OFD1, the Gene Mutated in Oral-Facial-Digital Syndrome Type 1, Is Expressed in the Metanephros and in Human Embryonic Renal Mesenchymal Cells

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Abstract. Oral-facial-digital syndrome type 1 (OFD1) causes polycystic kidney disease (PKD) and malformations of the mouth, face and digits. Recently, a gene on Xp22, OFD1, was reported to be mutated in a limited set of OFD1 patients. This study describes mutation analysis in six further OFD1 families. Additionally, gene expression was sought in human development. In two OFD1 kindreds affected by PKD, a frameshift mutation and a splice-site mutation were detected. In four apparently sporadic cases, three frameshift and a missense mutation were found. Using RT-PCR of RNA from first-trimester normal human embryos, both alternative splice forms of mRNA (OFD1a and OFD1b) were found to be widely expressed in organogenesis. Northern blot detected OFD1 mRNA in metanephros, brain, tongue, and limb, all organs affected in the syndrome. A polyclonal antibody directed to a C-terminal OFD1a epitope detected a 120-kD protein in the metanephros and in human renal mesenchymal cell lines. In normal human embryos, OFD1a immunolocalized to the metanephric mesenchyme, oral mucosa, nasal and cranial cartilage, and brain. Moreover, using normal human renal mesenchymal cell lines, the immunoreactive protein colocalized with γ-tubulin, suggesting that OFD1 is associated with the centrosome. First, it is concluded that OFD1 mutations would generally be predicted to result in unstable transcripts or nonfunctional proteins. Second, OFD1 is expressed in human organogenesis; on the basis of the metanephric expression pattern, the results suggest that OFD1 plays a role in differentiation of metanephric precursor cells.

Malformations of the face, oral cavity and digits characterize the oral-facial-digital (OFD) syndromes (1,2). OFD1 (MIM311200) is the most common, occurring in 1:250,000 births. Most cases appear to be sporadic, but others are inherited in an X-linked dominant manner. Affected females have malformations of oral cavity (cleft palate, lip and tongue, abnormal dentition, hamartomas), face (hypertelorism and milia), and digits (syndactyly, brachydactyly, polydactyly). Affected XY males almost invariably die before birth (3,4); the only OFD1 male reported to have prolonged postnatal survival being XXY (5). When OFD1 was first delineated, renal disease was not considered to be characteristic (1,6). More recently, several OFD1 patients have been reported to have a form of polycystic kidney disease (PKD) that often leads to end-stage renal failure in late childhood or adulthood (7–12). In fact, OFD1 should be suspected when PKD occurs in females with dysmorphic features or when PKD is transmitted from generation to generation—affecting only females. Limited histologic studies suggest that glomerular cysts predominate (8,10). Brain malformations also occur (9,12), accompanied by learning difficulties, as do pancreatic cysts (10). On the basis of this clinical spectrum, the gene mutated in OFD1 is anticipated to have important roles in normal growth of diverse tissues, including the kidney.

Feather et al. (13) mapped OFD1 to Xp22.2 to 22.3, and Ferrante et al. (14) identified Cxorf5 (renamed OFD1; accession number Y15164) as the mutated gene in seven kindreds. OFD1 has an open reading frame of 3033 bp over 23 exons (15). Due to the use of alternative 3’ splice sites in intron 9, two mRNA species, here designated OFD1a and OFD1b, are generated. Although the OFD1b transcript is the longer form, OFD1a has a longer translated region. The two putative OFD1 proteins have different C-termini; the larger form (here designated OFD1a) is predicted to have 1011 amino acids (aa), whereas OFD1b has 367 aa. Although many OFD1 anomalies are developmental aberrations, no published data exists on...
**OFD1** expression in human development. We here describe further **OFD1** mutations in six kindreds, including two families with several members affected by PKD and report, for the first time, an overview of **OFD1** expression in human organogenesis and subcellular localization in human embryonic renal cell lines.

**Materials and Methods**

Chemicals were obtained from Sigma-Aldrich Chemical Company (Poole, UK), unless otherwise stated.

