Zonal expression of *dickkopf-3* and components of the Wnt signalling pathways in the human adrenal cortex

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Abstract

The mechanisms underlying the differentation of the adrenal cortex into zones are unclear. Microarray studies on RNA from microdissected zona reticularis (ZR) and zona fasciculata/zona glomerulosa (ZF/ZG) derived from adult human adrenal glands showed that a gene of the *dickkopf* family (*DKK*), *DKK3*, is differentially expressed in the zones. The Dickkopf proteins are morphogens involved in Wnt signalling. Northern blotting showed higher *DKK3* transcript levels in ZF/ZG than ZR samples. *In situ* hybridization on adult human adrenal gland sections showed that *DKK3* expression was much higher in the ZG than in the ZF or ZR. *DKK3* expression was also higher in the medulla. We screened for expression of other members of the *DKK* family and the related *Wingless-type mouse mammary tumor virus integration site gene family* (*WNT*), *frizzled* (*FZD*), and *dishevelled* (*DVL*) gene families. Among *dickkopf* family members, only *DKK3* was expressed at a detectable level in both human and mouse adrenocortical RNA samples. Consistent with previously published data on the effects of *Wnt4* gene disruption in the mouse, we found only *WNT4* expression within the *WNT* family in both human and mouse RNA. Northern blotting showed that *WNT4* was expressed at a higher level in ZF/ZG cells than in ZR. The higher level of *DKK3* and *WNT4* expression in ZF/ZG cells was confirmed by real-time PCR. In the *frizzled* and *dishevelled* families we found *FZD1*, *FZD2* and *DVL3* transcripts in human adrenocortical RNA, and *FZD2* and *DVL3* in mouse adrenocortical RNA. These data show that a variety of genes of the Wnt signalling pathways are expressed in the adrenal cortex. The zonal distribution of *DKK3* expression suggests that it could be involved in zonal differentiation or growth.

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Introduction

The zones of the adrenal cortex have been recognized as morphological entities since the middle of the 19th century. At various times in the past they have been thought of as entirely separate cell types, sustained by proliferation within each zone, but currently it is generally thought that the three zones of the cortex represent forms of differentiation of a single cell type (Wolkersdorfer & Bornstein 1998, Mitani *et al.* 1999, Rainey 1999, Whitworth & Vinson 2000, Alesci & Bornstein 2001). However, questions of the nature of stem cells within the adrenal cortex, if they exist, have not yet been resolved (Hornsby 2001). The concept that cells of one of the three zonal cell types may re-differentiate into another zonal cell type has been derived from observations on the growth of the adrenal cortex and experiments involving adrenocortical regeneration and cell transplantation (Okamoto *et al.* 1998, Wolkersdorfer & Bornstein 1998, Mitani *et al.* 1999, Teebken & Scheumann 2000, Hornsby 2001).

The zones have long been known to have distinct physiological roles. The concept that the zona glomerulosa is the part of the cortex responsible for the secretion of aldosterone has long been accepted; more recently, it has become clear that the zona reticularis is responsible for the biosynthesis of dehydroepiandrosterone (DHEA), whereas the zona fasciculata secretes cortisol, but not DHEA (Endoh *et al.* 1996). There is an increasing understanding of the biochemical and molecular biological differences among the zones (Rainey 1999, Hanley *et al.* 2001, Raza *et al.* 2001, Takemori *et al.* 2001, Bassett *et al.* 2002), but little is known of the mechanisms by which the zones develop in the fetal and postnatal adrenal gland, or of the

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processes that maintain the zones and regulate their width in the adult. However, recent observations on mice with a disruption of the *Wnt4* gene have implicated the Wnt⋆ signalling pathways in adrenocortical zonation (Heikkila *et al.* 2002). *Wnt4* expression is confined to the glomerulosa in newborn wildtype mice; newborn *Wnt4*-deficient mice had a smaller number of glomerulosa cells and lower plasma aldosterone (Heikkila *et al.* 2002).

Previously, to address questions of differential gene expression in the zones of the human adrenal cortex, we developed a microdissection technique for the separation of the zona reticularis (ZR) away from the outer zones of the adult human adrenal cortex (Endoh *et al.* 1996). This dissection produces relatively pure ZR tissue, as shown by the absence of expression of type II 3β-hydroxysteroid dehydrogenase (3β-HSD), which is not expressed in the ZR (Endoh *et al.* 1996). The remaining tissue comprises a mixture of zona fasciculata (ZF) and zona glomerulosa (ZG) cells. Because of the limited extent of the ZG in the adult human adrenal cortex, separation of ZF from ZG was not feasible. These zonal tissue preparations (ZF/ZG and ZR) were used to prepare cell cultures, and RNA was prepared from the cultured cells (Endoh *et al.* 1996). We investigated patterns of gene expression in these separated cell populations (Wang *et al.* 2001). RNA from ZR and ZF/ZG cells was amplified by PCR; the PCR products were then labelled with ^{32}P and hybridized to arrayed cDNA clones (Wang *et al.* 2001). We identified 16 genes that showed an average of > twofold differences in hybridizations of four different pairs of ZF/ZG- and ZR-derived samples. Some of these genes were of known function and others had previously been identified as expressed sequence tags (ESTs).

