Regulation of Renal Hyaluronan in Water Handling

Studies in vivo and in vitro

SARA STRIDH
Hyaluronan (HA) is a negatively charged extracellular matrix (ECM) component with water-attracting properties. It is the dominating ECM component in the renal medullary interstitium, where the amount changes in relation to hydration status: it increases during hydration and decreases during dehydration. It has, therefore, been suggested that HA participates in the regulation of renal fluid handling by changing the permeability properties of the interstitial space. This thesis investigates potential mechanisms for such a role in renal fluid regulation.

The results demonstrate that the high renal HA content of late nephrogenesis decreases during the completion of kidney development in the rat, which takes place in the neonatal period. The heterogeneous distribution of HA is mainly established during the first three weeks after birth. On day 21, the HA content is similar to that in the adult rat. The process is dependent on normal Ang II function. It primarily involves a reduction of HA synthase 2 expression and an increase of medullary hyaluronidase 1.

The cortical accumulation of HA that results from neonatal ACE inhibition can partly explain the pathological condition of the adult kidney, which causes reduced urinary concentration ability and tubulointerstitial inflammation.

It is possible to reduce renomedullary HA with the HA synthesis inhibitor 4-MU, and the kidney’s ability to respond to a hydration challenge will then be suppressed, without affecting GFR.

The investigation of renomedullary interstitial cells (RMIC) in culture, shows that media osmolality and hormones of central importance for body fluid homeostasis, such as angiotensin II, ADH and endothelin, affect HA turnover through their effect on the RMICs, in a manner comparable to that found in vivo during changes in hydration status.

In established streptozotocin-induced diabetes, HA is regionally accumulated in the kidney, proteinuria and polyuria, reduced urine osmolality, and reduced response to ADH V2 activation will occur. As opposed to the proteinuria, the HA accumulation is not sensitive to mTOR inhibition, suggesting an alternate pathway compared to other ECM components.

Taken together, the data suggest that during normal physiological conditions, renomedullary interstitial HA participates in renal fluid handling by affecting the interstitial prerequisites for fluid flux across the interstitial space. This is possible due to the water-attracting and physicochemical properties of this glycosaminoglycan. During pathological conditions, such as diabetes, the elevated interstitial HA can contribute to the defective kidney function, due to the proinflammatory and water-attracting properties of HA.

Keywords: Kidney, water balance, fluid handling, diabetes, nephropathy, ACE fetopathy, reabsorption, hyaluronic acid, glycosaminoglycan, GAG, extracellular matrix, mTOR, rapamycin, streptozotocin

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ISSN 1651-6206
ISBN 978-91-554-8800-0
urn:nbn:se:uu:diva-209763 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-209763)
Till Larz

Numquam serius ceditur
Ad eundum quo nemo ante iit
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I  Angiotensin converting enzyme inhibition blocks interstitial hyaluronan dissipation in the neonatal rat kidney via hyaluronan synthase 2 and hyaluronidase 1.  

II  Inhibition of hyaluronan synthesis in rats reduces renal ability to excrete fluid and electrolytes during acute hydration.  

III  Hyaluronan turnover by renomedullary interstitial cells. Influence of fluid regulating hormones.  

IV  Inhibition of mTOR activity in established diabetes reduces proteinuria but not renal accumulation of hyaluronan.  

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Duc, sequere, aut de via decede
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>4-MU</td>
<td>4-methylumbelliferone</td>
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<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
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<td>ACEI</td>
<td>ACE inhibitor</td>
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<tr>
<td>Actb</td>
<td>beta-actin</td>
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<tr>
<td>ADH</td>
<td>vasopressin, antidiuretic hormone</td>
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<tr>
<td>Ang II</td>
<td>angiotensin II</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>Asc-P</td>
<td>L-ascorbic acid 6-hexadecanoate</td>
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<tr>
<td>bw</td>
<td>body weight</td>
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<tr>
<td>dAVP</td>
<td>desmopressin</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ET-1</td>
<td>endothelin</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>HA</td>
<td>hyaluronan</td>
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<tr>
<td>HAS</td>
<td>hyaluronan synthase</td>
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<td>Hyal</td>
<td>hyaluronidase</td>
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<td>G6PDH</td>
<td>glucose-6-phosphate dehydrogenase</td>
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<td>GAG</td>
<td>glycosaminoglycan</td>
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<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
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<tr>
<td>MAP</td>
<td>mean arterial pressure</td>
</tr>
<tr>
<td>mTAL</td>
<td>medullary thick ascending loop of Henle</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>RAAS</td>
<td>renin-angiotensin-aldosterone system</td>
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<td>RMIC</td>
<td>renomedullary interstitial cells</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>STZ</td>
<td>streptozotocin</td>
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<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
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*Lagunculae leydiana* 
*non accedunt*
Parturient montes,
nascetur ridiculus mus
Introduction

Hyaluronan (HA) is present in most parts of the body, but in higher concentrations in soft connective tissues and the renal medulla (Fraser 1997). The average human body contains approximately 15 g of HA, of which one third is turned over every day (Stern 2004).

HA has gained a lot of attention in the areas of cosmetics, wound healing, cancer treatment and osteoarthritis, but what is its function in the kidney? In 1958, Ginetzinsky found that HA-degrading proteins, hyaluronidases (Hyal) are excreted with the urine in higher amounts during dehydration, and vanish during hydration (Ginetzinsky 1958). This led to the suggestion that Hyal could change the environment in the kidney interstitium - a perhaps theoretically neglected area between the tubuli and the capillaries - where the medullary concentration gradient exists. It was further suggested that the effect of ADH was exerted through the action of Hyal.

Later experiments have shown that HA in the medullary interstitium changes with hydration status: it increases during acute hydration, and decreases during dehydration (Hansell 2000). HA is also heterogeneously distributed in the kidney, with much higher amounts in the medulla than in the cortex (Decleves 2006, Hallgren 1990, Hansell 2000, Wells 1990). As urine concentration is made possible by the interstitial environment of the medulla, these results strengthen the theory that HA is involved in kidney water handling.

Hyaluronan structure and properties

HA is a molecule of high molecular weight. It consists of a linear polysaccharide, formed of repeating units of D-glucuronic acid and N-acetyl-D-glucosamine (Figure 1). HA is a glycosaminoglycan (GAG), but differs from other GAGs by not containing any sulphate groups, by consisting of much longer polysaccharide chains, and by not containing any peptide (Laurent and Fraser 1992). Other GAGs are approximately <50kDa, and usually even 15-20 kDa, but HA ranges between 200 and 2000 kDa, in long carboxylated chains.

A key property of HA is its ability to attract water. One gram of HA attracts one liter of water, and it forms a gel in concentrations higher than 0.2 mg/ml. The gelatinous network of HA occupies space and excludes larger
molecules in the solution. This steric exclusion can affect water transport and osmotic activity in the extracellular matrix (Comper & Laurent 1978).

Besides the steric exclusion, HA also exerts electrostatic exclusion by its negative charges. This will affect the flow of positively charged ions (Wiig 2008).

HA is involved in processes of basic biology such as cell proliferation, differentiation and migration. It is also of importance in pathological processes such as rheumatoid arthritis and cancer, where the extracellular matrix metabolism is enhanced (Toole 2001), and also in transplant rejection (Hällgren 1990), and diabetes (Rügheimer 2008).

Figure 1. The molecular structure of hyaluronan.

Hyaluronan turnover

In Figure 2 the cellular HA turnover is depicted with HA synthases (HAS) and the degradation pathway including the hyaluronidases (Hyal) 1 and 2.

Synthesis

HA is synthesized in the plasma membrane (Markovitz 1959, Prehm 1983), which makes it unique among GAGs, otherwise being produced in the Golgi. So far, three vertebrate HAS have been identified: HAS 1-3 (DeAngelis 1999, Spicer 1996, Weigel 1997). The three HAS produce different HA chain lengths (Itano 1999, Pummill 2003). The HAS3 HA polymers are up to 1 MDa, and the HAS1 and HAS2 produce polymers up to 2 MDa (Itano 1999). In the kidney, the three HAS have different expression levels – HAS2 > HAS1 > HAS3 – with generally more expression in the medulla than in the cortex (Rügheimer 2009).
Degradation

The HA turnover is tissue-dependent (Fraser 1997), so that in dense structures, HA will tend to be degraded in situ, and not drained and degraded by the lymphatic system. In situ degradation might also occur for renomedullary HA, as there are no lymphatics in the renal medulla, just as in dense structures (review by Stridh 2012). The importance of lymphatic drainage of HA has been debated.

HA half-life varies between 12 and 72 hours, depending on the tissue, and most is degraded by the liver (85-90%), and only approximately 1-2% is excreted by the kidneys (Fraser 1997).

There are six different hyaluronidases (Hyal). Hyal1 mRNA is present in the heart, kidney, liver, lung, placenta and skeletal muscle, but cannot be found in the brain (Frost 1997). It is the predominant form of Hyal, and the only one found in plasma. Hyal2 is acid-active, and expressed in various tissues (Csóka 2001). Hyal3 is not as studied, and much is yet unknown.
about it. It is found in mammalian testis and bone marrow. The PH-20/SPAM is important during egg fertilization, and exerts Hyal activity (Gmachl 1993). It is known as testicular Hyal. Hyal 4 is primarily expressed in placenta and skeletal muscle (Csóka 2001), but might not exert Hyal activity. The sixth and final Hyal is Hyal P1, which is a pseudogene in humans, due to premature termination codon (Csóka 1999).

The cellular degradation pathway of HA is depicted in Figure 2. Breakdown is initiated on the plasma membrane by Hyal 2, before it is internalized for further degradation by the HA scavenger receptor CD44 (Bourguignon 2004). Hyal 2 reduces the high molecular weight HA into low molecular weight HA, but will not degrade the low molecular weight HA further (Lepperdinger 1998). The low molecular weight HA fragments of approximately 20 kDa are then delivered into low pH lysosomes, where they are degraded further by Hyal1 (Formby 2003).

Hyaluronan receptors

The most important HA cell surface receptor is the scavenging receptor CD44 (Aruffo 1990, Lee 2000). CD44 participates in matrix-to-cell signaling, cell migration, cell-to-cell aggregation, and receptor-mediated internalization/degradation of HA (Culty 1992, Hua 1993).

CD44 in the kidney is mostly found in the inner stripe of the outer medulla, on the basolateral membranes of the collecting ducts, and of the descending limb of loop of Henle, and on macula densa cells (Decleves 2006). In cultured RMIC from rats, CD44 is downregulated during hyposmolality and upregulated during hyperosmolality (Göransson 2001). During renal disease or damage to the kidney tissues, CD44 is upregulated together with increased interstitial HA (Decleves 2006, Göransson 2004, Melin 2006).

HA turnover increases during inflammation, when low molecular weight HA fragments accumulate (McKee 1996). Most likely, the HA fragments during inflammation will induce the expression of macrophage genes, which among other things will maintain the inflammatory response. The pathway is dependent on the binding of CD44 to HA.

The alteration in HA in response to changes in hydration status could be achieved through altered CD44 expression. CD44 expression increases when osmolality increases, and decreases in the opposite case (Göransson 2001). A CD44 increase would, in this case, point to an HA decrease, just as is observed during high interstitial osmolality in vivo: dehydration.

Other HA receptors are the receptor for HA-mediated motility (RHAMM; Hardwick 1992), the Ca²⁺-independent endocytic HA receptor of hepatic endothelial cells (Yannariello-Brown 1992), the hyaluronan receptor for endocytosis (HARE; Zhou 2000), layilin (Bono 2001) and the lymphatic endothelial receptor 1 (LYVE-1; Banerji 1999, Prevo 2001).
The Kidney

The kidney’s main task is to maintain the constant volume and composition of body fluids, essential for upholding homeostasis.

The kidney will maintain the excretion of water and electrolytes, to match intake and metabolic production. This makes it possible to control arterial blood pressure via several pathways, involving both hormones, nerves, structural components and intrarenal mechanisms.

The entire body’s plasma volume is filtered in about 20 min, which makes it possible to filter the plasma sixty times in a day (Guyton and Hall 2000). The filtration takes place in the nephron’s glomerulus, which is a hank of capillaries where fluid can be filtered from the blood coming from the arterioles, into the Bowman’s space beyond, and then into the tubular system. The nephron is the functional unit of the kidney, and consists of the glomerulus and the tubule, making it essentially a pipe with a filter and the ability to secrete and reabsorb along the line.

The medullary thick ascending limb of the loop of Henle (mTAL, counter current multiplier) is important for the medullary osmotic gradient, which makes it possible to concentrate urine (Guyton 2000). The vasa recta also contribute to the osmotic gradient through the “counter current exchanger”, which maintains the gradient.

Renomedullary interstitial cells

Renomedullary interstitial cells (RMIC) are major HA-producing cells in the kidney (Göransson 2001, Hansell 1999, Pitcock 1988). The renocortical interstitial cells produce HA in much lower amounts (Pedagogos 2001). This can partly explain the low amount of HA in the cortex, and the much higher amounts in the medulla, during normal physiological conditions.

Cultured RMIC produce differential amounts of HA depending on media osmolality (Göransson 2001, Hansell 1999). In hyperosmolar media conditions, which would be compared to dehydration in vivo, the HA content of the media is reduced. During the opposite conditions, in hyposmotic conditions when media osmolality is decreased – hydration in vivo – HA production by RMIC increases.

