EXPRESSION AND DISTRIBUTION OF RENAL GROWTH FACTORS IN SQUALUS ACANTHIAS AND LEUCORAJA ERINACEA

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We have previously shown that the kidneys of spiny dogfish *Squalus acanthias* and the little skate *Leucoraja erinacea* exhibit distinct areas where new renal tissue is induced and functioning glomerular nephrons develop. Remarkably, the mesenchymal cells in these nephrogenic areas display stem cell characteristics and resemble embryonic stem cells (Hentschel, *Am J Anat.*, 190(4):309-33, 1991). These findings suggest that in the adult elasmobranch kidney nephrogenesis occurs and that these processes may be controlled by developmentally regulated programs. We established the hypothesis that the same set of genes involved in mammalian embryonic kidney formation are also involved in nephron development of the adult elasmobranch.

To confirm this hypothesis we decided to analyze the gene expression and tissue-specific distribution of several developmentally regulated genes known to be involved in kidney development, such as the transcription factors pax2 and wnt4 and the VEGF receptor neuropilin1 (NRP1). These findings should allow us to gain further insight into the molecular mechanisms responsible for nephron formation in the adult elasmobranch kidney.

To clone the skate and shark homologues of wnt4, pax2 and neuropilin1 total RNA was extracted from shark and skate kidney. The RNA was reverse transcribed using random primers and poly(dT) oligonucleotides to obtain cDNA. Specific primers for PCR were designed by aligning protein sequences of these growth factors from mammals, teleosts and amphibians. The alignments were put together in homologous blocks using a web-based program Blockmaker. The resulting blocks were then used to design degenerate primers using another web-based program Code Hop. A special heat regime known as touchdown-PCR was used for amplification (Don et al., *Nucleic Acids Res.*, 19(14): 4008, 1991). After checking the PCR product on an agarose gel, it was ligated into the pCRII vector (Invitrogen), cloned into *E.coli* and sequenced at the MDIBL DNA Sequencing Facility. Using BLAST search algorithms (http://www.ncbi.nlm.nih.gov/BLAST/), the sequence data was analyzed and compared with database entries for homology.

A pax2 cDNA fragment of 738 base pairs was isolated from the shark and skate kidney. This cDNA fragment lies in the coding region of pax2 and codes for a 246 amino acid peptide. As seen in figure 1, the pax2 protein of shark and skate show a very high homology to pax2 of other organisms including mammals and teleosts.

Fig. 1. Protein alignment of Pax2. Amino acid sequences of Pax2 of little skate (Leucoraja erinacea), dogfish shark (Squalus acanthias), chicken (Gallus gallus), clawed frog (Xenopus laevis), human (Homo sapiens), mouse (Mus musculus), zebrafish (Danio rerio) and medakafish (Oryzias latipes) were aligned using ClustalW algorithms. Homology is seen in red, areas of dissimilarity are seen in blue and black.

An NRP1 cDNA fragment of 1600 base pairs was isolated from skate kidney. As seen in figure 2, the NRP1 protein sequence of skate revealed a weaker homology of NRP1 with mammals, teleosts and amphibia as compared with pax2. At present, we are sequencing the remaining base pairs in order to get more sequence homology information for the skate NRP1 protein.

Fig. 2. Protein alignment of NRP1. Amino acid sequences of NRP1 of little skate (Leucoraja erinacea), zebrafish (Danio rerio), clawed frog (Xenopus laevis), rat (Rattus norvegicus), human (Homo sapiens), mouse (Mus musculus), and chicken (Gallus gallus) were aligned using ClustalW algorithms. Homology is seen in red, areas of dissimilarity are seen in blue and black.

A wnt4 cDNA fragment of 511 base pairs was isolated from skate kidney that codes for a 170 amino acid peptide. As seen in figure 3 the wnt4 protein alignment has a high homology between skate, mammals, teleosts and amphibians.