Although these experiments revealed novel differences in gene expression among the zones, the small numbers of genes that can be arrayed on membrane filters limited the conclusions that could be drawn, and the differentially expressed genes that were identified did not appear likely to be involved in zonal differentiation. Therefore, as glass-slide microarrays with much larger numbers of arrayed human genes became available, we applied this technology to increase the number and variety of genes surveyed. We report here that these microarray studies identified a gene of the *dickkopf* family (*DKK*), *DKK3*, as differentially expressed in the zones of the human adrenal cortex. The Dickkopf proteins are secreted factors that act as morphogens by modulating Wnt protein signalling (Glinka *et al.* 1998, Monaghan *et al.* 1999, Nusse 2001, Zorn 2001). A major function of the Dickkopf proteins is head induction and limb morphogenesis in the mouse embryo (Mukhopadhyay *et al.* 2001). Our finding of zonal differences in *DKK3* expression prompted us to carry out the studies described here, in which we investigated the expression of *DKK3* in more detail and assessed the expression of genes of the Wnt signalling pathway in the human adrenal cortex.

Materials and Methods

Cell separation and culture

Adult human adrenal glands were obtained from kidney organ donors under an approved protocol at Baylor College of Medicine. We are grateful to the staff of Lifegift, Houston, for their assistance in providing adrenals from kidney transplants. Donors were of both sexes and were in the range of 20 to 40 years of age.

Zonal tissue preparations were obtained by microdissection (Endoh *et al.* 1996). The ZR was separated from the rest of the cortex on the basis of colour (the ZR is brown and the ZF is bright yellow). The remainder of the cortex was then separated from the capsule, resulting in a tissue preparation comprised largely of ZF with some ZG. Tissue fragments were dissociated to cell suspensions using enzymatic and mechanical dispersal (3 h incubation with 1 mg/ml type I-A collagenase and 0·1 mg/ml DNAse, both from Sigma Chemical Co., St Louis, MO, USA) (Hornsby & McAllister 1991).

ZR and ZF/ZG cells were placed in culture for several days before RNA was prepared from them. The culture conditions were as previously described (Hornsby & McAllister 1991). After 5 days the serum-containing medium was replaced with defined serum-free medium comprising Dulbecco's Eagle's Medium/Ham's F-12 1:1 (Invitrogen) with 200 μg/ml bovine serum albumin (Bayer Pentex cell culture grade), 40 μM vitamin E, 2 mM ascorbate, 20 nM selenite (Sigma), without insulin or other hormonal additions. After a 48-h incubation in defined medium, RNA was prepared from the cells using RNAzol B (Tel-Test Inc., Friendswood, TX, USA) following the manufacturer's instructions.

cDNA clones

cDNAs used as probes for Northern hybridization, for *in situ* hybridization, and for arraying on nylon membranes were obtained as plasmids produced by the I.M.A.G.E. (Integrated Molecular Analysis of Genomes and their Expression) Consortium project (Lennon *et al.* 1996) and purchased from Research Genetics (Huntsville, AL, USA). The cDNA clones represented the various members of the *DKK*, *WNT*, *FZD*, and *DVL* gene families. The cloned fragments were derived from the 3′ untranslated regions of the mRNAs and lacked any sequence similarity to other members of the respective gene families.

Northern blots

Northern blotting was performed using the NorthernMax kit (Ambion, Austin, TX, USA) following the manufacturer's instructions, with some exceptions. Briefly, RNA samples were separated on formaldehyde gels and blotted

onto BrightStar-Plus positively charged nylon membranes (Ambion). cDNAs were excised from the I.M.A.G.E. plasmids and were labelled with ^{32}P using random oligonucleotide primers (Feinberg & Vogelstein 1983). The hybridization conditions were those specified for the NorthernMax kit, but the final wash used was 0·2 × SSC (saline sodium citrate), 0·5% sodium dodecyl sulphate, at 65 °C. Blots were exposed to phosphor screens and imaged using a Cyclone storage phosphor system (Packard Bioscience, Meriden, CT, USA).

Microarrays

RNA obtained from several pairs of ZF/ZG- and ZR-derived cell populations were reverse transcribed and amplified as described below. The amplified products were labelled with Cy5 or Cy3 and hybridized to glass slide microarrays bearing 6000 arrayed human cDNAs (Baylor College of Medicine Microarray Core Facility, Houston, TX, USA).

The amplification method used was previously employed to prepare labelled DNA for hybridization to immobilized cDNAs on nylon membranes (Wang *et al.* 2001); similar procedures have been developed independently and validated for use with microarrays (Puskas *et al.* 2002). The amplification protocol is an adaptation of the SMART PCR technique developed by Chenchik *et al.* (1998) (SMART=switch mechanism at the 5′ end of RNA templates). The method for reverse transcription and PCR amplification has been described previously (Wang *et al.* 2001). SMART PCR products were labelled with Cy3-dCTP or Cy5-dCTP using random oligonucleotide primers (Feinberg & Vogelstein 1983). Conditions for hybridization and washing were standard for microarrays (Puskas *et al.* 2002). Slides were scanned using a GSI Lumonics ScanArray 5000 (Ottawa, Ontario, Canada). Scanned images were analysed using 'ScanAlyze' and hierarchical clustering analysis (Eisen *et al.* 1998).

