images at higher magnification. Scale bar shown, images of the pelves were taken at 20× magnification and stitched together via imaging J, whereas the single images were taken at 40× magnification using Nikon DS-Ri1 or with Dragonfly spinning disc confocal microscope, 3 independent rats per genotype at day 8 were used.

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Author contribution statement

Author GS performed experiments, analyzed data, designed the figures and wrote the manuscript. TB and SAA cut and sections from the paraffin blocks. RL organized the transport of the paraffin blocks, AC processed the paraffin blocks and BM and BJ provided the genotyping and tissue samples from Toledo, Ohio, USA, and JH provided critical revision. None of the authors has any conflict interest to disclose.

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