**Patient Identification and Collection of Samples**

Clinical details of two families with multiple members affected by **OFD1** and associated PKD have been described (7,10). For mutation analyses, we used these families and four other kindreds which are described in Results; all were assessed at Clinical Genetics centers and patients were studied with renal ultrasonography (US). Fifteen terminated normal human fetuses were collected under research ethical committee permission and were staged by external morphology (16) as between 7 and 14 wk of gestation, spanning early organogenesis. Sections of mature kidney tissue were also examined from areas that appeared histologically normal adjacent to three Wilms tumors, removed at 28 to 72 mo of age. The metanephros forms in the sixth week from the ureteric bud, which branches to form collecting ducts, and the mesenchyme, which differentiates into S-shaped bodies and glomeruli, the first of which form by eight weeks; this process reiterates until 34 wk. New nephrons are not formed after birth.

**Mutation Detection**

DNA was isolated from native or transformed leukocytes by standard techniques, after obtaining written consent. PCR amplified the entire **OFD1a** coding region (exons 1 to 23) including splice junctions (14). Exon 10a, which confers the additional genetic information in the **OFD1b** transcript, was not analyzed for mutations. PCR reactions contained 30 ng genomic DNA, 2 mM MgCl2, 200 μM dTTP, dGTP, dCTP, dATP, 25 pmol of each primer, 1 × PCR buffer, 1 × Q solution, and 0.1 U of HotStartTaq DNA polymerase (Qiagen, Crawley, UK) in 25 μl final volume. Cycling parameters were: 94°C for 10 min, 30 cycles of amplification (30 s at 94°C, 30 s at 58°C, and 1 min at 72°C) with final elongation of 5 min at 72°C. PCR products from one proband and, where available, one unaffected family member of each pedigree, were screened for sequence changes by denaturing HPLC (dHPLC), using the WAVE DNA fragment analysis system (Transgenomic Inc, Crewe, UK). Column temperatures and acetonitrile gradients were determined using WaveMaker v. 3.4.4 (Transgenomic Inc). Proband PCR products showing altered elution properties to controls were sequenced on both strands using ABI Big Dye Terminator Sequencing Kit and an ABI 377 Sequencer (Perkin Elmer, Forster City, CA). Sequences were analyzed using Sequence Analysis v. 3.4.1 (Perkin Elmer). Sequence changes in probands were then assessed to determine co-segregation with clinical disease.

**Cell Culture**

Human embryonic kidney (HEK) 293 cells were transfected to express full-length **OFD1**. This line was generated by adenoviral transformation of primary cultures, but it is uncertain what type of metanephric cell, if any, it faithfully represents. Indeed, Shaw et al. (17) reported that they express neuronal proteins as well as cytokeratins. Cells were cultured in Dulbecco Modified Eagle Medium (DMEM, Invitrogen) with 10% fetal calf serum (FCS), 1000 U/L penicillin G, and 1 g/L streptomycin. The day before transfection, cells were plated at subconfluent density. They were incubated for 6 h in serum-free medium containing 30 μl of Lipofectamine reagent (Invitrogen) and 10 μg of DNA per 10-cm-diameter plate. DNA used were: (1) myc-tag/enhanced green fluorescence protein (EGFP/OFD1 in pcDNA3 vector (Invitrogen), with **OFD1** cDNA corresponding to the **OFD1a** open reading frame; (2) vector with the myc-EGFP construct only. Cells were analyzed at 36 h after transfection. To produce renal mesenchymal lines, metanephiroi were dissected from normal human embryos (two at 10, one at 11, and one at 12 wk gestation), diced into five pieces, placed onto plastic tissue culture plates, and fed DMEM/Hams F12 medium (Invitrogen) supplemented with 5% FCS, epidermal growth factor (10 μg/L), hydrocortisone (36 μg/L), 3,3',5-triiodo-L-thyronine sodium salt (4 μg/L), insulin/transferrin/selenium Sigma media supplement, and antibiotics. Outgrowths were passaged, and monolayers were characterized at passages four-eight (data not shown and reference 18).

