The Ets Transcription Factor \textit{ELF5} Functions as a Tumor Suppressor in the Kidney

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Renal cell carcinoma is an important clinical disease with poorly understood etiology. \textit{ELF5} is an epithelial-specific member of the Ets family of transcription factors, characterized by the 80 amino acid Ets domain that binds the purine-rich GGAA/T Ets motif found in the promoter regions of a variety of genes. Since \textit{ELF5} is highly expressed in kidney and has been postulated to function as a tumor suppressor, at least in the context of the breast, we investigated its role in kidney cancer. In renal cell carcinoma \textit{ELF5} expression was consistently decreased in tumor samples versus normal. \textit{ELF5} mRNA was decreased in 94\% of lesions tested and \textit{ELF5} protein was undetectable in 40/40 kidney-derived carcinomas. Re-expression of the \textit{ELF5} gene in 786-O renal carcinoma cells suppressed their tumorigenic capacity in vitro and in vivo. This work is the first to suggest that \textit{ELF5} has tumor suppressor activity in the kidney.

\textbf{Keywords:} \textit{ELF5}, Ets transcription factor, renal carcinoma

The Ets transcription factors are a large family of proteins implicated in the control of cellular proliferation and tumorigenesis (reviewed in Seth & Watson, 2005). The Ets genes are characterized by a region of conserved sequence, called the Ets domain. This 80 amino acid region forms the winged helix-turn-helix (wHTH) DNA binding domain that recognizes a core GGAA/T sequence in the promoters of target genes (Donaldson et al., 1994). Most of the Ets genes are expressed in hemopoietic cells and are important in subtypes of leukemia and lymphoma. The isolation of an epithelial-specific subset of Ets transcription factors, comprising \textit{ELF3}, \textit{ELF5}, \textit{ESE}-3 and the prostate-specific \textit{PSE/PDEF}, opened a new avenue in Ets biology and considering the prevalence of epithelial-derived cancer in the human population, these genes may be of clinical significance.

The epithelial-specific Ets transcription factor \textit{ELF5} was first described by this laboratory (Zhou et al., 1998). We postulate that \textit{ELF5} may function as tumor suppressor based on its lack of expression in a large variety of carcinoma cell lines (Oettgen et al., 1999; Zhou et al., 1998) and primary breast cancers (Ma et al., 2003), and on its chromosomal localization to 11p13, a region that frequently undergoes LOH and hypermethylation in carcinomas of the breast, prostate, bladder and lung (Gudmundsson et al., 1995; Karnik et al., 1998; Ludwig et al., 1991; Shipman et al., 1993; Winqvist et al., 1995). \textit{ELF5} is expressed strongly in mammary epithelium and in other epithelium-rich organs such as the kidney (Lapinskas et al., 2004; Oettgen et al., 1999; Zhou et al., 1998). We reported that the loss of one functional allele of the \textit{ELF5} gene in the mouse results in developmental arrest of the mammary gland during pregnancy and established that \textit{ELF5} is required for differentiation of mammary epithelial cells into milk-producing units (Zhou et al., 2005). Relatively little is known about the signal transduction pathways in which Elf5 participates. In the mammary...
gland ELF5 has been placed downstream in the prolactin/Jak/Stat signaling pathway (Harris et al., 2006; Zhou et al., 2005) and in embryonic mouse lung is downstream of fibroblast growth factor (FGF) signaling, acting via FGFR2b and the PI3-Kinase/Akt pathway (Metzger et al., 2007). ELF5 is also induced during keratinocyte differentiation (Oettgen et al., 1999) and may act in a signal transduction pathway involving keratinocyte-restricted factor (KRF) (Tummala & Sinha, 2006). Finally, ELF5 was shown to induce expression of the parotid gland PSP promoter and the prostate-specific PSA promoter (Oettgen et al., 1999) and our studies have shown that Elf5 is also required for expression of a panel of genes encoding milk proteins (Rogers et al., 2010; Zhou et al., 2005).

Renal cell carcinoma is an increasingly important clinical disease. Apparent incidence has increased due to improvements in detection, but treatment has not made equivalent progress and the etiology of the disease is not well understood (Rathmell & Godley, 2010). Metastatic renal cell carcinoma is refractory to chemotherapy, hormonal therapy and radiation therapy (reviewed in Liou et al., 2004). Increased risk of kidney cancer is associated with specific diseases such as kidney infection and diabetes type I and II (Schlehofer et al., 1996) and there is an unidentified genetic component, with an increased relative risk of the cancer if a first-degree relative has also been diagnosed with kidney cancer (Schlehofer et al., 1996) but despite this knowledge, the molecular mechanisms involved are poorly understood.

