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Partially redundant functions of *Adamts1* and *Adamts4* in the perinatal development of the renal medulla

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Abstract

Adamts4 encodes a widely-expressed proteinase thought to be involved in processes ranging from cartilage metabolism to ovarian follicle development. To study its physiological roles, *Adamts4*-null mice were created by gene targeting. Unexpectedly, these were found to be phenotypically normal, suggesting that other gene(s) may compensate for its loss. *Adamts4*^{-/-} mice were therefore crossed with a strain lacking *Adamts1*, whose pattern of expression and substrate specificity overlap that of *Adamts4*. Most (>95%) *Adamts1*^{-/-};*Adamts4*^{-/-} mice died within 72h after birth with a marked thinning of the renal medulla. The renal defect was not observed in embryonic *Adamts1*^{-/-};*Adamts4*^{-/-} kidneys, but became apparent around birth. The few (<5%) *Adamts1*^{-/-};*Adamts4*^{-/-} animals to reach adulthood had the same renal phenotype seen in newborns. This study is thus the first to report *Adamts4* expression and function in the mammalian kidney, and to demonstrate that *Adamts1* and *Adamts4* play redundant and essential roles in perinatal kidney development.

Keywords

Adamts1; Adamts4; renal medulla; renal development; knockout mice

Introduction

Adamts1 and *Adamts4* are members of the *Adamts* (a disintegrin and metalloproteinase with thrombospondin motifs) family, a group of 20 secreted zinc metalloproteinases (Salter et al., 2010; Shiomi et al., 2010). Whereas all ADAMTS proteins share a high degree of homology and specific structural characteristics, they can be subdivided into specific clades according

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to their functional properties and substrate specificities (Salter et al. 2010; Apte, 2009). ADAMTS1 and ADAMTS4 are part of the proteoglycanase clade due to their ability to cleave proteoglycan-rich lecticans such as aggrecan (ACAN), versican (VCAN), brevican and neurocan, the expression of the latter two being largely restricted to the CNS (Apte, 2009; Porter et al., 2005; Nakamura et al., 2000). ADAMTS1 is able to cleave both ACAN and VCAN, and also has the ability to inhibit VEGF (Vascular Endothelial Growth Factor) and FGF-2 (Fibroblast Growth Factor 2) induced angiogenesis under certain conditions (Porter et al., 2005). *Adamts1* knockout mice have multiple phenotypic abnormalities including an increased rate of perinatal death, poor growth, developmental kidney defects and reduced fertility (Shindo et al., 2000; Mittaz et al., 2004; Mittaz et al., 2005). ADAMTS4 is able to cleave ACAN, VCAN and brevican (Gao et al. 2002). Due to its inducible expression in articular cartilage and ability to degrade articular ACAN, ADAMTS4 was notably proposed to be involved in the pathogenesis of osteoarthritis (Glasson et al., 2004). However, more recent studies involving the generation of *Adamts4* and *Adamts5* knockout mice showed that *Adamts5* is of much greater importance in the metabolism of cartilage ACAN, whereas *Adamts4* appears expendable (Glasson et al., 2004; Majumdar et al., 2007; Stanton et al., 2005). Indeed, *Adamts4*^{-/-} mice have been reported to develop normally and are devoid of morphological or physiological defects, despite the widespread expression and known physiological roles of *Adamts4* (Glasson et al. 2004).

In mice as in all mammals, the kidneys develop in 3 distinct phases during embryogenesis: the pronephros, the mesonephros and finally the metanephros (Kuure et al., 2000). Only the metanephros persists and differentiates completely to become the functional, adult kidney. In mice, the metanephric kidney first appears at dpc (day post-conception) 10.5 (Bard, 2002). Its development originates from two transient intermediate structures known as the metanephric mesenchyme and the ureteric bud. At dpc11.5, the ureteric bud invades the metanephric mesenchyme, which initiates a phase of reciprocal induction of both structure's development, and starts branching morphogenesis (Dressler, 2006). The ureteric bud differentiates into epithelial branches, at the tips of which metanephric mesenchyme cells form clumps that will eventually differentiate into epithelial cells, which in turn will become the glomeruli and convoluted tubules of the nephrons (Dressler, 2006). As branching morphogenesis proceeds, the ureteric bud splits countless times to form the collecting ducts of the kidney, growing in a centrifugal pattern and giving rise to most of the renal medulla (Lechner and Dressler, 1997).

The molecular biology of kidney branching morphogenesis is an area of intense investigation, and a number of molecular mediators have been identified (Bard, 2002). Among these are heparan sulfate and chondroitin sulfate proteoglycans, for which biochemical and genetic evidence exists that suggests their involvement in the branching of the ureteric bud (Steer et al. 2004). VCAN is a chondroitin sulfate proteoglycan that is expressed in the kidney, and is thought to be a component of the extracellular matrix (ECM) of renal proximal tubule epithelial cells (Selbi et al. 2006) and the distal straight tubules (Kawashima et al., 1999). ADAMTS1 can cleave VCAN, and is expressed in the developing convoluted tubules and loops of Henle during embryogenesis (Mittaz et al., 2005). *Adamts1*^{-/-} mice suffer from a developmental dysgenesis of both the renal medulla and cortex, which has been proposed to be the result of the loops of Henle failing to descend normally into the medulla during the perinatal period (Mittaz et al., 2005). It is therefore possible that a defect in proteolytic processing of VCAN could contribute to the renal dysgenesis observed in *Adamts1*^{-/-} mice. Whether other proteoglycanases and the proteolytic processing of their substrates may be required for kidney development remains unknown.

