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Effects of PAX2 expression in a human fetal kidney (HEK293) cell line

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Abstract

PAX2, a member of the ''paired-box'' family of homeotic genes, is a nuclear transcription factor expressed in the early stages of nephrogenesis by induced blastemal cells as they progress from mesenchymal condensates to the ''S-shaped'' stage and also by the ureteric bud. Spontaneous mutations in one copy of PAX2 in humans causes a syndrome of proteinuric renal failure and coloboma of the eye (P. Sanyanusin et al., Nat. Genet. 9 (1995) 358–363); transgenic mice with disruption of the PAX2 gene are anephric (M. Torres et al., Development 121 (1995) 4057–4067. Although PAX2 is clearly critical for normal kidney development, its direct effects on kidney cell phenotype are unknown. To address this issue, we developed stable transfectants of the HEK293 human fetal kidney epithelial cell line expressing human PAX2 protein under tetracycline-regulatable promoter. In these cells, PAX2 had no effect on the proliferative rate, but increased the expression of the Wilms' tumor gene (2-fold) and E-cadherin (7-fold). PAX2 had a strong inhibitory effect on vimentin; vimentin/GAPDH mRNA ratio was suppressed to 8% of control whereas cytokeratin-18/GAPDH mRNA ratio was unchanged. During nephrogenesis, loss of vimentin and onset of low-level WT1 and E-cadherin expression occur in mesenchymal condensates. Our observations suggest that these events may be, in part, regulated by PAX2. © 1998 Elsevier Science B.V.

Keywords: PAX2; Kidney development; Cadherin; Cytokeratin; Wilms' tumor; Human

1. Introduction

The metanephric kidney develops as a result of reciprocal interactions between branches of the invading ureteric bud and adjacent cells of the undifferentiated metanephric blastema. In response to signals from the ureteric bud, clusters of blastemal cells are induced to aggregate and undergo conversion from their mesenchymal phenotype into an organized layer of polarized epithelial cells with a central fluid-filled lumen. In turn, signals from the induced mesenchyme cause the ureteric bud to branch repeatedly, forming a system of collecting ducts which fuse with each emerging renal vesicle. Each early renal vesicle then elongates to form the glomerulus, proximal tubule, loop of Henle and distal convoluted tubule of the final nephron.

The molecular mechanisms which orchestrate conversion of mesenchyme to epithelium are not well

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understood. However, it is likely that the inductive signals from ureteric bud elicit expression of a new panel of transcription factor genes in blastemal cells. Among these is PAX2, a member of the ''paired-box'' (PAX) family of homeotic genes [3]. Interest in the PAX genes as key developmental regulators has been intensified by the fact that PAX gene mutations consistently produce developmental malformations [4]. Mutations of PAX3 and PAX6 have been associated with several different human malformations including Waardenburg syndrome (PAX3) [5,6] and aniridia (PAX6) [7]; PAX2 mutations produce a syndrome of renal dysplasia with proteinuric renal failure and colobomas of the eye (PAX2) [1,8].

In the developing kidney, PAX2 is detected in the condensing mesenchyme and early epithelial structures derived from these cells (the ''comma'' and ''S-shaped'' bodies) [9,10]. As the tubule elongates and begins to differentiate into more mature nephron segments, PAX2 is rapidly downregulated [10,11]. PAX2 is also detected in the branching ureteric bud, and Dressler has postulated that it is a key transcription factor orchestrating the pattern of gene expression during reciprocal interactions between ureteric bud and blastema [12]. In fetal kidney organ culture, antisense PAX2 oligonucleotides disrupt normal nephrogenesis from mesenchymal cell aggregation onward [13]. Deregulated overexpression of PAX2 in transgenic mice results in cystic renal malformation with congenital nephrotic syndrome and death in the first days of life [12], and transgenic mice homozygous for disrupted PAX2 genes lack kidneys, ureters and genital tracts [2].

Although PAX2 clearly plays an important role in kidney development, its precise influence on cell phenotype is a matter of speculation. To address this issue, we stably transfected a human fetal kidney cell line (HEK293) with a tetracycline-regulated PAX2 expression vector and examined the effect on cell phenotype. Although this adenovirus-immortalized cell line did not provide a system in which we were able to assess putative effects of PAX2 on cell proliferation, we could demonstrate that PAX2 modified vimentin, E-cadherin and Wilms' tumor gene expression in a pattern reiterating some of the events which occur during the mesenchyme-epithelium transition of early nephrogenesis in vivo.

2. Materials and methods

2.1. Stable tetracycline-regulatable HEK293 PAX2 / tTA transfectants

In order to generate HEK293 cell lines expressing variable amounts of PAX2 protein under tetracycline regulation, we subcloned the full-length human PAX2a cDNA (the gift of Dr. M. Eccles, Otago, NZ) into the multicloning site of the p10-3 regulatable expression plasmid, the gift of Gossens and Bujard [14]. Initially, the hPAX2a cDNA was digested with Nco1, filled in with Klenow enzyme, dephosphorylated with CIAP and attached to *Bgl*-II adaptors (Boehringer Mannheim). The construct was further digested with *Xba*1, and the resultant 1.3 kb *Bgl*-II/*Xba*1 fragment containing the whole human PAX2a coding sequence was inserted into a pCMV5 expression vector (gift of J. Pelletier). Integrity of the nucleotide sequence and expression of hPAX2a protein were confirmed by direct sequencing and Western blotting with anti-mPAX2 antibody before and after subcloning the hPAX2a cDNA into the *Eco*R1/*Xba*1 sites of p10-3.

