THREE-DIMENTIONAL ARCHITECTURE OF THE RENAL INNER MEDULLA OF THE DESERT RODENT DIPODOMYS MERRIAMI: POTENTIAL IMPACT ON THE URINARY CONCENTRATING MECHANISM.

By

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A Thesis Submitted to The Honors College

In Partial Fulfillment of the Bachelors degree
With Honors in

Health Sciences

THE UNIVERSITY OF ARIZONA

December 2010

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Abstract:

The objective of the following research project was to analyze the methods behind the urinary concentrating mechanism in *Dipodomys merriami*, Merriam's kangaroo rat. We hypothesize that the inner medulla of *Dipodomys merriami* contains extreme examples of various architectural features as well as transport properties which enable it to produce concentrated urine at over 6000mOsm/Kg water. The three-dimensional architecture of the vasculature and nephron segments inside the renal inner medulla (IM) was assessed using digital reconstruction from tissue sections. Descending thin limbs (DTLs), ascending thin limbs (ATLs), collecting ducts (CDs), and ascending vasa recta (AVR) were identified with indirect immunofluorescence using antibodies and lectins that recognize segment-specific proteins associated with solute and water transport (AQP1, ClC-K1, and AQP2). Electron microscopy shows close contact between CDs, AVR, and ATLs at adherence areas. The CDs, AVR, and ATLs are sufficiently close together to form discrete interstitial compartments. This architectural arrangement and apparent isolation of these compartments raise questions regarding their function. One possibility is that lateral solute diffusion from ATLs and CDs into AVR could be preferentially restricted to these areas. Interstitial cell architecture, which could increase and define compartmentalization, may further restrict diffusive exchange.

Introduction:

The kidneys are two bean shaped organs located behind the peritoneum and are thus known as retroperitoneal organs. They contain an abundant amount of individual components known as nephrons which are the actual functional units of the kidneys. Blood enters the glomeruli through afferent arterioles, then is forced through capillary networks before exiting through the efferent arteriole. Blood filtrate enters the proximal tubule as it exits the glomeruli. The blood pressure pressing the blood against the walls of the glomerulus is what filters the blood into urine which leaves via the proximal convoluted tubule. After the urine leaves the