The aim of the present study was to determine the physiological roles of *Adamts4*. In confirmation of a previous study (Glasson et al., 2004), we found *Adamts4*^{-/-} mice to be developmentally and physiologically normal, suggesting that other members of the *Adamts* proteoglycanase clade can compensate for its loss. Due to the structural and functional similarities between *Adamts1* and *Adamts4*, we tested the hypothesis that they may function in a redundant manner by generating *Adamts1*^{-/-};*Adamts4*^{-/-} mice. The latter were found to die perinatally due to severe developmental defects of the renal medulla. This work therefore defines a novel function of *Adamts4* in the mammalian kidney, and demonstrate that *Adamts1* and *Adamts4* act in a partially redundant manner to ensure proper perinatal development of the renal medulla.

Results

Perinatal lethality and kidney defects in *Adamts1*^{-/-};*Adamts4*^{-/-} mice

To determine the physiological roles of *Adamts4*, *Adamts4*^{-/-} mice were generated by gene targeting (Fig. 1). *Adamts4*^{-/-} pups were born from heterozygote parents in the expected Mendelian ratio, grew normally, had normal life expectancies and fertility, and were otherwise morphologically and behaviorally indistinguishable from their wild-type and heterozygote littermates (not shown). Extensive histopathologic evaluation of tissues from newborn and adult *Adamts4*^{-/-} mice of both sexes failed to identify differences with tissues from control mice (not shown). These results therefore confirm those published by another group (Glasson et al. 2004).

As *Adamts1* and *Adamts4* share overlapping substrate specificities and patterns of expression (Porter et al., 2005), we hypothesized that *Adamts1* may be able to compensate for the loss of *Adamts4* in certain tissues. We therefore generated *Adamts1*^{-/-} mice and mated them to the *Adamts4*^{-/-} strain to generate a double knockout. Although born in the expected Mendelian ratio, the vast majority of *Adamts1*^{-/-};*Adamts4*^{-/-} mice (>95%) died within 72h after birth. Thorough comparative histological examination of all organs was conducted on newborn wild-type, *Adamts1*^{-/-}, *Adamts4*^{-/-} and *Adamts1*^{-/-};*Adamts4*^{-/-} mice (n = 3 males and 3 females/genotype) to identify lesions that could cause the perinatal mortality phenotype. In every *Adamts1*^{-/-};*Adamts4*^{-/-} pup examined, the renal pelvis was dilated and the renal medulla was irregularly and markedly thinned (Fig. 2). Both kidneys were invariably affected. Conversely, the renal cortex seemed to be completely spared and retained its typical histological appearance, with normal glomeruli and tubules. In contrast to what was observed in the *Adamts1*^{-/-};*Adamts4*^{-/-} newborn pups, all wild-type, *Adamts1*^{-/-} and *Adamts4*^{-/-} mice had morphologically normal kidneys at birth (Fig. 2). Other than the kidneys, organs and tissues (brain, thymus, lungs, heart, liver, pancreas, spleen, stomach and intestinal tract, skeletal muscle, male and female gonads and reproductive tracts) from every individual of all 4 genotypes were devoid of morphological abnormalities.

In order to determine the onset of the renal phenotype observed in *Adamts1*^{-/-};*Adamts4*^{-/-} newborn mice, the kidneys of 15.5 dpc and 17.5 dpc *Adamts1*^{-/-};*Adamts4*^{-/-} and wild-type embryos were examined (Fig. 3A–D). We could not discern any sign of the renal lesions described above in the *Adamts1*^{-/-};*Adamts4*^{-/-} embryonic kidneys, indicating that the onset of the kidney defect occurs very near or shortly after the time of birth. A very limited number of *Adamts1*^{-/-};*Adamts4*^{-/-} animals survived beyond the initial 72h (<5%, n = 2 males and 1 female), and these lived to adulthood. Whereas the males seemed to grow normally and appeared healthy, the female grew poorly and had poor body condition. All organs and tissues from these individuals were collected and examined microscopically, but again no anomalies could be identified other than in the kidneys. The kidneys of adult *Adamts1*^{-/-};*Adamts4*^{-/-} individuals showed the same pelvic/medullary defects described above for the newborns (Fig. 3E, F).

Renal medullary thinness and pelvis dilation are known to occur in cases of hydronephrosis; the medullary atrophy resulting from the increased intrapelvic pressure induced by a urinary obstruction. To verify if urinary obstruction could explain the renal phenotype observed in *Adamts1*^{-/-};*Adamts4*^{-/-} mice, careful histological examination of the lower urinary tract of newborn mice was performed. All the structures examined, including the ureteropelvic junction, ureters, urinary bladders and urethras were found to be patent and indistinguishable from those collected from WT animals (Fig 3G–I and not shown). This suggested that the renal abnormality found in *Adamts1*^{-/-};*Adamts4*^{-/-} mice was not the consequence of a mechanical obstruction of the urinary tract. However, the latter could not be ruled out entirely, as we were unable to assess the patency of the urinary tract with a functional assay.

