

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.

Leucoraja 1 -----QNPTMFWE: RDRLLAEGICDNDVPSVSSINR: IRTKVQOPPHPSDGGPGSAVPAQHT IVPST
Squalus 1 -----QNPTMFWE: RDRLLAEGICDNDVPSVSSINR: IRTKVQOPPHPSDGGPGSAVPAQHT IVPST
Gallus 80 PKVIGGSKPKVATPKVVDKIAEYKRONPTMFWE: RDRLLAEGICDNDVPSVSSINR: IRTKVQOPPHPSDGGPGSAVPAQHT IVPST
Xenopus 80 PKVIGGSKPKVATPKVVDKIAEYKRONPTMFWE: RDRLLAEGICDNDVPSVSSINR: IRTKVQOPPHPSDGGPGSAVPAQHT IVPST
Homo s. 80 PKVIGGSKPKVATPKVVDKIAEYKRONPTMFWE: RDRLLAEGICDNDVPSVSSINR: IRTKVQOPPHPSDGGPGSAVPAQHT IVPST
Mus 79 PKVIGGSKPKVATPKVVDKIAEYKRONPTMFWE: RDRLLAEGICDNDVPSVSSINR: IRTKVQOPPHPSDGGPGSAVPAQHT IVPST
Danio 83 PKVIGGSKPKVATPKVVDKIAEYKRONPTMFWE: RDRLLAEGICDNDVPSVSSINR: IRTKVQOPPHPSDGGPGSAVPAQHT IVPST
Oryzias 81 PKVIGGSKPKVATPKVVDKIAEYKRONPTMFWE: RDRLLAEGICDNDVPSVSSINR: IRTKVQOPPHPSDGGPGSAVPAQHT IVPST

Leucoraja 66 NSPPVSSASNDPVGSYSINGILGIPRNGEAKRKDEDTQDNGSGPNSGQTAESVXQLRSEA?ACQQLAERLVRPQHYSDVFNSE
Squalus 66 NSPPVSSASNDPVGSYSINGILGIPRNGEAKRKDEDTQDNGSGPNSGQTAESVXQLRSEA?ACQQLAERLVRPQHYSDVFNSE
Gallus 170 ASPVSSASNDPVGSYSINGILGIPRNGE-KRKDEDSVSGSVFNGSSQSSVSLRKHRLADNTTCQQLAERLVRPQHYSDVFNSE
Xenopus 170 ASPVSSASNDPVGSYSINGILGIPRNGE-KRKDEDSVSGSVFNGSSQSSVSLRKHRLADNTTCQQLAERLVRPQHYSDVFNSE
Homo s. 170 ASPVSSASNDPVGSYSINGILGIPRNGE-KRKDEDSVSGSVFNGSSQSSVSLRKHRLADNTTCQQLAERLVRPQHYSDVFNSE
Mus 169 ASPVSSASNDPVGSYSINGILGIPRNGE-KRKDEDSVSGSVFNGSSQSSVSLRKHRLADNTTCQQLAERLVRPQHYSDVFNSE
Danio 173 ASPVSSASNDPVGSYSINGILGIPRNGE-KRKDEDSVSGSVFNGSSQSSVSLRKHRLADNTTCQQLAERLVRPQHYSDVFNSE
Oryzias 169 ASPVSSASNDPVGSYSINGILGIPRNGE-KRKDEDSVSGSVFNGSSQSSVSLRKHRLADNTTCQQLAERLVRPQHYSDVFNSE

Leucoraja 156 HIKSEQANEYSALALASGLDVKSSLSAATPDLGANSVSGPSYVVTGRMASTTLPGYPPHVPPTGCGSYSTSLAGMVFGEFSGNP
Squalus 156 HIKSEQANEYSALALASGLDVKSSLSAATPDLGANSVSGPSYVVTGRMASTTLPGYPPHVPPTGCGSYSTSLAGMVFGEFSGNP
Gallus 259 HIKSEQANEYSALALASGLDVKSSLSAATPDLGANSVSGPSYVVTGRMASTTLPGYPPHVPPTGCGSYSTSLAGMVFGEFSGNP
Xenopus 259 HIKSEQANEYSALALASGLDVKSSLSAATPDLGANSVSGPSYVVTGRMASTTLPGYPPHVPPTGCGSYSTSLAGMVFGEFSGNP
Homo s. 259 HIKSEQANEYSALALASGLDVKSSLSAATPDLGANSVSGPSYVVTGRMASTTLPGYPPHVPPTGCGSYSTSLAGMVFGEFSGNP
Mus 257 HIKSEQANEYSALALASGLDVKSSLSAATPDLGANSVSGPSYVVTGRMASTTLPGYPPHVPPTGCGSYSTSLAGMVFGEFSGNP
Danio 262 HIKSEQANEYSALALASGLDVKSSLSAATPDLGANSVSGPSYVVTGRMASTTLPGYPPHVPPTGCGSYSTSLAGMVFGEFSGNP
Oryzias 258 HIKSEQANEYSALALASGLDVKSSLSAATPDLGANSVSGPSYVVTGRMASTTLPGYPPHVPPTGCGSYSTSLAGMVFGEFSGNP