For use with the tTA-responsive hPAX2a expression vector above, the human embryonic kidney cell line, HEK293, was obtained from ATCC (#CRL-1573) and grown in plastic culture dishes in Dulbecco Modified Eagle's Medium (DMEM) (Gibco-BRL) supplemented with 10% FBS (In Vitrogen, NY) and 1% Pen/Strep Solution (Gibco-BRL). Cells were plated at 500 000 per 100 mm dish, grown to about 50% confluency and co-transfected by the calcium-phosphate precipitation method with: (A) various amounts of the tetracycline-repressor/VP16 plasmid (tTA) of Gossens and Bujard; (B) 1 μg of pSV2Neo (ATCC #37149). At 16–20 h, the cells were washed and fresh DMEM medium added. At 48 h, cells were trypsinized, split into 150 mm Petri dishes and grown in the presence of 500 mg/ml G-418 (Gibco-BRL). After 2–4 weeks, individual clones were selected, expanded and analyzed for tTA expression by transient transfection in the presence or absence of tetracycline (1 μg/ml) with 0.1 μg/dish of a test-plasmid, p10-3 (gift of Gossens and Bujard), containing a β-galactosidase reporter cDNA under control of the tTA-responsive minimal CMV pro-

moter. A clone, HEK293/tTA(#3), exhibiting tightly regulated β-galactosidase activity in these transient transfection assays was selected for the studies below.

HEK293/tTA-3 cells were used for a second round of stable transfection with 10 μg/dish of p10-3/hPAX2a and 1 μg/dish of pCMVHygro (gift of Dr. J. Pelletier); the transfected cells were detached and replated in medium containing 180 mg/ml hygromycin (Sigma). After 2–4 weeks, individual clones were selected for tetracycline-regulatable hPAX2a protein expression.

2.2. Stable HEK293 murine PAX2 transfectants

HEK293 cells were also transfected with: (A) 10 μg of plasmid expression vectors containing murine mPAX2a or mPAX2b isoforms under the control of pCMV (gift of Dr. G Dressler); (B) 1 μg of pSV2Neo. Individual clones were selected in the presence of 500 mg/ml G-418.

2.3. Western immunoblotting

Protein extracts were prepared from mPAX2a, mPAX2b and hPAX2a/tTA stable transfectants by boiling cell pellets in 2× SDS loading buffer (100 mM TRIS base/2% SDS/ 20% glycerol/ 2% mercaptoethanol/0.01 mg/ml bromophenol blue). Samples were resolved on SDS/10% polyacrylamide gels, electroblotted onto ECL membranes (Amersham) and probed with polyclonal rabbit anti-mPAX2 antibody (1:3000) (gift of Dr. G. Dressler) followed by peroxidase-linked anti-rabbit IgG and ECL detection (Amersham).

2.4. Northern analysis

Total RNA from PAX2-expressing cells was isolated using the NP-40/urea method [15]. Total RNA (20 μg) from each sample was resolved on 1% agarose/formaldehyde gels and transferred to nylon Hybond N membranes (Amersham) and cross-linked with UV light. cDNA probes were radiolabeled using the DECAprime II DNA labelling kit (Ambion). Northern blots were prehybridized and then probed at 42°C overnight in 50% formamide, 2.5× Denhardt's solution, 25 mM KPO4 (pH 7.4), 0.1% SDS, 10% (w/v) dextran sulfate, 5× SSPE, and 200 μg/ml herring sperm DNA (Sigma). The blots were then washed twice in 2× SSPE, 0.2% SDS at RT for 20 min, and twice in 0.1× SSPE, 0.2–0.4% SDS at 60–65°C for 20 min and exposed for 1–4 days to BioMax MR X-ray film (Kodak) at −80°C. Band intensity was estimated by densitometry using NIH Image 1.49 software and normalized for RNA loading by reprobing each blot with GAPDH cDNA.

The following cDNA probes were used for Northern analysis of RNA from the transfected HEK293 cells: 0.45 kb *Pst*1 fragment of human vimentin [16]; 0.8 kb *Eco*R1 fragment of human cytokeratin 18 [17]; 2.4 kb *Eco*R1 fragment of murine E-cadherin [18]; 1.8 kb *Eco*R1 fragment of human WT1 [19]; 0.75 kb *Pst*1–*Xba*1 fragment of human GAPDH [20], human PAX8 [21], SLC3A1 [22].

2.5. Semi-quantitative reverse transcriptase-PCR analyses

The reverse transcription reaction was performed using 2.5 μg total RNA in the presence of 1× First-strand buffer (Gibco-BRL), 10 mM DTT (Gibco-BRL), 500 μM dNTP mix (Pharmacia), 1.6 μg Oligo(dT)12-18 (Pharmacia), and 2 units of Superscript reverse transcriptase (Gibco-BRL) in a total volume of 20 μl. The RT mix was incubated for 10 min at 25°C followed by 50 min at 42°C. Reverse transcriptase was then inactivated by heating the reactants to 70°C for 15 min.

PCR amplification was performed in 50 μl of 1× PCR buffer (Gibco-BRL) containing 200 μM dNTPs, 2.5 units of Taq polymerase (Gibco-BRL), 2 μl of each RT product and 200 pmol of each primer. The following conditions were established for DNA amplification in the linear range:

For human E-cadherin: Primers: 5′-CCTTCCTCCCAATACATCTCCC and 3′-TCTCCGCCTCCTTCTTCATC 22 PCR cycles: (94°C × 30 s, 58°C × 30 s, 72°C × 45 s).