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Leucoraja    1 ----------CLAIEECQYQFRNRRWNCSTVDTLPVFGKVVTQGTREAAFVYAISSAGV
Xenopus     61 LEVMDSVRRGAQLAIEECQYQFRNRRWNCSTLDTLPVFGKVVTQGTREAAFVYAISSAGV
Gallus      61 LEVMDSVRRGAQLAIEECQYQFRNRRWNCSTLDTLPVFGKVVTQGTREAAFVYAISSAGV
Mus         61 LEVMDSVRRGAQLAIEECQYQFRNRRWNCSTLDSLPVFGKVVTQGTREAAFVYAISSAGV
Homo s.     61 LEVMDSVRRGAQLAIEECQYQFRNRRWNCSTLDSLPVFGKVVTQGTREAAFVYAISSAGV
Rattus      61 LEVMDSVRHGAQLAIEECQYQFRNRRWNCSTLDSLPVFGKVVTQGTREAAFVYAISSAGV
Danio       61 VEVMDAVRRGAQLAIDECQYQFKNRRWNCSTLESVPVFGKVVTQGTREAAFVYAISAASV

Leucoraja   50 ALVVTRACSSGELEKCGCDRTVHGVSPDGFQWSGCSDNIAYGVAFSQSFVDVRERSKGAS
Xenopus    121 AFAVTRACSSGDLEKCGCDRTVHGVSPQGFQWSGCSDNILYGVAFSQSFVDVRERSKGGS
Gallus     121 AFAVTRACSSGELDKCGCDRTVQGGSPQGFQWSGCSDNIAYGVAFSQSFVDVRERSKGAS
Mus        121 AFAVTRACSSGELEKCGCDRTVHGVSPQGFQWSGCSDNIAYGVAFSQSFVDVRERSKGAS
Homo s.    121 AFAVTRACSSGELEKCGCDRTVHGVSPQGFQWSGCSDNIAYGVAFSQSFVDVRERSKGAS
Rattus     121 AFAVTRACSSGDLEKCGCDRTVHGVSPQGFQWSGCSDNIAYGVAFSQSFVDVRERSKGAS
Danio      121 AFAVTRACSSGELDKCGCDRNVHGVSPEGFQWSGCSDNIAYGVAFSQSFVDIRERSKGQS

Leucoraja  110 SSRPLMNLHNNEAGRKAILNNMRVECKCHGVSGSCEVKTCWKAMPPFRKVGNVLKEKFDG
Xenopus    181 SSRALMNLHNNEAGRKAILNNMRVECKCHGVSGSCEVKTCWKAMPTFRKVGNVLKEKFDG
Gallus     181 SNRALMNLHNNEAGRKAILNNMRVECKCHGVSGSCEFKTCWKAMPPFRKVGNVLKEKFDG
Mus        181 SSRALMNLHNNEAGRKAILTHMRVECKCHGVSGSCEVKTCWRAVPPFRQVGHALKEKFDG
Homo s.    181 SSRALMNLHNNEAGRKAILTHMRVECKCHGVSGSCEVKTCWRAVPPFRQVGHALKEKFDG
Rattus     181 SSRALMNLHNNEAGRKAILTHMRVECKCHGVSGSCEVKTCWRAVPPFRQVGHALKEKFDG
Danio      181 SNRALMNLHNNEAGRKAILNHMRVECKCHGVSGSCEVKTCWKAMPPFRKVGNVIKEKFDG

Leucoraja  170 AT----
Xenopus    241 ATEVEQ
Gallus     241 ATEVEQ
Mus        241 ATEVEP
Homo s.    241 ATEVEP
Rattus     241 ATEVEP
Danio      241 ATEVEL
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Fig. 3. Protein alignment of Wnt4. Amino acid sequences of Wnt4 from little skate (Leucoraja erinacea), clawed frog (Xenopus laevis), chicken (Gallus gallus), mouse (Mus musculus), human (Homo sapiens), rat (Rattus norvegicus), and zebrafish (Danio rerio) were aligned using ClustalW algorithms. Homology is seen in red, areas of dissimilarity are seen in blue and black.

