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Short sequence paper

FoxP4, a novel forkhead transcription factor

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Abstract

Forkhead proteins have been demonstrated to play key roles in embryonic development, cell cycle regulation, and oncogenesis. We report the characterization of a new forkhead transcription factor, which is a member of the FoxP subfamily. In adult tissues *FoxP4* is expressed in heart, brain, lung, liver, kidney, and testis. By Northern hybridization, very low levels of *FoxP4* expression were found as early as E7 during embryonic development. Embryonic expression was highest at E11 and subsequently decreased at E15 and E17. In situ hybridization revealed expression of *FoxP4* in the developing lung and gut, suggesting a role for *FoxP4* during the development of these organs. In addition, *FoxP4* was found to be significantly reduced in patients with kidney tumors. Lastly, *FoxP4* matches an uncharacterized human EST that has previously been shown to be down-regulated in larynx carcinoma.

Keywords: Development; Winged helix; Kidney cancer; Gut

Members of the forkhead gene family share a highly conserved domain, containing approximately 100 amino acids. This domain was first identified in the *Drosophila* forkhead gene [1] and the mammalian *Hnf3* transcription factors were found to contain highly similar domains [2]. Over the past several years, many different forkhead domain-containing genes have been identified in species as diverse as yeast and human [3]. Forkhead domain proteins have been demonstrated to regulate transcription by direct DNA binding. Gel mobility-shift assay experiments have further confirmed that the forkhead domain is crucial for the DNA binding activity of these proteins [2]. X-ray crystallographic analysis demonstrated that the structure of the forkhead domain consisted of a central part derived from the α-helical and β-sheet domains which was flanked by two wings. This structure gave the domain its synonymous name, the winged helix domain [4].

Mutations in mouse as well as in zebrafish and *Drosophila* demonstrated a wide range of functions and key roles of these genes during embryonic development. Forkhead genes have been shown to play integral roles in early developmental processes such as node formation, anterior–posterior and left–right axis patterning [5,6] and, in addition, for cell cycle regulation [7]. Furthermore, these genes regulate the development of major organ systems and many different tissues including the brain, cardiovascular system, lung, gut, kidney, and others [8–11]. In addition to their roles in normal developmental events, members of this family also participate in mammalian oncogenesis. Chromosomal translocations that include forkhead transcription factors have been shown to occur in patients with acute leukemia and rhabdomyosarcoma [12–15]. Recently, *FoxP1* was found to be misexpressed in gastrointestinal cancers [16]. Interestingly, forkhead genes are also target genes of the PKB signaling pathway which is involved in carcinogenesis [17].

Recently, a new forkhead protein family was identified. FoxP1 and FoxP2 proved to be important regulators of lung epithelial transcription and were shown to act as transcriptional repressors [10]. Frameshift mutations in *FoxP3*, resulting in a product lacking the forkhead domain, were found to cause the X-linked phenotype of the scurfy mouse with overproliferation of CD4+CD8− T lymphocytes and multi-organ infiltration [18]. In humans, mutations in *FoxP3* cause a similar X-linked syn-

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drome characterized by immune dysfunction, polyendocrinopathy, and enteropathy (IPEX-syndrome [19]). In addition to their similarity within the forkhead domain, all three FoxP proteins contain a zincfinger structure N-terminal to the forkhead domain. Here, we report the characterization of a new FoxP family member, which was identified by BLAST searches against the mouse genomic database.