RMICs express receptors for angiotensin II (AT1; Zhuo 1998), ADH (V1; Serradeil-Le 1996, Zhuo 2000), and for endothelin (ET)-A and –B (Zhuo 2000). The infusion of ADH reduces renomedullary interstitial HA in vivo, and ADH and angiotensin II (Ang II) can both reduce HA production by RMICs (Rügheimer 2008). The HA amount changes in the medulla, but not in the cortex, where the HA amounts are low (Hansell 2000, Rügheimer 2008), pointing to the RMICs, and not their cortical cousins, as the active producing cells.
Hormones – ADH, Endothelin and RAAS

Hormones are involved in the kidney’s maintenance of blood pressure and the renal excretion rate. They are mainly divided into diuretic and antidiuretic, based on function.

The antidiuretic hormones include the renin-angiotensin-aldosterone system (RAAS), norepinephrine, and vasopressin/antidiuretic hormone (ADH). ADH is the most powerful of all the hormones for decreasing water excretion, and in its absence, as is the case during diabetes insipidus, urine volume can reach 20 l/day.

ADH works by increasing water permeability of the late nephron, the collecting ducts, where the main regulation of urine volume occurs. ADH does this by inserting aquaporins (pores for water) into the apical cell membrane (Nielsen 1993). With maximum ADH presence, urine output can decrease to 0.5 l in a day, and with minimal ADH, it can be up to 20 l/day (Guyton and Hall, 2000).

In the RAAS system, angiotensinogen is converted by renin to angiotensin I, which is converted by angiotensin-converting enzyme (ACE) into Ang II. Renin is released from juxtaglomerular cells, and ACE can be found in the epithelial lining of the lungs, kidney and heart.

Ang II has many functions, but as a part of RAAS, it is responsible for physiological functions. It is a powerful vasoconstrictor and a key player in blood pressure regulation. Ang II also increases sodium reabsorption, causing decreased excretion. It can increase sodium via two pathways, either via the tubular epithelial cells, or through increased aldosterone secretion, the second A in RAAS (Guyton and Hall 2000). The RAAS system is upregulated during the perinatal development of the kidney (Guron 2000), when Ang II operates as a growth hormone.

ACE inhibition is a common treatment for hypertensive patients. The inhibitors reduce the conversion of angiotensin I to Ang II, thereby excluding the effects of Ang II and aldosterone on the system. There are risks with ACE inhibitor treatment during pregnancy (Shotan 2004), due to the involvement of Ang II in kidney development.

Endothelin (ET-1) is a peptide, and the most powerful endogenous vasoconstrictor known, being ten times more potent than Ang II (Yanagisawa 1988).

ET-1 has a concentration-dependent biphasic response on the vascular smooth muscles; a low concentration results in dilation and high concentration results in contraction (Harris 1991, Edwards 1992).

ET-1 has two receptors, which are responsible for the biphasic response. There is a receptor antagonist for each variant: the ET-A receptor antagonist BQ123, and the ET-B receptor antagonist BQ788. It has been hypothesized that low concentration ET-1 primarily affects ET-B receptors, which in turn increase HA by increasing NO production (Deliu 2012, Chenevier-Gobeaux
Endothelins are also known to enhance the release of prostaglandins by stimulation of ET-B receptors located on vascular endothelial cells (De Nucci 1988, Warner 1989), which also elevates HA production (Honda 1993, Mahadevan 1995, Rügheimer 2005). The ET-A receptors have been shown to increase CD44 expression with a BQ123-sensitive mechanism (Tanaka 2000). We have previously demonstrated an inverse relationship between elevated levels of surface CD44 on RMICs and supernatant HA, suggesting increased internalization (Göransson 2001) and thereby reduced levels of HA in the supernatant. ET-B activation reduces fluid reabsorption via NO, while ET-A activation increases fluid reabsorption (review by Hyndman 2013).

The mammalian target of rapamycin (mTOR) is a mediator of hyperglycemic information from the extracellular space to the cell nucleus (Lloberas 2006, Mariappan 2012, Brosius & Alpers 2013). It has also been suggested to be involved in hyperglycemia-induced increased production of extracellular matrix components, but there has been no investigation that clarifies the connection between hyperglycemia, mTOR and the accumulation of HA. As HA does not contain protein or sulphate groups, as other ECM components do, the regulatory pathways may differ. Other glycosaminoglycans are synthesized in the Golgi apparatus, while HA is synthesized in the plasma membrane.

**Hyaluronan and the kidney**

HA composes part of the extracellular space, lends structural support and contributes to homeostasis, and regulates the distribution and transport of plasma proteins in tissues. HA also regulates cell functions such as cell proliferation, locomotion and protection, regulation of inflammation, and recognition functions (Laurent and Fraser 1992).

HA can influence the tissue hydration through transvascular fluid balance (Armstrong and Bell 2002). It stabilizes the extracellular matrix by binding hyaladherins. By binding to cell surface receptors, HA can activate signal pathways and regulate cell motility, invasion and proliferation (Noble 2002).

The action of HA is size-dependent (Noble 2002). Low molecular fragments of HA accumulate in injured tissue and cause cell proliferation and migration. Accumulation of larger polymers of HA will instead promote inhibition and dormancy. The fragments of HA are not synthesized, but are the products of degradation of high molecular weight HA. HA degradation can be caused by reactive oxygen species (ROS), just like other GAGs, but HA is more susceptible than the sulphated varieties (Moseley 1997).

HA is mainly found in the inner medulla of the healthy kidney, with much smaller amounts in the cortex (Decleves 2006, Hällgren 1990, Hansell 2000).
As mentioned above, HA is heterogeneously distributed (Figure 3). The HA amount is 50-100 times larger in the papilla than in the cortical parts of the kidney. Therefore, the papillary interstitium has the highest tissue hydration and HA content. The actual amount of HA in the papilla is 0.6 mg/ml (Hansell 2000), sufficient to form a gel with water. The heterogeneous distribution of kidney HA suggests that HA not only lends structural support to the interstitium, but also has a physiological function.

**Figure 3.** The heterogeneous hyaluronan (HA) distribution in the kidney. Data from Study IV.

**Figure 4.** Schematic view of the possible role of hyaluronan (HA) in urine concentration. HA increases in water loading (hydration), and thus decreases reabsorption of water and increases urine volume. From Stridh et al. (2012).

HA in the medulla increases in hydration, and decreases in dehydration (Hansell 2000; Figure 4). Together with heterogeneous distribution, with
high amounts where urine concentration is made possible, this suggests that HA is involved in kidney water handling.

After 2 hours of hydration, HA increased by approximately 50% in the papilla and outer medulla (Hansell 2000). A 24 hours dehydration period decreased HA in the papilla by 17%. The HA amount matches the body hydration status, with a peak at the maximum diuretic response to hydration and minimum osmolality of excreted urine. The HA involvement is that of changing the physico-chemical characteristics of the interstitium, perhaps together with altered interstitial hydrostatic pressure (Hansell 2000, Lai-Fook and Brown 1991, Zawieja 1992).

It is possible that the repulsion between the negatively charged carboxylate groups of HA attracts positively charged ions and increases osmosis, thus attracting water. The water becomes immobilized within the HA by forming a gel, which in turn counteracts water reabsorption. The increased HA content and gel formation of the interstitium might also affect the function of the medullary thick ascending limb of the loop of Henle (mTAL), where the medullary osmotic gradient originates. The vasa recta might also be affected, thus affecting the function of the “counter current exchanger”, which maintains the osmotic gradient. Lastly, the interstitial swelling that follows an increased HA content, will increase diffusion distances between tubuli and capillaries, and thus affect reabsorption (Figure 4).

Nephrogenesis

The extracellular matrix surrounding the migrating and proliferating cells in embryonic development contains high levels of HA, which decrease as differentiation continues (Toole 1997). The development of the kidney is different from that of other organs (Spitzer 1992), and follows its phylogenic history. During its development, three different organs form: pronephros, mesonephros and metanephros (Kanwar 1997). The metanephros remain to become the adult kidneys (Spitzer 1992). These early structures emerge in the fifth week of gestation and induce the differentiation of the kidneys. The successive process of kidney development sets the conditions for possible malformation due to faulty interactions. The severity of the malformation depends on when in the development the faulty interaction occurs.

The extracellular matrix is a mediator of morphogenic branching (Pohl 2000b), and HA is implicated in embryogenesis (Pohl 2000a). HA accumulates in the early metanephros, but decreases as development continues with branching and increase of Hyals (Belsky 1983). The changes are different in different developing kidney regions, so a change in HA cannot be assigned to a specific morphological change in the kidney, although HA seems to accumulate as the mesenchyme develops into epithelium.
HA stimulates early branching, such as cellular process formation (Pohl 2000b). If HA is absent, cell survival and morphogenesis decrease. HA, CD44 and HAS, all expressed in a spatial pattern during this phase, might together form a pathway for the morphoregulation of the developing kidney.

As mentioned above, HA is heterogeneously distributed, a prerequisite for normal kidney function (Göransson 2002, Hansell 2000, Johnsson 1996, Wells 1990). The distribution is established postnatally in the rat, which is the final stages of nephrogenesis. Studies of what leads up to this heterogeneous distribution suggest an increased Hyal activity (Belsky 1983, Nilsson 2001).

The newborn infant has a fluid intake and excretion of approximately seven times greater than that of the adult, in relation to body weight (Guyton 1996). The low capacity has several probable reasons, such as few vasopressin V2-receptors in the collecting duct, low reabsorption capacity of sodium in the mTAL, a low amount of urea in the medullary interstitium (Alpern 2008), and also the relatively high HA levels in both cortex and medulla (Nilsson 2001).

ACE inhibitors have been shown to affect nephrogenesis and HA turnover (Friberg 1994, Guron 1999, Nilsson 2001). The ACE inhibitors are used to treat hypertension and diabetes complications. Friberg et al. (1994) discovered, while testing vascular responses to ACE inhibition, that it also caused kidney damage. Histological abnormalities persisted after the end of the treatment, such as inflammation of the cortical interstitium, papillary atrophy and pelvic dilation, causing decreased urine concentrating ability. An Ang II AT1 receptor blocker gave the same results. The window of vulnerability for these abnormalities is within 14 days after birth in rats. Treatment starting after this point will not cause any of the damages. Expression and activity of ACE peaks around day 14 (Costerousse 1994, Jung 1993). Both angiotensinogen (Darby 1995) and AT1-receptor expression (Norwood 1997, Tufro-McReddie 1993) peak in the newborn period, possibly explaining the window of vulnerability.

The structural abnormalities in the investigated rats and pigs are similar to those in human fetuses with ACE inhibitor fetopathy (Shotan 1994). For this reason, antagonists to the renin angiotensin system are contraindicated during pregnancy and lactation. In spite of this, the use of RAAS inhibitors during pregnancy increases (Bowen 2008, Schaefer 2012).

Upon study of the specific kidney damage, it was found that the urinary concentrating deficiency is caused by impaired tubular water reabsorption in the medullary collecting duct (Guron 1999, Nilsson 2001). This impairment is likely due to the papillary atrophy and elevated interstitial HA, which leads to edema and inflammation. There is also a decreased medullary tissue hyperosmolarity. Paired with reduced aquaporin-2 expression, this will result in reduced tubular reabsorption of water.
Diabetes – animal model, mTOR

Insulinopenic diabetes mellitus is a chronic autoimmune disease, marked by the absence of insulin-producing beta-cells. Patients with diabetes often acquire secondary complications due to the diabetes’ sustained hyperglycemia and elevated oxidative stress (Diabetes Control and Complications Trial Research Group 1993, Leon 2005).

Eventually, the patient will develop nephropathy, neuropathy and retinopathy. Approximately one fourth of patients with type 1 diabetes develop diabetic nephropathy (Andersen 1983), the leading cause of end-stage renal failure.

Diabetic nephropathy causes accumulation of extracellular matrix components and renal hypertrophy (Steffes 1992, Mason & Wahab 2003, Lloberas 2006, Mariappan 2012). When the ECM increases, the basement membranes of tubules and glomeruli will thicken, the mesangium will expand, and there will be glomerular hypertrophy causing scarring and fibrosis (Gilbert & Cooper 1999, Molitch 2004, Najafian & Mauer 2009) and interstitial edema (Melin 2006).

Diabetic nephropathy displays both functional and structural abnormalities. Reduced GFR and urinary leakage of proteins are observed, which indicate decreased kidney function. Early on in the disease, there is also often a transiently increased GFR (Marre 1992), which could be important for the progression of the disease (Yip 1996). There are also structural changes, which together encompass just about every renal structure. Extracellular matrix accumulation is central to the structural changes in diabetic nephropathy (Steffes 1992). The traditionally noted changes are thickening of the glomerular and tubular basement membranes, glomerulosclerosis, expansion of the mesangium, and hyalinosis of afferent and efferent arterioles.

Extracellular matrix components such as collagen types IV and VI, laminin, fibronectin and HA are present in excess, due to increased synthesis or decreased removal (Falk 1983, Kim 1991, Rügheimer 2008, Melin 2006).

Hyperglycemia causes several kidney cells in vitro to produce more HA (Jones 2001, Mahadevan 1995, Takeda 2001, Wang 2004). This implies that diabetes promotes HA production in the kidney, which could be involved in the development of diabetic nephropathy, due to the changed matrix, which in turn can affect kidney function.

Interstitial fibroblasts and mesangial cells have been shown to be stimulated into HA production by elevated glucose concentration (Takeda 2001, Wang 2004). Increased kidney HA content is a result of diabetes (Malathy 1972, Lewis 2008, Berenson 1970, Wang 2004).

Is increased HA in hyperglycemia directly involved in the development of diabetic nephropathy? The diabetic patients and animal models for research often both show osmotic diuresis due to hyperglycemia, and high diuresis
can lead to volume depletion. During hyperglycemia, HA is elevated, but the medullary HA also varies with hydration status.

In a study on diabetic rats, the elevated HA was primarily located to the medulla, and correlated to an increased HAS2 mRNA expression (Rügheimer 2008). A hydration of these animals could not increase HA further, but they did not have a normal hydration response. This suggests that the renomedullary HA is maximized and cannot be further increased by hydration. The abnormal regulation of medullary HA could have a direct functional effect on diuretic response/volume regulation. With a longer time of diabetic disease, there would probably also be a change in cortical HA.