**RNA Analyses**

For RT-PCR, RNA (500 ng), extracted from isolated organs with Tri-reagent, was treated with deoxyribonuclease I and subjected to reverse transcription with Superscript II (Life Technologies BRL). cDNA was diluted to a volume of 100 μL and 2 μl was used as a template with the following primers sets synthesized by MWG-Biotech AG:

1. **OFD1** primer set 1: sense (5'TGAATTGAACCAGAAGCTCC3', exon 9/10) and antisense (5'CCTCCTCTTGCAAATGAACAG3', exon 12)
2. **OFD1** primer set 2: sense (5'ATGGTGTCTGCTGGCTTGTTG-3', exon 10a) and antisense (5'GACTGTTATCATGTCAGGACG3', exon 10a)
3. hypoxanthine-guanine phosphoribosyltransferase (HPRT), a ‘house-keeping’ gene: sense (5'CCACGAAAGTTGGTTGGATAACG3') and antisense (5'GGCCATGTGCAATTAGCCGATG-3')

All PCR reactions were performed in a volume of 50 μl, with 0.25 units of HotStartTaq polymerase, 200 μM dNTPs, 2 mM MgCl2, and 3 pmol of primers. PCR conditions were: 10 min at 94°C followed by 30 cycles of amplification (30 s at 94°C, 30 s at 58°C, and 1 min at 72°C) with final elongation of 5 min at 72°C. PCR products were electrophoresed through 2% agarose gels containing ethidium bromide and sequences of the PCR products confirmed using the ABI Prism Big Dye Terminator Cycle Sequencing kit and an ABI 377 automated sequencer. **OFD1** primer set 1 amplifies cDNA derived from both **OFD1** cDNA isoforms to generate a 225-bp **OFD1a** band, and an 850-bp **OFD1b** band. In preliminary experiments, the former band was preferentially amplified in the PCR conditions used, while the product from **OFD1b** was barely visible. Therefore, we designed a second set of **OFD1** primers (primer set 2) on the region of 630 bp in exon 10a unique to **OFD1b** cDNA. For Northern blot, 25 μg RNA was run on a formamide agarose gel and transferred to a Nylon membrane (Hybond N+: Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was hybridized with either the **OFD1**/GFP cDNA, described above, or a 18S rRNA probe labeled with 35PdCTP by random priming, using the Megaprime DNA labeling system (Amersham Pharmacia Biotech). Hybridization was performed with Quikhybolution (Stratagene, La Jolla, CA) at 68°C. X-ray films were exposed to filters for up to 7 d at −70°C.

**Protein Analyses**

Whole **OFD1** proteins were not available, so we selected an oligopeptide, CDVQKIQEEQKEEKIREQQVKER (867 to 891 aa in
OFD1a), to generate antibodies in rabbits. Oligopeptide synthesis and production of antisera were undertaken by Sigma-Genosys (Cambridge, UK). Bleeds from two animals were tested by ELISA against the immunizing peptide to ensure that antisera had high titer and avidity. Both antisera gave similar results, and for simplicity in Results, we have not distinguished between results derived from either. For Western blot, tissues were lysed in RIPA buffer with protease inhibitors (19). Protein concentration was quantified (BCA kit; Pierce, Rockford, IL), and 50 μg was mixed with an equal volume of sample buffer (1 ml glycerol, 0.5 ml mercaptoethanol, 3 ml 10% sodium dodecyl sulfate, 1.25 ml Tris, pH 6.7, and 1 mg of bromophenol blue), heated at 95°C for 5 min, and loaded onto SDS-polyacrylamide gels. After electrophoresis and transfer by electroblotting, membranes were stained with Ponceau S to confirm equality of total protein transfer (data not shown). Blots were washed with deionized water, placed in blocking solution (5% nonfat milk, 0.1% Tween-20 in PBS) for 1 h, then incubated overnight at 4°C with primary antibodies directed against the following proteins: OFD1a antisera (1:500); OFD1a antisera (1:500) preincubated for 2 h at room temperature with immunogenic peptide as a negative control; preimmune serum (1:500) as another negative control; WT1 (1:250; Santa Cruz); PAX2 (1:500; Zymed); hepatocyte growth factor (HGF; 1:200; R&D Systems); glial cell line-derived neurotrophic factor (GDNF; 1:200; R&D Systems). Appropriate horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:5000, DAKO, High Wycombe, UK) were applied, and bound second antibody was detected with ECL reagent (Amersham Life Science). Some blots were stripped and reprobed with either mouse anti-human β-actin (1:10,000), a housekeeping protein, or anti-myc (1:5000; Invitrogen).