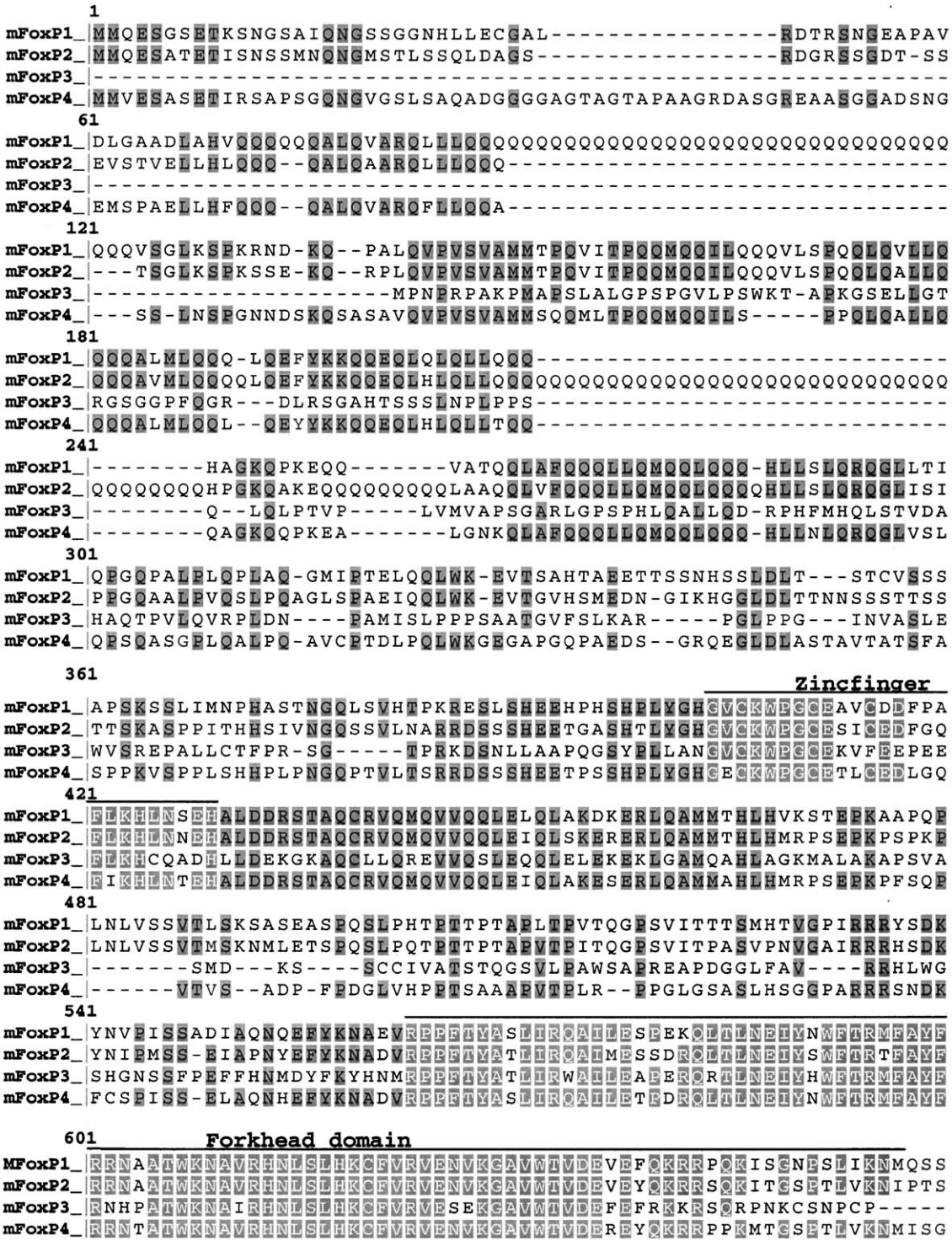

Fig. 1. Comparison of the amino acid sequences of mouse FoxP1, FoxP2, FoxP3, and FoxP4. Amino acids 404 to 429 of the aligned sequences comprise the zincfinger structure. The forkhead domain spans amino acids 563 to 657. Identical amino acids between at least three proteins are shaded. Dashes represent gaps in the sequence alignment.