Leucoraja 246 YTHPOYTTYNEARFSTNP-----
Squalus 246 YTHPOYTTYNEARFSTNP-----
Gallus 349 YSHPOYTTYNEARFSTNPALLSSPYYSATSRGSAFPTAAAYDRH
Xenopus 349 YSHPOYTTYNEARFSTNPALLSSPYYSATSRGSAFPTAAAYDRH
Homo s. 349 YSHPOYTTYNEARFSTNPALLSSPYYSATSRGSAFPTAAAYDRH
Mus 347 YSHPOYTTYNEARFSTNPALLSSPYYSATSRGSAFPTAAAYDRH
Danio 350 YSHPOYTTYNEARFSTNPALLSSPYYSATSRGSAFPTAAAYDRH
Oryzias 344 YSHPOYTTYNEARFSTNP-----

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.

Leucoraja 1 -----EKEVRSVLVIQGVKQNDNKVYMRKFKLGYSTNGSDWKLVTSFGSPKPKKI
Danio 478 YADENLQIDLAEKLVKGLIIQGGKHIRDNKVFMRKFKLGYSTNGSDWKLVMDATGNKPKKI
Xenopus 481 YTKEWLQIDLAEKLVIRGVIIQGGKHRENKVFMRKFKLGYSTNGSDWKLMDDSKRKAKS
Rattus 480 YINEWLQVDLGEKLVIRGVIIQGGKHRENKVFMRKFKLGYSTNGSDWKLMDDSKRKAKS
Homo s. 480 YINEWLQVDLGEKLVIRGVIIQGGKHRENKVFMRKFKLGYSTNGSDWKLMDDSKRKAKS
Mus 480 YINEWLQVDLGEKLVIRGVIIQGGKHRENKVFMRKFKLGYSTNGSDWKLMDDSKRKAKS
Gallus 478 YINEWLQVDLGEKLVIRGVIIQGGKHRENKVFMRKFKLGYSTNGSDWKLMDDSKRKAKS

Leucoraja 49 FEGNTNYDTPEVRKFEPLLTRIRVYPERASAGLGLRMELGCDIEVPTAAPTAVET---
Danio 538 FEGNLNYDTPEVRKFEPLTRFVRIYPRDGTGAGMGLRLLELLGCMEVPTVPTTFAAST
Xenopus 541 FEGNTNYDTPELRFAHITTFIRIYPERASAGLGLRLLELLGCCEVETPTTPTTPEVN-
Rattus 540 FEGNNNYDTPELRFAHITTFIRIYPERATHSGGLGLRMELGCEVETAGTPTTNGN-
Homo s. 540 FEGNNNYDTPELRFAHITTFIRIYPERATHSGGLGLRMELGCEVETAGTPTTNGN-
Mus 540 FEGNNNYDTPELRFAHITTFIRIYPERATHSGGLGLRMELGCEVETAGTPTTNGN-
Gallus 538 FEGNTNYDTPELRFAHITTFIRIYPERATHSGGLGLRMELGCEVETAGTPTTNGN-

Leucoraja 106 ---DECDEDGNGCHSGTGDGDFDTGSSSTAVGTEAPTLPESSQYFDRILIPINEVPGFNCKF
Danio 598 TPSDECDDDDQANCHSGTGDGDFDTGSSSTAVGTEAPTLPESSQYFDRILIPINEVPGFNCKF
Xenopus 600 -GGDECEGDLANCHSGTGDGDFDTGSSSTAVGTEAPTLPESSQYFDRILIPINEVPGFNCKF
Rattus 599 -PVDECDDDQANCHSGTGDGDFDTGSSSTAVGTEAPTLPESSQYFDRILIPINEVPGFNCKF
Homo s. 599 -LVDECDDDQANCHSGTGDGDFDTGSSSTAVGTEAPTLPESSQYFDRILIPINEVPGFNCKF
Mus 599 -PVHECDDDQANCHSGTGDGDFDTGSSSTAVGTEAPTLPESSQYFDRILIPINEVPGFNCKF
Gallus 597 -PVDECDDDQANCHSGT-----GGTTVLNTEKPTVIDNTVQPELPP-----YNLNCGF

