Lectins as markers for tubule regions in the kidney of *Squalus acanthias*, with further observations on SGLT2 immunohistochemistry

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After presenting the first results of immunohistochemical studies on the localization of the Na^+/D-glucose cotransporter (SGLT) in the kidney of the spiny dogfish (*Squalus acanthias*) in last year's bulletin[3], we can now refine these results with more details. During analysis of the tissue sections we encountered some problems in identifying individual tubule segments. Here we present a new valuable approach to identify distinct nephron regions in tissue sections in a reproducible manner.

For immunohistochemistry we used frozen sections of shark kidney preferentially from female animals (simply because the kidneys are easier to prepare). The tissues were fixed during preparation by a drip of ice cold 4% paraformaldehyde (in 3x PBS, pH6.8), cut into small pieces and then further fixed over night at 4°C. After rinsing the samples in PBS the tissue was embedded in Tissue-Tek OCT compound (Sakura Finetek U.S.A., Inc., Torrance, CA) and frozen in liquid nitrogen. Cryosections of 6 µm thickness (obtained with a Leica Microsystems cryostat) on slides were blocked for one hour in 3% dry skim milk (in PBS) and subsequently incubated for another hour with specific primary antibodies (diluted 1:300 in PBS + 1% Triton-X-100). The primary antibodies were detected with fluorescent-labelled secondary antibodies (Alexa-Fluor 488 or Alexa-Fluor 555 anti rabbit IgG, Molecular Probes), diluted as described above. The DNA stain 4',6-Diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei. To label the endogenous alkaline phosphatase, a marker for the brush border membrane (BBM), the Vectastain ABC-AP kit (Vector Laboratories) was applied according to the manufacturers manual. Fluorescein-labeled lectins, also obtained from Vector Labs, were used at 1:150 dilution in 1x PBS and incubated for 20 min at room temperature. In detail these were Sophora japonica agglutinin (SJA), Griffonia simplicifolia lectin I (GSL I), Ricinus communis agglutinin I (RCA I), Soybean agglutinin (SBA), Dolichos biflorus agglutinin (DBA), Ulex europaeus agglutinin (UEA I), peanut agglutinin (PNA) and Concanavalin A (Con A). The stained sections were mounted with ProLong antifade solution (Molecular Probes). Microscopy was performed on an Axiovert 200M microscope equipped with Apotome and CCD camera Axiocam MR (Carl Zeiss, Göttingen, Germany) with Plan-Neofluar lenses 25x/0.8 and F-Fluar 40x/1.3.

The complex renal architecture of *Squalus acanthias*, and other marine cartilaginous fish, involves a distinct zonation of the tissue (for review see Elger et al.[2]). Thus cross sections through the excretory opisthonephric kidney generally reveal three characteristic regions: (1) The zone of lateral countercurrent bundles, (2) the mesial tissue zone, and (3) a region between the two zones, where glomeruli abound (Figure 1).
An individual nephron displays the following segments: neck segment NS, proximal tubule segments PI and PII, intermediate segment IS, early distal tubule segment EDT, and late distal tubule segment LDT. According to microdissection combined with histology, spiny dogfish display a specific subdivision of the proximal tubule segment PI and PII (PIa in the bundle, PIb in the region of the glomeruli, PIIa and PIIb exclusively in the mesial tissue) (Elger and Hentschel[1]). The early distal tubule EDT, which is present exclusively in the lateral bundle zone, is contiguous with the late distal tubule, which thereafter performs numerous bends in mesial tissue. The late distal tubule LDT is present in mesial tissue, where it courses along the pathway of PIIa tubules.

Table 1: Lectin binding to renal structures of *Squalus acanthias* and localization of SGLT2. Results were obtained from 2 animals. As we did not attempt semi-quantitative evaluation, scoring was done with an arbitrary scale with:
- no signal; +/- very weak signal; + weak signal; ++ medium signal; +++ strong signal; ++++ very strong signal

	SJA	GSL I	RCA I	SBA	DBA	PNA	UEA I	SGLT2
Glomerulus	-	++	+	+ to ++	-	-	-	-
Neck segment	-	-	-	+	-	-	-	-
Proximal tubule segment								
PIa	-	+	-	++	-	+	-	+ to ++
PIb	-	+	+++	+++	-	+++	-	++ to ++++
PIIa	-	+	+	(+)	-	-	-	-
PIIb	-	+	+++	+	(+)	++	-	++ to +++
Intermediate segment	+	+	-	+	++	-	(+)	-
Early distal tubule	-	+++	-	++	+	-	(+)	+/-
Late distal tubule	-	++	-	++	(+)	-	++ to +++	+++
Collecting tubule/collecting duct	-	++	++	++ to +++	-	-	+ to ++	+++