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      661
mFoxP1_|HAYCTPLNAALQASMAENSIPLYTTASMGNP-TLGSLASAIREELNGAMEHTNSNESDSS
mFoxP2_|LGYGAALNASLQAALAESSLPLLSNPGLINN-ASSGLLQAVHEDLNGSLDHIDSN-GNSS
mFoxP3_|------------------------------------------------------------
mFoxP4_|LSYG-ALNASYQAALAESSFPLLSNPGMLNPGSASSLLPLSQEDLGVPGEPLPSNGSSSP
      721
mFoxP1_|PGRSPMQAVHPIHVKEEPLDPEEAEGPLSLVTTANHSPDFD-HDRDYEDEPVNEDME---
mFoxP2_|PGCSPQPHIHSIHVKEEPVIAEDEDCPMSLVTTANHSPELE-DDREIEEEPLSEDLE---
mFoxP3_|------------------------------------------------------------
mFoxP4_|PRLSPPQYSHQIQVKEEPAEAEEDRRPGPPLGAPNPSTVGPPEDRDLEEDLGGEDMPSQP
      781
mFoxP1_|------------------------------------------------------------
mFoxP2_|------------------------------------------------------------
mFoxP3_|------------------------------------------------------------
mFoxP4_|CPLIPGWKPSLLHLSYCVKPKFTVSVGSKTPSSPLPPPPRVQGSYSLPPCSYLAYGDMRG
      841
mFoxP1_|------------------------------------------------------------
mFoxP2_|------------------------------------------------------------
mFoxP3_|------------------------------------------------------------
mFoxP4_|QNPAPSPGLLSGVGGGLFRCLHRTKSPSLPGVWILAAELETMRFHRPPMGDPQPKTADWV
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Fig. 1 (*continued*).

FoxP4 was identified by BLAST search of FoxP1 (acc. no. P58462) against the NCBI non-redundant database (tblastn [20]). In addition to known family members Foxp2 and FoxP3, the FoxP1 search found a match to a new, uncharacterized cDNA transcript (acc. no. AK004693) and its corresponding protein sequence (acc. no. BAB23479). In order to further investigate the structure of the new protein and to confirm its homology to other FoxP family members, FoxP4 protein sequence was searched for conserved domains using the Pfam database [21]. Pfam search revealed a forkhead domain between amino acids 459 and 553 as well as a zincfinger structure between amino acids 312 and 337 of the FoxP4 protein sequence. The existence and upstream location of a zincfinger structure, as well as a coiled-coils structure between these two domains, as predicted by the COILS algorithm [22], have been found in all three known family members and further confirms the homology of FoxP4 to these proteins. Using the ClustalW webserver [23], all four protein sequences were aligned. Highest homology was found in the zincfinger and forkhead domains (Fig. 1). Considerable homology was also found between FoxP1, FoxP2, and FoxP4 outside these domains, making it obvious that FoxP4 is much more closely related to FoxP1 and FoxP2 as they share 54% and 60% identity, respectively. The FoxP3 and FoxP4 protein sequences are only 47% identical in the aligned sequence region, which in addition is shorter compared to alignments with FoxP1 and FoxP2.

BLAST search against the mouse genomic database localized *FoxP4* to chromosome 17. Similar to other family members, the *FoxP4* gene spans a large genomic region of more than 37 kb. It was further predicted that the gene is organized in 16 exons with lengths between 67 and 379 bp (Ensembl [24]). All the splicing donor and acceptor sites follow the GT/AG rule [25]. The zincfinger structure is encoded by exons 6 and 7 and the forkhead domain by exons 10 to 13. Due to these high structural similarities, we have subsequently not only evaluated the expression pattern of *FoxP4* in wild-type mouse tissues but also in human neoplastic tissues.