Leucoraja 163 GGIIQSRFWR-----
Danio 647 GWANDPSFCGWISE-DSGFRWQIQSSGTPTLNTCPNMDHTGGSGNFIYTLATGAQETEA
Xenopus 654 GWGSHKTFCHWEHDNHVQLKWSVLTS-----KTGPVQDHTG-DGNFIYSQADENQKGVAA
Rattus 653 GWGSHKTFCHWEHDNHVQLKWSVLTS-----KTGPVQDHTG-DGNFIYSQADENQKGVAA
Homo s. 653 GWGSHKTFCHWEHDNHVQLKWSVLTS-----KTGPVQDHTG-DGNFIYSQADENQKGVAA
Mus 653 GWGSHKTFCHWEHDNHVQLKWSVLTS-----KTGPVQDHTG-DGNFIYSQADENQKGVAA
Gallus 644 GWGSHKTFCHWEHDNHVQLKWSVLTS-----KTGPVQDHTG-DGNFIYSQADESQKGVAA

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.

Leucoraja	1	-----CIAIEECQYQFRNRWNCSTVDLTPVFGKVVTCGTREAAFPVYAISSAGV
Xenopus	61	LEVMDSVRRGAQLAIEECQYQFRNRWNCSTLTLTPVFGKVVTCGTREAAFPVYAISSAGV
Gallus	61	LEVMDSVRRGAQLAIEECQYQFRNRWNCSTLTLTPVFGKVVTCGTREAAFPVYAISSAGV
Mus	61	LEVMDSVRRGAQLAIEECQYQFRNRWNCSTLDSLPVFGKVVTCGTREAAFPVYAISSAGV
Homo s.	61	LEVMDSVRRGAQLAIEECQYQFRNRWNCSTLDSLPVFGKVVTCGTREAAFPVYAISSAGV
Rattus	61	LEVMDSVRRGAQLAIEECQYQFRNRWNCSTLDSLPVFGKVVTCGTREAAFPVYAISSAGV
Danio	61	VEVMDAVRRGAQLAIDECCYQFRNRWNCSTLESVPVFGKVVTCGTREAAFPVYAISSAGV
Leucoraja	50	ALVVTRACSSGELEKCGCDRTVHGVSFQGFQWGGCSDNIAYGVAFSQSFVDVRRERSKGAS
Xenopus	121	AFVTRACSSGDLEKCGCDRTVHGVSFQGFQWGGCSDNIAYGVAFSQSFVDVRRERSKGAS
Gallus	121	AFVTRACSSGELEKCGCDRTVHGVSFQGFQWGGCSDNIAYGVAFSQSFVDVRRERSKGAS
Mus	121	AFVTRACSSGELEKCGCDRTVHGVSFQGFQWGGCSDNIAYGVAFSQSFVDVRRERSKGAS
Homo s.	121	AFVTRACSSGELEKCGCDRTVHGVSFQGFQWGGCSDNIAYGVAFSQSFVDVRRERSKGAS
Rattus	121	AFVTRACSSGDLEKCGCDRTVHGVSFQGFQWGGCSDNIAYGVAFSQSFVDVRRERSKGAS
Danio	121	AFVTRACSSGELEKCGCDRTVHGVSFQGFQWGGCSDNIAYGVAFSQSFVDVRRERSKGAS
Leucoraja	110	SSRLMNLHNEAGRKAILLNMRVECKCHGVSGSCEVKTCKWAMPFRKVGNVLEKFPDG
Xenopus	181	SSRALMNLHNEAGRKAILLNMRVECKCHGVSGSCEVKTCKWAMPFRKVGNVLEKFPDG
Gallus	181	SSRALMNLHNEAGRKAILLNMRVECKCHGVSGSCEVKTCKWAMPFRKVGNVLEKFPDG
Mus	181	SSRALMNLHNEAGRKAILLNMRVECKCHGVSGSCEVKTCKWAMPFRKVGNVLEKFPDG
Homo s.	181	SSRALMNLHNEAGRKAILLNMRVECKCHGVSGSCEVKTCKWAMPFRKVGNVLEKFPDG
Rattus	181	SSRALMNLHNEAGRKAILLNMRVECKCHGVSGSCEVKTCKWAMPFRKVGNVLEKFPDG
Danio	181	SSRALMNLHNEAGRKAILLNMRVECKCHGVSGSCEVKTCKWAMPFRKVGNVLEKFPDG
Leucoraja	170	AT----
Xenopus	241	ATEVEQ
Gallus	241	ATEVEQ
Mus	241	ATEVEP
Homo s.	241	ATEVEP
Rattus	241	ATEVEP
Danio	241	ATEVEL