After incubation with the fluorescence-labelled lectins we found that individual lectins bound preferentially to distinct regions of renal tubules. Three patterns of lectin-binding were observed: (1) lectins labelled a variety of structures, glomeruli and various tubule segments (GSL-I, SBA). (2) lectins bound preferently to certain tubular regions, proximal (PNA, RCA-I) or distal (DBA, UEA-I). (3) lectins showed specificity for a distinct tubule segment (SJA, DBA) (Table 1 and Figure 1).

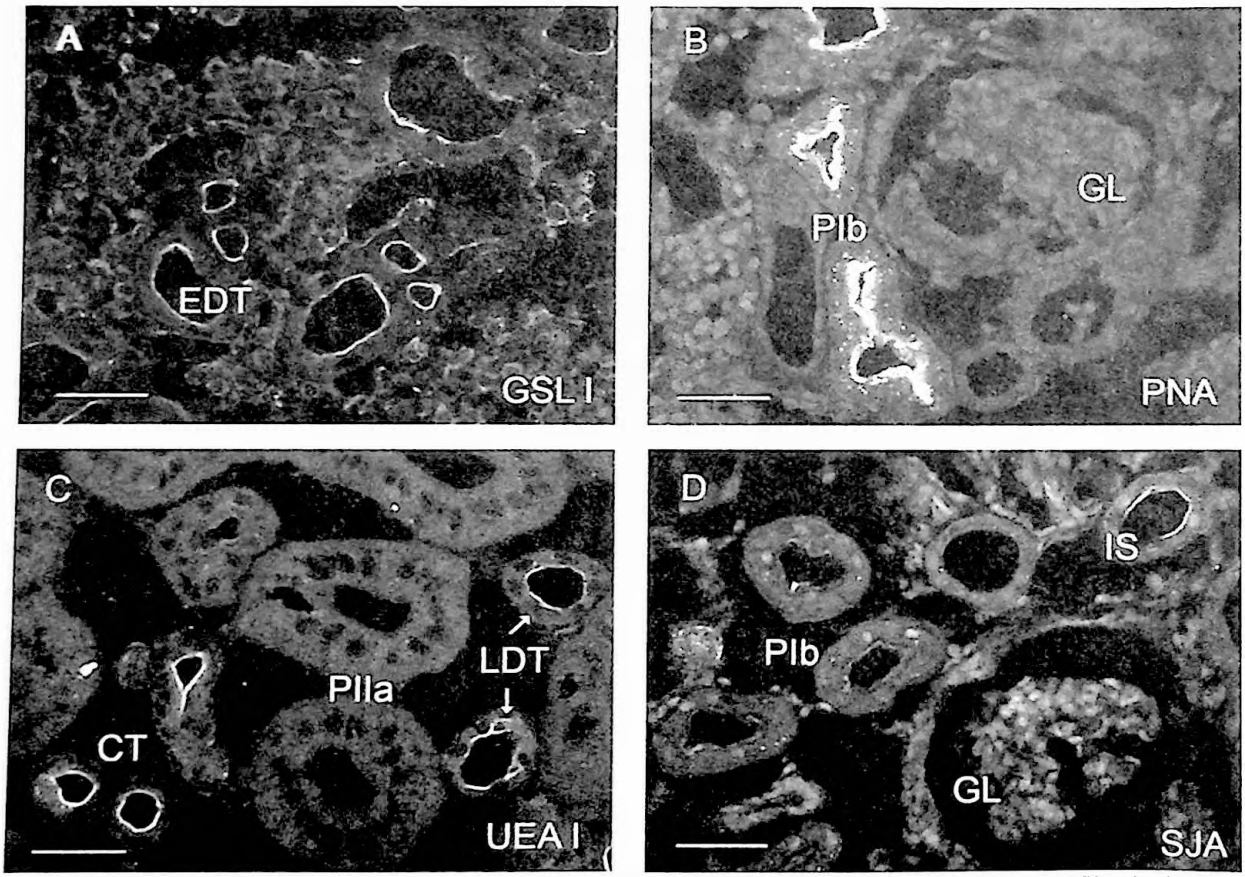

Fig.1. Lectin binding to renal structures. **A**. Cross sections through lateral bundles. Several tubular profiles including early distal tubule (EDT) are labeled by Griffonia simplicifolia lectin (GSL I).**B**. Selective staining of proximal tubule segment PIb near glomerulus (GL) by Peanut agglutinine (PNA) fluorochrome. **C**. Mesial tissue with large profiles of proximal tubule segment PIIa, small profiles of late distal tubule (LDT) and collecting tubule (CT). LDT and CT bind Ulex europaeus agglutinin (UEA-I). **D**. Staining by Sophora japonica agglutinin (SJA) at the apex of epithelial cells of intermediate segment (IS) is highly specific. Nuclear counterstain DAPI. Bar equals 50 µm. Shown are typical images from 2 animals.

Anti-SGLT2 antibody labelled specific nephron subsegments and the collecting tubule-collecting duct system in all regions of dogfish kidney: lateral bundle zone (LB), glomerular region (GL) and mesial tissue (MT) (Figure 2).The PIa segment in the lateral bundle zone displayed apical SGLT2 fluorescence signal within the region of its brush border. The PIb segment, which performs a tortuous course with many bends in the