STZ-diabetic rats display increased kidney Hyal activity from day 3 (Ikegami-Kawai 2003). The activity increased until the third week, which corresponded to a doubled level compared to controls. Cortical Hyal activity increased more than the medullary activity. The heterogeneous enzyme activities suggest different effects for HA in different regions of the kidney. Hyal activity did not increase at all in the spontaneously diabetic Goto-Kakizaki rat, which did not have progressed nephropathy. This raises the suggestion that Hyal activity could be used as a marker for diabetic nephropathy.

Finally, a treatment of diabetic mice with high molecular HA, decreased diabetic nephropathy (Campo 2010). The conclusions drawn were that HA decreased CD44 and protein kinase C gene expression, which in turn would reduce inflammation and other secondary diabetic pathologies.

In summary, diabetes causes the renal content of HA to increase. HAS2 mRNA and Hyal activity are elevated, and coincide with proteinuria, diuresis and decreased kidney function.

The pro-inflammatory and water-attracting properties of HA may be involved in the progression of diabetic nephropathy.
Aims of the investigation

The overall aim was to gain new insights into the regulation and function of kidney HA by a) elucidating its regulation during completion of nephrogenesis; b) testing the concept of HA involvement in renal fluid handling; c) determining the effects of hormones involved in normal regulation of renal fluid balance on HA turnover; and d) investigating the possible pathways of HA accumulation in diabetes.

Study I
This study aimed to elucidate the regulation and normal turnover of HA during completion of nephrogenesis in the rat. This was done to elucidate the sequence of events leading to the heterogeneous intrarenal distribution of HA in the adult kidney, which is a prerequisite for normal kidney function. Also, to identify possible mechanisms underlying the HA accumulation in the adult kidney after neonatal treatment with RAAS inhibition.

Study II
Study II aimed to test the concept of an involvement of renomedullary interstitial HA in renal fluid handling. This was achieved through reduction of HA synthesis by administration of 4-MU to rats, and then testing the consequences for the kidney’s ability to respond to a hydration challenge.

Study III
The aim was to elucidate the mechanisms by which renal HA content changes during physiological and pathophysiological conditions by determination of the effects of low osmolality, and hormones involved in normal regulation of renal fluid balance on HA turnover by RMICs. Hormones of central importance for fluid homeostasis should affect HA turnover in vitro similarly to previous in vivo findings. The importance of Hyal activity and of the scavenging receptor CD44 expression for HA turnover were also investigated.

Study IV
The aim of this study was to test if interstitial HA accumulation during diabetes involves mTOR signaling. Established streptozotocin (STZ)-diabetic rats were treated with the mTOR inhibitor rapamycin, and measurements were carried out regarding kidney function and regional renal HA content.
Materials and methods

All chemicals were from Sigma-Aldrich (Saint Louis, MI, USA) and of highest grade unless otherwise stated.

Animals

All experiments were performed in accordance with the NIH guidelines for the use and care of laboratory animals, and approved by the local animal care and use committee. All animals were kept in a room with a controlled temperature of 24 °C and a 12 h dark–light cycle, and had free access to standard rat chow and tap water.

In Study I, a total of 120 Wistar pups were used, locally delivered from pregnant females purchased at gestation day 15–16 (Møllegaard Breeding Centre, Copenhagen, Denmark).

In Study II, a total of 30 male Sprague-Dawley rats (Charles River, Sulzfeld, Germany; body weight 274 ± 32 g) were used. Half received an HA synthesis inhibitor, and half the corresponding vehicle in the drinking water.

For Study III, male Sprague-Dawley rats weighing 80-90 g (Charles River, Sulzfeld, Germany) were used for cell harvesting.

In Study IV, 40 male Sprague-Dawley rats (body weight ~270g; Charles River, Sulzfeld, Germany) were used.

Study I

Treatment with ACEI/Enalapril

The Wistar pups, were given daily intraperitoneal (i.p.) injections of enalapril maleate (10 mg/kg, MSD, Sollentuna, Sweden, n=60) or the corresponding vehicle (saline, 10 ml/kg, n=60) from days 3–13 after birth (before completion of nephrogenesis in the rat).
Gene expression analysis / mRNA and PCR

In groups of 16–26 animals, the kidneys were excised on days 5, 9, 12 and 17 for subsequent evaluation (n=78 in total). In these animals, urine was also collected for analysis of Hyal activity.

After excision, specimens were placed in Eppendorf tubes with RNAlater® (Ambion Inc., Houston, TX, USA). The pieces of cortex and medulla were used for total RNA isolation with RNAquous®-4PCR (Ambion). cDNA was obtained from RNA using iScript™cDNA Synthesis Kit (Bio Rad Laboratories, Hercules, CA, USA). The following semi-quantitative real-time PCR was performed by DyNAmo™ Capillary SYBR® Green qPCR kit (Finnzymes, Espoo, Finland) and a Lightcycler system (Roche Diagnostics, Mannheim, Germany). Samples were run in duplicates, and PCR products were verified by agarose gel electrophoresis. The genes analyzed were Hyal1-4 and HAS1-3. All values were normalized for reference genes TATA-binding protein (TBP) and beta-actin (Actb). Values were then expressed as normalized values for the means of the two reference genes, by this formula: 2Ct(reference genes)−Ct(gene of interest), where Ct is the cycle number, and Ct for the reference genes is a mean of the cycle numbers for the two reference genes, which did not differ much from each other. Primers were obtained from MWG Biotech (Ebersberg, Germany).

Urine hyaluronidase activity and creatinine

In groups of 16–26 animals (n=78 in total), urine was collected by puncture of the bladder and aspiration into a syringe on days 9, 12 and 17 for analysis of Hyal activity.

Urinary Hyal activity was determined by quantitative zymography (Ikegami-Kawai 2004) with a slight modification. All values were normalized for creatinine in urine, which was determined colorimetrically according to standard procedures.

Lymph vessels – analysis of podoplanin

Kidney sections were stained by indirect immunoperoxidase, as previously described (Regele 2000). The number of (non-glomerular) positive cells in each sample was counted in at least 10 random medium power fields, each 0.5 mm² in diameter, with an epifluorescence microscope and a 10× lens. The mean of the randomly selected fields was used as n=1 for each sample.
Study II

The animals received the HA synthesis inhibitor 4-MU (1.45 ± 0.07 g/kg body weight/24 h) in the drinking water or the corresponding vehicle for five consecutive days. The drinking water was made fresh every day with 4-MU. The dose was calculated after the experiments from the concentration in the drinking water (15 mg/ml), and by measuring daily water consumption.

Anesthesia

After five days of treatment with 4-MU, the rats were anesthetized with an intraperitoneal injection of thiobutabarbital (Inactin, 5-ethyl-5-(1-methylpropyl)-2-thio-barbiturate sodium, 120 mg/kg body weight), and were placed on a servo-controlled heating pad to maintain the core temperature at 37.5°C.

Surgery

After tracheotomy, polyethylene catheters were inserted into the right femoral vein and artery, the former for infusion of isotonic saline (0.9% NaCl) and hypotonic glucose-saline (0.25% NaCl, 0.5% glucose) containing 3H-inulin, and the latter for measurement of mean arterial blood pressure (MAP). The urinary bladder was catheterized through a suprapubic incision for urine sampling.

Experimental protocol

After a post-surgery equilibration period of 45 min, another 45-min period followed for determination of the baseline glomerular filtration rate (GFR) estimated from inulin clearance. Therefore, 3H-inulin (185 kBq/ml; Bionuclear Scandinavian AB, Bromma, Sweden) dissolved in isotonic saline was infused (5 ml/h/kg body weight) intravenously from the start of the equilibration period. Urine and arterial blood samples were taken for subsequent analyses. After this control period, the rats were hydrated by changing the infusion to a hypotonic glucose-saline solution (approximately 100 mOsm/kg H2O) and increasing the infusion rate (15 ml/h/kg body weight). Then, three consecutive periods followed, lasting 45 min each, resulting in 135 min of hypotonic infusion corresponding to hydration of the animal.

After completion of the experimental procedure, the kidneys were excised and weighed, and samples from cortex and inner medulla (papilla) were taken and frozen for subsequent analysis of HA content.
Urine and plasma analysis - GFR and RBF measurement

GFR was estimated from the clearance of 3H-inulin. The radioactivity of 3H-inulin in plasma (10 µL) and urine (1 µL) was measured by liquid scintillation counting. Urine volumes were measured gravimetrically, osmolality by use of a freezing point technique (Model 210, The Fiske Micro-Sample Osmometer Advanced Instruments, Boston MA, USA), and urinary sodium concentrations by use of flame photometry (IL943, Instrumentation Lab, Milan, Italy).

Study III

Cell isolation and culture

RMICs were isolated from kidneys of young Sprague-Dawley rats as previously described by Maric et al. (1996) and cultured as described by Fontoura et al. (1990).

Isolation procedure

A catheter was inserted into the carotid artery for retrograde perfusion with 30 ml of ice cold HBSS. During the perfusion, the kidney vein was punctured. The kidneys were then excised and placed into ice cold HBSS.

The papilla was carefully excised from the kidneys and finely cut into a mixture of HBSS and collagenase. The tissue pieces were then left to digest in 37 °C for 30 min. The reaction was stopped by placing the sample tubes on ice for 10 min.

The digested fragments were then sieved in a 150 µm metal sieve and rinsed with 3 ml HBSS. The mixture was centrifuged for 3 min at 200g, after which the supernatant was aspirated, and the centrifugation was repeated three more times with new HBSS.

Finally the cells were resuspended into a 1:1 solution of RPMI and conditioned DMEM in culture flasks. The cultures were kept in incubators with 5 % CO₂ in 37 °C.

For the first few weeks, the culture is primarily epithelial, but after a few weeks it consists of a homogenous non-epithelial population. The cells were used for experiments no earlier than at passage 7.

Experimental protocol

RMICs were plated at a density of about 5x10⁴ cells/cm² for 48h in a 1:1 mixture of RPMI 1640 culture medium and DMEM culture medium conditioned by 3T3 mouse fibroblasts, and the mixture contained a total of 15%
fetal bovine serum, as previously described by Fontoura (1990). The cells were then treated for 24h with different compounds described below.

Following 24h treatment, the supernatant was collected and analyzed for HA content and Hyal activity. Cells were harvested and analyzed for CD44 expression and gene expression of HAS and Hyals. The amount of protein was determined using a routine method (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA).

Ang II (Bachem, Bubendorf, Switzerland), ADH, or a combination of the two were given in concentrations of $10^{-6}$M. ET-1 was administrated to provide final concentrations of $10^{-10}$M, $10^{-8}$M or $10^{-6}$M. The highest ET-1 concentration ($10^{-6}$M) was also given together with the selective ET-A receptor antagonist BQ123 ($10^{-6}$M). The lowest ET-1 concentration ($10^{-10}$M) was also given together with the selective ET-B receptor antagonist BQ788 ($10^{-6}$M). The Hyal inhibitor L-ascorbic acid 6-hexadecanoate (Asc-P) was administered in a final concentration of $10^{-7}$M. The HA synthesis inhibitor 4-methylumbelliferone (4-MU) was used in a concentration of $10^{-6}$M. Growth media osmolality was reduced to 200 mOsm/kg H$_2$O by 2:3 dilution with distilled water.

Hyaluronidase activity in supernatants
Supernatant Hyal activity was determined by quantitative zymography (Ikegami-Kawai 2004) with a slight modification because of its very low activity.

CD44 western blot
Prior to CD44 analysis by western blot, the surface proteins were isolated (Pierce® Cell surface protein isolation kit, Pierce Biotechnology, Rockford, IL, USA). Molecular weight separation was performed on 10% Tris-HCl gels with Tris/glycine/SDS buffer, the proteins transferred to nitrocellulose membranes, and CD44 detected with sheep anti-rat CD44 (0,1 µg/ml; R&D Systems, Minneapolis, MN, USA) and HRP-conjugated rabbit anti-sheep (1:5,000; Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). The luminescent signal was captured on an ECL-camera system (Kodak image station 2000; New Haven, CT, USA). β-actin was detected with mouse anti-rat β-actin antibody (1:20,000) and secondary HRP-conjugated goat-anti mouse antibody (1:10,000; Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). CD44 western blot analysis of samples from isolated surface proteins was normalized to the β-actin expression.
Gene expression analysis
Total RNA was isolated from the cells (RNAquous®-4PCR, Ambion, Austin, TX, USA). cDNA was obtained like in Study I. The following semi-quantitative real-time PCR was performed by Lightcycler FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics, Mannheim, Germany) in a Lightcycler system (Roche Diagnostics, Mannheim, Germany). The following procedure took place as in Study I. The genes analyzed were Hyal1 and 2, and HAS2. All values were normalized for reference genes TATA-binding protein (TBP), beta-actin (Actb), and Glucose-6-phosphate dehydrogenase (G6PDH).

Study IV

Treatment with mTOR inhibitor
The rats were divided into four groups. Half the animals were made diabetic. The diabetic and control groups were given either rapamycin (0.45g/kg body weight/day; Rapamune, Pfizer, New York City, NY, USA) or sham treatment by oral gavage once per day, from diabetic week seven through eight. After eight weeks of diabetes, including two weeks of rapamycin or sham treatment, the rats were anesthetized.

Induction of diabetes
Half the animals were made diabetic by an intravenous dose of STZ (50 mg/kg bw) into the tail vein. The diabetic state (blood glucose above 20 mmol/l) was monitored by blood glucose test strips (MediSense, Bedford, MA, USA) in blood from a small cut in the tail tip. Blood glucose was tested two days after STZ, and weekly throughout the experiment, together with body weight, for a total of eight weeks.