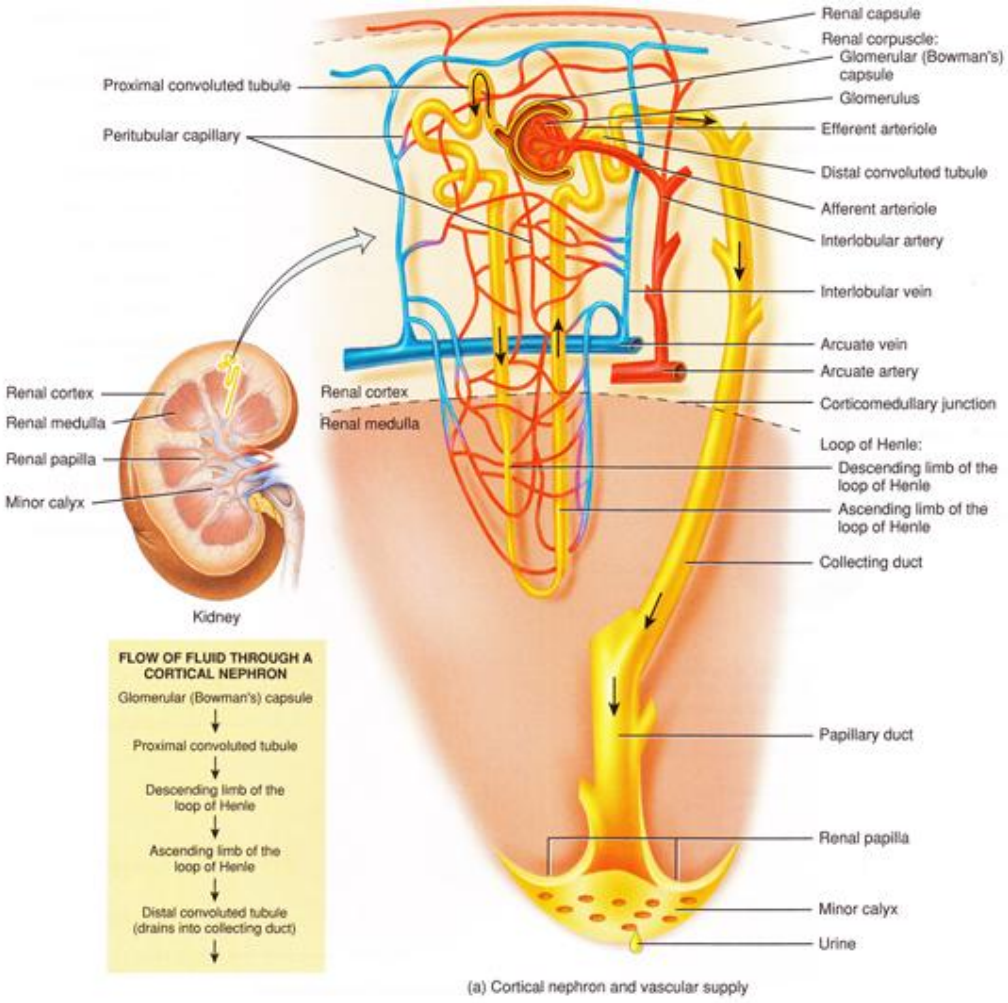

Figure 1. shows how the blood and urine flows through the individual nephrons units.

glomeruli it travels deeper within the kidney from the renal outer medulla to the renal inner medulla via the descending limb of the loop of Henle and then passes back up to the renal cortex via the thick and thin ascending limbs of the loop of Henle. It is important to note that while the urine is in the descending limb of the loop of Henle and ascending limb of the loop of Henle many capillaries known as peritubular capillaries surround the loops of Henle and provide the necessary architecture for tubular reabsorption as well as tubular secretion. From the ascending limb of the loop of Henle the urine travels through the distal tubule into a series of collecting ducts which connect with other nephrons and together flow into the papillary duct, minor calyx, major calyx, renal pelvis, ureter, and finally into the bladder (Tortora and Derrickson 2009).

The main task of the kidneys is to filter the circulating blood, however they are not simply limited to this task. The kidneys regulate various ion concentrations, blood pH, blood volume, blood pressure, blood osmolarity, hormone production, blood glucose level, and the excretion of wastes and foreign substances (Tortora and Derrickson 2009). The primary regulation site occurs within the proximal convoluted tubule where epithelial cells along the renal tubules all participate in some form of reabsorption and secretion. The proximal convoluted tubule is responsible for reabsorbing solutes such as glucose, amino acids, and urea. It is also responsible for reabsorbing ions such as sodium, potassium, calcium, and chloride. Once the fluid passes the proximal convoluted tubule the fluid becomes more balanced as to what is homeostatically necessary as it proceeds through the rest of the renal tubules. This occurs because of the fact that water follows solutes by the process of osmosis. Most of the water that is reabsorbed in the renal tubules is reabsorbed within the proximal convoluted tubule (Tortora and Derrickson 2009). Reabsorption is not the only process involved within the renal tubules; secretion from the blood via the peritubular capillaries into the renal tubules also plays a role in

maintaining homeostasis. Ions such as hydrogen, potassium, and ammonium along with certain drugs are also secreted from the blood to the renal tubules (Tortora and Derrickson 2009).

The *Dipodomys merriami*, Merriam's kangaroo rat is a small mammal that is located within the southwestern United States and northern Mexico. It is for the most part nocturnal to prevent predators from killing it. Its diet consists mostly of various seeds from different species of plants with very little if any water intake. The kangaroo rat is one of the only species in the world that obtains almost all of its water from the seeds it eats. It is thought that the water is obtained through metabolic processes. The kangaroo rat is able to survive the harsh desert environment it inhabits due to various processes. Its ability to retain what little water it obtains from its diet is remarkable. A hypothesis for the rats ability to retain this water is the architecture of the renal inner medulla of the kidney (Tremor S. and Haas B., 2000).

Several important techniques are required to make the tubules being followed visible to obtain the images used to reconstruct the architecture of the inner and outer medulla. The first technique is learning how to properly perform immunohistochemistry on tissues from the kidney. Immunohistochemistry uses antibodies to bind to specific target proteins located within a section of tissue. The antibodies themselves are attached to a specific fluorescent tag which allows the molecule and subsequently the specific tissue to be seen under a specific wavelength of light using fluorescence microscopy. Primary antibodies are used to tag the protein. The secondary antibodies, which are coupled to the fluorescent tag are used to detect the primary antibodies. In the case of the kangaroo rat, the primary antibodies used are the chicken AQP1 antibody, the rabbit ClCK antibody, and the goat AQP2 antibody. The chicken AQP-1 antibody attaches to a water channel protein or aquaporin, found within the DTL. The rabbit ClCK antibody attaches to a chloride channel protein found within the ATL and associated pre-bend segments. The goat

AQP2 antibody attaches to an aquaporin protein found within the CD's. Secondary antibodies that fluoresce are then used to detect the primary antibodies.