Expression of Adamts1, Adamts4, Vcan and Acan in the mouse kidney

To study the perinatal expression of *Adamts1* and *Adamts4* and its potential substrates in the murine kidney, we conducted a real-time RTPCR timecourse analysis of *Adamts1*, *Adamts4* and *Vcan* expression in WT kidneys collected from 15.5 and 17.5 dpc embryos, as well as in newborn and 7 day-old animals (Fig. 4A–C). *Adamts1* and *Adamts4* mRNA were detected at high and statistically unvarying ($P > 0.05$) abundance at all stages examined. *Vcan* mRNA was detected at highest levels at 15.5 dpc, and decreased gradually thereafter ($P < 0.05$). *Acan* mRNA was also detectable by RTPCR in the newborn kidney (Fig. 4D), but only at very low levels that did not permit reliable quantitative analyses.

As *Adamts4* expression in the kidney had not been previously reported, immunohistochemical analyses were done to localize its expression within the kidney. In both newborn (Fig. 5B) and adult (Fig. 5D, F) kidneys, ADAMTS4 was predominantly detected in the proximal and distal convoluted tubules of the renal cortex, and little or no signal was detected in glomeruli or the collecting ducts and loops of Henle of the renal medulla.

Absence of VCAN and ACAN proteolytic processing defects in *Adamts1*^{-/-};*Adamts4*^{-/-} mice

To determine if the kidney defects observed in *Adamts1*^{-/-};*Adamts4*^{-/-} mice could be attributed to abnormal proteolytic processing of ACAN and/or VCAN at their ADAMTS cleavage points, Western blotting was performed on WT, *Adamts1*^{-/-}, *Adamts4*^{-/-} and *Adamts1*^{-/-};*Adamts4*^{-/-} newborn kidneys. Levels of full-length VCAN appeared lower in *Adamts1*^{-/-};*Adamts4*^{-/-} samples, possibly related to the loss of medullary tissue (Fig. 6). More importantly however, abundant cleaved ACAN and VCAN could be detected in all *Adamts1*^{-/-}, *Adamts4*^{-/-} and *Adamts1*^{-/-};*Adamts4*^{-/-} samples, suggesting that ACAN and VCAN processing in the kidney was not perturbed by the loss of either or both enzymes (Fig. 6).

Discussion

Roles have been proposed for *Adamts4* in processes as wide-ranging as ovulation, osteoarthritis and tumor invasion (Glasson et al., 2004; Held-Feindt et al., 2006; Richards et al., 2005). The finding that the targeted deletion of *Adamts4* in mice is apparently without consequence was therefore unexpected and somewhat surprising (Glasson et al., 2004 and the present study). As the ADAMTS gene family encodes a number of related proteases with overlapping substrate specificities and patterns of expression, we tested the hypothesis that *Adamts1*, the *Adamts* gene that is most highly phylogenetically related to *Adamts4* (Jones and Riley, 2005), could compensate for its loss. Our finding that *Adamts1*^{-/-};*Adamts4*^{-/-} mice suffer from a severe developmental defect of the renal medulla defines for the first time a functional redundancy between these two enzymes. *Adamts1*^{-/-} mice are

characterized by developmental kidney defects that are highly similar to those observed in *Adamts1*^{-/-};*Adamts4*^{-/-} animals, but that are much less severe, as most *Adamts1*^{-/-} mice survive to adulthood (Mittaz et al., 2004). Indeed, in our study, *Adamts1*^{-/-} mice did not have obvious kidney defects at birth, consistent with the results of a previous study (Shindo et al., 2000), but contradicting those of another (Mittaz et al., 2004). The reason for these discrepancies are unclear, but may be related to factors such as the background strain of the knockout mice. Notwithstanding, the redundancy of function in the case of *Adamts1* and *Adamts4* could be best described as partial, as loss of *Adamts4* can be completely compensated for by *Adamts1*, whereas loss of *Adamts1* results in phenotypic anomalies that cannot be compensated for by *Adamts4*, but that are markedly less severe than when both genes are inactivated.

The precise pathogenesis of the renal lesions observed in *Adamts1*^{-/-};*Adamts4*^{-/-} mice remains unclear, but they are morphologically similar to what is observed in cases of hydronephrosis (Cotran et al., 1999). In these cases, a mechanical obstruction of the outflow of urine (which can originate anywhere from the ureteropelvic junction to the urethra) leads to the dilation of the renal pelvis and calyces and the progressive atrophy of the kidney parenchyma. *Adamts1*^{-/-} mice have been reported to have a partial fibrous obstruction of the ureteropelvic junction (Shindo et al., 2000). We carefully examined all structures of the lower urinary tract in embryonic, newborn and adult *Adamts1*^{-/-};*Adamts4*^{-/-} mice and found them to be normal, thus eliminating the possibility of mechanical urinary obstruction leading to hydronephrosis. Similarly, the study of Mittaz et al. (2004) conducted on *Adamts1*^{-/-} mice did not reveal any evidence of ureteropelvic fibrous obstruction. The normal outflow of urine from the kidneys to the urinary bladder is dependent on pyeloureteric peristalsis (Lang et al., 2008), and a defect in this process can create a functional obstruction that can also lead to hydronephrosis. Theoretically, the lack or a decrease of pelvis peristaltic movements would create lesions similar to those observed in our affected mice. While we consider this to be an unlikely scenario, we cannot exclude that such a functional urinary obstruction may contribute to the *Adamts1*^{-/-};*Adamts4*^{-/-} renal phenotype.