The majority of renal cell carcinomas are sporadic in origin but a small percentage of cases arise due to a predisposition associated with a heritable syndrome. Seven hereditary renal cell cancer syndromes have been recognized and the genes for five of these identified: von Hippel–Lindau (VHL); the MET proto-oncogene; fumarate hydratase (FH); Birt–Hogg–Dubé (BHD); and hyperparathyroidism 2 (HRPT2) (reviewed in Pavlovich et al., 2004). The VHL gene, the first gene identified for hereditary renal cell carcinoma and defective in 75% of sporadic renal cancers, was also discovered along with two other tumor suppressor genes, a Ras effector known as RAS-association domain family 1 (RASSF1A) and the DNA repair gene, OGG1 in studies examining allele loss at chromosome 3p, the most frequent genetic alteration in renal cell carcinoma (Audebert et al., 2000; Cliford et al., 1998; Gnarra et al., 1995; Morrissey et al., 2001). The diverse nature and functions of the genes so far identified in renal cell carcinoma implies that various biological pathways and mechanisms are involved. Other genes such as TRC8 (translocation in renal cancer from chromosome 8), JAB1 (Jun activation domain binding protein 1) and HIF-1α (Hypoxia-inducible factor-1alpha) have been implicated due to their participation in a cellular pathway involving VHL (Gemmill et al., 2002; Tanimoto et al., 2000).

In this study we tested the hypothesis that ELF5 behaves as a tumor suppressor in cancer of the kidney. We clearly demonstrate that in renal cell carcinoma, ELF5 expression was consistently decreased and that re-expression of the ELF5 gene in a renal carcinoma cell line suppressed their tumorigenic capacity. Thus, ELF5 may function as a tumor suppressor in the kidney.

Method

Quantitative RT-PCR Analysis of Renal Carcinomas

Eighteen renal cell carcinoma cDNAs were obtained from the Ludwig Institute for Cancer Research Oncology Unit (Melbourne). All samples were obtained according to appropriate human ethical guidelines. Normal reference kidney RNA was obtained from Stratagene. QRT-PCR was performed on the LightCycler (Roche) using FastStart DNA Master SYBR Green I (Roche). ELF5 transcript was detected using oligonucleotides ELF5-18 (5′-TGGCCTTTTGAGCATCAGACAG-3′) and ELF5-19 (5′-AGTATCATCTTGGTCGG-AGG-3′), in 3 mM MgCl2, with 10 sec denaturation at 95°C, 5 sec annealing at 55°C and 11 sec extension at 72°C. Cytokeratin 8 was used as a marker for simple epithelium. Oligonucleotide sequences were CK8F (5′-CTTGGAGTCTGGCCTTGGGGA-3′) and CK8R (5′-CCTCCTGACTCTGCTTGACC-3′). Amplification was performed as for ELF5 but with 5 sec annealing at 59°C and 8 sec extension at 72°C.

Protein Expression of ELF5 in Cancer Sections

Renal cancer biopsy slides containing punch biopsies 2 mm in diameter and sectioned at 4 μm were obtained from SuperBioChips Laboratories (Seoul, South Korea). Anti-ELF5 immunohistochemistry was performed on this formalin-fixed, paraffin-embedded tissue using a polyclonal anti-ELF5 antibody along with the TSA Indirect kit (NEN Life Sciences) essentially as described (Lapinskas et al., 2004).

Production of 786-O Renal Carcinoma Cell Lines Expressing ELF5

Full length ELF5 cDNA was cloned in the pEF-BOS expression vector (Mizushima and Nagata, 1990). 786-O cells were maintained in RPMI 1640 medium with 10 % FCS. 786-O cells were co-transfected (FuGene 6) with ELF5-pEF-BOS and a puromycin-resistance plasmid (Ramirez-Solis et al., 1995). Clonal lines were selected with 3 μg/ml puromycin (ICN Biochemicals). After selection, puromycin was removed for all subsequent procedures. Expression of ELF5 cDNA was confirmed by RT-PCR using primer pair ELF5-18 and ELF5-19.

Growth of Transfected Cells Under Anchorage-Independent Conditions

Two ml of 0.7 % (w/v) agarose in culture medium was plated in 6 well plates (BD Labware). 1x10⁴ cells were resuspended in 1 ml of 0.35 % (w/v) agarose in culture

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medium and layered upon the 0.7% (w/v) bottom agarose. Wells were overlayed with culture medium and incubated at 37°C/5% CO₂ for 21 days. Each of 3 clones was plated in triplicate and the assay repeated three times independently. For scoring, three fields of each well were counted as small colonies (cells that had not divided more than twice) or large colonies (cells that had divided more than twice).