Using a probe specific to *FoxP4*, expression of the gene was analyzed by Northern hybridization (Fig. 2). Transcripts

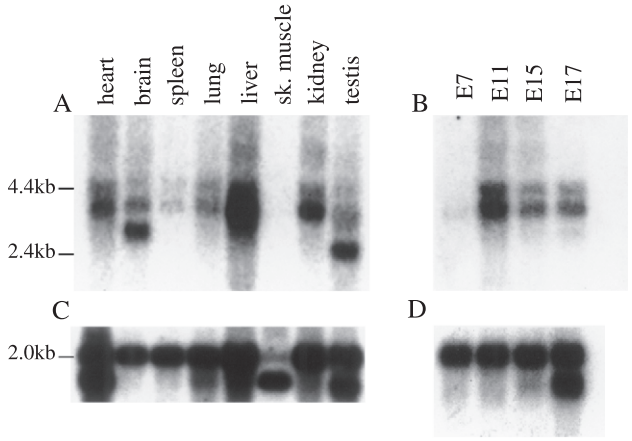

Fig. 2. (A) Northern blot showing the expression of *FoxP4* of adult mouse tissues. A main transcript was detected at approximately 3.5 kb in heart, brain, spleen, lung, liver, kidney, and testis. Also, a larger, but less expressed, transcript of about 4.4 kb was observed in these tissues. Smaller transcripts of 3.0 and 2.4 kb were found in brain and testis, respectively. (B) Very low levels of *FoxP4* expression were found as early as E7 during embryonic development. Embryonic expression was highest at E11 and subsequently decreasing at E15 and E17. (C, D) Control hybridizations with *β-actin* demonstrated equal mRNA amounts in each lane. Northern hybridizations were performed on adult and embryonic mouse Multiple tissue Northern (MTN) blots (Clontech) following the manufacturer's instructions. The mouse *FoxP4* probe was obtained by PCR (m*FoxP4*—primers: 5′-GTCAGCCTGCAGCCAAGCCAAGCCTC-3′ (forward) and 5′-GGAGCTGTCTCTCCGAGATGTGAGCAC-3′ (reverse)) from full-length mouse brain cDNA (Clontech) and subcloned using the TOPO PCR Cloning Kit (Invitrogen).

were found in adult heart, brain, spleen, lung, liver, kidney, and testis. The main transcript was about 3.5 kb in size and strongest expression was detected in liver tissue. However, a larger but less expressed transcript of about 4.4 kb was observed in heart, brain, spleen, lung, liver, kidney, and testis, and smaller transcripts of 3.0 and 2.4 kb were found in brain and testis, respectively. The *β-actin* control hybridization demonstrated equal amounts of mRNA per lane. The presence of multiple transcripts is suggestive of alternative splicing (Fig. 2A and C). In comparison to *FoxP1*, *FoxP4* was expressed at higher levels in kidney and liver tissue but not in skeletal muscle, whereas *FoxP2* expression was not detected in the heart [10]. Together, these genes show partially overlapping but distinct expression profiles in the adult mouse, suggesting that each gene may have unique functions.

mRNA transcripts were found at embryonic days E7, E11, E15, and E17, by means of Northern hybridization (Fig. 2B and D). However, only very low expression was detectable at E7. Highest expression of the gene was found at E11 and with expression subsequently decreasing at E15 and E17.

As *FoxP1* and *FoxP2* had been demonstrated to be important for lung and gut development, expression of *FoxP4* was further investigated by in situ hybridizations on frozen sections of E13.5 and E15.5 embryos (Fig. 3). At E13.5 *FoxP4* is expressed in a very specific pattern around the developing airway epithelium of the lung. At E15.5 expression was reduced, but still concentrated around the airway epithelium (Fig. 3A, C, E and G). This expression pattern is comparable to that of *FoxP2*, which is also localized around the airways. Strong expression was also observed in the developing gut. At both stages E13.5 and E15.5, *FoxP4* was expressed in the epithelium of the developing gut (Fig. 3B, D, F and G). This expression pattern is similar to that of *FoxP1* and distinct from that of *FoxP2*, which is predominantly expressed in the muscular layer of the developing gut.