A more likely explanation for the renal medullary defect in *Adamts1*^{-/-};*Adamts4*^{-/-} mice could be a developmental dysgenesis, as has been proposed for *Adamts1*^{-/-} mice (Mittaz et al., 2004). *Adamts1* and *Adamts4* are secreted proteoglycanases that associate with the ECM, where they act to remodel it (Porter et al., 2005). Both genes are expressed in the developing convoluted tubules (Mittaz et al., 2004 and the present study). In all likelihood, there is remodeling of the kidney's ECM around birth that occurs in conjunction with the elongation of medullary tubules (Cha et al., 2001). The descent of the loops of Henle into the medullary region during postnatal kidney development appears to be inhibited in *Adamts1*^{-/-} and *Adamts1*^{-/-};*Adamts4*^{-/-} mice (Mittaz et al., 2004 and the present study). Therefore, an extracellular matrix defect caused by ADAMTS1/4 deficiency may prevent the proper expansion of the renal medulla during postnatal kidney development, ultimately leading to a medullary dysgenesis. For this reason, we theorized that loss of ADAMTS1/4 activity would result in abnormal processing of the kidney ECM components ACAN and VCAN, which are known substrates of both ADAMTS1 and ADAMTS4 (Porter et al., 2005). Surprisingly however, we were not able to observe meaningful differences in the abundance of ACAN and VCAN that had been cleaved at the ADAMTS cleavage site in *Adamts1*^{-/-}, *Adamts4*^{-/-} and double knock-out newborn kidneys. This would suggest that proteoglycanases other than ADAMTS1/4 are responsible for ACAN and VCAN cleavage in the developing kidney, and that ACAN and VCAN processing defects are not involved in the pathogenesis of the kidney defects observed in *Adamts1*^{-/-} and *Adamts1*^{-/-};*Adamts4*^{-/-} mice. The mechanism by which *Adamts1* and *Adamts4* promote normal renal medullary development therefore remains unclear. Both enzymes may have proteolytic activity on substrates other than

VCAN or ACAN. Another possibility is that they may exert their functions via non-catalytic activities. ADAMTS1 and ADAMTS4 both possess disintegrin, cysteine-rich and spacer domains as well as thrombospondin-type 1 motifs, all of which likely have important biological functions that have not yet been well defined (Jones and Riley, 2005, Nagase and Fushimi, 2008). Further work will therefore be required to identify catalytic and non-catalytic mechanisms of ADAMTS1 and ADAMTS4 action in the developing kidney.

Interestingly, the failure of renal medullary development that we observe in *Adamts1^{-/-};Adamts4^{-/-}* mice bears a strong resemblance to the phenotype observed in a mouse model featuring conditional inactivation of the *Wnt7b* gene (Yu et al., 2009). *Wnt7b* encodes a secreted signaling molecule that is strongly expressed in the collecting duct epithelium, and its expression is thought to be regulated by a signaling mechanism involving the heterodimeric laminin receptor $\alpha 3\beta 1$ integrin (Yu et al., 2009; Liu et al., 2009). Accordingly, the conditional inactivation of the genes encoding the $\alpha 3$ or $\beta 1$ integrin subunits in the ureteric bud also results in medullary developmental defects (Liu et al., 2009; Wu et al., 2009). One of the main functions of integrins is to relay to the cell information originating from adjacent cells and the ECM. It is therefore tempting to speculate that ECM defects that arise from ADAMTS1/4 deficiency could perturb $\alpha 3\beta 1$ integrin/WNT7b signaling, thereby giving rise to the medullary defect. Investigation of these signaling pathways in the *Adamts1^{-/-};Adamts4^{-/-}* model will be required to test this hypothesis.

In summary, this study describes for the first time that *Adamts4* is expressed in the kidney, and plays a role in perinatal renal development that is partially redundant with that of *Adamts1*. The mechanism of ADAMTS1 and ADAMTS4 action in this context remains undefined, but apparently does not involve the proteolytic processing of ACAN or VCAN.

Experimental procedures

Gene targeting and animal models

As the phenotype of *Adamts4^{-/-}* mice had not been reported at the beginning of this study, a mouse strain bearing a floxed *Adamts4* allele was created in order to circumvent potential embryonic lethality in *Adamts4^{-/-}* mice by conditional gene targeting if necessary. The *Adamts4^{fllox}* allele was created using standard gene targeting techniques, as previously described (Boyer et al., 2010). Briefly, a targeting construct was built so as to flank the second and third exons of the *Adamts4* gene with loxP sites. Homologous sequences were isolated from R1 embryonic stem (ES) cell genomic DNA by PCR using the Expand long template PCR system (Roche Molecular Biochemicals, Laval, QC, Canada), and inserted into the targeting vector so as to generate the construct illustrated in Fig. 1Ab. Targeting construct DNA was introduced into the R1 ES cell line (Nagy et al., 1993) by electroporation, and recombinant colonies were selected by addition of 400mg/ml Geneticin (Invitrogen, Burlington, ON, Canada). Colonies were analyzed by Southern blotting to screen for proper homologous recombinants as previously described (Sambrook et al., 1989). Sixteen of 384 colonies screened showed a second band consistent with the expected size for the targeted allele (Fig. 1B). Two of these lines were subsequently used to generate chimeric males according to standard protocols. Chimeras derived from both cell lines sired pups heterozygous for the targeted allele (Fig 1Ac). To generate a null *Adamts4* allele (Fig. 1Ad), *Adamts4^{fllox/+}* mice were mated to the B6.C-Tg(CMV-cre)1Cgn/J strain (The Jackson Laboratory, Bar Harbor, ME) which expresses Cre in the germline. Following PCR verification of the *Adamts4^{-/-}* allele (Fig. 1C), the Cre transgene was bred out from *Adamts4^{+/-};Tg(CMV-cre)1Cgn* mice on the following generation. Genotyping analyses were performed by PCR on DNA obtained from tail biopsies as previously described (Boyer et al., 2010) using the oligonucleotide primer sequences CAGCCTCTAGTCCTACTCCACACA, CCCCTAACCCCAAATCCCAGTGT and