For human actin: Primers: 5′-GATTCCTATGTGGGCGA and 3′-CTGGAAGAGTGCCTCAGGGC 20 PCR cycles: (94°C × 60 s, 60°C × 60 s, 72°C × 90 s).

PCR products were resolved on 1.5% agarose gels and blotted onto Hybond N nylon membranes. Southern hybridization was carried out according to the protocol described by Maniatis [23]. Southern blots

were probed with the cDNAs for human E-cadherin [24] or human β-actin and exposed to BioMax X-ray film for 8–12 h prior to densitometry using NIH Image 1.49 software as above.

For detection of human WNT4: Primers: 5′-GGTAAAGACGTGCTGGCGAG and 3′-CTGCAGCGTTCAGCCAGGTC 30 PCR cycles: (94° × 60 s, 60° × 60 s, 72° × 60 s).

Alkaline phosphatase assay: Alkaline phosphatase activity was measured colorimetrically as described by Borgers et al. [25].

3. Results

3.1. Characterization of HEK293 cells

After screening a panel of renal cell lines for properties relevant to early development, the embryonic kidney cell line, HEK293, was selected because of several advantageous characteristics (Table 1). Like the induced blastema, HEK293 cells express Wilms' tumor gene (WT1), Wnt4 and low levels of PAX2 mRNA (but no detectable PAX2 protein by Western immunoblotting). Like cells of the early ''S-shaped'' body during nephrogenesis, they express mRNA for the epithelial cell marker, cytokeratin, but lack markers of highly differentiated tubular segments such as alkaline phosphatase activity or cystinuria gene (SLC3A1) mRNA (proximal tubule markers) and PKD1 or vasopressin receptor (collecting duct markers). The cells are of human origin and are readily transfectable. One disadvantage, however, is that these cells were originally immortalized with an adenovirus construct, bypassing normal regulators of the cell cycle.

3.2. Generation of an HEK293 cell line expressing tetracycline-inhibitable hPAX2a

We used the tetracycline-regulated expression system of Gossens and Bujard [14] to establish an HEK293 cell line expressing human PAX2a in the absence of antibiotic. This system allows one to regulate the level of hPAX2a expression by varying the amount of tetracycline in the medium. The system consists of two plasmids: p15-3/tTA (constitutive expression of a tRepressor/tTA fusion protein is driven by the cytomegalovirus promoter); and p10-3 (which carries a minimal CMV promoter and bacterial tetracycline operon sequences). The human PAX2a cDNA was cloned into the multicloning site of the p10-3 plasmid.

HEK293 cells were first co-transfected with p15-3/tTA and SVNeo plasmids; thirty neomycin-resistant colonies were selected and screened for optimal tTA activity. In transient transfection assays with a test reporter plasmid p10-3βGal, clone#3 exhibited fully repressable βGal activity and was chosen for second round of transfection with p10-3/hPAX2 and pCMV/Hygro. Eighteen hygromycin-resistant colonies were selected and screened for tetracycline-repressable hPAX2a expression by Western immunoblotting. As seen in Fig. 1(A), clone HEK/tTA/hPAX2a #3-3 expresses hPAX2a protein, but in the presence of increasing concentrations of tetracycline, hPAX2a expression is fully repressed. Northern blots of HEK/tTA/PAX2a #3–3 cell total RNA probed with the hPAX2a cDNA showed the same correlation (data not shown). When cells are transferred to medium without tetracycline, the cells rapidly re-express hPAX2a protein within 3–6 h (Fig. 1(B)).

Table 1

Markers	293 cells	Condensed mesenchyme	''S-shaped'' body	Proximal tubules	Distal tubules	Ref.
WT1	+	+	+	−	−	[26]
PAX2	+	+	+	−	−	[10]
Wnt4	+	+	+	−	−	[27]
Cytokeratin	+	−	+	+	+	[28]
PAX8	−	−	+	+	−	[21]
Alkaline Phosphatase	−	−	−	+	−	[25]
SLC3A1	−	−	−	+	−	[22]
PKD1	−	−	−	+	+	[29]
V2R	−	−	−	−	+	[30]

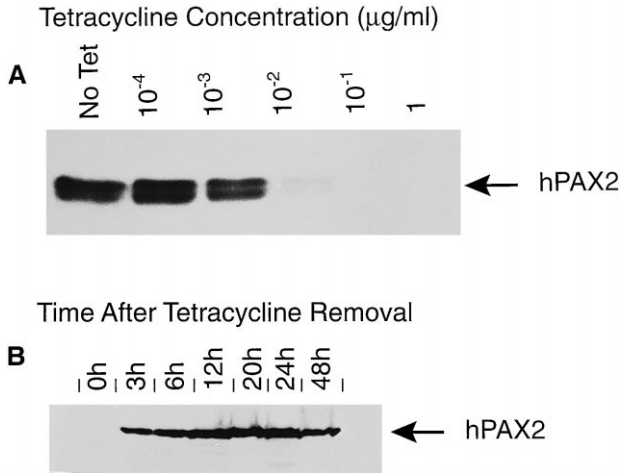

Fig. 1. (A) Western analysis of the PAX2 protein expression in tetracycline-regulatable 293 tTA/hPAX2 #3-3 cell line. Total protein was extracted from cells grown in 0 μg/ml; 0.0001 μg/ml; 0.001 μg/ml; 0.01 μg/ml; 0.1 μg/ml and 1 μg/ml of tetracycline for three days. Each lane contains 20 μg of total protein. Arrow points to the PAX2 43–45 kDa protein. (B) Western analysis of the PAX2 protein expression in 293 tTA/hPAX2 #3–3 cell line after tetracycline removal. 293 cells were grown in 1 μg/ml of the tetracycline-containing media for three days. Tetracycline was removed from the media and cells were collected for Western analysis at various times thereafter. Each lane contains 20 μg of total protein. Arrow points to the appearance of PAX2 protein expression in cells grown in tetracycline-free media.

