PARTIAL CLONING OF GENES EXPRESSED IN THE KIDNEY AND INVOLVED IN THE CALCIUM SIGNALING PATHWAY FROM *SQUALUS ACANTHIAS*

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The kidney of the shark *squalus acanthias* provides a model system for kidney development because elasmobranchs are able to develop new glomeruli throughout life. Light and electron microscopy have shown immature and mature nephrons and distinc developmental stages of nephrons (Hentschel (1990) *Am .J. Anat* . 190(4):309-33, 1990). It can be assumed that the developing renal tissue expresses genes involved in kidney development and regeneration. Furthermore, the glomerular structures of the spiny dogfish resemble glomeruli in mammals Lacy et al. *Anat Rec.* 18(3):294-305, 1987). We have therefore started to search for molecular markers which may play a role in signal transduction and differentiation of the developing kidney . Since we and others have recently demonstrated that protein kinase C (PKC) plays a role in the differentiation of endothelial cells in vitro (Wellner et al. *Arterioscler Thromb Vasc Biol.* 19(1):178-85,1999) we have first analyzed related proteins and tested the hypothesis that PKC and other proteins involved in the calcium-signaling pathway may be differentially expressed in the developing renal tissue.

We have used PCR based strategies to clone kidney proteins from the shark involved in differentiation. The shark kidney was immediately frozen in liquid nitrogen and subsequently powdered. Total RNA was isolated using an established protocol (Qiagen) and cDNA synthesis was performed. Using highly degenerated primers to the endothelial specific molecule ESM-1 and protein kinase C. we isolated and sequenced more than 100 candidate clones. Analyses were performed using BlastN and BlastX (Database of National Institute of Health). Five gene fragments involved in Ca^{++}-mediated signal transduction were described.

The first fragment was related to the T-type calcium channel CACNA 16. We isolated a 368 bp cDNA fragment. The corresponding amino acid sequence contains 57 aa (amino acid); of these 47 aa show 36 % identity and 55 % similarity to the T-type calcium channel suggesting that we have cloned a fragment from the *squalus acanthias* T-type calcium channel. The gene of the T-type calcium channel CACNA16 has been identified as a target for hypermethylation in several tumors. The expression was detected in normal colon and bone marrow, but expression was only absent in five tumor cell lines in which methylation was found. The methylation inhibitor 5-deoxy-aza-cytidine restored the expression of CACNA16 in the corresponding tumor cell lines. Therefore, inactivation of CACNA16 may play a role in cancer development by modulating calcium signaling which potentially affects cell proliferation and apoptosis (Toyota et al. *Cancer Research* 59: 4535 - 4541, 1999).

The second fragment was related to the human Ca-calmodulin-dependent protein kinase β. We isolated a 418 bp fragment which contains an open reading frame encoding for more than 60 aa. From these, 31 aa show 45 % identity and 57 % similarity to the human CaMKKβ (Anderson et al. *J. Biol. Chem.* 273, 31880 - 31889, 1998). Our cloning shows the expression of the corresponding *S. acanthias* homologue in the kidney. The Ca-calmodulin-dependent protein kinase β is a nuclear protein and regulates transcription through phosphorylation of several transcription factors (Soderling *TIBS* 24, 232-6, 1999). CaMKKβ is broadly distributed among rat tissues with highest levels in brain, thymus, spleen and testis.

Transcription factors are DNA-binding proteins which are able to identify specific nucleotide sequences. By binding to the corresponding nucleotides they regulate the gene expression at the level of transcription. The eukaryotic RNA polymerase II transcribes each gen together with a set of general transcription factors, which are basically the same for each gene. Furthermore, more than 100 specific transcription factors have been identified so far. These specific transcription factors regulate the expression pattern of inducible genes during growth and differentiation (for a review, see Blume, A. and Unger, T., *J Mol Med* 77: 339 - 357, 1999).
The third novel fragment which we cloned from the shark kidney is related to the forkhead family of transcription factors. We amplified a 532 bp fragment which shows 64 % homology to the human FREAC-1 and 63 % homology to the mouse forkhead gene LUN. The deduced amino acid sequence shared 75% identity with the human FREC-1 and 73 % identity with the mouse lun protein. Transcription factors of the forkhead family, named according to the structural motif involved in DNA binding, are involved in embryogenesis, in tumorigenesis or in the maintenance of differentiated cell states (for a review, see Kaufmann, E. and Knoechel, W., *Mech. Dev.* 57 3 - 20, 1996). Forkhead related activator 1 (FREAC-1) is a mammalian transcriptional activator and is expressed in adult tissues mainly in lung and placenta (Mahlapuu et al., *Dev. Biol.* 202 183 - 195, 1998). The homologue mouse forkhead gene LUN encodes a transactivator that acts in the lung (Miura et al., *Genomics* 50: 346 - 56, 1998). During human renal morphogenesis, HFH-4 is expressed in the developing epithelial cells of the ureteric duct, glomerulus and epithelial vesicles. The unique pattern of HFH-4 expression during human fetal development suggests a role for this forkhead/winged-helix factor during pulmonary and renal epithelial development (Pelletier et al. *Am. J. Physiol.* 274 (3 Pt 1):L351-9, 1998).