The second technique is epifluorescence microscopy. The microscope used to obtain the images was the Applied Precision DeltaVision Olympus IX70. This microscope incorporates a motorized stage that facilitates production of montages of large tissue sections. To become familiar and properly trained required a total of 2 hours of instruction and 4 hours of supervised microscopy. Once proper training is completed the microscopy and imaging process can begin.

Three-dimensional reconstruction software such as Amira software, in combination with conventional image analysis software, such as Adobe Photoshop, is used to then take the two-dimensional images from the microscope and create a three dimensional reconstruction. Amira allows the input of individual circles which are placed on top of the tubules being followed and can later be layered to produce and display a three-dimensional representation of the tubules and their relationship to the other tubules around them.

The purpose of the study was to try and determine which structural features of the Merriam Kangaroo Rat kidney are important for concentrating its urine to an amount that is very high. In particular, we were interested in understanding the architectural layout of the various tubules within the inner medulla, where the cellular mechanism for the generation of the osmotic gradient is not as well known as the outer medulla. In addition, we wanted to localize transporters for fluid and solutes that reside within cell membranes of nephron segments, so as to better understand the dynamic flows of fluid and solutes through the many compartments of the inner medulla.

Methods:

In order to begin the project several kangaroo rats were caught in order to have a small population of kidneys for this project, but also for others down the line. To begin the project an adult male kangaroo rat (*Dipodomys merriami*) was euthanized using carbon dioxide. After the animal was euthanized the kidney was perfused through the aorta of the heart. Once perfused the kidney was dissected out along with the inner medulla and placed in a periodate-lysine-paraformaldehyde (PLP) fixative for three hours at 4 °C. The inner medulla was fixed for 3 hrs in PLP at 4 °C, then washed in phosphate buffer solution (PBS) and then dehydrated using a series of ethanol rinses (30 min each: 50, 70, 85, 90, ending with two 100% rinses). The inner medulla was then cut to a smaller size and placed in a 1:1 ratio of Spurr's epoxy resin and ethanol for 48 hrs at room temp, then placed into 100% Spurrs at 4 °C for 24 hrs, then placed into 100% Spurrs and baked at 60 degrees for 12 hrs. Once embedded in the resin, serial transverse sections were cut at 1 μm with every fifth section placed on a microscope slide and a total of four sections placed on the slide. Immunohistochemistry was the performed on the slide by first creating a wax border around the tissue sections in order to keep the solutions from running off the slide. Once the wax border had dried tissue sections were identified with a diamond pen. The diamond pen was used to etch the back of the slide to identify where the tissue sections were. Once completed an etching solution of pulverized 0.5 grams NaOH, 5 mL ETOH, and 5 mL of propylene oxide was made. This solution was placed into a small beaker and set on a stir plate to mix gently for 10 minutes. The etching solution is used for dissolving the Spurrs. A blocking solution of BDT was then made using 5% BSA, 1% donkey serum, and 0.2% triton. A total of 200 μL of this solution is needed for each slide. The total amount of solution to be made varies with the amount of slides being prepared. Once the 10 minutes has passed by the etching solution was used to

flood each slide for 3 minutes. Careful precaution was taken to insure the slides did not dry out. After the 3 minute period the slides were then placed in Coplin jars with 100% ethanol and then shaken gently for 1 minute. After the minute passed the Coplin jars were emptied of the ethanol and replaced with distilled water and gently shaken for 1 minute. This step was repeated another two times for a total of three washes with distilled water. The slides were then flooded with a solution of PBS with 0.2% Triton for 2 minutes. The slides were then flooded with a solution of 1% SDS in PBS for 5 minutes being careful to make sure the slides did not dry out. The SDS treatment serves as an antigen retrieval technique that exposes molecular binding sites and facilitates binding of primary antibody to the protein of interest. The slides were then flooded again with the solution of PBS with 0.2% Triton for 5 minutes. This was then repeated two more times for a total of three washes with the PBS and 0.2% Triton. A moist paper towel was then placed onto the bottom of a slide box to prevent the slides from drying out during the longer incubation periods. The wax border was then dried and the slides placed into the slide box with the moistened paper towel. 50 µL of the BDT was placed onto the slide for 10 minutes. While waiting during this time the solution for the primary antibodies can be made. Once the antibodies are obtained they should be vortexed and then centrifuged to ensure a proper mixture. A 1.5 mL centrifuge tube was used to hold the solution of BDT and the three antibodies. The amount of BDT to be added to the centrifuge tube needs to be enough for 50 µL of BDT per slide. Once the total amount of BDT is added the primary antibodies can be added as well. The first primary antibody used was Chicken AQP1, CHIP, COOH, which labels for DTL's and was used in a 1:100 concentration. The second primary antibody used was Goat AQP2 which labels for CD's and was used in a 1:200 concentration. The third primary antibody used was Rabbit ClCK which labels for ATL's and was also used in a 1:200 concentration. Once the 10 minutes have passed