Forkhead domain proteins are further known to participate in oncogenesis and *FoxP1*, in particular, has been demonstrated to be misexpressed in gastrointestinal tumors, suggesting a possible role of FoxP1 in gastrointestinal cancer. Due to its high homology with *FoxP1*, expression of *FoxP4* in human cancer tissues was investigated. A human *FoxP4* probe was hybridized to a blot containing cDNA from matched tumor and normal tissues of various patients with different tumors (Clontech). Significant reduction of expression was observed in at least 8 out of 14 patients with kidney tumors (Fig. 4A), suggesting a role of *FoxP4* in the pathogenesis of kidney cancer. Other tumor tissues tested did not demonstrate clear differential expression compared to normal tissue. Since the family members *FoxP1* and *FoxP2* are thought to act as transcription repressors, it is likely that *FoxP4* has a similar function and a reduction or loss of *FoxP4* expression may lead to excessive proliferation, resulting in subsequent increase of

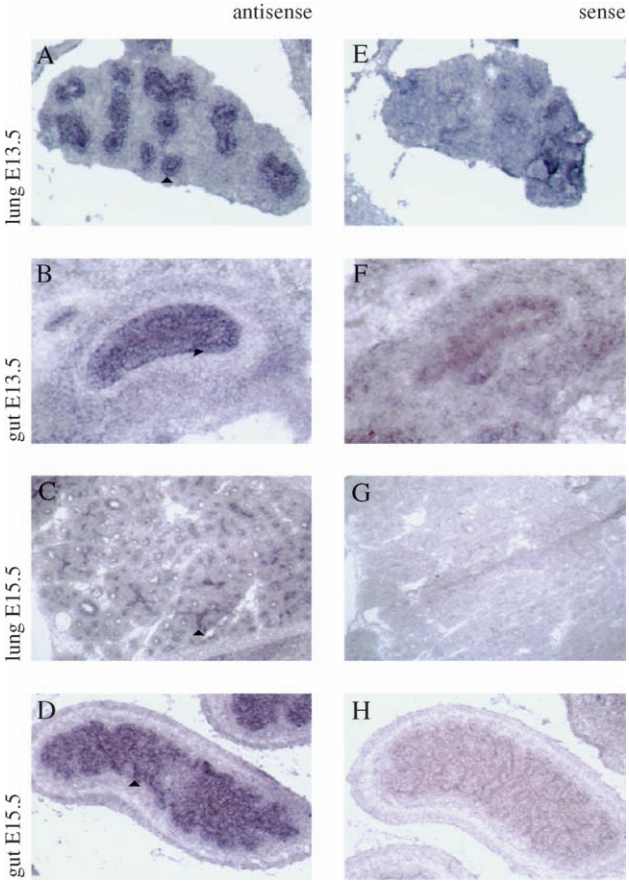

Fig. 3. (A–D) In situ hybridizations of *FoxP4* on frozen sections of lung and gut of E13.5 and E15.5 mouse embryos. At both stages *FoxP4* is expressed in the gut epithelium and around the developing airway epithelium of the lung. Sites of *FoxP4* expression are indicated by filled arrowheads. (E–H) Control hybridizations with a sense Probe demonstrated no distinct expression pattern in these tissues. Embryos were frozen in Tissue-Tek (Sakura Finetek, CA, USA). Fourteen-micron sections were cut with a cryostat and mounted on Superfrost plus slides. Hybridization was performed as described [26] and conducted in hybridization chambers from Grace Bio Labs (Bend, OR, USA). Hybridization and washes were performed at 70 °C. Sense and antisense RNA probes transcribed from the TOPO/*mFoxP4* probe (Fig. 2) were quantified using the Ribogreen assay from Molecular Probes (Eugene, OR, USA). DIG-labeled sense and antisense probes were detected using the DIG-detection kit from Roche.

the risk of tumorigenesis. In order to further quantify and verify these results, cDNA from Patient No. 7 (HP107K) was obtained and analyzed by quantitative PCR using the LightCycler system (Fig. 4C). Using primers specific to the human *FoxP4* gene, a reduction of average $2^{4.3}$ (approximately 95%) was found between the normal and tumor tissue in three identical experiments. Equal amounts of cDNA in the tumor and normal tissue samples were verified by control primers amplifying the human ribosomal protein S9.