For immunohistochemistry, tissues were fixed in 4% paraformaldehyde overnight, dehydrated, wax-embedded, and sectioned at 5 μm. Sections were dewaxed in Histoclear (National Diagnostics, Atlanta, GA) and rehydrated through graded ethanol. Antigens were unmasked by digestion in 1 mg/ml trypsin for 10 min and then treated with 3% hydrogen peroxide in PBS (pH 7.4) for 30 min to quench endogenous peroxidase activity. Nonspecific binding was blocked by incubation with 10% FCS in PBS for 30 min before overnight incubation at 4°C with either OFD1a anti-serum or preimmune serum (1:350) or antisera preincubated with immunizing peptide. Washed slides were exposed to a streptavidin-biotin peroxidase system followed by diaminobenzidine (Dako, ABC Kit) and counterstained with hematoxylin.

For immunocytochemistry, mesenchymal cells were plated on four-well Lab-Tek chamber slides (Nunc, Naperville, IL) at a density of 50,000 per well, cultured overnight, then fixed for 10 min in methanol at −20°C. After blocking for 1 h with 10% FCS in PBS, they were incubated overnight at 4°C with OFD1 antisemur (1:100), preimmune serum or anti-serum preincubated with immunizing peptide. In some experiments, for double labeling, the cells were additionally incubated with mouse monoclonal γ-tubulin antibody (1:50). The cells were washed three times in PBS and 0.1% Tween-20, and incubated for 1 h with donkey anti-rabbit FITC (1:100) or donkey anti-mouse Cy5 (1:500) secondary antibodies; the emission spectra for these fluorochromes is non-overlapping. After immunocytochemistry, some slides were also incubated for 5 min with the nuclear marker propidium iodide (4 mg/L). Fluorescence was visualized on a confocal laser scanning microscope (Leica Aristoplan, Heidelberg, Germany).

### Results

#### OFD1 Mutations

Mutations were defined in two OFD1 pedigrees affected by PKD, previously clinically described (7,10). Both families had been used to map the disease locus (13). In the first pedigree (see family 1 in Figure 1 of reference 13) an insertion of A in exon 8 (702insA) was detected in one allele, a frameshift predicted to lead to a premature stop codon in exon 8. In the next pedigree (see family 2 in Figure 1 in reference 13), a splice-site mutation was detected; it was caused by the substitution of A for T in the acceptor splice-site of exon 11 leading to the abolition of the splice-site (IVS10–2A>T). Tracking the mutations through families showed that the females with mutations had classic OFD1 dysmorphic features, while several clinically unaffected females carried normal alleles only. Table 1 depicts clinical and genetic details of four newly described affected individuals with no first-degree or other relatives with OFD1 dysmorphology. Sequence analysis revealed three deletions (one in exon 3 and two in exon 9) and one missense mutation in exon 3. In patients 3, 4, and 6, mutations were not detected in maternal DNA, consistent with a de novo genetic change causing the disease; maternal DNA was not available for the fourth family. Ninety normal chromosomes were screened by dHPLC, and none showed shifts in exon 3, con-