the slides should be taken out of the slide box and the BDT removed by tipping the slide. The wax border should then be dried again before the slides are placed into the slide box and the primary antibodies are added onto the slide. Once the primary antibodies are added the slide box should be placed into a 4°C cold room overnight. Once the slides have been in the cold room overnight they are taken out of the cold room and out of the slide box and washed three times with the PBS and 0.2% Triton. Once the washes are complete the wax border is dried again and the slides placed into the slide box. The secondary antibodies used include: donkey α rabbit TRITC which labels the ATL's in a 1:200 ratio, donkey α goat DAPI which labels the CD's in a 1:200 ratio, donkey α chicken Cy5 which labels the DTL's in a 1:200 ratio, and Wheat Germ FITC which labels all tubules and vessels in a 1:50 ratio. These antibodies are combined with a 50 µL per slide allotment of BDT in another 1.5 mL centrifuge tube. Once the contents are mixed 50 µL of the solution is placed onto each of the slides. Once the secondary antibodies have been added to each of the slides the slide box must be covered from light, the slide box was therefore placed inside a level drawer which was isolated from all light for a two hour period. Once the two hours had passed another three washes were done on the slides with the PBS and 0.2% Triton solution. Once the slides are washed slide covers are fixed to the slides and the slides are labeled with the initials of the individual doing the immunohistochemistry and the date the immunohistochemistry was performed. Once the immunohistochemistry was completed the slides were ready for imaging. The four sections on the slide are then imaged in an order that is as follows: upper left, upper right, lower left, and lower right. These sections are labeled a, b, c, and d respectively. This allows each slide and section to be properly accounted for and properly named. The image below shows what one of the slides looks like. The white square includes the

slide ID along with the initials of the person performing the immunohistochemistry and the date that it was performed.

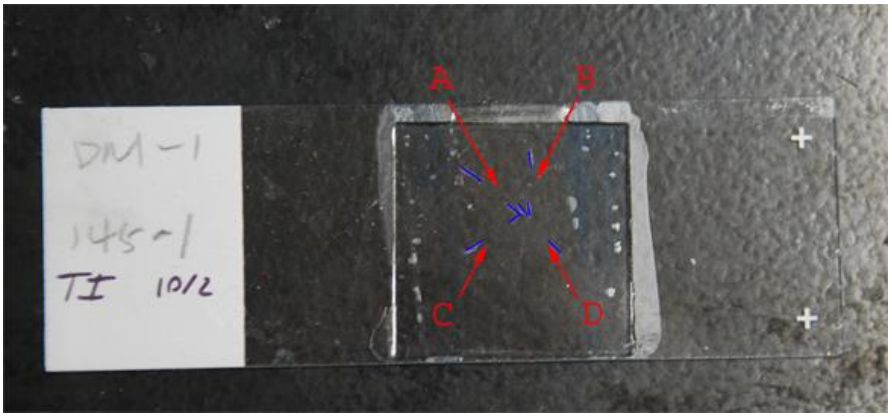

Figure 2. Image of a typical microscope slide with 4 inner medullary tissue sections labeled a,b,c,d

The slides were then imaged using an Olympus IX70 epifluorescence microscope using the FITC, DAPI, Cy-5, and TRITC excitation and emission filters and a 20X air objective. FITC used a wavelength of 528 nm to identify the wheat germ lectin which labels all the tubules. DAPI used a wavelength of 457 nm which was used to identify the CD's. Cy-5 used a wavelength of 685 nm to identify the ATL's. TRITC used a wavelength of 617 nm to identify the DTL's. Due to the fact that the sections were fairly large, a montage of all the images was needed to collect the full image. To accomplish this, a motorized stage and the stitching application within the Applied Precision software were used. Standard defaults were used with the exception of the Rotation in degrees set to -0.5. This corrected the angle of the images so that all tubules aligned up perfectly with each other. Once the slides were imaged they were saved onto a compact disk or USB drive and moved to the computer in the lab. From here the images are separated into their individual wavelengths 457-CD, 528-ALL, 685-ATL, and 617-DTL and renamed into incremental numbers, eg. 151, 152, 153 etc. The images are then aligned and cropped using Photoshop 6.0. Once the images are aligned and cropped they are placed into the Amira program and voxels (labeled circles) are assigned to the individual tubules. Voxels belonging to the same

tubule are given the same name while voxels belonging to a different tubule are given a different name. This process continues throughout the entire set of tissue images collected. Once the individual tubules are followed through the inner medulla analysis of the results can begin.