Filter arrays

Arrays of selected plasmid cDNA clones were prepared using a published procedure (Jin *et al.* 1997). SMART PCR products were labelled with ^{32}P and hybridized to these filter arrays as described previously (Wang *et al.* 2001).

Immunocytochemistry

Portions of human adrenal glands were fixed in 4% paraformaldehyde and were dehydrated and embedded in paraffin using standard techniques. Sections (4 μm) were deparaffinized and rehydrated using graded alcohol concentrations. Antigen retrieval was performed by incubation in 100 mM sodium citrate pH 6·0, and were subjected to three cycles of heating in a microwave oven for 3 min followed by 10 min of cooling. After nonspecific binding was blocked with 10% horse serum (10 min), sections were incubated with goat anti-human Dkk-3 (C-19, sc-14959, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:10 dilution for 40 min at room temperature. Bound primary antibody was visualized using the Biotinylated Universal Antibody Vectastain ABC kit (Vector Labs, Burlingame, CA, USA) according to the manufacturer's instructions.

In situ *hybridization*

In situ hybridization was carried out on paraffin sections of human adrenal glands using standard procedures (Angerer & Angerer 1992). ^{35}S-Labelled antisense and sense probes against DKK3 and other mRNAs were prepared from I.M.A.G.E. Consortium plasmids using T7 and T3 RNA polymerases. Following hybridization and washing, sections were exposed to single-sided X-ray film (Hyperfilm, Amersham Pharmacia Biotech, Piscataway, NJ, USA). Autoradiographs were scanned at 4800 d.p.i. using a Canoscan D2400UF film scanner (Canon USA, Lake Success, NY, USA).

RT-PCR using gene-specific primers

RNA was reverse transcribed and subjected to PCR amplification as previously described (Suwa *et al.* 2001). Levels of expression of selected mRNAs were assessed by semi-quantitative competitive RT-PCR using β-actin as a control mRNA. First-strand cDNA products were used in a PCR reaction with specific primers (FZD1, 5′-CCG ACTGCCAGAGGGAGGATG-3′; 5′-TGACGCTGGC CAAACCCATAC-3′; FZD2, 5′-GCACTACACGCCG CGCATGTC-3′; 5′-CCCACCCCGGGCGGAGGAA AG-3′; DVL3, 5′-CTCCACCAGCTCCTCCATC AC-3′; 5′-CTGGTACGGGAAAGCCATGGG-3′). The reaction was allowed to proceed for 40 cycles, each cycle comprising 20 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C. The products were separated by agarose gel electrophoresis, stained with ethidium bromide and imaged using a gel documentation system (Alpha Innotech, San Leandro, CA, USA).

RT-PCR using gene-family primers

We used three sets of primers designed to amplify all mRNAs of the *WNT*, *FZD*, and *DVL* families respectively. The sequences of the primers used were those described by Gavin *et al.* (1990) (*WNT* family) and Helmbrecht *et al.* (2001) (*FZD* and *DVL* families). SMART PCR products (90 ng) derived as described above were used as templates in PCR reactions. For the WNT family primers we used 40 cycles, each cycle comprising 20 s at 95 °C, 1·5 min at 50 °C, 1 min at 72 °C; for primers for the FZD and DVL families, we used