TAGGAACCTTCAATTCCCCGCAAGA. This protocol results in PCR products of approximately 220bp for the wild-type *Adamts4* allele, 260bp for the floxed allele, and 200bp for the knockout allele (Fig. 1C). Loss of *Adamts4* expression in *Adamts4*^{-/-} mice was confirmed by RT-PCR and immunoblotting performed on adult kidney RNA and protein samples as detailed below (Fig. 1D, E). The *Adamts1*⁻ allele was generated from a previously-described *Adamts1*^{fllox} allele (Mittaz et al, 2004) by mating *Adamts1*^{fllox/+} mice to the B6.C-Tg(CMV-cre)1Cgn/J strain, as described for *Adamts4* above. Genotyping for *Adamts1* was performed as described (Mittaz et al., 2004). All animal procedures were approved by the Institutional Animal Care and Use Committee and were conform to the USPHS Policy on Humane Care and Use of Laboratory Animals.

Quantitative and semi-quantitative RT-PCR

For quantitative measurement of *Adamts1*, *Adamts4* and *Vcan* mRNA during kidney development, RNA was isolated from wild-type mouse kidneys using the RNeasy Mini kit (Qiagen, Mississauga, Ontario, Canada). Real-time RT-PCR was done using the SuperScript™ III Platinum® Two-Step qRT-PCR Kit (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions, and using 200ng RNA samples and the following primers: *Adamts1*; CATGGTGATCACTCATCTCACATG (sense) and CTCATCAGATTTCTTGCCCAGAAG (antisense), *Adamts4*; CCAGACCCTACGCAGCTTCTG (sense, located in exon II) and CAGTTCATGAGCAGCAGTGAAGG (antisense, located in exon III), *Vcan*; GAAACGGGAGATGGGCAATGCCT (sense) and CTGAAATGATGAGTGAAGCACATGT (antisense). Primers for the housekeeping gene *Rpl19* were as previously described (Boyer et al., 2010). Thermal cycling and data capture were performed using a Rotor-Gene RG-3000 apparatus (Corbett Research, Mortlake NSW 2137, Australia) and using to the manufacturer's recommended conditions. Relative gene expression was calculated using Rotor-Gene 6.0 software (Corbett Research) by comparing amplification curves to a series of standard curves obtained by amplification of serial dilutions of a reference cDNA fragment. All data were subsequently normalized by dividing expression levels of individual genes by corresponding *Rpl19* values.

Semi-quantitative RT-PCR was performed to detect *Acan* (Fig. 4) and *Adamts4* (Fig. 1) expression. RNA samples (200ng per RT reaction) were isolated from newborn mouse kidney, adult brain and adult articular cartilage as described above, and RT-PCR performed using the Superscript one-step RT-PCR kit (Invitrogen) as directed by the manufacturer. Primers used for *Adamts4* and *Rpl19* were as described above, primers for *Acan* were CACTGGGAAGAGCCTCGAATCACCT (sense) and CTTCTTTAATGGATTCCTTATATGACA (antisense). Cycling conditions were 94°C for 3 minutes followed by 25 cycles (*Rpl19*) or 40 cycles (*Adamts4*, *Acan*) of 94°C for 45 seconds, 55°C for 30 seconds, 72°C for 1 minute. Preliminary experiments done for *Rpl19* ensured that the cycle number selected fell within the linear range of PCR amplification (data not shown), whereas 40 cycles were done for *Adamts4* and *Acan* to ensure maximum sensitivity of the assay. PCR products were separated by electrophoresis on 1.8% TAE-agarose gels containing ethidium bromide and photographed under UV light.

Western Blotting

Proteins were extracted from newborn mouse kidneys using T-PER tissue protein extraction reagent (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's instructions, and 20µg protein samples incubated for 1h at 37°C with 0.01 units/sample chondroitinase ABC (Sigma, St. Louis, MO, catalog #c3667). Samples were separated by SDS-PAGE (10% acrylamide), transferred to polyvinylidene difluoride membranes, blocked in 5% non-fat dried milk in tris-buffered saline with 0.1% Tween-20, and incubated

overnight with the primary antibody at 4°C. Primary antibodies were used to detect either intact forms of ACAN and VCAN (Shi et al. 2004), or neoepitopes generated by ADAMTS1/4-mediated cleavage of ACAN (Sahebjam et al., 2007) and VCAN (Abcam, Cambridge, MA, catalog # 19345). Following washes and incubation with a horseradish peroxidase-conjugated secondary antibody (GE Amersham, Piscataway, NJ), the protein bands were visualized by chemiluminescence using ECL Plus Western Blotting Detection Reagents (GE Amersham) and High Performance Chemiluminescence film (GE Amersham). The membranes were then stripped and reprobed with a mouse anti-ACTB (β -actin) antibody (Santa Cruz Biotechnology Inc., catalog # sc-47778). The molecular weight of immunoreactive bands was estimated by comigration of a ladder of rainbow molecular weight markers (GE Amersham). To confirm loss of full-length ADAMTS4 protein in *Adamts4*^{-/-} mice (Fig 1E), blotting was done as described above, except chondroitinase treatment was omitted. The primary antibody was raised against the ADAMTS4 C-terminus (Thermo Fisher Scientific catalog # PA5-14340).