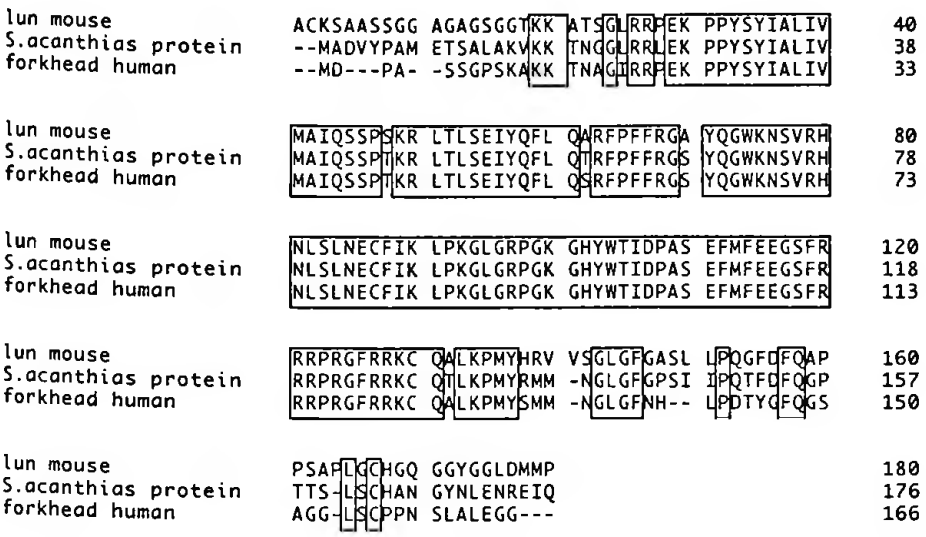

Fig.1: Protein Alignment of the isolated *S.acanthias* protein sequence to the human forkhead and mouse lun protein (Miura et al., *Genomics* 50: 346 - 56, 1998)

We isolated a 527 bp cDNA clone which shows homologies to the XLIM-1, a LIM protein, isolated from *Xenopus laevis*. The corresponding amino acid sequence contains 50 aa; of these 31 aa show 45 % identity and 54 % similarity to XLIM-1. The LIM domains of XLIM-1 show 37 - 54 % identity to the LIM domains of *caenorhabditis elegans* or rat (Taira et al., *Genes & Development* 6: 356-366, 1992). Therefore, we suppose that the isolated fragment contains a lim exon rather than a pseudogene. The LIM class of homeobox genes is defined by the association of two copies of a cystein-rich domain with a homeobox domain. Homeobox genes are involved in the control of various steps in embryogenesis such as body axis determination and tissue- or cell-type specification. LIM1, a LIM protein isolated from mouse, appears to be required for the formation of the nephric duct and the pro- and mesonephric tubules (reviewed in Lechner, M. and Dressler, G. *Mech. Dev.* 62: 105 - 120, 1997), indicating that LIM1 plays a fundamental role in the early steps of kidney formation. Recently, it has been suggested that the homeobox transcription factor LIM-1 may be a co-factor for Pax-8 1 in order to efficiently direct cells to form pronephric kidneys (Carroll et al. *Dev Biol.* 214(1):46-59, 1999). These two genes are initially expressed in overlapping domains in late gastrulae, and cells expressing both genes will go on to form the kidney.

The last novel fragment we report here also belongs to the transcription factor family. We isolated a 643 bp cDNA clone, which contains an open reading frame. The deduced amino acid sequence (49 aa) shows 34 % identity and 52 % similarity to the transcription factor Spt 16. This factor has also been described in human tissue. Recently, it could be demonstrated that the human and *S. cerevisiae* sequence share 36 % identity (Orphanides et al., *Nature* 400:284-288, 1999). Interestingly, spt 16 may be part of the transcription elongation factor FACT. Both factors seem to play a role in regulating chromatin structure. The regulation of gene expression depends upon chromatin structure. The minimal system, naked DNA with general transcription factors and RNA polymersae II, cannot transcribe DNA packaged into chromatin. Therefore, accessory factors may facilitate access to DNA. FACT (facilitates chromatin transcription) comprises the human homologue of the *Saccharomyces cerevisiae* Spt16 and the high-mobility group-1-like protein structure-specific recognition protein-1. FACT specifically interacts with nucleosomes and histon dimers, indicating that it may work by promoting nucleosomes disassembly upon transcription.

In summary, using degenerated primers we isolated cDNA fragments from genes expressed in *S. acanthias* kidney. We found a T-type calcium channel, a calcium-calmodulin-dependent protein kinase kinase β and three transcription factors indicating that molecules from the Ca-mediated signal transduction may play a role in developing renal structures.