BLAST search against the human EST database (NCBI [20]) identified an additional role of *FoxP4* in carcinogenesis. The *Foxp4* cDNA was found to match a human EST (acc. no. AJ403110) that has previously been

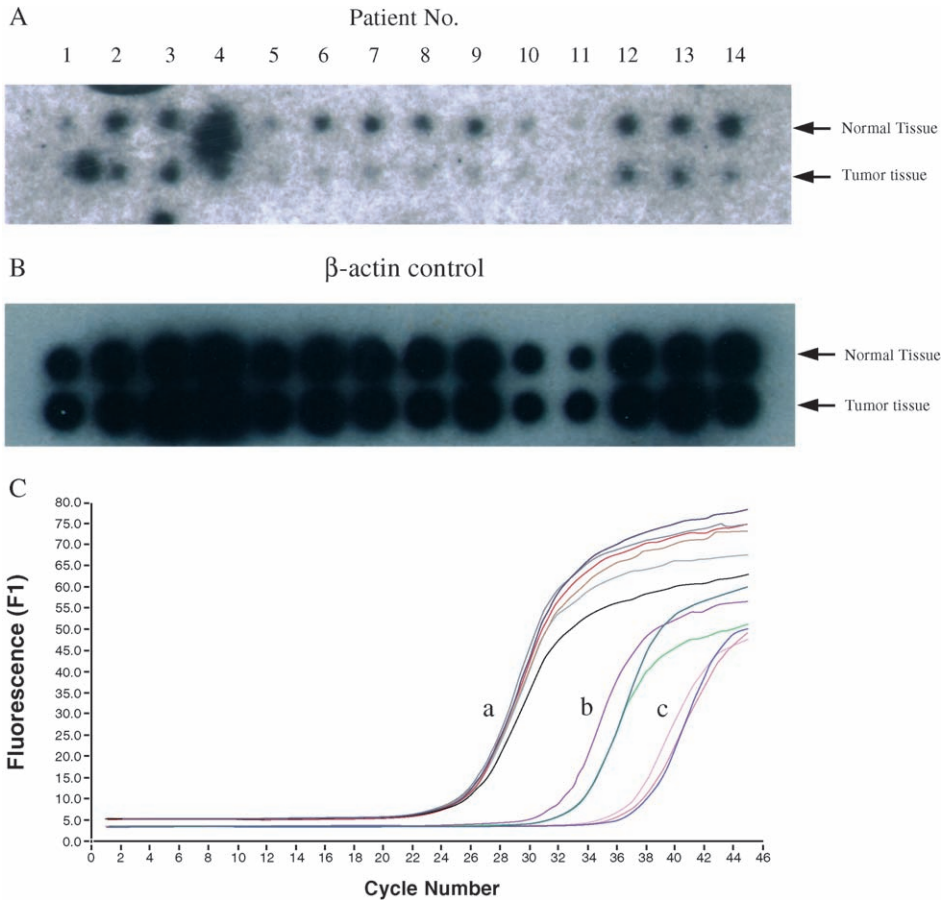

Fig. 4. (A) Northern hybridization of matched tumor and normal tissue samples from patients with kidney tumors. Notice the reduced expression in tumor tissues of patient nos. 2, 6, 7, 8, 9, 12, 13, and 14. (B) To confirm equal loading, the same blot was hybridized with a human *β-actin* probe as a control. Northern hybridizations were performed on a human Matched Tumor/Normal Expression Array (Clontech) following the manufacturer's instructions. The human *FoxP4* probe was obtained by PCR (*hFoxP4*—primers: 5′-GTCAGCCTGCAGCCCAACCAAGCC-3′ (forward) and 5′-GAG CTG TCT CTC CGA GAT GTG-3′ (reverse)) from human testis cDNA (Origene) and subcloned using the TOPO PCR Cloning Kit (Invitrogen). (C) Quantitative PCR on normalized cDNA from patient no. 7 (see A). The graph shows the results of three identical PCR runs. (a) Control primer amplifying human ribosomal protein S9 demonstrates equal cDNA amounts in tumor and normal tissue samples. (b) Reaction amplifying human *Foxp4* in normal kidney tissue. (c) Amplification of human *FoxP4* in the corresponding tumor tissue, indicating reduced expression. Quantitative PCR on cDNA HP107K (Clontech) was performed on 1 ng of cDNA using the LightCycler system (Roche) and the FastStart DNA Master SYBR Green I Kit according to the manufacturer's instructions. Amplification conditions were as follows: 95 °C for 10 min followed by 45 cycles of 95 °C 15 s, 62 °C 5 s, 72 °C 10 s. Control primers amplifying the human Ribosomal Protein S9 were used as supplied by Clontech.