Results:

The series of tissues used for reconstructions throughout this experiment belonged to a group called Kangaroo Rat 1. Electron micrographs were obtained from the kidney of a second kangaroo rat. This series of tissues consisted of 135 slides which yielded 524 images that were each 5 μm apart. Data were collected for comparison between the kangaroo rat and the Munich Wistar rat which included: loop of Henle length, pre-bend length, and aquaporin 1 positive and aquaporin 1 negative DTL length.

I. Comparison between Kangaroo rat and Munich Wistar rat for loop of Henle lengths.

In the Kangaroo rat the aquaporin positive DTL segment is approximately 53% of the AQP1-positive DTL length of tubule, while in the Munich Wistar rat the aquaporin positive DTL segment is approximately 40% of the AQP1-positive plus AQP1-negative DTL length of the tubule (Pannabecker et al. 2004). The pre-bend region for the kangaroo rat was approximately 118.46 μm on average with the pre-bend region for the Munich Wistar rat being approximately 163.71 μm on average. Table 1 reflects the values from the kangaroo rat for the tubules which have been reconstructed so far.

Table 1. Loop of Henle length, Pre-bend length, AQP Null length, and AQP1 Positive DTL length for Kangaroo Rat

Tubule Number	Loop Length (µm)	Pre-Bend Length (µm)	Aquaporin Null Length (µm)	AQP1 Length (µm)
1	2535	120	685	1730
2	2575	270	665	1640
7	2525	95	790	1640
8	2530	145	745	1640
9	2565	120	630	1815
12	2455	95	900	1460
17	2610	60	740	1810
19	2585	90	655	1840
21	2440	105	755	1580
25	2415	85	750	1580
27	2365	120	740	1505
28	2525	140	705	1680
29	2570	120	720	1730
30	2615	100	765	1750
77	1370	155	615	600
78	1300	135	725	440
79	1540	150	775	615
80	1505	110	755	640
83	1880	130	775	975
85	1025	65	660	300
98	1160	110	715	335
112	1115	120	675	320
119	1025	95	655	275
140	1490	120	655	715
141	1960	110	550	1300
145	1480	115	805	560
Average	**2006.15**	**118.46**	**715.58**	**1172.12**