Anesthesia
After eight weeks of diabetes and two weeks of rapamycin or sham treatment, the rats were anesthetized with an intraperitoneal injection of thiopentobarbital (Inactin, 120 mg/kg body weight), and were placed on a heating pad to maintain a core temperature of 37.5°C.
Surgery

The anesthetized rats were tracheotomized, and polyethylene catheters were inserted into the right femoral vein and artery. The venous catheter was used for infusion. The arterial catheter was used for measurement of mean arterial blood pressure (MAP) and blood sampling. The urinary bladder was catheterized through a suprapubic incision for urine sampling. The left kidney was exposed through a subcostal flank incision and immobilized in a plastic cup lined with saline-soaked cotton wool. The kidney surface was covered with paraffin oil (Apoteksbolaget, Gothenburg, Sweden). The surgery was followed by a post-surgery 45 min equilibration period.

Experimental protocol

The equilibration period was followed by a 30 minute measurement period for establishment of baseline parameters. The glomerular filtration rate (GFR) was estimated from fluorescein isothiocyanate (FITC) inulin clearance. For this purpose FITC-inulin dissolved in Ringer acetate (Fresenius Kabi, Bad Homburg, Germany) was infused (Controls: 5 ml/kg body weight/hr; Diabetic animals: 10 ml/kg body weight/hr) from the start of the equilibration period, in a final concentration of 0.375%.

The FITC-inulin infusion was prepared from a stock solution of 1.5% concentration, prepared in PBS, and filtered through a 0.45 µm syringe filter. The solution was then dialyzed in 2000 ml PBS at 4°C overnight, using a 1000 Da cut-off dialysis membrane (Spectra/Por 6 Membrane, Spectrum Laboratories Inc, Rancho Dominguez, CA, USA). The dialyzed inulin solution was filtered through a 0.22 µm syringe filter before its dilution in Ringer to the final concentration, in preparation for use. The FITC solution was protected from light at all times during preparation, use and after the experiment.

After the baseline period followed two measurement periods of 30 min each, with the infusion supplemented with desmopressin (dAVP; Minirin, Ferring, Saint-Prex, Switzerland) to challenge the water-concentrating ability. The dAVP infusion was kick-started by a bolus dose of 0.1 ml of a 60 ng/ml concentration. The bolus dose was flushed into the system with 0.3 ml, and followed by a maintenance infusion of 4 ng/ml of dAVP dissolved in the FITC-inulin Ringer solution. After completion of the two experimental periods, the kidneys were excised and weighed. Samples were taken from the cortex, outer medulla and inner medulla (papilla), and frozen for subsequent analysis of HA content.
Measurement of GFR, RBF and urine parameters

GFR was approximated from clearance of FITC-inulin. The FITC-inulin was dissolved in a Ringer solution and given as continuous infusion. GFR was approximated from inulin clearance and calculated by the formula \( \text{GFR} = U \cdot \frac{V}{P} \), where \( U \) and \( P \) denote the fluorescence of FITC-inulin in the urine and plasma, respectively, and \( V \) denotes the urine flow rate. The original plasma samples of 2 µl were diluted with 68 µl HEPES buffer (pH 7.4), and the urine samples were loaded similarly, but diluted another ten times before adding HEPES. The samples were loaded onto a black 384-well microplate (Greiner Bio-One GmbH, Kremsmuenster, Austria) and measured in the plate reader (Safire II, Tecan Austria GmbH, Grödig, Austria) at 496 nm excitation and 520 nm emission.

The renal blood flow (RBF) was measured by an ultrasound probe placed around the renal artery of the left kidney (Transonic systems, Ithaca, NY, USA). Both RBF and MAP were measured continuously and recorded by a PowerLab instrument (AD Instruments, Hastings, UK). The urine volume was measured gravimetrically. Urine osmolality was estimated from the depression of the freezing point (Model 210, The Fiske Micro-Sample Osmometer Advanced Instruments, MA, USA).

Urine hyaluronidase activity and creatinine

Urinary Hyal activity was determined by quantitative zymography (Ikegami-Kawai 2004) with a slight modification. Creatinine in urine was determined colorimetrically using a commercial assay kit and following the instructions provided by the manufacturer (LabAssayTM Creatinine, Wako Pure Chemical Industries). The blank values originating in FITC-inulin were subtracted.

Hyaluronan quantifications

Different HA quantifications have been used in the studies, due to changing availability of commercial kits.

Study I

The HA content of the final supernatants was analyzed by radiometric assay (Pharmacia Diagnostics, Uppsala, Sweden).

In groups of three animals, each of the kidneys were excised on days 6, 8, 10, 12, 14, 17 and 21 for subsequent evaluation (n=42 in total). Lyophilized specimens were ground and HA extracted from the tissues with 0.5 M NaCl for a period of 16 h. The samples were centrifuged for 15 min at 2000×g and the HA content of the supernatants was analyzed by radiometric assay.
The technique is based on the binding of HA to specific HA-binding proteins (HABP). Briefly, a 100 μl sample was incubated for 60 min at 4–7 °C with 200 μl ^125^I-labelled HABP. HA-Sepharose (100 μl) was added and incubation continued for 45 min at 4–7 °C, followed by centrifugation at 2000×g for 10 min, together with 2 ml washing solution. The radioactivity of the pellet was measured in a gamma counter. A standard curve was constructed from known amounts of HA. Each sample was analyzed in duplicate and the variability was <10%. The relative water content, expressed as a percentage of the total tissue wet weight, was calculated as 100×(ww−dw)/ww. Cortex wet weight (ww) is defined as weight immediately after excision, and 3 min on filter paper. Dry weight (dw) is defined as kidney weight after lyophilisation.

Study II and IV
The frozen kidney samples were dried at 68°C overnight, then disrupted in 0.5 M NaCl (FP120, Thermo Electron Corporation, Marietta, OH, USA). The disrupted HA samples were left to extract overnight in 4°C. Protein content of the samples was measured using a commercial assay (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA). The HA samples were then centrifuged at 2700xg for 15 min. The HA content of the supernatant was measured by a commercial ELISA kit and followed the instructions provided by the manufacturer (Echelon Biosciences Inc., Salt Lake City, UT, USA).

Study III
HA content in supernatants from RMICs in culture was measured using the commercially available ELISA (Echelon Biosciences Inc., Salt Lake City, UT, USA) and related to the amount of protein (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis
All values are expressed as mean ± SEM. A P-value of <0.05 was considered statistically significant.

Study I
One-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests were used for comparing effects over time within each group. Bonferroni's multiple comparison tests were used for comparing for treatment effects at each time point.
Study II
One-way ANOVA and Dunnett’s multiple comparison tests were used for comparing effects over time within each group. Bonferroni’s multiple comparison tests were used for comparisons of treatment effects at each time point. Kidney HA content at the end of the study protocol was analyzed using unpaired Student’s t-test.

Study III
The comparison between groups was evaluated with one-way ANOVA followed by Fisher LSD test. Parameters presented as percentage in graphs were statistically analyzed using the original absolute values.

Study IV
The comparison between groups was evaluated with two-way ANOVA followed by Fisher LSD test.
Results

Study I
Hyaluronan content
In 6–8 day old control rats, cortical HA content was high, and was then reduced by 93% on days 10–21, reaching adult low levels similar to those previously reported (Hansell 2000, Johnsson 1996, Nilsson 2001). The greatest reduction took place between days 8 and 13, when 80% of the HA dissipated.

In neonatally ACEI-treated rats, the normal reduction in HA was abolished (Figures 5, 6). During days 10–21, no cortical reduction occurred, and at day 21, the HA content of the cortex was 97±26 μg/g dw, which is more than 13-fold higher than vehicle-treated animals (p<0.05; Figure 5).

![Figure 5](image_url)

*Figure 5.* Renal cortical hyaluronan (HA) in days 6 to 21 after birth of the rat, treated neonatally with vehicle or ACE-inhibitor enalapril. Values are means±SEM, and * denotes p<0.05 vs corresponding control value.

Medullary HA content in control animals was high on days 6–8, about 6-fold higher than cortical levels, and then reduced by 85% to a level still 12-fold above cortical at day 21 (Figure 6).
In ACEI-treated animals, the high HA values at 6–8 days after birth also gradually decreased after day 10, but leveled out during 17–21 days to about 3-fold higher than vehicle-treated animals (308±61 μg/g dw, p<0.05).

Though qualitatively clear, caution must be taken regarding exact absolute values of HA during the first days after birth, due to the small specimen size, which is evident from the variation in data.

**Figure 6.** Medullary hyaluronan (HA) in days 6 to 21 after the birth of the rat, treated neonatally with vehicle or ACE-inhibitor enalapril. Values are means±SEM, and * denotes p<0.05 vs corresponding control value. The large variations in the first time points are due to small specimen sizes, causing low measurement accuracy.

**Gene expressions**
The relative mRNA expression of synthases was HAS2>HAS1>HAS3 (data not shown). The medullary expressions were higher than those of the cortex at all time points investigated, mirroring the intrarenal heterogeneous HA content.

The mRNA expression of HAS2 dominated both in the cortex and medulla of the untreated 5 day old pups. Temporal expression of HAS2 corresponded with the reduction of HA content in the normal kidney. The cortical expression was reduced by 72% from day 5 to 17 (Figure 7), while the corresponding medullary reduction was 51% (p<0.05; Figure 8).

In ACEI-treated animals, cortical HAS2 remained twice the expression of controls, as opposed to the medullary expression, which diminished.
Figure 7. Gene expression of hyaluronan synthase 2 (HAS2) in the cortex of control and ACEI-treated rats 5–17 days after birth. * denotes p<0.05 vs. day 5 (baseline) in the same group, and † denotes p<0.05 when compared to the control group at the corresponding time.

Figure 8. Gene expression of hyaluronan synthase 2 (HAS2) in the medulla of control and ACEI-treated rats 5–17 days after birth. * denotes p<0.05 vs. day 5 (baseline) in the same group, and † denotes p<0.05 when compared to the control group at the corresponding time.
The medullary mRNA expression of each hyaluronidase was only slightly higher than the corresponding cortical expression. Hyal3 expression dominated in the cortex and medulla of untreated 5 day old pups. The relative expressions were Hyal3>Hyal2>Hyal4>Hyal1 (data not shown).

Medullary Hyal1 increased in controls, but decreased in ACEI-treated animals, which could explain the difference in HA amounts (Figure 9).

The expression of Hyal1 in controls was 26% higher day 17 after birth compared to that after day 5. In ACEI-treated animals, Hyal1 expression reduced by 36% from day 5 to day 17 after birth.

Figure 9. Gene expression of medullary Hyaluronidase 1 (Hyal1) in control and ACEI-treated rats 5–17 days after birth. * denotes p<0.05 vs. day 5 (baseline) in the same group, and † denotes p<0.05 when compared to the control group at the corresponding time.

Hyaluronidase activity

Urine Hyal activity decreased with time in control animals, while in ACEI-treated animals, it was initially 50% lower, and did not change over time (Figure 10).

At day 9 after birth, the urine Hyal activity was almost twice as high in control animals as in those treated with ACEI (0.28±0.02 vs. 0.14±0.02 RI/mg creatinine, p<0.05). With time, Hyal activity was reduced in control animals (to 0.09± 0.02 RI/mg creatinine at day 17, ns), while no change occurred in ACEI-treated animals (to 0.17±0.02 RI/mg creatinine, ns).
Figure 10. Hyaluronidase activity in the urine of control animals (solid line) and ACEI-treated animals (dashed line) days 9, 12 and 17 after birth. RI, relative intensity. * denotes p<0.05 vs. corresponding value in control group. # denotes p<0.05 vs. day 9 of same group.

Podoplanin labeling
Lymphatic cells can be quantified by measurement of the lymphatic endothelial mucoprotein podoplanin (Kerjaschki 2004).

The number of podoplanin-positive cells in the renal cortex (excluding podocytes in the glomeruli) of vehicle-treated animals decreased from day 6 to days 17–21 (p<0.05). In ACEI-treated animals, the number of podoplanin-positive cells increased 18-fold compared to controls (p<0.05), suggesting compensation.

CD44
There was no apparent up-regulation of CD44 in ACEI-treated animals (data not shown).

Morphology
ACEI treatment caused degeneration (atrophy) of the papilla at postnatal day 21, but otherwise, minimal changes in histological appearance were observed (data not shown).

Study II
During the five days of treatment prior to acute experiments, the animals ingested similar amounts of fluid, suggesting similar hydration (control group 21.8 ± 1.4 ml/day; 4-MU group 25.7 ± 1.2 ml/day).
Hyaluronan content
The control medullary and cortical HA contents were 7.85 ± 1.29 ng/mg protein and 0.08 ± 0.01 ng/mg protein, respectively (Figure 11).

Medullary HA content after 4-MU was 38% of that in controls (2.98 ± 0.95 ng/g protein, p<0.05), while the low cortical levels were unaffected (Figure 11).

Figure 11. Hyaluronan (HA) content in cortex and inner medulla (papilla) in control rats and in rats treated with the HA synthesis inhibitor 4-MU. * denotes p<0.05 vs control.

Diuretic response to hydration
In control animals, a 135 minute hypotonic hydration period increased diuresis and osmotic excretion, while sodium excretion and glomerular filtration rate (GFR) remained unchanged. Baseline urine flow in 4-MU treated rats was not different from that in controls, but the diuretic response to hydration was only 51% of that in controls (157 ± 36 versus 306 ± 54ml/g kidney weight/135 min, p < 0.05; Figure 12) and the osmolar excretion only 47% of that in controls (174 ± 47 versus 374 ± 41 mOsm/g kidney weight/135 min, p < 0.05; Figure 12). Sodium excretion, GFR, and arterial blood pressure were similar to those of control rats, and unaltered during hydration (Table 1).
Figure 12. Accumulated urine excretion (left) and accumulated osmolar excretion (right) during hydration in control rats and in rats treated with the hyaluronan synthesis inhibitor 4-MU. * denotes p<0.05 vs control. kw= kidney weight.