PAX2 is normally expressed as two alternatively spliced isoforms, PAX2a and PAX2b, which differ only in the presence of a 23 amino acid sequence (outside the critical paired domain) [10]. To ascertain whether effects of hPAX2a were isoform-specific, we also examined the phenotype of HEK293 cells stably transfected with expression vectors encoding the two murine isoforms (mPAX2a and mPAX2b). In the experiments below, all effects of hPAX2a expression on HEK/tTA/PAX2a #3–3 cell phenotype were reproduced in cells stably overexpressing either mPAX2a or mPAX2b. For simplicity, the results below focus on data with hPAX2a in the tetracycline-regulatable system.

3.3. Effect of PAX2 on HEK293 cell proliferation

To determine whether the rate of HEK293 cell proliferation is influenced by PAX2 expression, HEK/tTA/hPAX2a #3–3 cells were plated at low density, grown for 1–5 days in various concentrations of tetracycline, trypsinized and counted. Neither cell number (Fig. 2) nor the rate of thymidine incorporation (data not shown) was affected by the level of hPAXa protein expression. Similarly, cell number and thymidine incorporation were equivalent in HEK293 cells overexpressing mPAX2a, mPAX2b and in controls (data not shown).

3.4. Effect of PAX2 on expression of cytoskeletal molecules

During the transition from undifferentiated mesenchyme to polarized epithelium, vimentin expression falls off before cytokeratins are induced [28,31]. To determine whether PAX2 might be involved in regulating these cytoskeletal changes, we assessed steady state levels of vimentin and cytokeratin-18 transcripts by Northern analysis of total RNA from HEK/tTA/hPAX2a #3-3 in the presence or absence of tetracycline. As seen in Fig. 3, the level of vimentin mRNA is inversely proportional to the level of hPAX2a protein. When assessed by densitometry and normalized for GAPDH mRNA, maximal vimentin expression was only 8% of control at high

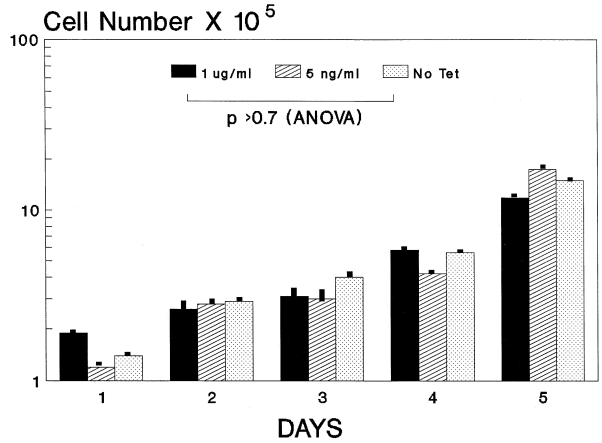

Fig. 2. Effect of PAX2 protein expression on cell proliferation. 293 tTA/hPAX2 #3-3 cells were plated 100 000 per dish in either tetracycline-free media, 0.005 μg/ml or 1 μg/ml of tetracycline (to allow high level, low level or no PAX2 expression, respectively). Cells were collected at 1–5 days and counted in a hemocytometer. Each time point was done in duplicate. Each experiment was repeated three times. No significant differences in cell number were detected by ANOVA over the 5 days.

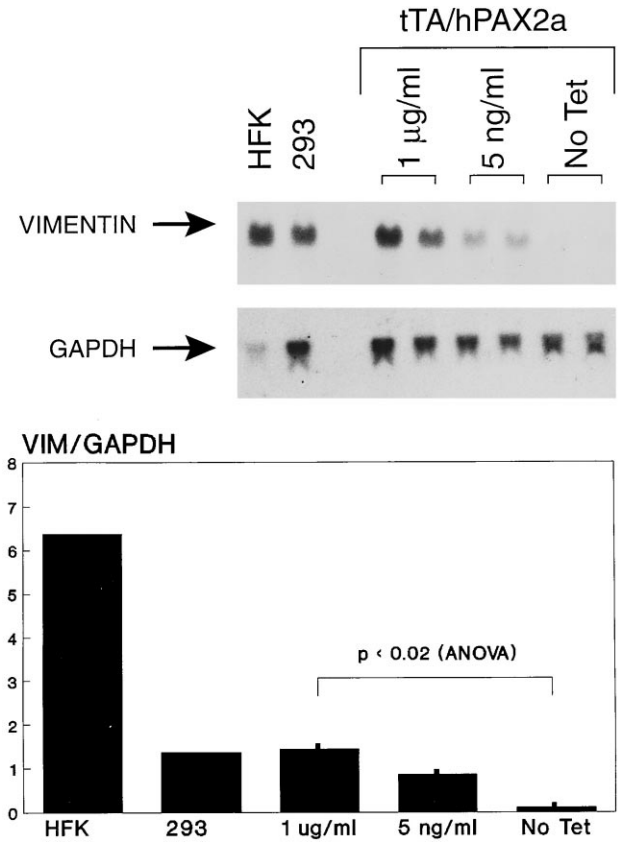

Fig. 3. Northern Analysis: Effect of PAX2 on human vimentin mRNA expression. Upper panel: In two separate experiments, 293 tTA/hPAX2 cells were grown in the presence of 1 µg/ml (no PAX2 expression); 0.005 µg/ml (low level of PAX2 expression) or no tetracycline (high level of PAX2 expression) containing media and harvested at ~90% confluency. 20 µg of total RNA were loaded into each lane: HFK (human fetal kidney total RNA); 293 (293/neo cells grown in the same tetracycline-free media); tTA/hPAX2 samples as indicated. The blot was probed with human vimentin cDNA and then reprobed with human GAPDH to control for mRNA loading. Bottom panel: Densitometric analysis of human vimentin mRNA expression standardized for GAPDH. Vimentin was significantly ($P < 0.021$, ANOVA; $P < 0.05$ Student-Newman-Keuls) inhibited in dose-dependent fashion by PAX2.