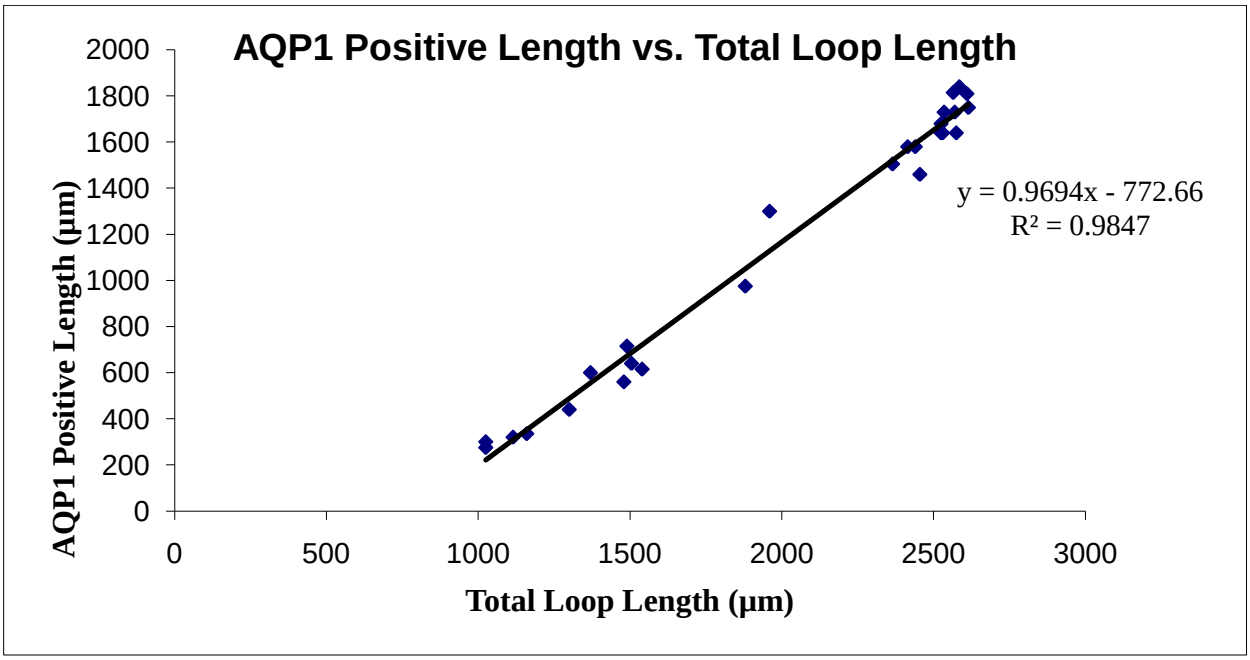

Figure 3. Graph showing AQP1 Length vs. Total Length in microns.

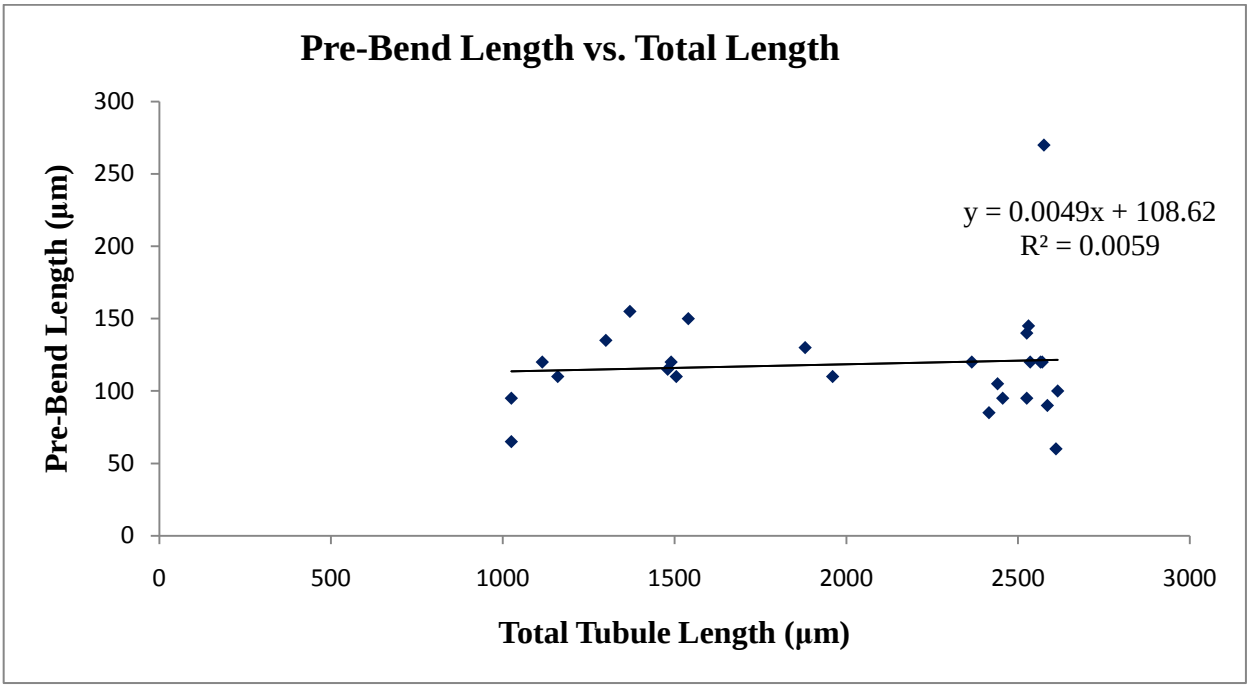

Figure 4. Graph showing Pre-Bend Length vs. Total Length in microns.

Figure 3 show a comparison between AQP1 length verses Total tubule length in microns. Figure 3 shows an R^2 value of 0.9847 which suggests a strong correlation between AQP1 length and total length. Assuming a linear relationship holds for loops of Henle having a shorter length than those examined, all loops with a length less than 797 microns express no detectable AQP1 in their inner medullary descending segment. Interestingly, for those loops that form their bend at 1500 microns or less below the OM-IM boundary, the mean AQP1-positive length (as a percent fraction of total loop length) is 34.6%. For those loops that form their bend at greater than 1500 microns below the OM-IM boundary, the mean AQP1-positive length (as a percent fraction of total loop length) is 62.7%. Figure 4 shows a comparison between pre-bend length versus tubule length in microns. Figure 4 shows an R^2 value of 0.0059 which suggests no correlation between pre-bend length and total tubule length.