Immunohistochemistry

Embryonic and adult mouse tissues were fixed in 10% buffered formalin, embedded in paraffin, and cut at a thickness of 6 μ m. Deparaffinized and rehydrated sections were boiled for 10min in 10mM sodium citrate, pH 6.0. Endogenous peroxidase activity was quenched by 10min treatment with 3% hydrogen peroxide. Immunohistochemistry was then performed using the VECTASTAIN Elite ABC Kit (Vector Labs, Burlingame, CA, USA) according to manufacturer's instructions. The primary antibody against ADAMTS4 (Aviva systems biology, San Diego, CA, catalog # ARP41556) was used at a dilution of 1:200 for 2 hours at room temperature. Control sections were probed with non-specific rabbit IgG (10 μ g/ml final concentration). Staining was performed using the 3,3'-diaminobenzidine (DAB) peroxidase substrate kit (Vector Labs) as directed, and slides were lightly counterstained with hematoxylin prior to mounting.

Statistical methods

Real-time RTPCR results were analyzed by Tukey HSD's all pairs comparison in order to evaluate potential effects of developmental stage on *Adamts1*, *Adamts4* and *Vcan* mRNA levels. *P* values lower than 0.05 were considered statistically significant. Analyses were performed using JMP® software (SAS Institute, Cary, NC, USA).

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References

- Apte SS. A disintegrin-like and metalloprotease (reprolysin-type) with thrombospondin type 1 motif (ADAMTS) superfamily: functions and mechanisms. *J Biol Chem.* 2009; 284:31493–31487. [PubMed: 19734141]
- Bard JBL. Growth and death in the developing mammalian kidney: signals, receptors and conversations. *Bioessays.* 2002; 24:72–82. [PubMed: 11782952]
- Boyer A, Lapointe E, Zheng X, Cowan RG, Li H, Quirk SM, Demayo FJ, Richards JS, Boerboom D. WNT4 is required for normal ovarian follicle development and female fertility. *FASEB J.* 2010; 24:3010–3025. [PubMed: 20371632]

- Cha JH, Kim YH, Jung JY, Han KH, Madsen KM, Kim J. Cell proliferation in the loop of henle in the developing rat kidney. *J Am Soc Nephrol*. 2001; 12:1410–1421. [PubMed: 11423570]
- Cotran, RS.; Kumar, V.; Collins, T. Robbins pathologic basis of disease. 6th ed.. W.B. Saunders; Philadelphia, PA: 1999. Urinary tract obstruction; p. 988
- Dressler GR. The cellular basis of kidney development. *Annu Rev Cell Dev Biol*. 2006; 22:509–529. [PubMed: 16822174]
- Gao G, Westling J, Thompson VP, Howell TD, Gottschall PE, Sandy JD. Activation of the proteolytic activity of ADAMTS4 (Aggrecanase-1) by C-terminal truncation. *J Biol Chem*. 2002; 277:11034–11041. [PubMed: 11796708]
- Glasson SS, Askew R, Sheppard B, Carito BA, Blanchet T, Ma H, Flannery CR, Kanki K, Wang E, Peluso D, Yang Z, Majumdar MK, Morris EA. Characterization of and osteoarthritis susceptibility in ADAMTS-4-knockout mice. *Arthritis Rheum*. 2004; 50:2547–2558. [PubMed: 15334469]
- Held-Feindt J, Paredes EB, Blömer U, Seidenbecher C, Stark AM, Mehdorn HM, Mentlein R. Matrix-degrading proteases ADAMTS4 and ADAMTS5 (disintegrins and metalloproteinases with thrombospondin motifs 4 and 5) are expressed in human glioblastomas. *Int J Cancer*. 2006; 118:55–61. [PubMed: 16003758]
- Jones GC, Riley GP. ADAMTS proteinases: a multi-domain, multi-functional family with roles in extracellular matrix turnover and arthritis. *Arthritis Res Ther*. 2005; 7:160–169. [PubMed: 15987500]
- Kawashima H, Li YF, Watanabe N, Hirose J, Hirose M, Miyasaka M. Identification and characterization of ligands for L-selectin in the kidney. I. Versican, a large chondroitin sulfate proteoglycan, is a ligand for L-selectin. *Int Immunol*. 1999; 11:393–405. [PubMed: 10221651]
- Kuure S, Vuolteenaho R, Vainio S. Kidney morphogenesis: cellular and molecular regulation. *Mech Dev*. 2000; 92:31–45. [PubMed: 10704886]
- Lang RJ, Tonta MA, Zoltowski BZ, Meeker WF, Wendt I, Parkington HC. Pyeloureteric peristalsis: role of atypical smooth muscle cells and interstitial cells of Cajal-like cells as pacemakers. *J Physiol*. 2006; 576:695–705. [PubMed: 16945969]
- Lechner MS, Dressler GR. The molecular basis of embryonic kidney development. *Mech Dev*. 1997; 62:105–120. [PubMed: 9152004]
- Liu Y, Chattopadhyay N, Qin S, Szekeres C, Vasylyeva T, Mahoney ZX, Taglienti M, Bates CM, Chapman HA, Miner JH, Kreidberg JA. Coordinate integrin and c-Met signaling regulate Wnt gene expression during epithelial morphogenesis. *Development*. 2009; 136:843–853. [PubMed: 19176588]
- Majumdar MK, Askew R, Schelling S, Stedman N, Blanchet T, Hopkins B, Morris EA, Glasson SS. Double-knockout of ADAMTS-4 and ADAMTS-5 in mice results in physiologically normal animals and prevents the progression of osteoarthritis. *Arthritis Rheum*. 2007; 56:3670–3674. [PubMed: 17968948]
- Mittaz L, Russell DL, Wilson T, Brasted M, Tkalecic J, Salamonsen LA, Hertzog PJ, Pritchard MA. ADAMTS-1 is essential for the development and function of the urogenital system. *Biol Reprod*. 2004; 70:1096–1105. [PubMed: 14668204]
- Mittaz L, Ricardo S, Martinez G, Kola I, Kelly DJ, Little MH, Hertzog PJ, Pritchard M. Neonatal calyceal dilation and renal fibrosis resulting from loss of ADAMTS-1 in mouse kidney is due to a developmental dysgenesis. *Nephrol Dial Transplant*. 2005; 20:419–423. [PubMed: 15615810]
- Nagase H, Fushimi K. Elucidating the function of non catalytic domains of collagenases and aggrecanases. *Connect Tissue Res*. 2008; 49:169–174. [PubMed: 18661336]
- Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci U S A*. 1993; 90:8424–8428. [PubMed: 8378314]
- Nakamura H, Fujii Y, Inoki I, Sugimoto K, Tanzawa K, Matsuki H, Miura R, Yamaguchi Y, Okada Y. Brevican is degraded by matrix metalloproteinases and aggrecanase-1 (ADAMTS-4) at different sites. *J Biol Chem*. 2000; 275:38885–38890. [PubMed: 10986281]
- Porter S, Clark IM, Kevorkian L, Edwards DR. The ADAMTS metalloproteinases. *Biochem J*. 2005; 386:15–27. [PubMed: 15554875]