Table 1. Kidney and blood pressure data

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<tr>
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<th>Control period</th>
<th>Hydration 1</th>
<th>Hydration 2</th>
<th>Hydration 3</th>
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<td><strong>GFR</strong> (mL/min/g kw)</td>
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<td>C:</td>
<td>0.90 ± 0.27</td>
<td>0.78 ± 0.22</td>
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<td><strong>MAP</strong> (mm Hg)</td>
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<td></td>
</tr>
<tr>
<td>C:</td>
<td>109 ± 11</td>
<td>104 ± 11</td>
<td>108 ± 9</td>
<td>105 ± 8</td>
</tr>
<tr>
<td>4-MU:</td>
<td>97 ± 4</td>
<td>96 ± 4</td>
<td>96 ± 3</td>
<td>92 ± 3</td>
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<td><strong>Urine flow rate</strong> (µL/min/g kw)</td>
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<tr>
<td>C:</td>
<td>1.31 ± 0.45</td>
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<td>2.61 ± 0.89</td>
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<td>4-MU:</td>
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<td>1.16 ± 0.34</td>
<td>1.48 ± 0.41</td>
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<td><strong>Sodium excretion</strong> (µL/min/g kw)</td>
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<tr>
<td>C:</td>
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Glomerular filtration rate (GFR), mean arterial blood pressure (MAP), urine flow rate and sodium excretion in control rats (C) and in rats treated with the hyaluronan synthesis inhibitor 4-MU. Each period corresponds to 45 min in consecutive order, kw= kidney weight. * denotes p<0.05 vs control period of same group. Values are means ± SEM.
Study III

Osmolality change
The HA content in the supernatant of RMICs grown under iso-osmotic conditions was 0.29 ±0.11 ng HA/ng cell protein. A change of the RMIC growth media osmolality from normal to low (200 mOsm/kg H₂O), which simulates hydration *in vivo*, increased HA in the supernatant 4-fold, thereby corroborating our previous *in vitro* and *in vivo* studies regarding osmolality-regulated HA turnover (Figure 13).

HAS2 and Hyal2 mRNA expressions were unaltered by osmolality change (Figures 14, 15), while Hyal1 mRNA was reduced by 35% (Figure 16) and cell surface expression of scavenging receptor CD44 was reduced by 90% (Figure 17). Supernatant Hyal activity of the low osmolality group was reduced by 44% compared to iso-osmolar conditions (Figure 18).

![Figure 13. Hyaluronan (HA) content in supernatants of cultured RMICs during control conditions (normal osmolality), and after 24h exposure to either hypo-osmotic media conditions (200 mOsm/kg H₂O), a hyaluronidase inhibitor (Asc-P), or the HA synthesis inhibitor 4-MU. P<0.05 vs corresponding value of control cells.]

Changing the hyaluronan synthesis or degradation
Addition of L-ascorbic acid 6-hexadecanoate (Asc-P), an inhibitor of Hyal activity, elevated HA similarly as low osmolality, implying an important regulatory mechanism (Figure 13).

Using 4-methylumbelliferone (4-MU) to inhibit HAS, reduced HA by 52% (Figure 13).
Figure 14. mRNA expressions of hyaluronan synthase 2 (HAS2) in RMICs during different treatments. All values related to cells grown at normal osmolality=100%.

Figure 15. mRNA expressions of hyaluronidase 2 (Hyal 2) in RMICs during different treatments. All values related to cells grown at normal osmolality=100%.

Figure 16. mRNA expressions of hyaluronidase 1 (Hyal 1) in RMICs during different treatments. All values related to cells grown at normal osmolality=100%.

*P<0.05 vs control cells (normal osmolality).
Figure 17. Expression of the scavenging receptor CD44 on the cell surface of RMICs during different treatments.

Figure 18. Hyaluronidase activity in supernatants of cultured RMICs during different treatments. Values are related to the amount of total cell protein in each culture dish. RI= relative intensity. *P<0.05 vs control cells (normal osmolality).

Angiotensin II and ADH
Neither Ang II nor ADH alone reduced HA content in the supernatant significantly (-16% and -58%, respectively, ns), while simultaneous treatment with the two hormones reduced HA by 69% (Figure 19).

The combination of Ang II and ADH caused no change in the mRNA expressions of HAS or Hyals, while Hyal activity in the supernatant increased by 67% (Figure 18). CD44 expression was also only affected by the combination of Ang II and ADH (-42%; Figure 17).
Figure 19. Hyaluronan (HA) content in the supernatant of cultured RMICs during control conditions (normal osmolality), and after 24h exposure to either angiotensin II (Ang II, 10^{-6}M), vasopressin (ADH, 10^{-6}M), or a combination of Ang II and ADH. * P<0.05 vs corresponding value of control cells.

Endothelin
Endothelin (ET-1) at low concentrations (10^{-10} and 10^{-8}M) increased HA 3-fold (Figure 20). On the contrary, at a high concentration (10^{-6}M), ET-1 reduced HA by 47%, compared to controls.

The ET-A receptor antagonist BQ123 not only reversed the reducing effect of high ET-1 on HA, but elevated it to the same level as low concentration ET-1 (10^{-10}M), suggesting an important mechanism involving the active ET-B receptor and separate regulating roles for ET-A and ET-B receptors. This was corroborated by the addition of ET-B receptor antagonist BQ788 to low concentration ET-1, which abolished the HA increase.

HAS2 and Hyal2 mRNA expression did not change in most of the treatment groups, however, in the groups with low and medium concentration of ET-1, HAS2 increased (Figure 14), and BQ788 together with low concentration ET-1 increased expression of both HAS2 and Hyal2 (Figure 14, 15). Hyal1 mRNA increased at all ET-1 concentrations tested (Figure 16). This elevation was not affected by the ET-A receptor blocker BQ123, but was abolished by the ET-B receptor blocker BQ788.

Hyal activity was elevated the most by high ET-1 concentration (Figure 18). At 10^{-8}M, the activity increased by 54%, while the high concentration (10^{-6}M) elevated the Hyal activity by 137%. Blockade of ET-A receptors by BQ123 prevented about 30% of this response. When the ET-A receptor antagonist BQ123 was added, the elevation in activity after the high concentration of ET-1 (10^{-6}M) reduced and was thus similar to that of the lower concentrations used (55%).
CD44 expression was elevated by the low ET-1 concentrations to more than 4-fold above baseline (Figure 17). The intermediate dose of ET-1 (10^{-8} M) also increased the CD44 expression, but to a lesser extent.

![Figure 20. Hyaluronan (HA) content in the supernatant of cultured RMICs during control conditions and after 24h exposure to endothelin (ET-1), with or without the ET-A receptor antagonist BQ123 or the ET-B receptor antagonist BQ788. *P<0.05 vs control cells (normal osmolality).]

Study IV

Parameters of diabetes
Diabetic animals displayed classic symptoms: hyperglycemia, proteinuria, hyperfiltration, renal hypertrophy, overt diuresis with reduced urine osmolality, reduced weight gain, and also elevated renal cortical and outer medullary HA content.

Arterial blood pressure and renal blood flow were similar between diabetics and vehicle controls (Table 2). Rapamycin treatment did not affect these parameters during control or diabetic conditions, although a slight tendency was observed towards reduced arterial blood pressure.

Hyaluronan content
HA was regionally accumulated in the kidneys of diabetics. Cortical and outer medullary HA were elevated more than 2-fold and 3-fold, respectively (cortex: 0.0125±0.001 vs 0.0268±0.001 µg/mg protein, p<0.05; outer medulla: 0.711±0.120 vs 2.743±0.263 µg/mg protein, p<0.05; Figure 21, 22), while papillary HA was unaffected in diabetic rats (data not shown). Ra-
Pamycin treatment did not alter the diabetes-induced HA accumulation in any part of the kidney, and did also not affect the HA in controls.

The urine Hyal activity was 95% higher in diabetic rats compared to controls (p<0.05; Figure 23).

Figure 21. Cortical hyaluronan (HA) content in control rats treated with or without rapamycin and diabetic rats treated with or without rapamycin. * denotes P<0.05 vs corresponding control group.

Figure 22. Outer medullary HA content in control rats treated with or without rapamycin, and diabetic rats treated with or without rapamycin. * denotes P<0.05 vs corresponding control group.

Figure 23. Urine hyaluronidase activity in control rats treated with or without rapamycin, and diabetic rats treated with or without rapamycin. RI= relative intensity. * denotes p<0.05 vs corresponding control group.
Urine concentration challenge
The ability to respond to the vasopressin V2-receptor agonist desmopressin with increased urine osmolality was absent in the diabetic rats, whereas in controls, osmolality increased by 45-65% (p<0.05; Figure 24). Both control rats treated and untreated with rapamycin, elevated their urine osmolality by 46-64% (p<0.05). In control rats, urine osmolality increased from 1329±140 to 1917±178 (p<0.05), and in those treated with rapamycin, it increased from 1287±93 to 1624±147 mOsm/kg H₂O (p<0.05). In the diabetic rats, urine osmolality did not increase significantly when treated with desmopressin (diabetic rats from 894±45 to 1110±166 mOsm/kg H₂O, ns; diabetics+rapamycin from 874±46 to 1020±173 mOsm/kg H₂O, ns; Figure 24).

In diabetic rats treated with rapamycin, the proteinuria was reduced by 32% (controls 0.114±0.006; diabetics 0.279±0.040 mg/min, p<0.05; Figure 25), while all other parameters were unaffected (Table 2). Rapamycin did not alter protein excretion in the control rats.

Figure 24. Change in urine osmolality during challenge with the vasopressin V2-receptor agonist desmopressin in control and diabetic rats with or without rapamycin treatment. * denotes p<0.05 vs before desmopressin.

Figure 25. Urinary protein excretion in control rats treated with or without rapamycin, and diabetic rats treated with or without rapamycin. * denotes P<0.05 vs corresponding control group, whereas # denotes P<0.05 vs vehicle-treated diabetics.
Table 2. *Data from control and diabetic rats treated with vehicle or the mTOR inhibitor rapamycin.*

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + Rapamycin</th>
<th>Diabetes</th>
<th>Diabetes + Rapamycin</th>
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<tr>
<td><strong>Body weight (g)</strong></td>
<td>481±16</td>
<td>473±10</td>
<td>331±14*</td>
<td>317±11*</td>
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<tr>
<td><strong>Total kidney weight (g)</strong></td>
<td>2.97±0.04</td>
<td>2.84±0.06</td>
<td>3.91±0.09*</td>
<td>3.80±0.05*</td>
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<tr>
<td><strong>Blood glucose (mM)</strong></td>
<td>6±0.1</td>
<td>7±0.5</td>
<td>22±1*</td>
<td>23±1*</td>
</tr>
<tr>
<td><strong>Arterial blood pressure (mmHg)</strong></td>
<td>115±3</td>
<td>107±3</td>
<td>112±4</td>
<td>105±5</td>
</tr>
<tr>
<td><strong>Urine flow rate (µl/min/kidney)</strong></td>
<td>4±0</td>
<td>5±1</td>
<td>25±2*</td>
<td>30±4*</td>
</tr>
<tr>
<td><strong>Urine osmolality (mOsm/kg H₂O)</strong></td>
<td>1329±140</td>
<td>1287±93</td>
<td>894±45*</td>
<td>874±46*</td>
</tr>
<tr>
<td><strong>GFR (ml/min/kidney)</strong></td>
<td>1.59±0.11</td>
<td>1.56±0.14</td>
<td>2.71±0.32*</td>
<td>2.78±0.48*</td>
</tr>
<tr>
<td><strong>RBF (ml/min/kidney)</strong></td>
<td>5.6±0.4</td>
<td>5.0±0.2</td>
<td>5.1±0.5</td>
<td>4.4±0.3</td>
</tr>
</tbody>
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Values are means ± 1SEM. * denotes P<0.05 vs corresponding control group.
Discussion

The traditional view of regulating water reabsorption in the renal medulla, is primarily by changing the epithelial cell permeability for water i.e. ADH-regulated aquaporins. However, the past two decades have disclosed data, which infer that the renomedullary interstitial space also changes in its matrix composition during variations in hydration status, and also during pathological conditions (Stridh 2012). This would suggest that not only the epithelial cell, but also the interstitial space, change in terms of permeability characteristics, when fluid reabsorption needs to change in relation to intake. A dominating ECM component of the renomedullary interstitial space is the negatively charged glycosaminoglycan HA. The results of the present thesis are in favor of the concept that HA participates in renal fluid regulation, by changing in content with variations in hydration status, and thereby affecting interstitial permeability characteristics.

Ginetzinsky (1958) was the first to propose an involvement of HA in renal water handling. It was suggested that the antidiuretic hormone (ADH, arginine vasopressin) in the renal medulla, exerted its action through the activation of Hyals, which changes the interstitial properties for fluid transport by reducing the amounts of charged glycosaminoglycans. The findings were forgotten, and the discovery of aquaporins (Carbrey 2009) shifted the interest further from a possible involvement of HA in renal fluid regulation.