II. Collecting Duct Cluster.

The collecting duct cluster is simply a cluster of CD's which all merge to form a single CD which ultimately leads to the tip of the papilla. The collecting duct cluster that is illustrated in Figure 5 helps illustrate what is included in a collecting duct cluster. The white line delimits the outer perimeter or border of each collecting duct cluster. This border is created with the Euclidean distance mapping technique. By definition, all structures lying on this border lie as far as possible from any CD. The blue tubules are CD's which merge together to form a single collecting duct deeper in the inner medulla. The red tubules are DTL's which are typically located around the periphery of the collecting duct cluster as illustrated in the image. The green tubules are the ATL's and pre-bend segments, which are typically located around the collecting ducts themselves. These three components are used to identify a collecting duct cluster.

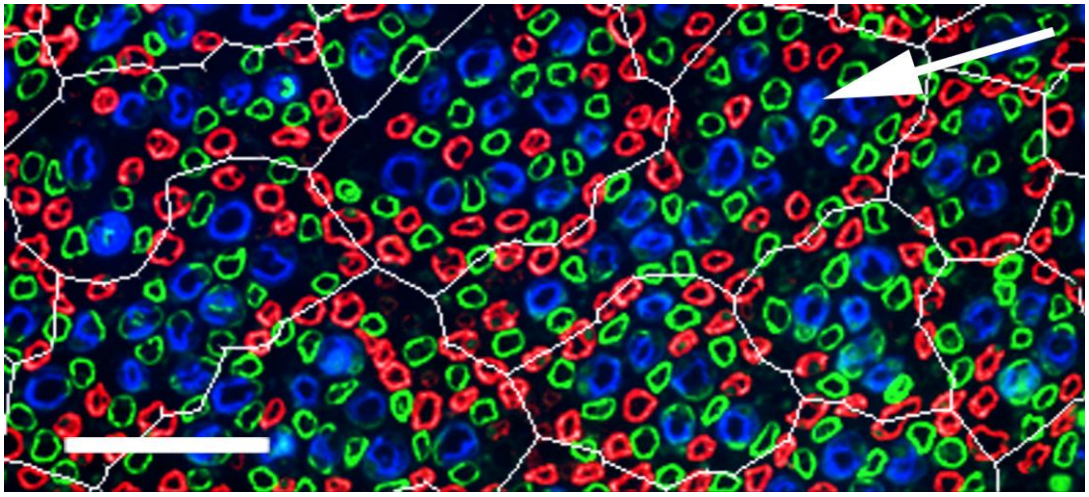

Figure 5. Image of a kangaroo rat inner medullary transverse section illustrating a collecting duct cluster. Structures were labeled with immunohistochemistry: collecting ducts/AQP1, blue; AQP1-positive DTLs/AQP1, red; ATLs and prebends/ClC-K1, green. Scale bar, 100 microns.

III. The proposed countercurrent exchange mechanism.

Using the reconstruction method a proposed hypothesis for urine concentration was established. This method required CD's, AVR, and ATL's. The ultimate goal of these tubules being so close together allows for the diffusion of solutes and water in and out of the various tubules. The function of this countercurrent system works when salt water and urea are transported down the CD. From the CD they are absorbed into the interstitium and eventually the AVR's by diffusion and close contact. Thus, there is a path of low resistance for the solutes to move out of the CD's and into the AVR. The AVR shuttles the water and solutes to the outer levels of the medulla, and back to general circulation where the water can be reabsorbed and the solutes recycled. The cycle continues in order to help the body retain as much water as possible.

IV. Interstitial Nodal Spaces

The interstitial nodal spaces are created by the close abutment of an ATL, CD, and an AVR. In the Munich Wistar rat, the ATL's, AVR's and CD's are all very close together and often touch one another. The Kangaroo rat exhibits the same abutment of tubules. These interstitial nodal spaces occur in both the Munich Wistar rat and Kangaroo rat.