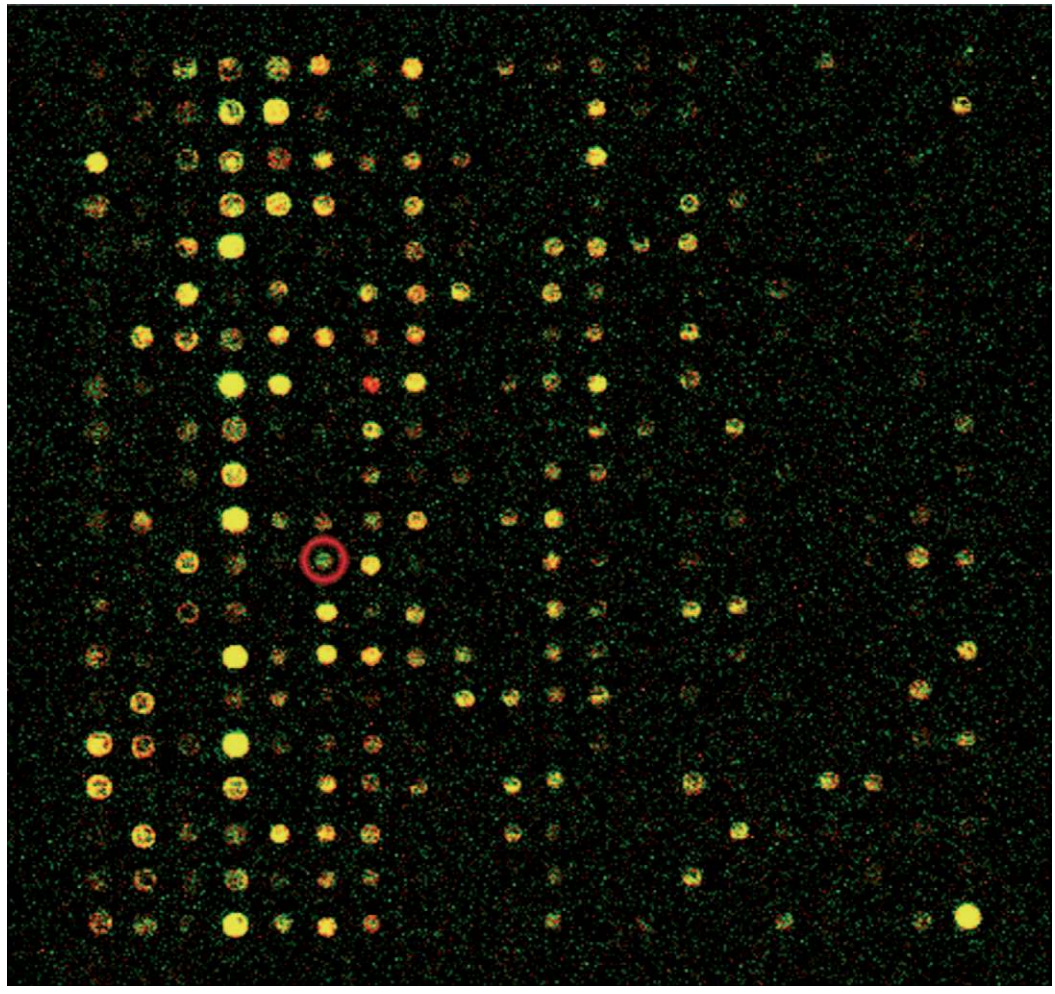

Figure 1 Microarray analysis of differentially expressed genes in the human adrenal cortex. Shown here is the image of a portion of a cDNA microarray following hybridization with Cy5-labelled ZR RNA (red) and Cy3-labelled ZF/ZG RNA (green). The arrayed *DKK3* cDNA spot (circled) appears green. Note that the level of hybridization to this spot on the array is low. The differential hybridization was discovered by systematic examination of all spots with greater than twofold differences in normalized green/red fluorescence.

40 cycles, each cycle comprising 20 s at 95 °C , 30 s at 55 °C, 1 min at 72 °C. The products of the reactions were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA) for sequencing.

Real-time PCR studies

Real-time PCR was performed on RNA samples from separated ZF/ZG and ZR cells. Primers were those predicted to be optimal by the program Primer Express (Applied Biosystems, Foster City, CA, USA). Reverse transcription and PCR were performed using the protocols supplied by Applied Biosystems, using a model ABI 7900 HT real-time PCR machine. Accumulation of PCR products was measured by SYBR green fluorescence. For each RNA sample, 3 to 5 cDNA concentrations were assayed, each cDNA concentration being used in triplicate wells. Analysis of data was performed using SDS software from the manufacturer. The number of cycles required to produce the threshold level of PCR product was determined. For each sample pair and for each gene, the difference in threshold cycles was calculated and was normalized with respect to the threshold cycle values for β-actin.

Results

We used microarray technology to examine differences in gene expression between ZR cells and cells from the outer zones (ZF/ZG). ZR and ZF/ZG cells were placed in culture before being used as a source of RNA. The

intention of this short period in culture was to allow them to recover from the processes of harvesting of the tissues and preparation of the cell suspension, which could affect gene expression. Culturing the cells also ensured that dead and damaged cells were not included in the population used for preparation of RNA, and allowed assessment of the contamination of the cell population by non-adrenocortical cells. Cell morphology and rounding in response to adrenocorticotrophin or cyclic AMP (McAllister & Hornsby 1987) showed that the cell populations comprised <5% of other cell types (principally endothelial cells and fibroblasts). No medullary cells survive the dissociation process used.

For microarray analysis we used slides bearing 6000 arrayed human cDNAs. RNA samples from several pairs of ZR- and ZF/ZG-derived preparations were labelled with Cy5 or Cy3 for hybridization to the arrays. This analysis revealed several novel differences in gene expression between the zones (T Suwa and P J Hornsby, unpublished observations). The present work was initiated when we observed that *DKK3* was expressed at a higher level in ZF/ZG samples (Fig. 1). This finding suggested that it would be fruitful to investigate the expression of this and other genes involved in Wnt signalling.

In order to validate the differential zonal expression of *DKK3*, we analysed ZR and ZF/ZG RNA samples by conventional Northern blotting (Fig. 2). Hybridization of blots with a specific *DKK3* probe showed a transcript of the expected size (~2·6 kb) (Krupnik *et al.* 1999). This transcript was present at much lower levels in the ZR samples than in the ZF/ZG samples. Rehybridization of blots with probes for steroidogenic enzyme genes showed that the zonal difference in expression of *DKK3* was specific. Type II 3β-hydroxysteroid dehydrogenase (*HSD3B2*) was much higher in ZF/ZG samples than in ZR, as expected (Endoh *et al.* 1996). Another steroidogenic enzyme gene, cholesterol side-chain cleavage enzyme (*CYP11A1*) was expressed in both zones, although it was noted to be variably somewhat higher in ZR samples. Rehybridization of the blots with an oligonucleotide probe for 28S ribosomal RNA verified the presence of equal amounts of RNA on the blots.