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.

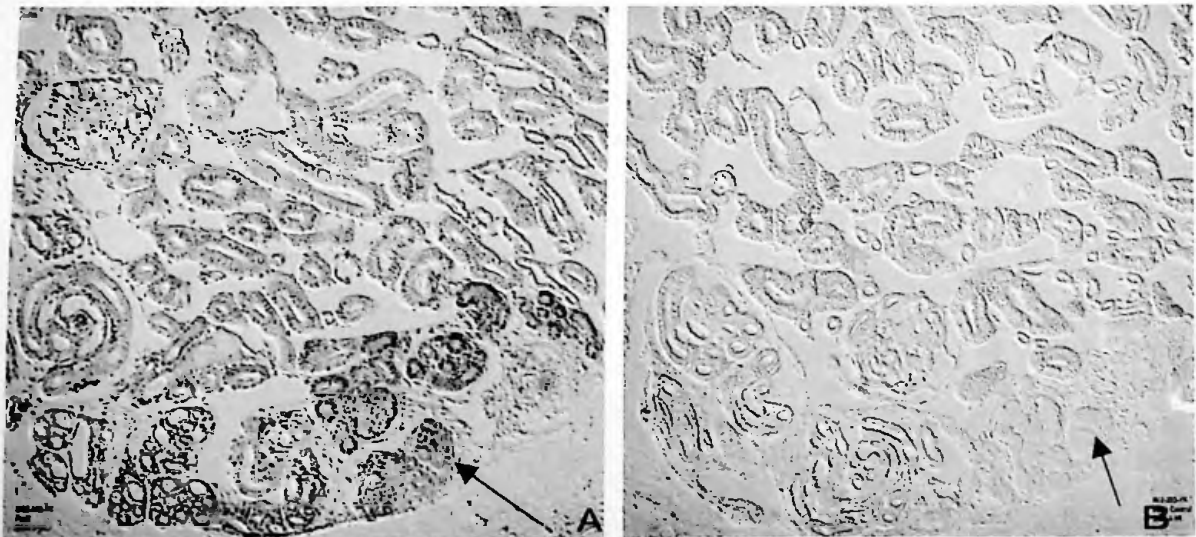


Fig. 4. Skate kidney *in situ* hybridization. A. *pax2*, B. Control with no probe. Slides were viewed with a light microscope (Leica), images were taken with a digital camera (SPOT). Arrows indicate the developing zone, the reaction is seen in developing tubules and glomerulus in figure A.

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, digoxigenin (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).

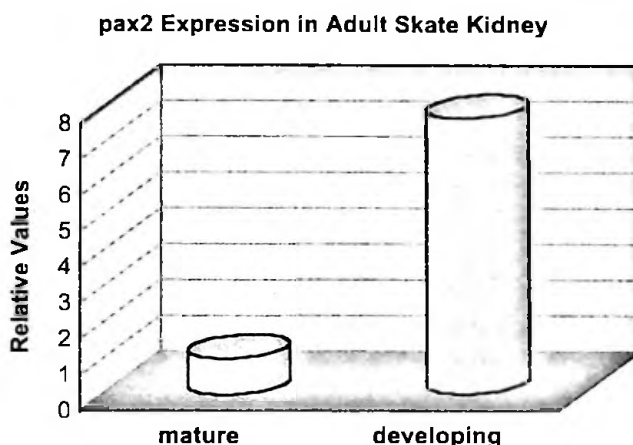


Fig. 5 Quantitative RT-PCR expression of pax2 mRNA in skate kidney. RNA was extracted from mature and developing zones of adult skate kidney, transcribed into cDNA using random primers and applied to quantitative RT-PCR using pax2-specific primers and SYBR-Green chemistry. As normalization control the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