- Richards JS, Hernandez-Gonzalez I, Gonzalez-Robayna I, Teuling E, Lo Y, Boerboom D, Falender AE, Doyle KH, LeBaron RG, Thompson V, Sandy JD. Regulated expression of ADAMTS family members in follicles and cumulus oocyte complexes: evidence for specific and redundant patterns during ovulation. *Biol Reprod.* 2005; 72:1241–1255. [PubMed: 15659705]
- Sahebjam S, Khokha R, Mort JS. Increased collagen and aggrecan degradation with age in the joints of Timp3^{-/-} mice. *Arthritis Rheum.* 2007; 56:905–909. [PubMed: 17328064]
- Salter RC, Ashlin TG, Kwan AP, Ramji DP. ADAMTS proteases: key roles in atherosclerosis? *J Mol Med Jul.* 2010; 22 Epub ahead of print.
- Sambook, J.; Fritsch, EF.; Maniatis, T. *Molecular Cloning: A Laboratory Manual.* 2nd ed.. Cold Spring Harbor Laboratory Press; Cold Spring Harbor: 1989.
- Selbi W, de la Motte CA, Hascall VC, Day AJ, Bowen T, Phillips AO. Characterization of hyaluronan cable structure and function in renal proximal tubular epithelial cells. *Kidney Int.* 2006; 70:1287–1295. [PubMed: 16900089]
- Shi S, Grothe S, Zhang Y, O'Connor-McCourt MD, Poole AR, Roughley PJ, Mort JS. Link protein has greater affinity for versican than aggrecan. *J Biol Chem.* 2004; 270:12060–12066. [PubMed: 14724283]
- Shindo T, Kurihara H, Kuno K, Yokoyama H, Wada T, Kurihara Y, Imai T, Wang Y, Ogata M, Nishimatsu H, Moriyama N, Oh-Hashi Y, Morita H, Ishikawa T, Nagai R, Yazaki Y, Matsushima K. ADAMTS-1: a metalloproteinase-disintegrin essential for normal growth, fertility, and organ morphology and function. *J Clin Invest.* 2000; 105:1345–1352. [PubMed: 10811842]
- Shiomi T, Lemaître V, D'Armiento J, Okada Y. Matrix metalloproteinases, a disintegrin and metalloproteinases, and a disintegrin and metalloproteinases with thrombospondin motifs in non-neoplastic diseases. *Pathol Int.* 2010; 60:477–496. [PubMed: 20594269]
- Stanton H, Rogerson FM, East CJ, Golub SB, Lawlor KE, Meeker CT, Little CB, Last K, Farmer PJ, Campbell IK, Fourie AM, Fosang AJ. ADAMTS5 is the major aggrecanase in mouse cartilage in vivo and in vitro. *Nature.* 2005; 434:648–652. [PubMed: 15800625]
- Steer DL, Shah MM, Bush KT, Stuart RO, Sampogna RV, Meyer TN, Schwesinger C, Bai X, Esko JD, Nigam SK. Regulation of ureteric bud branching morphogenesis by sulphated proteoglycans in the developing kidney. *Dev Biol.* 2004; 272:310–327. [PubMed: 15282150]
- Wu W, Kitamura S, Truong DM, Rieg T, Vallon V, Sakurai H, Bush KT, Vera DR, Ross RS, Nigam SK. Beta1-integrin is required for kidney collecting duct morphogenesis and maintenance of renal function. *Am J Physiol Renal Physiol.* 2009; 297:F210–217. [PubMed: 19439520]
- Yu J, Carroll TJ, Rajagopal J, Kobayashi A, Ren Q, McMahon AP. A Wnt7b-dependent pathway regulates the orientation of epithelial cell division and establishes the cortico-medullary axis of the mammalian kidney. *Development.* 2009; 136:161–171. [PubMed: 19060336]

Figure 1.