levels of hPAX2a expression ($P < 0.021$ ANOVA; $P < 0.05$ Student-Newman-Keul). In contrast to vimentin, cytokeratin-18 expression was unchanged by hPAX2a (Fig. 4). The same selective suppression of vimentin was seen in cells stably transfected with either mPAX2a or mPAX2b (data not shown).

3.5. Effect of PAX2 on transcription factors

In aggregating blastemal cells and the early ''comma'' and ''S-shaped'' body stages of nephron formation, PAX2 and WT1 genes are co-expressed [9,11]. To investigate a potential link between the two transcription factors, WT1 mRNA was assessed by Northern analysis in HEK/tTA/hPAX2a #3–3 cells in the presence or absence of tetracycline. As seen in Fig. 5, the level of endogenous WT1 mRNA rises as PAX2 expression is increased (2-fold in the absence

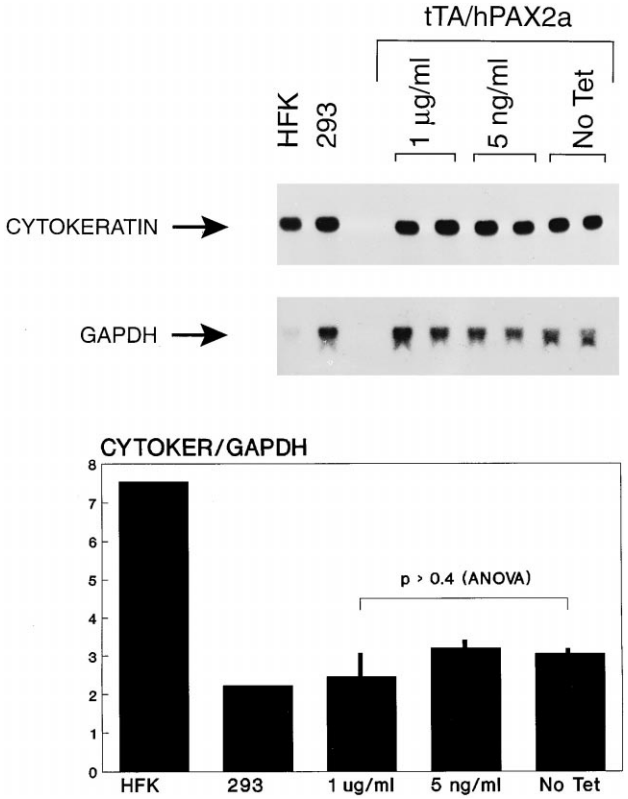

Fig. 4. Northern Analysis: Effect of PAX2 on cytokeratin expression. Upper panel: In two separate experiments, 293 tTA/hPAX2 cells were grown in media containing 1 and 0.005 µg/ml or no tetracycline corresponding to no PAX2, low level of PAX2 or high level of PAX2 protein, respectively. At ~90% confluency, cells were harvested and total RNA was extracted. Each lane contains 20 µg of total RNA: HFK (human fetal kidney total RNA); 293 (293/neo cells grown in tetracycline-free media); tTA/hPAX2 samples as indicated. The blot was probed with human cytokeratin 18 cDNA and then reprobed with human GAPDH as a loading control. Bottom panel: Densitometric analysis of human cytokeratin mRNA expression standardized for GAPDH. No significant effect of PAX2 on cytokeratin ($P > 0.39$, ANOVA) was detected.