### Table 1. Clinical and genetic details

<table>
<thead>
<tr>
<th>Family</th>
<th>Mutation</th>
<th>Renal Ultrasound</th>
<th>Renal Function</th>
<th>Oral/Facial Features</th>
<th>Digital Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>707del 13 (exon 8); Parents have normal sequence</td>
<td>PKD 15 y</td>
<td>ESRF</td>
<td>Hypertelorism, facial milia and clefting, crowded teeth</td>
<td>Syndactyly</td>
</tr>
<tr>
<td>4</td>
<td>S74F (221T&gt;C) (exon 3); Parents and female siblings have normal sequence</td>
<td>Normal 14 y</td>
<td>ND</td>
<td>Hypertelorism, oral frenulae, high arched palate, facial milia, hydrocephalus</td>
<td>Brachy- and syndactyly, clinodactyly</td>
</tr>
<tr>
<td>5</td>
<td>843 del TT (exon 9)</td>
<td>PKD 2nd decade</td>
<td>ESRF</td>
<td>Hypertelorism, cleft palate, multiple oral frenulae</td>
<td>Brachy- and syndactyly</td>
</tr>
<tr>
<td>6</td>
<td>837 del AA (exon 9); Parents have normal sequence</td>
<td>Normal 3 y</td>
<td>ND</td>
<td>Facial milia, cleft lip and tongue, high arched palate</td>
<td>Brachy- and syndactyly</td>
</tr>
</tbody>
</table>

* All index cases were female. ND, not documented; ESRF, end-stage renal failure.
sistent with the missense change discovered for patient 4. Figure 1 depicts the above mutations, together with those previously reported (14,20).

**OFD1 Transcripts in Human Development**

RT-PCR (Figure 2A) detected OFD1a and OFD1b transcripts in metanephros, brain, tongue, and limbs from normal human embryos at eight to 14-wk gestation. Both transcripts were also detected in heart, lung, and gut, which are not known to be clinically affected. Organs from two embryos (9 and 14 wk gestation) were analyzed by Northern blot (Figure 2B). A major OFD1 transcript was detected at 4.0 kb in metanephros, brain, tongue, and limb, and a faint band was noted at 4.5 kb; these sizes respectively correspond to OFD1a and OFD1b transcripts, as noted in Northern blot of adult tissues (15). While the signal for 18S rRNA was similar in all four tissues, OFD1 signal was most prominent in brain. Using a similar exposure time, transcripts were barely visible in gut, lung, and heart (not shown).

**OFD1a-Specific Signal in Transfected HEK293 Cells**

As assessed by expression of green fluorescence protein (GFP), transfection efficiency of HEK 293 cells with the myc/EGFP/OFD1a construct (Figure 3A) was approximately 70% (not shown). Western blot of lysates from these cells demonstrated that OFD1a antiserum recognized a major band at 140 kD (Figure 3B, lane 1), the predicted size of the fusion protein. It was absent in cells transfected with the vector alone (Figure 3B, lane 2). Fainter bands, approximately 120 kD, were noted in cells transfected with OFD1a and the vector alone (Figure 3B, lanes 1 and 2); these may represent a low level of endogenous OFD1a protein. Marked diminution of signal intensity was observed after preincubation of the antibody with immunizing peptide (Figure 3B, lanes 3 and 4) or when pre-immune serum was substituted for the antiserum (not shown). The 140-kD protein was also detected in OFD1a cDNA-transfected cells after immunoprecipitation with anti-OFD1 antibody and exposure to anti-myc antibody (Figure 3B, lane 5). Bands were not detected in nontransfected cells (Figure 3B, lane 6).