region of the glomeruli showed a very strong immunoreactivity in the apical cell region, including the brush border. The PIIa and PIIb segments of the proximal tubule present in mesial tissue diplayed markedly different patterns of anti SGLT2 labeling characteristics. PIIa cells exhibited no SGLT2 immunolabel. In contrast, PIIb cells consistently displayed specific SGLT2 immunoreactivity. The apical cell membrane of EDT epithelial cells was weakly labeled by anti-SGLT2 antiserum. LDT cells showed strong SGLT2-labeling at the apical zone. Significant anti-SGLT2 immunoreactivity was observed in the collecting tubules and collecting ducts. SGLT2 antibody binding was confined to the region of the apical cell membrane and its adjacent cytoplasmic zone (see Table 1). In summary, we consistently found SGLT2 labeling in nephron segments PIa, PIb, PIIb and CT.

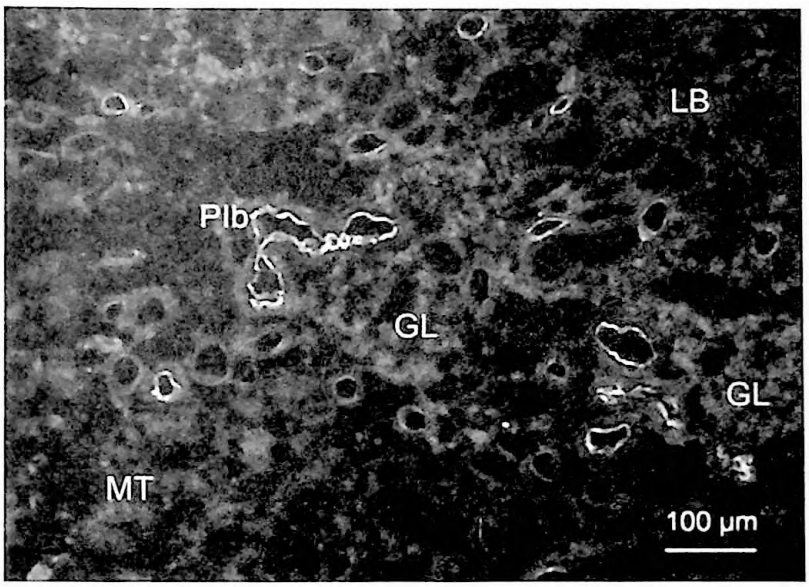

Fig. 2. Cross section through renal tissue of *Squalus acanthias* showing binding sites of anti-SGLT2 antibody. Frozen section (6µm) was incubated with rabbit anti-shark SGLT2 L13c antibody (1:300) and Alexa Fluor-488 anti rabbit secondary antibody. Shown is a typical image as seen in 2 animals.
GL = glomerular region; PIb = proximal tubule; LB = lateral bundle zone; MS = mesial tissue.

The knowledge about which lectin stains a specific segment of the nephron provides a powerful tool for further studies on transporter localization. Moreover, the anti-SGLT2 antibody has binding characteristics, which are useful to reveal epithelial cells of proximal tubule segment PIb and collecting tubule in tissue preparations of *Squalus acanthias*.

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