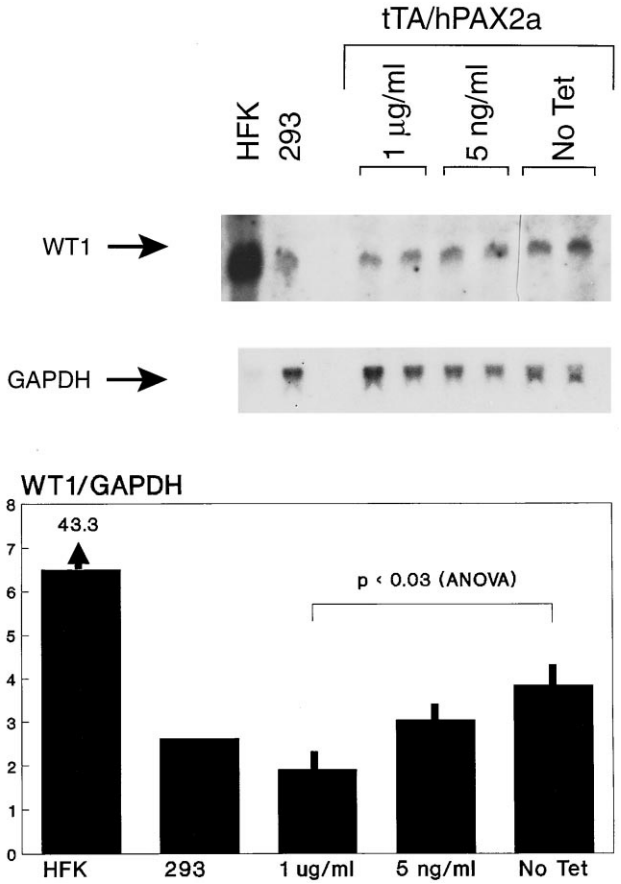

Fig. 5. Northern Analysis: Effect of PAX2 on WT1 expression. Upper panel: In two separate experiments, 293 tTA/hPAX2 cells were grown in media containing 1 and 0.005 μg/ml or no tetracycline-containing media. 20 μg of total RNA was loaded into each lane: HFK (human fetal kidney total RNA); 293 (total RNA isolated from 293, neo cells); tTA/hPAX2 samples as indicated. The blot was probed with human WT1 cDNA and then reprobed with human GAPDH to control for loading. Bottom panel: Densitometric analysis of WT1 mRNA expression standardized for GAPDH. WT1 mRNA was stimulated 2-fold ($P < 0.033$, ANOVA; $P < 0.05$ Student-Newman-Keuls) by PAX2.

of tetracycline; $P < 0.033$ ANOVA; $P < 0.05$ Student-Newman-Keuls).

3.6. Effect of PAX2 on cell adhesion molecules

Following induction, blastemal cells aggregate and then proliferate along spatial planes defining the tubular wall. Adjacent cells form specialized intercellular junctions characteristic of epithelia specialized for vectorial transport. In the transfilter model of kidney development, Ekblom has shown that following induction by spinal cord, metanephric cells begin to express E-cadherin [32].

Because levels of E-cadherin mRNA are relatively low in segments of the fetal kidney tubule derived from blastema, we used semi-quantitative RT-PCR to assess changes in E-cadherin expression associated with PAX2. Total RNA from HEK/tTA/hPAX2a #3-3 cells grown in the presence or absence of tetracycline was reverse-transcribed with dT-oligo primer, and E-cadherin cDNA was then amplified in duplicate with specific primers at low cycle number (within the linear PCR range) and quantified by densitometry of Southern blots. The same reverse transcriptase products were similarly assessed for β-actin expression, and the E-Cad/β-actin ratio was measured in three separate experiments. As seen in Fig. 6, a 7-fold increase in endogenous E-cadherin mRNA was associated with increasing hPAX2a expression ($P < 0.01$ Student *t*-test). We used the same method to assess E-cadherin expression in mPAX2b transfectants. Again, 5–10-fold stimulation of E-cadherin mRNA was associated with PAX2 expression (data not shown).

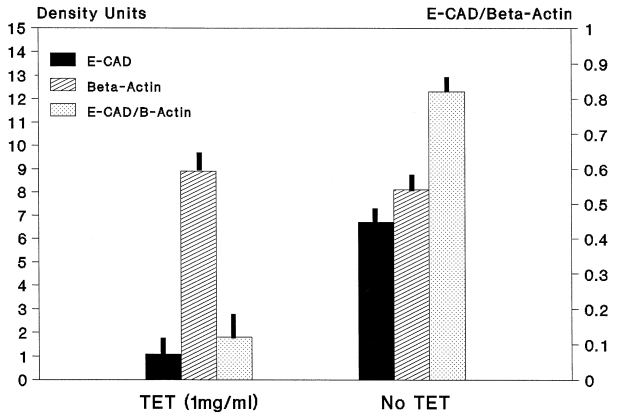

Fig. 6. Effect of PAX2 protein on E-cadherin mRNA expression. In three separate experiments, 293 tTA/hPAX2 cells were grown in medium containing tetracycline at 1 μg/ml (no PAX2 expression, control) or no tetracycline (high PAX2). Total RNA was prepared from all cell samples and RT-PCR reactions were carried out as described in Section 2. Southern blots of PCR products for E-cadherin or β-actin were probed with human E-cadherin and β-actin cDNAs, respectively. Films were analyzed densitometrically. Mean E-cadherin and β-actin signals and mean E-cadherin/β-actin ratios are depicted for the high-tetracycline or no-tetracycline conditions. E-cadherin expression was significantly ($P < 0.01$, Student *t*-test) stimulated 7-fold by PAX2.

4. Discussion

At the tips of each branch of the in-growing ureteric bud, clusters of blastemal cells are induced to aggregate and express a restricted set of new transcription factors (including PAX2) which are thought to activate the developmental cascade. Little is known about the initial effects of these transcription factors, but as a member of the ''paired-box'' homeodomain family, PAX2 is expected to act as a transcriptional regulator of other genes and is essential for normal nephrogenesis in rodents and humans [33]. In order to identify effects of PAX2 that might be of relevance to the early stages of nephrogenesis, we selected the HEK293 human fetal kidney cell line for in vitro studies. We found that these cells express markers (WT1, WNT4, cytokeratin) of condensing mesenchyme and ''S-shaped'' body stage but not of differentiated proximal or distal nephron segments and low levels of PAX2 mRNA but no detectable peptide. Taken together these features suggest that HEK293 cells are a lineage derived from the induced blastema and are suitable for eliciting PAX2 effects relevant to the early stage of nephrogenesis.