Other studies have suggested an important role for HA in renomedullary water handling. During normal physiological conditions in the rat, medullary HA levels, as opposed to the cortical, will change in relation to body hydration status. Acute hydration of the rat increase medullary interstitial HA content, while the opposite occurs during water deprivation (Hansell 2000, Göransson 2002). By changing the physicochemical characteristics of the interstitial space, which may involve alterations in interstitial hydrostatic pressure, HA will affect fluid handling (Lai-Fook & Brown 1991, Zawieja 1992, Wang 1999). The elevated medullary HA in the interstitium during excess water intake, antagonize medullary water reabsorption by changing the interstitial matrix properties, resulting in resistance to fluid flow. The opposite occurs during water deprivation in conjunction with increased ADH-regulated aquaporins.

A possible mechanistic view to the effect of HA on fluid transport is the following: 1) the repulsion between the negatively charged carboxylate...
groups of the sugar moieties (glucuronic acid) along the HA chain protrude outward at regular intervals, contributing to HA structure and size, 2) the negatively charged gel attracts positively charged ions, increasing osmosis, which will attract water into the gel matrix. Further water reabsorption will be antagonized once water is immobilized within the gel. Additionally, the medullary thick ascending limb of the loop of Henle (mTAL), which generates the medullary osmotic gradient (“counter-current multiplier”), may be functionally compromised when interstitial diffusion characteristics are changed due to elevated HA. And, when diffusion characteristics change, the efficiency of the vasa recta (“counter-current exchanger”), which help to maintain the osmotic gradient by recirculating fluid and electrolytes in the medulla, may also be affected, resulting in altered urine concentration capacity. Finally, interstitial swelling (“functional edema”) due to elevations in HA, increases the diffusion distances between the tubules and blood vessels, affecting the reabsorption rate.

Study I – Hyaluronan during final nephrogenesis

The study generated new insights into the intrarenal deposition of HA during the final stages of nephrogenesis. These insights clarify how the adult, heterogeneous HA levels arise, and their dependence on normal Ang II function.

Intrarenal HA content after birth is initially high throughout the kidney, but is rapidly reduced during days 8–13, primarily in the cortex, thus attaining very low cortical levels and high medullary levels. The rapid reduction is dependent on temporal regulation of primarily HAS2 and Hyal1, and requires an intact Ang II function, but does not rely on lymphatic vessels.

In the ACEI-treated kidney, the remaining high levels of HA might contribute to the pathological renal phenotype in the adult, i.e. tubulointerstitial inflammation and reduced urinary concentrating ability (Friberg 1994b, Guron and Friberg 2000) due to the pro-inflammatory and extreme water-attracting properties of HA.

It is well established that an intact RAAS is necessary for renal development. Interruption of the RAAS during nephrogenesis, results in severe structural and functional abnormalities (Friberg 1994b, Guron and Friberg 2000, Guron 1998, Nilsson 2001). Inhibition of the growth and proliferative properties by Ang II appears to be the primary mechanism for the prevailing renal defects (Guron and Friberg 2000). The structural abnormalities in rats and pigs resemble the renal histological changes in ACEI fetopathy observed in human fetuses and newborns (Shotan 1994). Guron et al. (1999) have demonstrated the consequences of papillary atrophy for urinary concentrating ability and handling of electrolytes and water. The inflammatory process in response to neonatal RAAS inhibition in the adult, is associated with
infiltrating cells in the interstitium, which appear first in the cortical regions and then deeper, in the renal medulla (Guron and Friberg 2000).

HA may have a role in inflammatory responses, where it directly stimulates the expression of cell adhesion molecules, such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mouse kidney epithelial cells (Oertli 1998). According to the previously reported pro-inflammatory activities of HA (McKee 1996), recruitment of leucocytes may be initiated by HA interaction with the cell surface HA scavenger receptor CD44. Furthermore, inflammatory cells produce growth factors that stimulate the synthesis of HA by fibroblasts (Hamerman and Wood 1984, Yaron 1978).

Ang II AT1-receptor antagonists are as equally effective as ACEI (Lassaitiene 2006). Angiotensin AT1-receptor expression increases during development and continues to increase postnatally, peaking at about 7 days after birth, with mRNA at about twice that as during adulthood (Norwood 1997, Tufro-McReddie 1993). Ang II AT2 receptor expression is not detectable at day 14 after birth (Aguilera 1994, Norwood 1997). This implies that the observed changes in ACEI-treated animals are primarily due to reduced AT1 receptor signaling. The high HA levels in the normal kidney cortex and medulla are reduced at an impressive rate, primarily between days 8 and 13 after birth. In absolute values, the reduction is largest in the medulla.

Nilsson et al. (2001) speculated that the inflammatory condition obtained during neonatal ACEI activates HAS, leading to the elevated HA levels found in the adult kidney. This speculation was based on previous data, proving that inflammatory cells release factors that induce HA synthesis via HAS (Hamerman and Wood 1984, Heldin 1989). From the results of Study I, it is obvious that the mechanism regulating the normal disappearance of HA was abrogated by neonatal inhibition of RAAS. What mechanisms underlie the normal rapid reduction of renal interstitial HA in these the final stages of nephrogenesis, and what causes the obstructed disappearance in the ACEI-treated rats? There are four main possibilities: a) reduced synthesis by HAS, b) increased degradation by Hyals, c) development of lymphatic vessels, or d) a combination of all three.

**Reduced synthesis by hyaluronan synthase**

Study I found the same expression pattern, mentioned above for HAS, during late nephrogenesis. HAS2 dominated both in the cortex and medulla, in line with Rosines et al. (2007). After birth, HAS2 gradually diminished with time, correlating with the reduction in interstitial HA. The medullary HAS2 expression in neonatally ACEI-treated animals was similar to that of control animals. However, the cortical HAS2 gene expression was downregulated similarly to controls in the neonatally ACEI-treated animals, and was almost
twice higher than that in controls. This can explain the high cortical HA levels in the ACEI-treated neonates.

There is not an exact overlap between the accumulation of HA, and changes in HAS2 and Hyal1 mRNA expression. This cannot be fully explained, but it should be noted that the animals in the HA analysis are not identical to those in the gene expression analysis. Additionally, the changes in activity of HAS and Hyals prior to the changes in actual protein amount can also be involved.

The mechanisms are unclear for the gradual expression reduction of HAS2 during the final phases of nephrogenesis. The effect does not seem to be directly Ang II AT1-receptor mediated, as angiotensin requires the insulin-like growth factor and its receptors for its maturing action on nephrogenesis (Nilsson 2000), which in itself will induce HAS (Kuroda 2001). Why loss of Ang II signaling results in an inability to implement this gradual reduction in HAS2 mRNA levels is not known, but possibly, the loss of Ang II signaling results in an inflammatory reaction. Cytokines such as tumor necrosis factor-alpha, interferon-gamma, and transforming growth factor-β, modulate HAS gene expression and protein activity (Hamerman and Wood 1984, Heldin 1989).

The cortical HAS1 shows a clear tendency of higher expression in ACEI-treated animals, which could contribute to the elevated HA. HAS3 expression in both cortex and medulla was lower in ACEI-treated animals compared to controls at day 17 after birth. The consequences are unclear, as HAS3 is only weakly expressed and should have a small impact on the total production of HA.

Increased degradation by hyaluronidases

In Study I, all Hyals were expressed in the neonatal kidney, but only the expression of medullary Hyal1 changed in a similar pattern as the HA content. The medullary Hyal1 expression increased with time after birth, thus possibly contributing to the medullary reduction of HA. In the ACEI-treated neonates, the decreased expression of medullary Hyal1 could contribute to the higher HA levels found in ACEI-treated kidneys. It is possible that during normal conditions, Hyal1 increases and degrades HA together with Hyal2. During ACE inhibition, Hyal1 decreases, but Hyal2 remains unchanged, which leads to reduced degradation of HA in lysosomes, and accumulation of a partially degraded HA by Hyal2. Thereby, the change in Hyal1 could contribute to both HA accumulation and inflammation following ACE inhibition. However, it cannot be ruled out that the change in Hyal activity could include a change in the activity of Hyal2 in spite of stable mRNA expression.

Ang II can reduce HA in the supernatant of RMICs in culture when combined with ADH (Rügheimer 2008a), which has been shown to activate the
Hyals (Ginetzinsky 1958, Gusev 1983, Ivanova and Melidi 1999). The infusion of ADH reduces renomedullary HA (Rügheimer 2008a). From these results, it is plausible to suggest that Ang II during completion of nephrogenesis will reduce renal interstitial HA content primarily by activating Hyals, without increasing gene expression or protein content. This would be in accordance with the urine Hyal activity, showing that ACEI-treated animals only had half the activity compared to that of controls, and remained stable, while control animals initially had high activity, which reduced with time.

Certainly, the high HA levels in the kidney during development are a prerequisite for normal organ development, but they are also suggested to have a role in antagonizing water reabsorption and limiting urine concentration performance prior to completion of nephrogenesis (Sulyok and Nyul 2005). The accumulation of HA in kidneys of neonatally ACEI-treated rats may affect renal fluid handling in both young and adult animals. These kidneys have reduced ability to concentrate urine (Nilsson 2001). This is similar to other models of kidney damage where HA is accumulated, such as ischemia–reperfusion injury, renal transplant rejection, and diabetes (Göransson 2004, Hallgren 1990, Rügheimer 2008b). An increased accumulation of water (edema) and presentation of ligands for receptors on inflammatory cells are two consequences of the HA accumulation (Gerdin and Hallgren 1997, Göransson 2004, Johnsson 1996). As described above in this thesis, elevated interstitial HA will counteract water transport across renal membranes due to the physicochemical properties of HA, thereby affecting urinary concentration ability.

Lymphatic vessel development

One of the questions posed in Study I was if the normal disappearance of HA could be due to the development of lymphatic vessels in the renal cortex, and if the accumulation of HA in ACEI-treated animals might be due to disrupted lymphatic development. The examination of lymphatic endothelial cell-specific mucoprotein podoplanin expression (Kerjaschki 2004), showed that the number of podoplanin-positive cells was 18-fold higher in the ACEI-treated group, which implies no inhibition of lymphatic development. It cannot, however, be concluded if the observed increased index of lymphatics were functional.

CD44 is the most studied HA receptor and found in a variety of cells (Toole 1990, Underhill 1992). In Study I, CD44 was not upregulated in ACEI-treated animals at day 21 after birth, but it is possible that the inflammatory process at this time was still in its very early phases and had not yet affected CD44 expression.

To summarize, the high renal HA content at birth is rapidly and evenly decreased during the completion of nephrogenesis, which takes place neonatally in the rat. At day 21 after birth, the HA content resembles the heteroge-
neous distribution found in the normal adult kidney. The process is dependent on normal Ang II function and primarily involves reduced expression of HAS2 and increased expression of medullary Hyal1.

The water-binding and pro-inflammatory properties of HA could, together with the accumulation of HA in the neonatally ACEI-treated kidney, partly explain the pathological renal phenotype of the adult kidney, which includes reduced urinary concentration ability and tubulointerstitial inflammation.

Study II – Hyaluronan effect on fluid transport

Study II demonstrates that if the HA synthesis inhibitor 4-MU is used to reduce renomedullary HA, the ability to respond to a hydration challenge will become suppressed, without affecting neither GFR nor arterial blood pressure. This suggests that the interstitial matrix component HA changes the physicochemical characteristics of the medullary interstitial space, which directly affects fluid transport from the tubular lumen through the interstitial space. Earlier findings on changes in medullary HA in relation to hydration status, corroborate this suggestion (Hansell 2000, Göransson 2002). During hydration, interstitial HA is elevated, which will reduce reabsorption in conjunction with reduced ADH-regulated aquaporins. During dehydration, lower interstitial HA and the upregulated aquaporins will increase fluid transport across the interstitial space of the medulla, leading to increased reabsorption.

It has been demonstrated that ADH infusion not only reduces renomedullary HA levels in vivo but also reduces the HA content in the supernatant of cultured RMIC (Rügheimer 2008a). As mentioned above, these cells are major producers of HA in the renal medulla. The effect on HA levels primarily involves the V1-receptor, since the V2-receptor agonist desmopressin did not alter the HA levels (Hansell 2000). The consequence is that ADH seems to exert its antidiuretic effect via two separate pathways; First, by regulating the number of aquaporins in the apical membranes of the medullary tubular system via the V2-receptor, thus changing the permeability. Second, by changing the physicochemical characteristics of the interstitial space by changing the HA content via the V1-receptor, which will reduce or increase fluid permeability.

Study II suggests that HA participates in renal water handling directly, by changing the physicochemical characteristics of the medullary interstitial matrix, and possibly the interstitial hydrostatic pressure (Hansell 2000, Lai-Fook & Brown 1991, Zawieja 1992). This enables HA to function as an important regulator of water diffusion in the interstitium, regardless of organ origin. A possible mechanistic view is mentioned above in this thesis. In Study II, 4-MU reduced the medullary HA content, which corresponded to an increased ability to reabsorb water, demonstrated by a reduced diuretic response upon hydration. The animals ingested the same amount of fluid
during the treatment period, but it cannot be ruled out that changed plasma ADH, or aquaporin expression, affected the response.

The exact mechanism underlying the reduced HA production by 4-MU is unclear. However, it is known that 4-MU has no direct influence on HAS, as studied in vitro by Kakizaki et al. (2002). Quite contrary, 4-MU is suggested to deplete the cellular stores of the HA substrate UDP-glucuronic acid, and thus reduce HA synthesis (Kakizaki 2004).

In summary, when renomedullary HA is reduced using the HA synthesis inhibitor 4-MU, the kidney cannot respond appropriately to hydration. The study strengthens the concept that HA modulates the properties of the medullary interstitium, and directly affects the transport of fluid.

Study III – Hormonal influence on hyaluronan turnover

Study III demonstrates an important regulatory role of reduced osmolality and hormones involved in renal fluid handling on HA turnover, by studying cultured RMIC. The results demonstrate an important mechanism via altered Hyal activity to regulate HA turnover. As opposed to primarily regulating synthesis, this provides rapid change of HA levels.