**Endogenous OFD1 Protein Expression**

Using the antiserum validated in transfected cells, we examined endogenous expression of OFD1a during normal human renal and craniofacial development. A doublet of around 120 kD, potentially representative of glycosylated or phosphorylated OFD1a forms, was detected on Western blot in lysates generated from normal human metanephroi at 8, 10, and 12 wk of gestation (Figure 3C, lanes 1 to 3). Note, signal intensity appeared weaker in the older specimen (Figure 3C, lane 3). We then stained normal metanephroi of 7, 10, 13, and 14 wk of gestation and the histologically grossly normal areas adjacent to three postnatal Wilms tumors. In the four normal fetal developing metanephroi, a consistent expression pattern was
observed: immunoreactive OFD1a protein was detected in the outer nephrogenic zone of the cortex in the mesenchyme (Figure 4A). From these wax-embedded sections, it was difficult to discern the exact subcellular localization of immunoreactivity, although positive signal was not detected in renal cell nuclei. Minimal positive signal was detected in later nephron precursors, including vesicles, comma, and S-shaped bodies. Consistent staining was also not observed in developing glomeruli (data not shown). Immunoreactive OFD1a protein was not detected in normal appearing areas of postnatal kidney sections (data not shown). Cranial OFD1 expression was also observed in an 8-wk embryo: positive signals were detected in nasal (Figure 4B) and developing skull (not shown) cartilage, oral mucosa (Figure 4C), skin (not shown), brain (not shown), and
Figure 4. Immunohistochemistry. Immunoperoxidase staining of normal human metanephros at 10 wk gestation (frames A and E) and normal head at 8 wk gestation (frames B through D and F through H). All sections counterstained with hematoxylin. Frames A through D were probed with OFD1a antiserum, and frames E through H are nearby sections probed with OFD1 antiserum preincubated with immunizing peptide (E) or with preimmune serum (F, G, H). A. Note the positive immunoreactive signal (brown color) in metanephric mesenchyme (m). Vesicles (v) and ureteric bud branches (u) also shown. (B) Positive signal in a subset of cells in the nasal cartilage. (C) Positive signal in oral mucosa. (D) Positive signal in myocytes of the tongue. Note that there is no significant signal in kidney mesenchyme in section E probed with preabsorbed antiserum; there is faint, probably unspecific signal in the ureteric bud branches. No significant signal is detected in the sections of the head probed with preimmune serum. Scale bar: 30 μm in A; 20 μm in E; 100 μm in D and H; 50 μm in the remainder.
tongue myocytes (Figure 4D). Adjacent metanephric sections probed with antiserum preincubated with immunizing peptide (Figure 4E) showed no mesenchymal signal. Other tissues probed with preimmune serum (Figure 4, F through H) showed no specific signals.

Expression of OFD1a Protein in Cultured Cells

Four cell lines were established from normal human metanephroi. Cells from all cultures appeared uniformly “mesenchymal” in shape, with irregular, elongated outlines; although uncloned, this phenotype was maintained for more than 20 passages. The mesenchymal phenotype of the cells was further examined by assessing the expression of proteins characteristic of this lineage in vivo (Figure 5A), including the WT1 (21) and PAX2 transcription factors (21), HGF (22) and GDNF (23). All lines expressed GDNF, as assessed by Western blot, with a prominent band at 18 kD (Figure 5A). In three of these lines (N75, N73, and N70: Figure 5A, lanes 2, 3, and 4), Western blotting additionally detected signals for another mesenchymal-derived growth factor, HGF, with a prominent band at 60 kD representing the α-chain. Furthermore, the three lines also expressed WT1 (52 kD) and PAX2 (doublet at 46 and 48 kD). In contrast, in the fourth line examined, HGF, WT1, and PAX2 were barely detectable using similar methodology (N84; Figure 5A). On the basis of these results, we speculate that lines N75, N73, and N70 represent “induced” renal mesenchyme, while N84 appears more like “uninduced” mesenchyme or stroma. Next, we examined OFD1a expression in the mesenchymal lines using Western blot (Figure 5B): OFD1a protein was not detected in line N84, whereas bands were observed at 120 kD in the other three lines (Figure 5B).

Subcellular localization of the protein was then investigated using confocal laser scanning microscopy of lines N84, N75, and N70. No signal was detected in line N84 (data not shown), whereas positive immunoreactive protein was observed in one or two discrete perinuclear dots in virtually all the cells of N75 and N70 lines (Figure 6A and not shown). Using Z-plane scanning, we confirmed that the discrete positive signal was within the cells rather than on the cell surface; nor was this appearance consistent with the cross section of an elongated structure such as the primary cilium. Significant staining was not detected in the cells incubated with preimmune serum or antiserum preincubated with immunizing peptide (Figure 6, B and C). The positive immunofluorescence was typical of centrosome-associated proteins; we therefore performed double labeling with anti-OFD1a (Figure 6D) and anti-γ-tubulin (Figure 6E), a well-characterized centrosomal marker (24); immunoreactive OFD1a and γ-tubulin co-localized exactly (Figure 6F). Although these cell lines are not clonal, this centrosomal localization was observed in over 98% of cells in cultures of the N75 and N70 lines when the whole thickness of the cell was examined by focusing up and down.