demonstrated to be down-regulated in larynx carcinoma [27].

As *FoxP4* is down-regulated in at least two tumor types and its homologs FoxP1 and FoxP2 act as transcriptional repressors, it is likely that *FoxP4* also regulates transcription and that subsequent down-regulation of this gene causes overproliferation of different tissues. Knock-out analysis will be used in order to test this hypothesis. Due to the fact that not all patients had a reduction of *FoxP4* expression, additional clinical studies will further investigate whether the level of *FoxP4* expression has an influence on the prognosis of patients with kidney or laryngeal tumors.

In conclusion, we characterized a novel family member of the *FoxP* family. The expression profile of *FoxP4* not only implies a role during embryonic development and in various tissues of the adult organism, but also suggests a role in kidney tumorigenesis.

References

[1] D. Weigel, G. Jurgens, F. Kuttner, E. Seifert, H. Jackle, The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the *Drosophila* embryo, Cell 57 (1989) 645–658.

[2] E. Lai, V.R. Prezioso, E. Smith, O. Litvin, R.H. Costa, J.E. Darnell Jr., HNF-3A, a hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally, Genes Dev. 4 (1990) 1427–1436.

[3] E. Kaufmann, W. Knöchel, Five years on the wings of fork head, Mech. Dev. 57 (1996) 3–20.

[4] K.L. Clark, E.D. Halay, E. Lai, S.K. Burley, Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5, Nature 364 (1993) 412–420.