The findings in Study III increase our understanding of how the composition of the renomedullary interstitial matrix changes in response to hydration status, and also sets the focus on Hyal activity for rapid responses in HA turnover.

Changes in HA content are due to changes in HA synthesis and/or HA degradation. Study III suggests that the regulation of the degradation is of major importance for the changes we observe in supernatant HA, both after a hypo-osmolar challenge as well as after hormonal action.

As mRNA expressions of HAS2 and Hyal2 in RMIC did not change after hypo-osmotic challenge, Ang II, or ADH, this suggests a change in Hyal activity. Previous in vivo data from rats show no change in the mRNA levels of HAS or Hyals after 2h hydration, when medullary HA is elevated (Rügheimer 2009), again suggesting a change in the activity of Hyals.

In the case of ET-1, mRNA levels of Hyal1 were elevated, while hypo-osmolality reduced the Hyal1 expression, which points to the importance of the degradation pathway as a way to change HA. In a previous in vitro study (Göransson 2001) in cultured rat RMICs, the HA-binding receptor CD44 is downregulated under hypo-osmotic conditions (mimicking in vivo hydration), while it is upregulated under hyperosmotic conditions (mimicking water deprivation). This if corroborated by Study III results, showing reduced CD44 expression when reducing growth media osmolality.

catalyzed by Hyal. This implies that during hypo-osmotic conditions, when low ionic strength applies, both the uptake of HA via CD44 and the breakdown of HA by Hyal, will be reduced. The elevation in HA (within 2h) by acute hydration in vivo suggests an important role via inhibition of Hyals and not primarily increased HAS expression or activity.

The addition of different ET-1 concentrations resulted in a biphasic response of supernatant HA. Such a concentration-dependent biphasic response is known for ET-1 in vascular smooth muscle, i.e. low concentration results in dilation and high concentration results in contraction (Harris 1991, Edwards 1992). Study III presented us with increased HA in the supernatant from low concentration ET-1, while high concentration reduced HA. When combining the high concentration with the ET-A receptor antagonist BQ123, HA returned to a level comparable with low concentration ET-1. As a hypothesis, low concentration ET-1 could primarily affect ET-B receptors, which increase HA by increasing NO production (Deliu 2012, Chenevier-Gobeaux 2004, Rügheimer 2005). This is corroborated by an abolished increase in HA by addition of the ET-B receptor antagonist BQ788 to low concentration ET-1.

Endothelins are known to enhance the release of prostaglandins by stimulation of ET-B receptors located on vascular endothelial cells (DeNucci 1988, Warner 1989), and prostaglandins elevate HA production (Honda 1993, Mahadevan 1995, Rügheimer 2005). High concentration of ET-1 may primarily affect ET-A receptors, which have been shown to increase CD44 expression through a BQ123-sensitive mechanism (Tanaka 2000).

An inverse relationship between elevated levels of surface CD44 on RMICs and supernatant HA was previously demonstrated, suggesting increased internalization (Göransson 2001), and thereby reduced levels of HA in the supernatant.

These changes in HA in Study III are in accordance with the demonstrated effects on medullary fluid handling of ET-A versus ET-B receptor activation. ET-B activation will reduce fluid reabsorption via nitric oxide, while ET-A activation will increase fluid reabsorption (review by Hyndman 2013).

The HA content in the supernatant of cultured RMICs was reduced by Ang II and ADH in combination, and it was previously shown that ADH infusion in vivo reduces papillary HA (Rügheimer 2008a). ADH presumably acts via the V₁-receptor, as the selective V₂-receptor agonist desmopressin fails to produce such a response (Hansell 2000). More corroboration for this is gained by the finding of V₁a receptors on RMICs (Hughes 1995), as opposed to the V₂-receptor, which has not been described in studies of these cells.

ADH stimulates the activity of Hyal in the rat renal papilla. The activation of these enzymes is associated with a decrease in the content of HA (Ivanova and Goryunova 1981). In homozygous Brattleboro rats lacking ADH, the urine osmolality and Hyal activity of renal papillary tissue were closely re-
lated after ADH treatment (Ivanova 1982). It is supported by Hansell (2000), that outer medullary HA content is increased in Brattleboro rats, inferring reduced breakdown. It has also been demonstrated that antisera against rat kidney Hyal blocks the hydro-osmotic effect of ADH (Law and Rowen 1981). In Study III, HA was not reduced with statistical significance by ADH alone, only when combined with Ang II. However, a previous study found a reduction of HA after ADH treatment (Rügheimer 2008a). The reason for this discrepancy is not clear. The effector mechanism underlying the reduction of HA by Ang II treatment (in combination with ADH) seems to be, at least partly, due to increased Hyal activity. This would be in accordance with data on neonatal ACE inhibition, showing reduced Hyal1 mRNA expression in the renal medulla and reduced urine Hyal activity early in the newborn rat (see Study I). It is also noteworthy that these two hormones are simultaneously elevated during dehydration.

In Study III on RMICs, a high dose ET-1 (ET-A receptor mediated) reduces the content of the ECM component HA, similarly to Ang II in combination with ADH. Both Ang II and ADH levels in plasma are elevated in antidiuresis, when medullary HA levels in vivo are reduced. Conversely, Ang II and ADH are reduced in plasma during water diuresis when medullary HA is elevated. However, it has previously been shown that ET-1, through the ET-A receptor, and Ang II, through the AT₁ receptor, will increase proliferation and ECM production by RMICs in culture (Maric 1999, Maric 2006). This presents an apparent contradiction, and what could be the underlying cause? The standard index for ECM production (i.e. ³⁵S-methionine/cysteine incorporation) is not a measure of HA production, as HA does not incorporate methionine/cysteine, due to HA being a sugar compound. This index is better suited for estimating collagen-related production, and the true relationship between different matrix components has not been demonstrated in parallel with the ECM-index. It has, however, been shown that laminin production by RMICs increases after Ang II treatment (Maric 2006). A reduced HA content in the medullary interstitium favors an increase in the permeability of glycosaminoglycan structures adjacent to the cell surface. As RMICs probably provide structural support for the renal medulla, it could be speculated that an increase in ECM production, like laminin, can maintain structural integrity when HA levels are reduced in parallel to increased in vivo interstitial water permeability. However, a reduced HA content coupled with elevation of collagen in a tissue, would provide for fibrosis during pathological conditions, as the HA reduction leads to reduced viscoelasticity and hydration.

CD44 is the main cell surface receptor for HA (Aruffo 1990). CD44 also provides a signal response to HA. It participates in HA endocytosis as a scavenger receptor (Lee & Spicer 2000). In Study III, low media osmolality reduced RMIC surface expression of CD44 when supernatant HA content
was concomitantly increased, which could provide a pathway for regulation via reduced internalization and degradation.

However, in other situations of altered CD44 expression (Ang II+ADH and low concentration ET-1, respectively), the supernatant HA content and CD44 expression changed in opposite directions, which does not correspond to a causal relationship. The underlying mechanism for reduced CD44 expression after Ang II+ADH, and elevated expression after low concentration of ET-1, is still unclear.

The Hyal inhibitor used in Study III (L-ascorbic Acid 6-hexadecanoate, Asc-P) is a documented, potent inhibitor of different Hyal activities (Botzski 2004). The importance of Hyal activity for regulating HA turnover in RMIC is distinct; inhibition of Hyal activity during iso-osmotic conditions, increases supernatant HA to similar levels as RMICs grown under hypo-osmotic conditions. As previously stated, this is in accordance with the suggested elevation in HA during hypo-osmotic conditions occurring through reduced intracellular Hyal activity.

In summation, Study III demonstrates an important regulatory influence of osmolality and hormones of central importance for renal fluid regulation on HA handling by RMICs, thereby supporting the concept of a dynamic involvement of interstitial HA on renal fluid handling.

Study IV – Mechanism for hyaluronan accumulation in diabetes

Study IV demonstrated accumulation of HA in the kidney during established diabetic conditions, which was not affected by treatment with the mTOR inhibitor rapamycin. However, rapamycin treatment did reduce diabetes-induced proteinuria.

The lack of effect on HA accumulation by mTOR suggests that HA, as opposed to other ECM components such as collagen, laminin, and fibronectin, is not induced via mTOR activation during hyperglycemic conditions. This discrepancy could be due to the unique structure of HA among the other ECM components, like not containing sulphate groups or any peptide, but this remains to be established. Another explanation is that the accumulation of HA, which is already established, is not reversible at this stage. Study IV could also show a reduced ability of the diabetic animals to respond with an elevation in urine osmolality when challenged with an ADH V₂-receptor agonist.
mTOR pathway

From the extracellular space to the cell nuclei, mTOR is a signaling pathway of hyperglycemia, like a metabolic sensor (review by Mariappan 2013). mTOR is a protein kinase with two distinct complexes, namely complex 1 (mTORC1) and complex 2 (mTORC2) (Wullschleger 2006). Rapamycin will primarily inhibit mTORC1, which in turn regulates several cellular processes, such as cell growth, proliferation and protein synthesis. Over-activation of mTORC1 in podocytes leads to albuminuria, glomerular basement membrane widening, expansion of the mesangium, and accumulation of fibronectin and collagen IV. These events can partially be prevented by rapamycin (Gödel 2011). In Study IV, rapamycin treatment in rats with established diabetes failed to reduce the HA accumulation, thus inferring a non mTOR-mediated response. If that is the case, then there are several other pathways that could induce renal HA during hyperglycemia in this study, of which the following are excellent contributors alone or in concert: oxidative stress, TGF-β, PKC, NF-κB, PGE₂, cytokines (e.g. IL-1), and PDGF (reviewed by Stridh 2012, Brosius & Alpers 2013, Dunlop 1996).

Another possibility is, that the already established accumulation of HA cannot be reversed by mTOR inhibition. This would infer that HA still might be dependent on the mTOR pathway, but that it can only be demonstrated if the inhibitor is introduced early in the disease process. Speaking against such a possibility, is the fact that an earlier study, with 4 weeks of STZ-diabetes, does not yet disclose elevations in cortical HA, while that of the medulla is increased (Rügheimer 2008b). In the present study, rapamycin is introduced after 6 weeks of STZ-diabetes, and administered during the following two weeks. This should be sufficiently early in the HA accumulation to disclose an effect of rapamycin.

Hyaluronan in diabetes

HA has potent water-attracting properties, and HA fragments are pro-inflammatory (Laurent & Fraser 1992). These qualities make HA a good candidate as a matrix component, for involvement in diabetic nephropathy and other kidney diseases (reviewed by Stridh et al. 2012). These HA traits will affect fluid transport in the interstitial space as mentioned above, and drive inflammatory pathways. Previous studies have demonstrated increased HA content in diabetic kidneys. A marginal increase of HA in kidney tissues from diabetic rats could be determined by Malathy and Kurup (1972). Also, Berenson et al. (1970) and Lewis et al. (2008) found increased HA content in diabetic human kidneys. Wang and Hascall (2004) demonstrated accumulation of HA in the glomeruli of diabetic kidneys, while Rügheimer et al. (2008b) found elevated levels of medullary HA in diabetic rats. Melin et al. (2006) found elevated kidney HA in diabetes, which was strongly induced
by ischemia-reperfusion. The effects of hyperglycemia on cellular HA production in vitro have also been demonstrated on: proximal tubular cells (Jones 2001), glomerular cells (Mahadevan 1995), renal interstitial fibroblasts (Takeda 2001), and mesangial cells (Wang and Hascall 2004), which all increase their production of HA when grown in elevated glucose conditions. High glucose has been shown to stimulate HA production through the PKC/TGF-β cascade in interstitial fibroblasts, whereas in proximal tubular cells, the HA elevation was associated with NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells)-activated transcription of HAS2. It remains to be seen whether it is a question of the same cellular events leading from hyperglycemia to increased HA production in vivo as in i.e. Study IV, but it appears plausible.

The elevated HA levels in the renal cortex and outer medulla of STZ-diabetic rats can be expected to affect fluid transport and inflammation, due to the inherent properties of HA (Laurent & Fraser 1992, review by Stridh 2012). HA of MDa size should normally only be found in very small amounts in the cortical tissue, while it is abundant in the papilla, and in medium amounts in the outer medulla.

Elevation of HA in the renal cortex is found in several pathological situations, such as ischemia-reperfusion injury, tubulointerstitial inflammation and renal transplant rejection (Johnsson 1996, Göransson 2004, Wells 1990, Nilsson 2001, Melin 2006, Decleves 2006, Study I). A more fragmented HA is expected in these pathological situations (Decleves 2012), thus rendering the HA pro-inflammatory. Also, an elevation of outer medullary HA is found in the Brattleboro rat with hereditary diabetes insipidus (Hansell 2000), which is unable to concentrate urine due to ADH deficiency. In all these studies, an inability to regulate fluid balance and concentrate urine is evident, as is in most cases, also interstitial inflammation. Here, it is of interest to note, the previously mentioned changes in medullary HA levels upon hydration. The regulation of renal HA levels in the normal kidney involves both fluid-regulating hormones such as ADH, Ang II, nitric oxide, prostaglandins, and endothelin, and osmolality (Rügheimer 2008a, Study III).

The diabetes-induced proteinuria was the only parameter that was significantly affected by rapamycin treatment in Study IV, and it was reduced by 32%, without changes in GFR or arterial blood pressure. A tendency for a hypotensive effect of rapamycin did not reach statistical significance in control or in diabetic rats. The reduction of proteinuria is a well-established feature of rapamycin (Lloberas 2006, Yang 2007, Mori 2009, Inoki 2011). The effect could be due to over-activation of the kinase mTOR complex 1 (mTORC1) on podocytes, like in hyperglycemic conditions, causing mislocalization of the proteins composing the filtration slits, giving rise to increased endoplasmatic reticulum (ER) stress in podocytes (Inoki 2011), which leads to increased glomerular permeability for macromolecules. Re-
ducing the mTOR activation by use of rapamycin will therefore reduce proteinuria.