Discussion

The Spectrum of OFD1 Mutations

OFD1 mutations described here occur throughout the gene, with no “hotspot,” hence confirming the previously reported pattern (14,20) (Figure 1). Most known mutations are small deletion/insertions, causing premature stop codons, while one other affects splicing, which will also lead to a premature stop codon. Because nonsense-mediated decay is the normal consequence of premature termination codons (25,26), it is perhaps not surprising that there are no obvious phenotype-genotype correlations with regard to the major external dysmorphic features of the syndrome. De Conciliis et al. (15) suggest that the OFD1 gene escape X-inactivation on the basis of studies using human-hamster cell hybrids. If correct, then normal females would be expected to have two active copies of the gene, whereas affected females would only have one copy. This is, however, equivalent to normal males because they would also only have one active copy; furthermore, individuals

Figure 5. Western blot of metanephric mesenchymal cell lines. (A) Cell lysates of metanephric cell lines (N84, N75, N73, N70) were probed for WT1, PAX2, HGF, and GDNF. In panel B, the upper panel is a representative blot probed with OFD1a antiserum; note the presence of a major band (approximately 120 kD) in all lanes except the N84 cells. In the middle panel, the 120-kD signals were abolished when the blot was probed with OFD1a antiserum pre-reacted with immunizing peptide; faint bands below 100 kD appeared as unspecific signals. The lower panel demonstrates that β-actin “housekeeping” protein was detected in all samples.
with Turner’s syndrome (XO) have only one X chromosome but do not develop the features of OFD1 syndrome. Hence, haploinsufficiency cannot be the only mechanism underlying OFD1 unless selective X-inactivation occurs in different organs during development. To prove this latter theorem, one would have to demonstrate that only one allele is expressed at specific time points using techniques such as RT-PCR of polymorphic markers within OFD1. One alternative possibility is that the defective gene product has a dominant negative effect, but there is currently no evidence to support this mechanism with OFD1. Virtually nothing is known about the function of OFD1 protein(s), although the predicted molecule contains LIS1 homology (LisH) motif (27); postulated functions for this motif include microtubule regulation, either by mediating dimerization or direct binding to cytoplasmic dynein heavy chains or microtubules. Notably, three of the 17 mutations previously published or in this report fall within this predicted site, including two missense mutations. A fourth mutation involves a splice site of exon 3, which contains the domain, and the protein product would be predicted to lack the domain. Such a concentration of mutations in a very short stretch of the protein suggests that this domain may genuinely have functional significance.

**OFD1 Mutations are Highly Penetrant for Dysmorphic Features**

Tracking mutations through two pedigrees showed that the mutation is highly penetrant; females with mutations had classic dysmorphic features, but three other females without such features did not have the mutant allele. This observation, also made by Ferrante et al. (14), is important for genetic counseling regarding prenatal diagnosis. It is also pertinent if a renal transplant from a living female relative is proposed for an OFD1 individual with renal failure.

**Does Occurrence of PKD Correlate with Genotype in OFD1?**

Two OFD1 proteins are predicted to be produced from two alternatively spliced mRNAs (14); the shorter one terminates at the beginning of exon 11. Four of the previously published mutations occur beyond this point (Figure 1); in these cases, the sequence of OFD1b, the shorter protein, would be predicted to be unchanged. Two of these patients did not have PKD on US (14), while the other two were not assessed by US (20). Conversely, most patients with mutations before this point have PKD (Figure 1). One interpretation is that the shorter protein is responsible for normal kidney growth, and the longer protein prevents dysmorphic features. Against this, it can be argued that premature stop codons in the later part of the molecule will lead to nonsense-mediated decay of the OFD1b mRNA. In future, exactly which mRNA alleles exist could be established if suitable mutant cells expressing OFD1 were available. A further argument against a simple renal phenotype/genotype correlation is that the severity of PKD (i.e., cyst numbers and severity of renal failure at certain ages) can vary markedly in one pedigree, all of whom will carry the