The microarray analysis and the Northern blot studies were performed using RNA prepared from microdissected adrenal cortex tissue. These results allowed the conclusion that *DKK3* mRNA levels are lower in ZR cells, but did not precisely determine the primary location of *DKK3* expression in the outer adrenal cortex. Because the ZG is very thin in most glands, it was not possible to reliably separate this layer from the ZF by microdissection. In order to address these issues, we performed *in situ* hybridization on adult human adrenal gland sections (Fig. 3). *In situ* hybridization performed on several glands showed that *DKK3* expression was in fact higher in the ZG than in the ZF or ZR. Comparisons of the autoradiographic images and conventional histological images showed an excellent correlation between the zone of *DKK3* expression and the morphological ZG. The expression in the ZF and the ZR was not zero, as shown by comparison with the level of radioactivity on sections hybridized with a sense probe. However, there was no detectable difference in *DKK3* expression level between the ZF and the ZR. The autoradiographic data also revealed that *DKK3* expression was higher in the medulla. Because medullary tissue is not present in most regions of the adult adrenal gland, as shown by the first section used in Fig. 3, we also used sections from portions of a gland adjacent to the central vein, where the medulla is more extensive (Fig. 3). These sections confirmed that the medulla also has a higher expression of *DKK3*. We then compared the pattern of *DKK3* mRNA, as determined by *in situ* hybridization, with the pattern of Dkk-3 protein, as determined by immunocytochemistry (Fig. 3). Dkk-3 protein was clearly detectable in the medulla, and here the extent of expression determined by immunocytochemistry

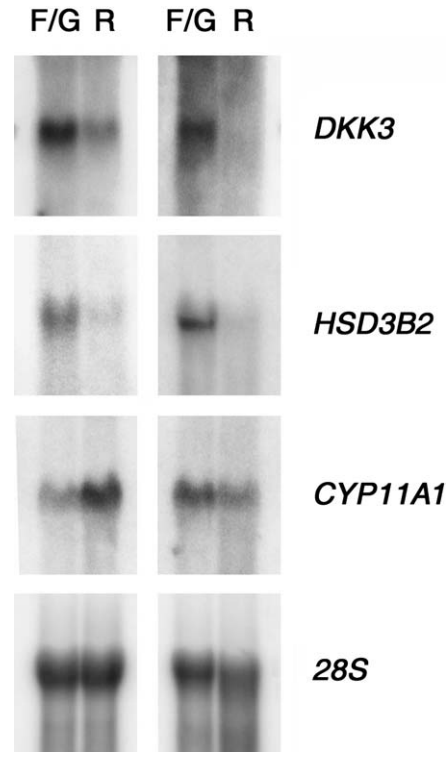

Figure 2 Expression of *DDK3* in microdissected zones of the human adrenal cortex. RNA from two different pairs of ZR (R)- and ZF/ZG (F/G)-derived samples (10 μg per lane) was hybridized with a *DKK3* cDNA probe. The blots were rehybridized with cDNA probes for type II 3β-hydroxysteroid dehydrogenase (*HSD3B2*), cholesterol side-chain cleavage enzyme (*CYP11A1*), and with an oligonucleotide against 28S ribosomal RNA. For the sample pair on the right, the hybridization with *HSD3B2* is from a separate blot, as the rehybridization of the *DKK3*-probed blot failed for technical reasons.