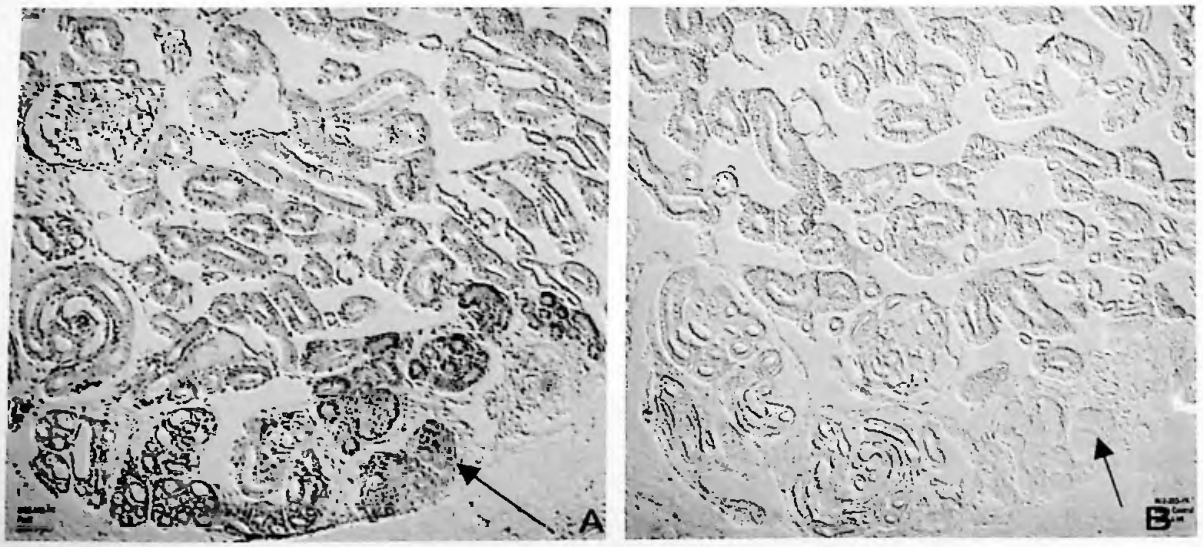

Fig. 4. Skate kidney *in situ* hybridization. A. pax2, B. Control with no probe. Slides were viewed with a light microscope (Leica), images were taken with a digital camera (SPOT). Arrows indicate the developing zone, the reaction is seen in developing tubules and glomerulus in figure A.

Tissue distribution of pax2 was analyzed by *in situ* hybridization. The pax2 DNA probe was generated from a cDNA fragment of 738 bp isolated from skate. Skate kidney tissue was perfusion-fixed with 4% paraformaldehyde (PFA), embedded in paraffin, cut and mounted onto

glass slides. For generation of the probe, digoxygenin (DIG, Boehringer) was incorporated into pax2 by PCR. The labeled probe was cleaned up by ethanol precipitation and hybridized to the tissue slides. Standard protocols from DAKO for DIG staining were followed. Tyramide amplification was performed according to protocols from Molecular Probes. Preliminary *in situ* results of pax2 expression showed the strongest signal in the region of the developing zone, as seen in figure 4.

To determine expression levels of pax2 in mature and developing skate kidney quantitative real time quantitative PCR was used. Using the MX4000 Real-Time PCR (Stratagene) and the SYBR Green Quantitac Kit (Qiagen) the relative mRNA expression was analyzed. As a normalization control, expression of 18S was determined. We found a greater than seven-fold increase of pax2 expression in skate developing tissue compared to mature tissue (fig.5).

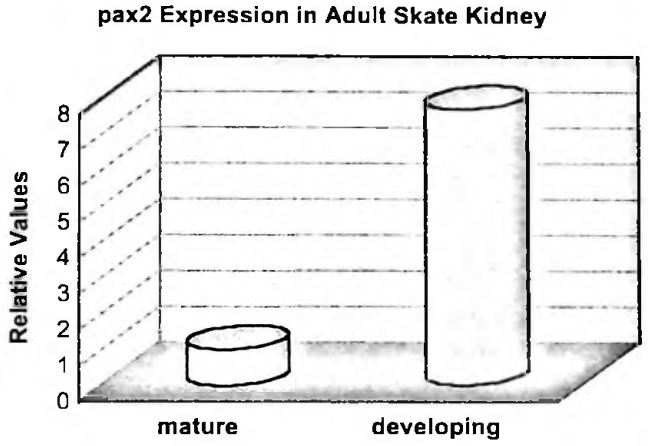

Fig. 5 Quantitative RT-PCR expression of pax2 mRNA in skate kidney. RNA was extracted from mature and developing zones of adult skate kidney, transcribed into cDNA using random primers and applied to quantitative RT-PCR using pax2-specific primers and SYBR-Green chemistry. As normalization control the contribution of 18S ribosomal RNA was measured.

These data showed that the developmentally regulated genes pax2, wnt4 and in part neuropilin1 displayed high levels of homology between elasmobranch and other species. *In situ* hybridization revealed a prominent distribution of wnt4, pax2 and neuropilin1 in the region of adult skate kidney where nephrogenesis occurs. For pax2 this distribution pattern could be confirmed by quantitative RT-PCR showing a greater than seven-fold increase in up-regulated mRNA in the developing zone of the skate kidney. These findings suggest, that the adult elasmobranch kidney is an ideal model to study factors and conditions that contribute to nephrogenesis.

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