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**Figure 6.** Subcellular localization of OFD1a. (A) OFD1a immunostaining of N75 cells showed localization in one or two perinuclear dots per cell (arrows). Note that these spots were also detected in cells that initially appeared negative (arrowhead) by scanning through different Z-planes of the cell. (B and C) No specific staining was observed when the cells were respectively incubated with the preimmune serum in the same conditions and with the antiserum preincubated with the immunizing peptide. (D to F) show labeling with anti-OFD1a antiserum in green (C), γ-tubulin in red (D), and the overlay image (E). OFD1a clearly colocalised with the centrosomal marker γ-tubulin, with the same positive spots indicated by arrows in each panel. Scale bar: 5 µm in A, B, and C; 10 µm in D, E, and F.
same mutation, as described previously by Feather et al. (10). In addition, we are aware of one individual with OFD1 dysmorphology who developed cysts on US over several years (SAF, personal observation); hence, the lack of cysts does not preclude their evolution.

**OFD1 Is Expressed in Developing Human Organs**

This study reports for the first time that OFD1 is expressed in human organogenesis. De Conciliis et al. (15) demonstrated by Northern blot that OFD1 was expressed in adult human organs. Transcripts corresponding to OFD1a RNA were found in kidney, pancreas, skeletal muscle, liver, lung, brain, heart, and placenta, while a larger species, postulated to be the OFD1b transcript, was noted in some organs, including kidney and brain. In situ hybridization studies in mice (14) demonstrated expression at embryonic day 12 in genital ridges, with transcripts detected later in gestation in craniofacial structures, including oral and nasal cavity epithelia, the nervous system, and metanephros. In the current study, using the highly sensitive technique of RT-PCR, OFD1a and OFD1b transcripts were found in a wide variety of developing human organs, including heart and lung. PCR is so sensitive that we may be detecting low levels of transcripts. In contrast, however, using less sensitive Northern blot analysis, significant signals were only observed in brain, metanephros, tongue, and limb, which is similar to previous in situ results in mice (14). We generated an antiserum to the carboxyterminal of the putative OFD1a protein and validated it in HEK293 cells transfected with OFD1a cDNA. In normal human embryos, OFD1a immunolocalized to oral mucosa, nasal cavities, cranial cartilage, and brain. Hence, during development, several cell types and lineages express the gene. OFD1a expression was observed in the mesenchyme of the outer metanephric cortex. These cells are highly proliferative (21), correlating with epithelial transformation. In fetal kidneys, OFD1a protein was apparently downregulated as the mesenchyme transformed into nephron epithelia and consistent glomerular staining was not observed. Moreover, OFD1a immunoreactivity was absent in sections of postnatal kidneys, consistent with the hypothesis that it may have a role in renal differentiation.

**OFD1 Is an Intracellular Protein**

In normal human embryonic renal mesenchymal lines, OFD1 protein immunolocalized to perinuclear “dots;” furthermore, γ-tubulin immunoreactivity overlapped with OFD1. These findings are consistent with OFD1 being a centrosome-associated protein. The centrosome is a small nonmembranous organelle, often associated with the nuclear membrane, composed of two centrioles surrounded by electron-dense pericentriolar matrix. Interestingly, the centrosomal matrix is the site of microtubule nucleation and contains a high proportion of coiled coil proteins (24). Further experiments, incorporating immunoelectron microscopy and biochemical studies to assess protein-protein interactions, will be necessary to determine the sub-centrosomal localization of OFD1. Although, the precise function of centrioles is unclear, they are known to be essential for the generation of the primary cilium (24). Expression of several proteins implicated in both human and murine PKD, including polycystin-1 and -2, polaris, and cystin, has recently been described in the primary cilium complex (28,29). Moreover, OFD1 contains a large number of coiled-coil domains and heptad repeats (15,27) and polycystins interact via coiled-coil domains (30). This raises the possibility that these molecules are involved in the same biologic pathway. Functional cell biology experiments are clearly required to assess potential roles of OFD1a during both nephrogenesis and in cystic kidney disease. In addition further studies are required to establish whether renal mesenchymal cells have primary cilia. Furthermore, while all developing organs that we studied expressed both OFD1a and OFD1b isoforms on RT-PCR, it remains possible that the two OFD1 proteins are not expressed synchronously, and the pattern of OFD1b protein remains to be determined.

**References**