Targeting of *Adamts4* in mice. A) Illustration of the targeting strategy used to generate the *Adamts4*^{fllox} and *Adamts4*^{-/-} alleles. The targeting strategy introduced a unique AflII restriction site, resulting in a novel 9.7kb restriction fragment that was detectable by Southern blotting using the indicated probe. Neo = neomycin resistance cassette, DTα = diphtheria toxin α-chain. Exons are illustrated as boxes and numbered with roman numerals. B) Generation of embryonic stem cell lines heterozygous for the *Adamts4*^{fllox} allele. The presence of the targeted allele was detected as a novel AflII restriction fragment by Southern blotting (clone BG3). C) Representative PCR genotyping analyses showing the relative migration of PCR products derived from the *Adamts4* wild-type, floxed and null alleles. The genotypes of the animals are indicated above each lane. D) RT-PCR analysis of *Adamts4* expression in kidneys from mice of the indicated genotypes. Results confirm the loss of *Adamts4* mRNA in *Adamts4*^{-/-} mice. E) Immunoblotting analysis of ADAMTS4 expression in kidneys from mice of the indicated genotypes. Results confirm the loss of a ~68kDa immunoreactive band in *Adamts4*^{-/-} mice.

Figure 2. Abnormal kidney development in *Adamts1*^{-/-};*Adamts4*^{-/-} mice. Photomicrographs of kidneys from newborn wild-type (A), *Adamts1*^{-/-} (B), *Adamts4*^{-/-} (C) and *Adamts1*^{-/-};*Adamts4*^{-/-} (D) mice. *Adamts1*^{-/-};*Adamts4*^{-/-} kidneys featured a thin medulla and dilated renal pelvis (arrow). Kidneys of the other 3 genotypes were histologically normal. Hematoxylin-eosin-phloxin-saffron (HEPS) stain, original magnification = 50×. Scale bar in panel A applies to all panels.

Figure 3.

Perinatal onset of the renal phenotype in *Adamts1*^{-/-};*Adamts4*^{-/-} mice.

Photomicrographs show kidneys from 15.5dpc (A, B) and 17.5dpc (C, D) wild-type and *Adamts1*^{-/-};*Adamts4*^{-/-} mice, all of which are morphologically normal. For both genotypes and embryonic stages, n = 3 animals (6 kidneys) were examined. Adult kidneys from wild-type (E) and *Adamts1*^{-/-};*Adamts4*^{-/-} (F) mice showed a markedly thinned medulla and pelvis dilation in the *Adamts1*^{-/-};*Adamts4*^{-/-} kidney (arrow). Analysis of the ureteropelvic junctions in adult wild-type (G) and *Adamts1*^{-/-};*Adamts4*^{-/-} (H) mice revealed no anomalies. Pap = renal papilla, pel = renal pelvis. Likewise, no evidence of obstruction, dilation or fibrosis was detected in the ureters (ure) of adult *Adamts1*^{-/-};*Adamts4*^{-/-} mice (I). HEPS stain, original magnifications 200× (A, B, I), 100× (G, H), 50× (C, D) or 25× (E, F).

Figure 4.

Adamts1, *Adamts4*, *Vcan* and *Acan* expression in perinatal kidneys. Real-time RTPCR timecourse analysis of *Adamts1* (A), *Adamts4* (B) and *Vcan* (C) expression in 15.5dpc, 17.5dpc, 1d and 7d wild-type mouse kidneys. *Vcan* was more highly expressed at 15.5dpc compared with 17.5dpc, 1d and 7d in the perinatal kidney ($P < 0.05$). (D) RT-PCR analysis of *Acan* expression demonstrates the presence of *Acan* mRNA in the newborn mouse kidney, albeit at low levels compared to brain and cartilage.

Figure 5.

Immunohistochemical localization of ADAMTS4 expression in newborn and 3 month-old adult wild-type mouse kidneys. (A) Negative control for newborn kidney. (B) ADAMTS4 was localized mainly to the developing distal and proximal tubules in newborn mice. (C, E) Negative control for adult kidney. (D, F) ADAMTS4 localized predominantly to the distal and proximal tubules (arrows), with minimal staining in the glomeruli and medulla. Staining in the area of the renal capsule was frequently observed in both control (IgG) and experimental samples, and is believed to be an edge artefact. *M*, medulla; *CT*, convoluted tubule; *G*, glomerulus.

Figure 6.

Absence of proteolytic processing defects of ACAN or VCAN in kidneys from newborn ADAMTS1/4-deficient mice. Western blotting was performed to detect full-length VCAN and ACAN, as well as neoepitopes created by the ADAMTS-mediated cleavage of ACAN and VCAN in their interglobular domains. Four animals were evaluated for each indicated genotype, representative results from 2 animals/genotype are shown. Approximate molecular weights are indicated on the right, ACTB was used as a loading control.