**Tumorigenic Capacity of Transfected Cells in vivo**

The tumor-forming capacity of ELF5-transfected renal carcinoma cell clones compared with mock-transfected cells was tested by subcutaneous cell inoculation into immuno-compromised SCID mice. 1 × 10⁶ 786-O derived cells were resuspended in 100 μl Matrigel (BD BioSciences) and injected subcutaneously under the hind leg of 5- to 6-week-old female SCID mice (Animal Research Council, WA, Australia). The tumor size (mm³) was evaluated by measurement of three perpendicular diameters with microcalipers and using the formula 4/3 L × W × H, where L is the longest diameter, W is the diameter perpendicular to L and H is the height of the tumor. When tumors reached an L diameter of 10 mm, or at 21 days post-inoculation, the mice were culled and regions of the tumor excised and examined by H&E staining.

**Results**

**ELF5 is Down-Regulated in Renal Cell Carcinoma**

Relatively high levels of ELF5 mRNA (Zhou et al., 1998) and protein (Lapinskas et al., 2004) are observed in the kidney so we reasoned that if ELF5 was a tumor suppressor, its loss may be involved in renal cell carcinoma. Also, loss of ELF5 transcript had been observed in kidney-derived carcinoma cell lines (Zhou et al., 1998). Since the expression of ELF5 mRNA in renal cell cancer had not been previously studied we quantified ELF5 expression levels in 18 renal cell cancers compared to normal kidney reference RNA by QRT-PCR (Figure 1). All but one sample showed a reduction in ELF5 expression.

To determine if the loss of ELF5 mRNA correlated with a loss of ELF5 protein, we examined protein expression of ELF5 in kidney carcinoma samples by immunohistochemistry. We used a polyclonal anti-ELF5 antibody that we had previously characterized for specificity (Lapinskas et al., 2004). The slides contained 9 normal samples matched to 9 of the cancer samples on the same slide (i.e., tumor and normal kidney tissue from the same person). In all cases, the normal tissue stained with the anti-ELF5 antibody and the tumor samples did not. ELF5 protein was not detectable in 40 kidney-derived carcinomas whereas it was detected in the 9 normal kidney tissue samples.

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**FIGURE 1**

ELF5 transcript levels are reduced in renal cell carcinoma.

Note: In the kidney all samples were compared with a reference normal sample as matched normal cDNA was not available. The amount of ELF5 in each sample was normalized to the amount of cytokeratin 8, which represents the quantity of epithelium present (n = 3). Data is presented as fold-change +/- SEM.
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These results concur with the loss of ELF5 mRNA expression in kidney-derived carcinomas and indicate that the loss of protein is due to a decrease in transcription rather than translation.

ELF5 Functions as a Tumor Suppressor in Renal Carcinoma Cells

Since the loss of ELF5 was associated with tumorigenesis in kidney carcinomas we investigated whether ELF5 was capable of functioning as a tumor suppressor in a renal carcinoma cell line. 786-O renal carcinoma cells, which do not normally express ELF5 (Figure 3A) were stably transfected with a construct expressing ELF5 (Figure 3A). The introduction of exogenous ELF5 altered the growth of these cells in three-dimensional, anchorage-independent conditions compared to the mock transfected control. Approximately 90% of mock transfected 786-O cells formed colonies in soft agarose (Figure 3B, C). The ELF5-expressing 786-O cells showed a significant 71% decrease in the proportion of cells that formed colonies compared to mock transfectants (p < .001) and the colonies that formed were smaller in size (Figure 3B, C).

Next we inoculated our 786-O transfectants into SCID mice to analyse the tumor-forming capability of the cells. Group size is cumulative for the number of mice injected with two clones over two independent experimental replicates. Ninety per cent of mock transfecant-inoculated mice (9 of 10) established tumors, while tumors developed in only 10% of the of ELF5-transfectant inoculated mice (2 of 20) at 21 days post-inoculation. Analysis of the inoculation sites of mock transfected 786-O cells showed formation of solid tumors containing closely packed...
epithelial-like cells and blood infiltrate. Small epithelial-like cell clusters, presumably of 786-O origin, dispersed among the subcutaneous fat were identified at the ELF5-expressing 786-O injection sites (Figure 4). Blood vessels and infiltrate were not observed at these sites.

Discussion

Ma and colleagues (2003) demonstrated decreased ELF5 expression in premalignant, preinvasive and invasive breast carcinomas compared to normal tissue. ELF5 mRNA expression was also decreased in some breast cancer cell lines (Zhou et al., 1998). In this study, we have shown in the kidney that ELF5 mRNA was decreased in 17 of the 18 renal carcinomas examined. The majority of the samples were classified as renal cell carcinoma (15 of 18 samples), the most common form of kidney cancer. Too few samples were available to enable us to draw any correlations between either the type or grade of renal cell carcinoma and the level of ELF5 mRNA. This reduction in ELF5 message was consistent with the lack of immunoreactivity of the anti-ELF5 antibody on kidney-derived carcinomas, whereas the normal kidney stained strongly throughout the tubules.