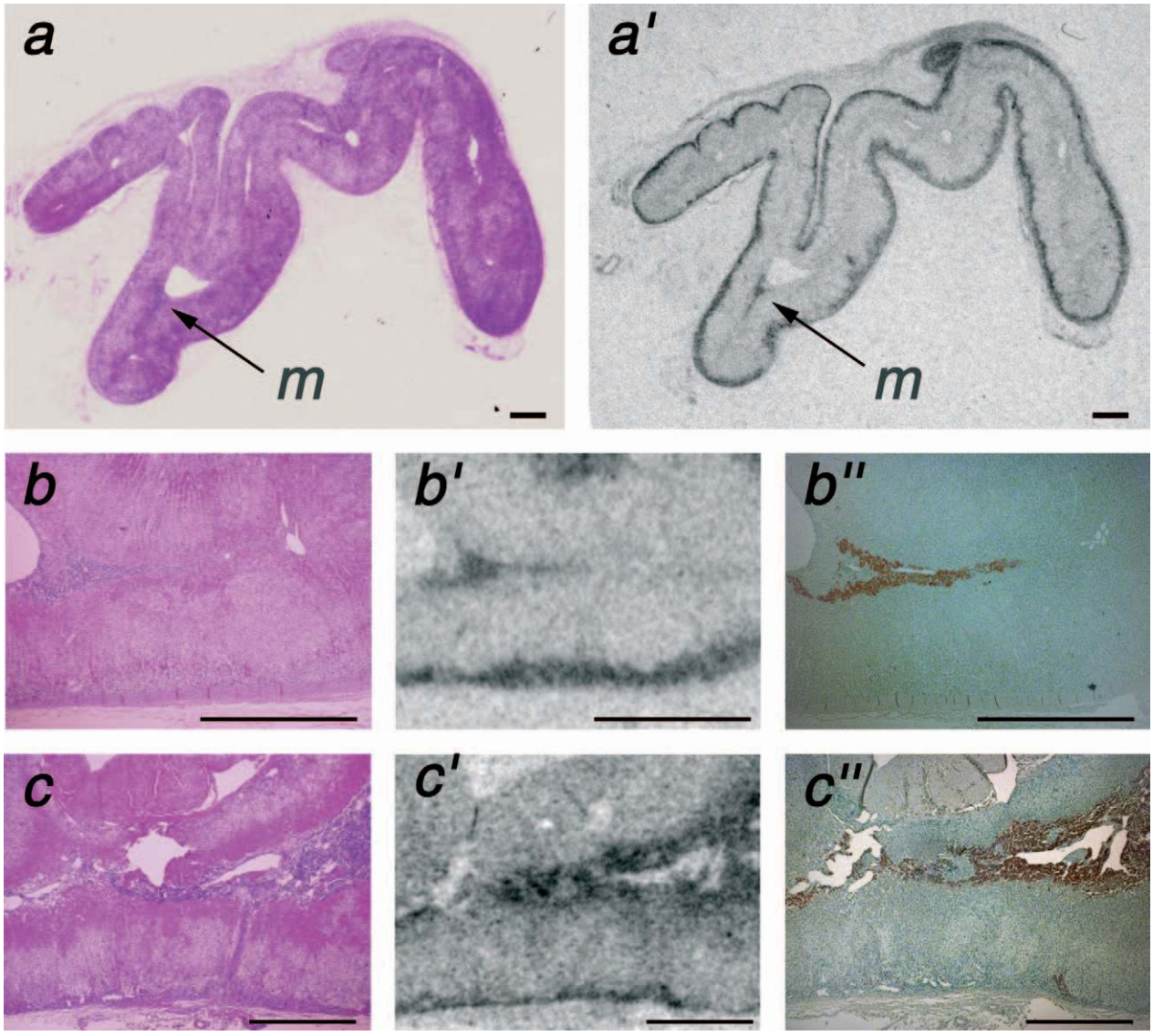

Figure 3 Patterns of expression of *DKK3* in the adult human adrenal gland. The sections in series a and b are from a female aged 40 years, and the sections in series c are from a male aged 36 years. (a, b, c) Sections stained with haematoxylin and eosin; (a′, b′ and c′) autoradiographic images resulting from *in situ* hybridization with an antisense probe for *DKK3*; (b″and c″) immunocytochemistry using an antibody against Dkk-3. The sections in each series are adjacent but not sequential, and there are, therefore, slight differences in the morphology. The sections in series a show only a very small amount of medullary tissue (marked m); the sections in series b and c were chosen to show the medulla. The bar in each photomicrograph is 0·1 mm.

was the same as the extent determined by *in situ* hybridization. However, Dkk-3 protein was not detected in the ZG. Reasons for this apparent discrepancy are addressed in the Discussion. The specificity of the antibody staining in the medulla was confirmed by preincubation of the polyclonal antibody with the peptide against which it was raised. In sections from 6 different human adrenal glands, preincubation with the peptide abolished all staining in the medulla.

The observed differential expression of *DKK3* in the zones of the human adrenal cortex indicated that it would be worthwhile to investigate the expression of other members of the Wnt signalling pathway. Using arrayed cDNA clones on nylon membranes, we assayed human adrenocortical RNA samples for levels of expression of members of the *DKK*, *WNT*, *FZD*, and *DVD* gene families, using methods we described previously (Wang *et al.* 2001). This screening showed that few of these genes

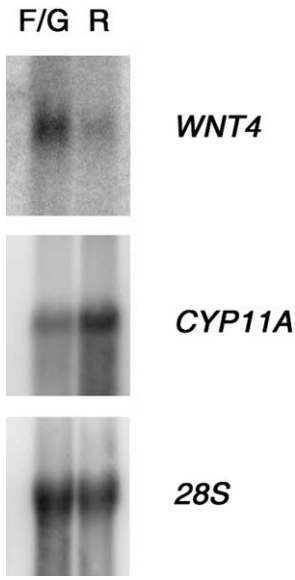

Figure 4 Expression of *WNT4* in microdissected zones of the human adrenal cortex. ZR (R)- and ZF/ZG (F/G)-derived RNA (10 μg per lane) was hybridized with a *WNT4* cDNA probe. The blot was rehybridized with a *CYP11A1* cDNA probe and with an oligonucleotide against 28S ribosomal RNA.

were expressed at levels above background. Within the *DKK* gene family (including the related gene *Soggy* (Krupnik *et al.* 1999)) only *DKK3* was expressed at detectable levels, whereas *DKK1*, *DKK2* and *DKK4* were not above background. In the *WNT* family, *WNT4* was expressed at above background levels; therefore we investigated *WNT4* expression by Northern blotting, as described below. However, none of the members of the *FZD* and *DVL* gene families were above background, necessitating the use of other detection methods, as also described below. Additionally, we assayed mouse adrenal cortex RNA for expression of these genes, using arrayed mouse cDNA clones of these families. In agreement with the data on human adrenal cortex RNA samples, in the *DKK* family only *DKK3* was expressed at above background levels in mouse adrenal cortex RNA. This was also confirmed by Northern blotting (not shown).