4.1. PAX2 and proliferation of fetal kidney cells

One feature of PAX2-expressing cells in fetal kidney is that they are highly proliferative. Intense staining for proliferating cell nuclear antigen (PCNA) is seen in the induced blastemal and in early stages of nephron formation, the ''comma'' and ''S-shaped'' bodies [34]. When stably transfected into NIH3T3 cells and rat 208 cells, PAX2 and other members of the PAX family are tumorigenic [35]. Cell proliferation was not directly assessed, but transfection with several PAX cDNAs promoted colony growth in soft agar and solid tumor formation when injected into nude mice [35]. Gnarra has shown that renal carcinoma cells (RCC) express high levels of PAX2 protein and that when RCC cells are exposed to antisense PAX2 oligonucleotides in vitro, the cells are growth inhibited [36]. Furthermore, PAX2, PAX5 and PAX8 were found to suppress transcription of p53, a critical regulator of the cell cycle in rodent fibroblasts [37]. In our experiments, however, HEK293 cells expressing PAX2 exhibited no change in basal proliferative rate. Conceivably, PAX2 might influence proliferation of fetal kidney cells in vivo by enhancing responsiveness to local growth factors not present in cultured HEK293 cells. However, it is more likely that the direct effects of PAX2 on HEK293 cell proliferation are masked by cell cycle effects of the adenovirus reagent initially employed to immortalize this particular cell line.

4.2. PAX2 and regulation of WT1

Because PAX2 and WT1 are co-expressed in the induced blastema and both transcription factors are expressed at high levels in Wilms' tumors, it has been postulated that PAX2 might normally participate in the transcriptional activation of WT1 expression. We found that the endogenous level of WT1 mRNA was moderately elevated by PAX2. Similarly, in HEK293 cells, both Dehbi et al. [38] and McConnell et al. [39] have reported stimulation of WT1 promoter activity when co-transfected with PAX2. Both authors reported even greater stimulation (8–35-fold) in non-renal cell lines and Dehbi noted marked stimulation of endogenous WT1 mRNA in the K562 keratinocyte line [38]. Taken together, these observations suggest that PAX2 might enhance WT1 expression in developing kidney. However, the situation in vivo appears to be complex. PAX2 mRNA is clearly present in cells of the branching ureteric bud which lack WT1 expression [9,10]. Conversely, PAX2 is absent in podocytes of the fetal glomerulus where WT1 expression is highest [40]. Thus, while PAX2 may influence WT1 transcription in some settings, possibly in condensing mesenchyme, alternative tissue-specific regulators of WT1 are likely to dominate at other sites.

4.3. PAX2 and the mesenchyme to epithelium transition

Following contact with the ureteric bud, metanephric mesenchymal cells undergo a radical phenotypic transformation to form the polarized epithelium lining an ''S-shaped'' vesicle [32]. These changes have been examined using the transfilter model of renal development in which induction of undifferentiated mesenchyme is initiated by co-cul-

ture with explants of fetal spinal cord [31]. In the uninduced mesenchyme, N-CAM is the only identifiable cell attachment molecule [41]; N-CAM is gradually replaced by E-cadherin as the ''S-shaped'' body forms [32,41]. In this study, we found that expression of PAX2 in HEK293 cells was associated with a 7-fold increase in steady state E-cadherin mRNA. This is consistent with the observation that PAX2 is expressed in condensing mesenchyme and the ''S-shaped'' body just as E-cadherin expression begins.

Conversion of mesenchyme to polarized epithelium also involves alteration of the cytoskeleton. Cultured human mesothelial cells proliferating in unsupplemented medium have a spindle-shaped morphology and express high levels of vimentin, but minimal cytokeratins [17]; in response to retinoic acid, the cells take on an epithelial appearance, suppress vimentin and express high levels of cytokeratins [17]. Lehtonen has examined the rearrangement of intermediate filaments in the transfilter model of nephron induction [28]. In the uninduced mesenchyme he noted strong immunoreactivity for vimentin. After two days, he was able to detect cellular aggregates corresponding to mesenchymal condensates associated with PAX2 expression in vivo [28]. By the 3rd day, early tubular structures were identifiable in which vimentin was completely absent. By the 4th day, cytokeratin was evident in the tubular structures. Similarly, in human fetal kidney, Matsell has shown that vimentin immunoreactivity is intense in uninduced blastema, barely detectable in the ''S-shaped'' stage and absent in maturing proximal tubule [42]. In our study, we have shown that PAX2 dramatically suppresses vimentin mRNA to 8% of controls, while cytokeratin-18 was unchanged or modestly increased. We speculate that, in condensing mesenchyme and the early ''S-shaped'' stage of nephrogenesis, intense PAX2 expression may serve to suppress the mesenchymal intermediate filament, vimentin, in anticipation of a switch to epithelial cytokeratins.

Our experiments do not determine whether PAX2 acts directly or indirectly, but its effects on expression of WT1, E-cadherin and vimentin in HEK293 cells mimick the changes which normally occur in PAX2-positive mesenchymal condensates and ''S-shaped'' bodies during the early stages of nephrogenesis.