[5] S.L. Ang, J. Rossant, HNF-3 beta is essential for node and notochord formation in mouse development, Cell 78 (1994) 561–574.
[6] S.L. Brody, X.H. Yan, M.K. Wuerffel, S.K. Song, S.D. Shapiro, Ciliogenesis and left–right axis defects in forkhead factor HFH-4-null mice, Am. J. Respir. Cell Mol. Biol. 23 (2000) 45–51.
[7] B.M. Burgering, G.J.P.L. Kops, Cell cycle and death control: long live Forkheads, Trends Biochem. Sci. 27 (2002) 352–360.
[8] S. Xuan, C.A. Baptista, G. Balas, W. Tao, V.C. Soares, E. Lai, Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres, Neuron 14 (1995) 1141–1152.
[9] T. Kume, H. Jiang, J.M. Topczewska, B.L. Hogan, The murine winged helix transcription factors, Foxc1 and Foxc2, are both required for cardiovascular development and somitogenesis, Genes Dev. 15 (2001) 2470–2482.
[10] W. Shu, H. Yang, L. Zhang, M.M. Lu, E.E. Morrisey, Characterization of a new subfamily of winged-helix/forkhead (Fox) genes that are expressed in the lung and act as transcriptional repressors, J. Biol. Chem. 276 (2001) 27488–27497.
[11] V. Hatini, S.O. Huh, D. Herzlinger, V.C. Soares, E. Lai, Essential role of stromal mesenchyme in kidney morphogenesis revealed by targeted disruption of Winged Helix transcription factor BF-2, Genes Dev. 10 (1996) 1467–1478.
[12] P. Parry, Y. Wei, G. Evans, Cloning and characterization of the t(X;11) breakpoint from a leukemic cell line identify a new member of the forkhead gene family, Genes Chromosomes Cancer 11 (1994) 79–84.
[13] A. Borkhardt, R. Repp, O.A. Haas, T. Leis, J. Harbott, J. Kreuder, J. Hammermann, T. Henn, F. Lampert, Cloning and characterization of AFX, the gene that fuses to MLL in acute leukemias with a t(X;11)(q13;q23), Oncogene 14 (1997) 195–202.
[14] N. Galili, R.J. Davis, W.J. Fredericks, S. Mukhopadhyay, F.J. Rauscher III, S. Mukhopadhyay, B.S. Emanuel, G. Rovera, F.G. Barr, Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma, Nat. Genet. 5 (1993) 230–235.
[15] R.J. Davis, C.M. D'Cruz, M.A. Lovell, J.A. Biegel, F.G. Barr, Fusion of PAX7 to FKHR by the variant t(1;13)(p36;q14) translocation in alveolar rhabdomyosarcoma, Cancer Res. 54 (1994) 2869–2872.
[16] A.H. Banham, N. Beasley, E. Campo, P.L. Fernandez, C. Fidler, K. Gatter, M. Jones, D.Y. Mason, J.E. Prime, P. Trougouboff, K. Wood, J.L. Cordell, The FOXP1 winged helix transcription factor is a novel candidate tumor suppressor gene on chromosome 3p, Cancer Res. 61 (2001) 8820–8829.
[17] G.J.P.L. Kops, B.M. Burgering, Forkhead transcription factors: new insight into protein kinase B (c-akt) signaling, J. Mol. Med. 77 (1999) 656–665.
[18] M.E. Brunkow, E.W. Jeffery, K.A. Hjerrild, B. Paeper, L.B. Clark, S.A. Yasayko, J.E. Wilkinson, D. Galas, S.F. Ziegler, F. Ramsdell, Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse, Nat. Genet. 27 (2001) 68–73.
[19] C.L. Bennett, J. Christie, F. Ramsdell, M.E. Brunkow, P.J. Ferguson, L. Whitesell, T.E. Kelly, F.T. Saulsbury, P.F. Chance, H.D. Ochs, The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3, Nat. Genet. 27 (2001) 20–21.
[20] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (1990) 403–410.
[21] A. Bateman, E. Birney, L. Cerruti, R. Durbin, L. Etwiller, S.R. Eddy, S. Griffiths-Jones, K.L. Howe, M. Marshall, E.L. Sonnhammer, The Pfam protein families database, Nucleic Acids Res. 30 (2002) 276–280.
[22] A. Lupas, M. Van Dyke, J. Stock, Predicting coled coils from protein sequences, Science 252 (1991) 1162–1164.
[23] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.
[24] T. Hubbard, D. Barker, E. Birney, G. Cameron, Y. Chen, L. Clark, T. Cox, J. Cuff, V. Curwen, T. Down, et al., The Ensembl genome database project, Nucleic Acids Res. 30 (2002) 38–41.
[25] L. Balvay, D. Libri, M.Y. Fiszman, Pre-mRNA secondary structure and the regulation of splicing, BioEssays 15 (1993) 165–169.
[26] N. Schaeren, A. Gerfin-Moser, A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes, Histochemistry 100 (1993) 431–440.
[27] M. Frohme, B. Scharm, H. Delius, R. Knecht, J.D. Hoheisel, Use of representational difference analysis and cDNA arrays for transcriptional profiling of tumor tissue, Ann. N.Y. Acad. Sci. 910 (2000) 85–104.