Re-expression of ELF5 in renal carcinoma cells resulted in decreased three-dimensional, anchorage-independent growth and in tumorigenicity in vivo. Sites where the kidney-derived 786-O-ELF5 cells are present in SCID mice lacked the tumor-associated blood vessels and blood cell infiltrations characteristic of aggressive tumors that promote formation of their own blood supply. Thus, ELF5 in the kidney appears to have tumor suppressor function. The mechanism whereby the loss of ELF5 expression contributes to tumorigenesis remains undefined but it is probable that like other Ets factors, aberrant expression of the ELF5 transcription factor in carcinomas leads to dysregulation of its target genes, and thus contributes to cellular transformation. This could conceivably represent a role for ELF5 in maintenance of growth suppression or terminal differentiation. Loss of ELF5 expression in this context could result in a selective growth advantage for tumorigenic cells.

There is some evidence to support Ets factors having a role in kidney carcinoma. For instance, one study reported the up-regulation of an ETS-like protein in renal cell carcinoma versus normal kidney (Rae et al., 2000) and ETS-1 was identified as a potential culprit in renal cell carcinoma because of its involvement in the angiogenic and matrix remodeling processes associated with carcinogenesis. ETS-1 is a target gene of VEGF and was found to be up-regulated and localized to carcinoma cells and endothelial cells in renal cell carcinoma (Mikami et al., 2006). ETS-1 regulates the expression of the matrix metalloproteinases MMP-1, MMP-3 and MMP-9 (Mizui et al., 2006), all of which have been found to be over-expressed in renal cell carcinoma (Bhuvarahamurthy et al., 2006). Suppression of the Tsc2 tumor suppressor gene promoter by the Ets factor Elf-1 was also linked to renal cell carcinoma in a study using a rat model of tuberous sclerosis (Honda et al., 2003) and Pea3 was found to transactivate the Wilms' tumor gene, WT1 (Discenza et al., 2004). Notably, all these studies invoke oncogenic properties of Ets transcription factors whereas our study supports a tumor suppressor function for ELF5. Although the mechanism whereby loss of ELF5 contributes to renal cell carcinoma remains undefined, based on the requirement of ELF5 for the differentiation of mammary epithelial cells and on its involvement in differentiated keratinocytes, we speculate that ELF5 is needed to maintain renal epithelial cells in a differentiated state. This role would also be consistent with its function as a transcription factor required for the expression of other tumor suppressor genes.

Dysregulation of a number of signal transduction pathways has been linked to the etiology of renal cell carcinoma. The most common pathway disrupted is centred around the loss of the VHL gene. The product of the VHL gene forms part of a ubiquitin ligase complex.
that targets proteins for degradation, so loss of VHL in renal cell carcinoma presumably disrupts normal cellular proteosome functions. Loss of VHL also increases the expression of hypoxia-inducible factor, HIF-1α which via its transcriptional regulation of vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), endothelial growth factor receptor (EGFR), glucose transporter protein 1 (GLUT1), erythropoietin and transforming growth factor-alpha (TGF α), creates a pseudohypoxic state in renal cells that promotes vascularisation of the tumor (Bratslavsky et al., 2007). A pathway known as the rapamycin-sensitive signal transduction pathway is also operative in renal cell carcinoma (Hidalgo & Rowinsky, 2000). The fungicide rapamycin exhibits its anti-tumorigenic properties by blocking the kinase activity of a protein called mTOR or mammalian target of rapamycin, which in turn leads to cell growth arrest. Recently, it was reported that inhibition of mTOR by rapamycin also inhibits tumor angiogenesis by blocking the mTOR-dependent activation of HIF-1 α and VEGF-A secretion (Land & Tee, 2007). How these pathways impinge on ELF5 or conversely, how ELF5 exerts its effects on these pathways, presently remains obscure. However, it is possible that the loss of ELF5 expression may represent an alternative carcinogenic mechanism.

Conclusions
In summary, we have shown for the first time that an Ets transcription factor, ELF5, behaves in a fashion consistent with a tumor suppressor gene in renal cancer. Further work is required to examine LOH, promoter hypermethylation or somatic mutation of the gene as potential explanations for the loss of ELF5 expression. Also, ascribing a role for ELF5 in normal kidney development and maintenance of function will assist in elucidating how dysregulation of this tumor suppressor gene can result in carcinogenesis.

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References