Hybridization of human adrenocortical RNA samples with a *WNT4* cDNA probe revealed a band of ~1·1 kb, the expected size of *WNT4* mRNA (Fig. 4). The transcript was present at a higher level in ZF/ZG RNA samples than in ZR samples. Rehybridization with *CYP11A1* and 28S probes showed that this result was not caused by uneven loading of RNA on the blot. We followed up on this result by performing *in situ* hybridization on human adrenal cortex sections using a *WNT4* probe, but the level of expression was too low to allow unambiguous determination that the higher level of expression of WNT4 in ZF/ZG samples was the result of higher expression in the ZG.

Because *WNT*, *FZD* and *DVL* are multi-gene families, we performed studies to determine which members of those families are expressed in the adult human adrenal cortex. To do this we performed RT-PCR on human adrenal cortex RNA using primers previously demonstrated to amplify all members of the respective gene family members (see Materials and Methods). When *WNT* family primers were used, *WNT4* was the only transcript amplified (3/3 clones sequenced). When *FZD* primers were used, both *FZD1* and *FZD2* transcripts were amplified (2/5 and 3/5 respectively), and when *DVL* primers were used only *DVL3* transcripts were amplified (4/4). Similar results were obtained when mouse adrenal cortex RNA was used as template with the same sets of primers. Using *WNT* primers, 2/2 clones sequenced were *WNT4*; using *FZD* primers, only *FZD2* was amplified (4/4); with *DVL* primers, only *DVL3* (4/4).

In order to confirm the expression of *FZD1*, *FZD2* and *DVL3* in the adrenal cortex, we performed RT-PCR using primers specific for these mRNAs, using both ZF/ZG and ZR RNA samples (Fig. 5). Products of the expected sizes were formed. These experiments demonstrated that all three genes are expressed in the human adrenal cortex. Based on these semi-quantitative RT-PCR results, we performed real-time PCR studies on five different pairs of ZF/ZG and ZR RNA samples, using primers for *DKK3*, *WNT4*, *FZD1*, *FZD2*, and *DVL3* (Fig. 5). These data confirm the results obtained by Northern blotting and RT-PCR. *DKK3* and *WNT4* were consistently expressed at higher levels in ZF/ZG samples. There was a trend for *FZD2* and *DVL3* also to be higher in ZF/ZG samples, but this was not as marked and did not reach statistical significance.

Discussion

The present studies reinforce the conclusions from recent studies on disruption of the *Wnt4* gene in the mouse (Heikkila *et al.* 2002) on the significance of Wnt signalling pathways in the adrenal cortex. Patterns of expression of the *DKK3* gene suggest that it could be a morphogen in the adrenal cortex. Previous studies of differences in gene expression among the zones have yielded information on receptors, signal transduction mechanisms, and steroid biosynthetic enzymes, but have so far not yielded clues as to the underlying mechanisms of zonal differentiation (Wolkersdorfer & Bornstein 1998, Mitani *et al.* 1999, Rainey 1999, Whitworth & Vinson 2000, Alesci & Bornstein 2001). In the present experiments we found that the *DKK3* gene was expressed in the ZG at a higher level than in the ZF and ZR. The Dickkopf proteins are secreted modulators of Wnt signalling. Consistent with data from the mouse (Heikkila *et al.* 2002), the *WNT4* gene was also expressed at a higher level in ZF/ZG tissue than in ZR tissue. The Wnt receptor genes of the *frizzled*