Importance of hyaluronidase activity
Urine Hyal activity was elevated in the diabetic rats, which may well be compensatory for the elevated HA levels in the tissue. Of the six Hyals, with different topicalities, Hyal1 is the predominant form and the only Hyal found in human plasma (Csóka 1999). It can therefore be hypothesized that Hyal1 dominates the urine activity. In an investigation of STZ-diabetic rats by Ikegami-Kawai et al. (2003), Hyal activity in the kidney tissue increased from day 3 of diabetes. The activity continued to increase until the third week, resulting in a more than doubled amount than that of the corresponding controls, which is similar to the findings in urine of Study IV. Hyal activity increases only in the STZ-induced diabetic rats, and not in spontaneously diabetic Goto-Kakizaki rats, which were without progressed nephropathy during the study. Hyal may thus potentially be used as a marker of diabetic nephropathy. This could provide possibilities for the diagnosis of patients, and a new clinical role for HA. The elevated Hyal activity in the diabetic kidney may also contribute to the inability to gain sufficient elevation in interstitial HA during hydration (Rügheimer 2008b).

Urine concentration ability
The reduced ability of diabetic rats to increase urine osmolality appropriately when challenged with the ADH V2-receptor agonist desmopressin, shows that the ADH-aquaporin function in these rats is suppressed. A relative inability of STZ-diabetic rats to respond with appropriate diuresis upon hydration challenge was demonstrated by Rügheimer et al. (2008b), which would be in line with the present results. The plasma level of ADH, and abundance of such medullary key proteins for urinary concentration, such as aquaporin 2 and urea transporter A1, are greater in diabetic rats, in spite of ongoing osmotic diuresis (Bardoux 2001; Zerbe 1985, Brooks 1989). These findings are evidence of plausible underlying mechanisms of the unresponsiveness of the diabetic animals to ADH V2-receptor stimulation in Study IV.

In summary, the established STZ-induced diabetes results in regional renal accumulation of the ECM component HA, which is not sensitive to mTOR inhibition by rapamycin. However, the diabetes-induced proteinuria is sensitive to rapamycin. Whether the diabetes-induced renal accumulation of HA occurs through different pathways than other ECM components containing protein and sulphate groups, or is irreversible when established, remains to be shown.
Conclusions

The high renal HA content of late nephrogenesis rapidly and evenly decreases in the neonatal completion of kidney development in the rat. The heterogeneous intrarenal distribution of HA in the adult rat kidney is mainly established during the first three weeks after birth. On day 21, the HA content is similar to that of the adult kidney.

The process of rapid HA reduction in the neonatal period is dependent on normal Ang II function. It primarily involves a reduction of HAS2 expression, and an increase of medullary Hyal1.

The accumulation of HA that results from neonatal ACE inhibition can partly explain the pathological condition of the adult kidney, with tubulointerstitial inflammation and defective papillary development, which causes reduced urinary concentration ability.

The renomedullary HA can be reduced with the HA synthesis inhibitor 4-MU, which will suppress the kidney’s ability to respond to a hydration challenge. The effect of a HA reduction in the kidney, points to the HA ability to change the physicochemical characteristics of the medullary interstitial space, which directly affects fluid transport from the tubular lumen through the interstitial space.

Media osmolality, and hormones of central importance for body fluid homeostasis, affect HA turnover through their effect on RMICs in a manner comparable to that found in vivo during hydration changes. This supports the concept of a dynamic involvement of interstitial HA in renal fluid handling.

Established diabetes, induced by STZ, will induce regional renal accumulation of HA, proteinuria, polyuria, reduced urine osmolality, and reduced response to ADH V2 activation. The HA accumulation is not sensitive to mTOR inhibition by rapamycin. The diabetes-induced proteinuria is, however, sensitive to rapamycin.

The diabetes-induced renal accumulation of HA might use different pathways than ECM components containing protein and sulphate groups. It might also be irreversible when established.

The suggestion is, that during normal physiological conditions, renomedullary interstitial HA participate in renal fluid handling by affecting the inter-
stitial properties for fluid flux across the interstitial space. This is possible due to the water-attracting and physicochemical properties of this glycosaminoglycan. Thus, not only is epithelial permeability changed during variations in hydration status, but also the permeability of the interstitial space, which can maximize the response. During pathological conditions, such as in diabetes, the elevated interstitial HA can contribute to the defective kidney function, due to the pro-inflammatory and water-attracting properties of HA.
Sammanfattning på svenska

Hyaluronan (HA) är en stor molekyl, en polysackarid av repeterande enheter. Den finns i nästan hela kroppen (bl. a. i njuren), men i högst koncentrationer i mjuk bindväv. HA har fått stor uppmärksamhet inom kosmetik, sårläkning, cancerbehandling och artros, men vilken betydelse har den för njurens funktion? Det började med Ginetzinsky 1958, som fann att HA-nedbrytande proteiner, hyaluronidaser, utsöndras med urinen i större mängder vid dehydrering (vätskebrist), och upphör under hydrender (vätskeöverskott). Utifrån detta föreslogs att HA kunde förändra miljön i njurens interstitium, det i allmänhet kanske lite bortglömda utrymmet mellan njurens rörsystem och blodkärlen. Senare försök har visat att HA i njurens inre, i märgen, ökar vid hydrender och minskar vid dehydrering. Dessutom har HA en oenhetlig, heterogen fördelning i njuren, med mycket större mängder i njurens märg, än i de yttre delarna, i barken. Detta stärker frågeställningen att HA kan ha betydelse för njurens vätskehantering, eftersom koncentrationen av urinen blir möjlig på grund av interstitiets miljö i märgen.

När HA ökar, fylls interstitiet av den stora, negativt laddade molekylen, med god vattentilldragande förmåga. HA och vatten bildar tillsammans en gel - interstitiet blir till en svårgenomtränglig gelé. Därmed blir det svårt för vattnet som tas upp från njurtubulus att transporteras/förflyttras över till blodkärlen på andra sidan interstitiet.

Eftersom teorin stämmer väl överens med HA-mängden vid hydrender och dehydrering, så återstår ”bara” att undersöka via vilka mekanismer som reglerar HA i njuren, och hur omsättningen av HA, dess bildning och nedbrytning, hänger ihop med redan kända hormoner som verkar i njuren, till exempel angiotensin II, antidiuretiskt hormon, endotelin med flera.

Den här avhandlingen syftar till att klargöra dessa samband och orsaksförhållanden, som gör att HA kan delta i njurens reglering av vattenhanteering.

Den första studien syftade till att utröna hur HA anläggs i njuren under organutvecklingen på fosterstadiet. Vid njurutvecklingens fullbordan uppstår en kraftig HA-gradient i njuren, med mycket lite i barken, och så mycket som 50-100 gånger mer i njurens inre, i märgen. Denna heterogena fördelning är en förutsättning för normal njurfunktion, och den kan störas vid behandling med ACE-hämmare (ett vanligt blodtryckssänkande läkemedel) under graviditeten. Vi ville utröna den normala regleringen av HA under

I studie 2, testade vi teorin att HA medverkar i njurens vätskehantering. Detta utfördes genom att hämma bildningen av HA, och sedan mätta urinsvaret på en hydreringsutmaning. Vi fann att HA minskade med hjälp av synteshämmaren, samt att njurens förmåga att svara på en hydering minskade, utan att påverka dess filtrationsförmåga. Dessa resultat stärkte teorin att HA deltar i regleringen av njurens vätskebalans genom att förändra miljön i interstitiet.

I studie 3 studerade vi HA i ett cellsystem, in vitro. Genom att studera de HA-producerande njurcellerna RMIC, i cellodling, och mäta effekterna av olika för vätskebalans relevanta hormoner på HA-produktionen, kunde mekanismerna för att reglera HA under sådana förutsättningar tydliggöras. Hypotesen var att om HA är viktig för njurens vattenhantering, så kommer vi att se effekter på HA-omsättningen i cellkulturerna, som liknar de fynd som gjorts in vivo. Särskilt intressanta var effekterna på hyaluronidas-aktivitet och på HA-receptorn CD44, som båda två resulterar i nedbrytning av HA. Resultaten visade att det finns viktig regulatorisk påverkan på HA från hormonerna, och att denna reglering verkar via RMIC. Detta stärkte vår teori, att HA är dynamiskt involverad i njurens vattenhantering.

I studie 4 undersökte vi om den HA-ansamling i njuren, som sker vid diabetes, kunde påverkas av mTOR-aktivering. Tanken bakom en koppling mellan mTOR och HA kom ifrån en föreslagen koppling mellan mTOR och ökad produktion av matrixkomponenter vid diabetes, men det var inte undersökt om detta även gällde HA, som är en matrixkomponent, men utan peptid eller sulfatgrupper. Däremot är det känt att HA ökar vid diabetes. Ökad HA-mängd vid diabetes kan bidra till utvecklingen av njurskador. Vi sökte ett samband mellan ökad HA-mängd vid diabetes och mTOR-aktivering genom att behandla diabetiska råttor med en mTOR-inhibitor, rapamycin, som vanligtvis används för att hämma avstötning vid transplantation. Vi följde däref-

Sammanfattningsvis visar avhandlingen, dels att HA bidrar till njurens vätskehantering under normala förhållanden, dels på de negativa effekter som förhöjda HA-nivåer kan ge vid sjukdomstillstånd såsom diabetes.
Acknowledgements

This work was carried out at the Department of Medical Cell Biology, Section of Integrative Physiology, Biomedical Center, Uppsala University, Sweden, at the Department of Molecular and Clinical Medicine, Wallenberg Laboratory, Sahlgrenska University Hospital, Gothenburg, Sweden.

The project was supported by the Swedish Medical Research Council (Project # 9047, 10840, 11133 and 7172) and Fonds zur Foerderung der Wissenschaftlichen Forschung (Project # 05, Contract 007).

The technical assistance of Silvia Hellström, Charlotta Jonsson and Angelica Fasching, and help with the CD44 analysis by Faranak Azarbayjani and Barbro Einarsson, and with the rapamycin treatment by Ebba Sivertsson is gratefully acknowledged.

This thesis was made possible by a lot of people, and I want to thank each and every one of my colleagues and friends, who all contributed in different ways.

**I would especially like to thank a few people:**

Thank you, Peter Hansell for supervising along the years. And thank you Fredrik Palm, co-supervisor and constant source of information.

Thank you, Donscho Kerjaschki, Louise Rügheimer, Tomoko Takahashi and Mayumi Ikegami-Kawai for rewarding collaborations.

A special thank you to Yun Chen for receiving me in Gothenburg and making it a great memory.

Another special thank you to Johan Olerud for showing me the ways of PCR and cell culture, and for hollering HELLO STRIDH all the time.

Thank you, Daniel Färnstrand for being as flexible as a contortionist. And as helpful too.

Thank you, all of SF.
Thank you, kidney research group:
Angelica Fasching is a super hero. Malou Friederich-Persson will receive a Nobel Prize someday. Ebba Sivertsson can be my physician any day. Patrik Persson is the essence of calm and effective. Per Liss for the bliss. Micke Hultström of the Fluff and Lulz. Liselotte Pihl is a blast (of yellow). Stephie Franzén has enough energy for the whole group. Sara Lycke is probably the one who’s got it all.

Thank you, too temporary members, Daniela Patinha and Carla Carvalho!

Thank you, department of Medical Cell Biology, I can’t mention you all:
Super-Nils Welsh, you’re indispensable. Camilla Sävmarker, Shumin Pan, Marianne Ljungkvist, Erik Gylfe, Göran Ståhl, Lina Thorvaldson, Gunilla Westermark, you keep the place running. Lena Holm, you always somehow manage to crack the nut of teaching distribution.

Thank you, most excellent roommate Sara Massena!

People of the lab! Gustaf Christoffersson, Andreas Ejdesjö, Ulf Eriksson, Rikard Fred, Xiang Gao, Antoine Giraud, Liza Grapensparr, Karin Gustafsson, Liyue Huang, Ulrika Pettersson, Monika Sandberg, Jan Saras, Evelina Vägesjö, Tomas Waldén, Parri Wentzel, David Ahl, Björn Åkerblom. And everybody else!

Thank you bestest friends in the world:
The metal avengers of Malmö – Michaela and Fredrik Lundhag

The Elitist Darkness Club – Anna Sellin, Helena Dahlgren, Anna Bark Persson, Sara Bergmark Elfgren, Maria Nygård, Merit Ljusberg, Eva-Lotta Ljung, Susanne Johansson, Siv Andersson, Marie Lännerstrand, Sandra Wändesjö and more.

The proud honorary member of EMK – Sofia Karlsson.

Ye Olde Brown Cloaks – You know who you are.

Thank you, Marta Axner, Jenny Ehnberg and Rickard Eriksson of Nämnden.

Thank you, #sweden.

Thank you, Samuel R Delany, Donna Tartt, Kristin Cashore, Steven Brust, Steven Erikson, Steven Moffat and many more.
Thank you dearest family:

Mamma och pappa, you’re the best in the whole world.

Mormor och morfar, puss och kram och kram och kram och kram.

Lina, let’s have a fika about all this.

Rune, Margaretha, Katarina, Ludde, Bobo and the gnutts! ;)

Larz, you know it already.

Rufus, Ozzy, Elvis and Okie <3

Yorrick.

Sincerely, with absolutely no panic whatsoever,
Uppsala, October 2013

Me transmitte sursum, caledoni
References


Carbrey JM, Agre P. Discovery of the aquaporins and development of the field. Handb Exp Pharmacol, 190: 3-28, 2009.


Valui ad satanam in computatrum meum invocandum
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