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References

[1] P. Sanyanusin, L. Schimmenti, L. McNoe, T. Ward, M. Pierpont, M. Sullivan, W. Dobyns, M. Eccles, Nat. Genet. 9 (1995) 358–363.
[2] M. Torres, E. Gomaz-Pardo, G.R. Dressler, P. Gruss, Development 121 (1995) 4057–4067.
[3] E.T. Stuart, P. Gruss, Cell Growth Differ. 7 (1996) 405–412.
[4] A. Read, Nat. Genet. 9 (1995) 333–334.
[5] C.T. Baldwin, C.F. Hoth, J.A. Amos, E.O. da-Silva, A. Milunsky, Nature 355 (1992) 637–638.
[6] M. Tassabehji, A.P. Read, V.E. Newton, R. Harris, R. Balling, P. Gruss, T. Strachan, Nature 355 (1992) 635–636.
[7] T. Glaser, D.S. Walton, R.L. Maas, Nat. Genet. 2 (1992) 232–239.
[8] P. Sanyanusin, L.A. McNoe, M.J. Sullivan, R.G. Weaver, M.R. Eccles, Hum. Mol. Genet. 4 (1995) 2183–2184.
[9] M.R. Eccles, L.J. Wallis, A.F. Fidler, N.K. Spurr, P.J. Goodfellow, A.E. Reeve, Cell Growth Differ. 3 (1992) 279–289.
[10] G. Dressler, U. Deutsch, K. Chowdhury, H.O. Nornes, P. Gruss, Development 109 (1990) 787–795.
[11] G. Dressler, E. Douglass, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 1179–1183.
[12] G. Dressler, E. Wilkinson, U. Rothenpieler, L. Patterson, L. Willams-Simons, H. Westphal, Nature 362 (1993) 65–67.
[13] U. Rothenpieler, G. Dressler, Development 119 (1993) 711–720.
[14] M. Gossen, H. Bujard, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 5547–5551.
[15] A.J. Berk, P.A. Sharp, Cell 12 (1977) 721–732.
[16] S. Ferrari, R. Battini, L. Kaczmarek, S. Rittling, B. Calabretta, J.K. de Riel, V. Philiponis, J.F. Wei, R. Baserga, Mol. Cell. Biol. 6 (1986) 3614–3620.

[17] K.H. Kim, V. Stellmach, J. Javors, E. Fuchs, J. Cell. Biol. 105 (1987) 3039–3051.
[18] A. Nagafuchi, Y. Shirayoshi, K. Okazaki, K. Yasuda, M. Takeichi, Nature 329 (1987) 341–343.
[19] K.M. Call, T. Glaser, C.Y. Ito, A.J. Buckler, J. Pelletier, D.A. Haber, E.A. Rose, A. Kral, H. Yeger, W.H. Lewes, C. Jones, D.E. Housman, Cell 60 (1990) 509–520.
[20] J.Y. Tso, X.H. Sun, T.H. Kao, K.S. Reese, R. Wu, Nucleic Acid Res. 13 (1985) 2485–2502.
[21] Z. Kozmik, P. Kurzbauer, P. Dopfler, M. Busslinger, Mol. Cell. Biol. 13 (1993) 6024–6035.
[22] W.-S. Lee, R.G. Wells, R.V. Sabbag, T.K. Mohandas, M.A. Hediger, J. Clin. Invest. 91 (1993) 1959–1963.
[23] T. Maniatis, E.F. Fritsch, J. Sambrook, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1982
[24] M.J. Bussemakers, A. van Bonhoven, S.G. Mees, R. Kemler, J.A. Schalken, Mol. Biol. Rep. 17 (1993) 123–128.
[25] M. Borgers, J. Histochem. Cytochem. 21 (1973) 812–824.
[26] K. Pritchard-Jones, S. Fleming, D. Davidson, W. Bickmore, D. Porteous, C. Gosden, J. Bard, A. Buckler, J. Pelletier, D. Housman, V. van Heyningen, N. Hastie, Nature 346 (1990) 194–197.
[27] K. Stark, S. Vainio, G. Vassileva, A.P. McMahon, Nature 372 (1994) 679–683.
[28] E. Lehtonen, I. Virtanen, L. Saxen, Dev. Biol. 108 (1985) 481–490.
[29] C.J. Ward, H. Turley, A.C.M. Ong, M. Comley, S. Biddolph, R. Chetty, P.J. Ratcliffe, K. Gatter, P.C. Harris, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 1524–1528.
[30] N.L. Ostrovski, W.S. Young 3-rd., M.A. Knepper, S.J. Lolait, Endocrinology 133 (1993) 1849–1859.
[31] P. Ekblom, FASEB J., 3, 2141–2150
[32] L. Sorokin, P. Ekblom, Kidney Int. 41 (1992) 6642–6657.
[33] L.T. Patterson, G. Dressler, Curr. Opin. Genet. Dev. 4 (1994) 696–702.
[34] A. Yoshimura, K. Gordon, C.E. Alpers, J. Floege, P. Pritzl, R. Ross, W.G. Couser, D.F. Bowen-Pope, R.J. Johnson, Kidney Int. 40 (1991) 470–476.
[35] C. Maulbecker, P. Gruss, EMBO J. 12 (1993) 23661–23673.
[36] J.R. Gnarra, G.R. Dressler, Cancer Res. 55 (1995) 4092–4098.
[37] E.T. Stuart, R. Haffner, M. Oren, P. Gruss, EMBO J. 14 (1995) 5638–5645.
[38] M. Dehbi, M. Ghahremani, M. Lechner, G.R. Dressler, J. Pelletier, Oncogene 13 (1996) 447–453.
[39] M.J. McConnell, H.E. Cuncliffe, L.J. Chua, T.A. Ward, M.R. Eccles, Oncogene,1997, in press
[40] G. Rayn, V. Steele-Perkins, J.F. Morris, F.J. Rauscher III, G. Dressler, Development 121 (1995) 867–875.
[41] E. Nouwen, S. Dauwe, I. Van Der Biest, M. de Broe, Kidney Int. 44 (1993) 147–158.
[42] D.G. Matsell, T. Bennett, R.A. Armstrong, P.R. Goodyer, C.G. Goodyer, V.K.M. Han, J. Am. Soc. Nephrol. 8 (1997) 85–94.