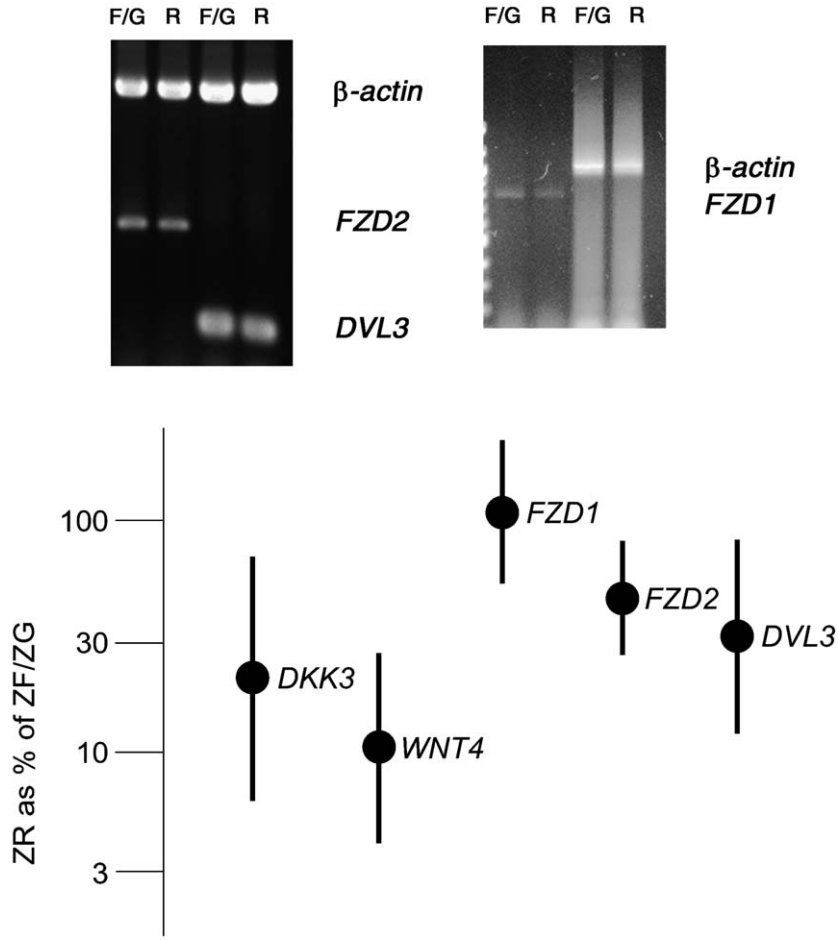

Figure 5 (Top) Expression of *FZD1*, *FZD2* and *DVL3* in the human adrenal cortex. ZF/ZG (F/G) and ZR (R) RNA samples were reverse transcribed and used as templates for PCR with gene-specific primers. The PCR products were separated by gel electrophoresis. Primers for β-actin were used as internal controls in the reactions with *FZD2* and *DVL3* primers. Because less product was produced in the reaction with *FZD1* primers, β-actin primers were used in separate reactions so that the *FZD1*-specific product could be clearly observed. (Bottom) Data from real-time PCR measurements, performed as described in Materials and Methods. Five different ZF/ZG and ZR samples were used with primers for the indicated cDNAs. The levels for the ZR samples have been plotted as percentages of the ZF/ZG levels (means ± standard error). The values for *DKK3* and *WNT4* are significantly different from 100% ($P < 0{\cdot}05$) whereas the others are not significantly different.

family (*FZD1* and *FZD2*) were expressed in the human adrenal cortex, as was also *DVL3*, one of the *dishevelled* genes which encode proteins that are acted on intracellularly by Frizzled proteins. The observed zonal patterns of expression of Wnt-related genes in the adrenal cortex suggest that the Wnt signalling pathway may be involved in zonal differentiation.

The Wnt family of morphogens was originally characterized as being involved in the early fate of cells within the *Drosophila* embryo, and subsequently was found to be involved in mammalian embryogenesis (Huelsken & Birchmeier 2001). More recently these proteins have been shown to be involved in adult stem cell maintenance and tissue regeneration (Austin *et al.* 1997, Huelsken & Birchmeier 2001). Moreover, they are now known to play a major role in many cancers, possibly by maintaining cells in a state closer to the stem cell state (Barker & Clevers 2000, Taipale & Beachy 2001).

The Dickkopf proteins (Dkk-1, -2, -3, and -4) have similar structural domains (Krupnik *et al.* 1999, Brott & Sokol 2002) but it has not yet been established that they all act via common mechanisms (Krupnik *et al.* 1999, Mao *et al.* 2001, Brott & Sokol 2002, Li *et al.* 2002, Rothbacher & Lemaire 2002). A study of the domains of the Dkk

protein family showed that the C-terminal domains of Dkk-1 and -2 form complexes with LDL receptor-related protein 6 (LRP6), whereas the similar C-terminal domain of Dkk-3 does not (Brott and Sokol 2002). Similarly the C-terminal domains of Dkk-1 and -2 activate the *Siamois* promoter when co-expressed with LRP6, but the C-terminal domain of Dkk-3 does not (Brott & Sokol 2002). The possible modes of action of Dkk-3 in mammalian cells therefore remain to be determined. Dkk-3 inhibits cell proliferation in several immortalized cell types, and the *DKK3* gene was independently described as *reduced expression in immortalized cells* (*REIC*), a possible tumour suppressor gene (Tsuji *et al.* 2000, 2001). Inhibition of Wnt signalling can also exert a stimulatory effect on cell proliferation (Taipale & Beachy 2001), but no evidence as to the mechanism of the growth effects of Dkk-3 has yet been presented.

A higher level of *DKK3* mRNA was detected in the ZG and in the medulla in these studies, but immunocytochemistry using an antibody against Dkk-3 showed the presence of Dkk-3 protein only in the medulla. An explanation for this result is that it is possible that a tissue such as the adrenal cortex, which is not specialized for protein storage and secretion, may immediately release secretory proteins, whereas part of the normal differentiated function of the medulla is the storage of proteins in secretory granules. However, further studies on the function of Dkk-3 in both the adrenal cortex and the adrenal medulla will be needed to clarify these possibilities.

To our knowledge, this is the first report of compartmentalization of expression of a *DKK* gene within an endocrine organ. We have preliminary data suggesting that the *dickkopf* genes may have roles in other endocrine organs. In the mouse ovary, *in situ* hybridization with a *DKK3* probe showed hybridization to follicles but not to the stroma (M Chen and P J Hornsby, unpublished observations). The latter observation adds to published data showing compartmentalization of *WNT* and *FZD* expression in the rat ovary (Ricken *et al.* 2002). The mechanism by which Dkk-3 may act on adrenocortical cell function, and possibly adrenocortical cell proliferation, certainly cannot be currently determined. Further experiments will be required to elucidate its mechanism of action, and to elucidate the potential role of *DKK3* and related genes in adrenocortical zonal differentiation.

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