V. Electron Microscopy.

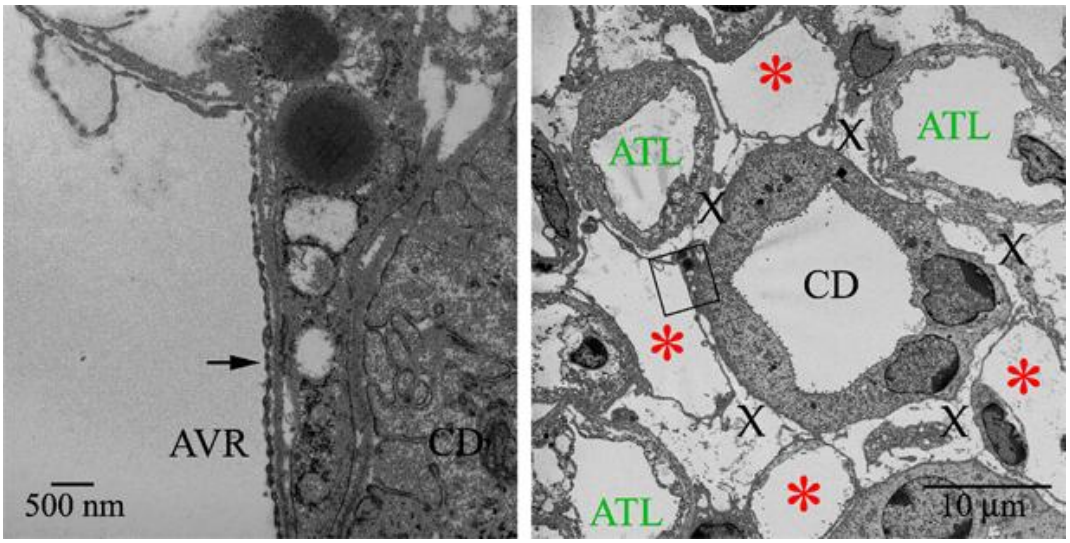

Figure 6. Electron micrograph image of a transverse section from the kangaroo rat inner medulla.

Figure 6 shows an electron micrograph image of an AVR and CD on the left and a CD with ATL's, AVR, and interstitial nodal spaces on the right. The image to the right shows a single collecting duct labeled with the black CD, the ascending thin limbs labeled with the green ATL, and the ascending vasa recta(AVR) labeled with the red asterisks. What is important to see with this image is the close abutment of each tubule which forms a pocket labeled with the black X. This pocket is a part of the interstitium and forms the interstitial nodal space which may play a very key role in the kangaroo rats ability to concentrate its urine. In the image on the left an AVR and CD are shown in close abutment with each other. The arrow pointing to the AVR wall shows the fenestrations in the AVR.

Discussion:

The Kangaroo rat is an amazing mammal which is able to live in some of the hottest driest regions on earth without drinking even a drop of water. This small mammals ability to obtain its water intake from the seeds it eats suggests an incredible ability to regulate water loss. One hypothesis for its ability to minimize water loss is though the architecture of the various tubules and blood vessels within the kidney.

Our study showed that the Kangaroo rat has a longer segment of AQP1-positive DTL than that of the Munich Wistar rat. In addition to this we found that the interstitial nodal spaces occur in the Kangaroo rat much like they do in the Munich Wistar rat.

Perhaps the single most important aspect from our findings is that the AQP1-positive DTL segment is about 13% longer than that of the Munich Wistar rat. Notably, the longest tubules (those greater than 1500 microns in total length) have even longer AQP1-positive segments, compared to the Munich Wistar rat. The longer lengths of AQP1-positive DTLs, relative to those of Munich-Wistar rats, may be important in enabling tubular fluid to equilibrate with fluid of the interstitium. Equilibration by fluid reabsorption in thin limbs of Henle is believed important in the urine concentrating mechanism. This is one mechanism for increasing osmolality of tubular fluid. This may suggest that although the Kangaroo rat has a more efficient kidney, there may be some other physiological adaptations that allow this particular mammal to survive in the harsh desert climate.

The results of this study can be used to an extent in clinical adaptations of certain drugs. If we can understand the reasons behind the function of the kidney and the associated architecture we can better target drugs which can take advantage of the architecture within the kidneys.

This particular study was very computer intensive. It required performing immunohistochemistry on a number of slides, imaging them, and finally reconstructing selected tubules by image analysis. The greatest limitation of this particular study was the potential for human error. Although we tried our very best at making sure each tubule was correctly marked from one image to the next, because of the very nature of human error it is very possible that some of the tubules may be incorrect. With this being said there were several of us looking over each tubule and double checking each other's work to make sure as best as possible that what we labeled was indeed the correct tubule.

Further experiments dealing with isolating more collecting duct clusters as well as some more tubules from other species may be beneficial for comparison. Furthermore comparing what is seen with mammals such as the Kangaroo rat and the Munich Wistar rat with the architecture of the human kidney would also be interesting to compare.

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