Hoxa 11 is upstream of *Integrin α8* expression in the developing kidney

M. Todd Valerius*†, Larry T. Patterson‡, Yuxin Feng*, and S. Steven Potter*§

Divisions of *Developmental Biology and ‡Nephrology and Hypertension, Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229

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Mutation of the functionally redundant *Hoxa 11*/*Hoxd 11* genes gives absent or rudimentary kidneys resulting from a dramatic reduction of the growth and branching of the ureteric bud. To understand better the molecular mechanisms of *Hoxa 11*/*Hoxd 11* function in kidney development, it is necessary to identify the downstream target genes regulated by their encoded transcription factors. To this end, we conducted a screen for *Hoxa 11*-responsive genes in two kidney cell lines. *HEK293* cells, which usually do not express *Hoxa 11*, were modified to allow inducible *Hoxa 11* expression. The *mK10* cells, derived specifically for this study from *Hoxa 11*/*Hoxd 11* double-mutant mice, were also modified to give cell populations with and without *Hoxa 11* expression. Differential display, Gene Discovery Arrays, and Affymetrix GENECHIP probe arrays were used to screen for genes up- or down-regulated by *Hoxa 11*. Nine genes, *PDGF A*, *Cathepsin L*, *annexin A1*, *Mm.112139*, *Est2 repressor factor*, *NrCAM*, *ZNF192*, *integrin-associated protein*, and *GCM1*, showed reproducible 3-fold or smaller changes in gene expression in response to *Hoxa 11*. One gene, the *Integrin α8*, was up-regulated approximately 20-fold after *Hoxa 11* expression. The *Integrin α8* gene is expressed together with *Hoxa 11* in metanephric mesenchyme cells, and mutation of *Integrin α8* gives a bud-branching morphogenesis defect very similar to that observed in *Hoxa 11*/*Hoxd 11* mutant mice. *In situ* hybridizations showed a dramatic regional reduction in *Integrin α8* expression in the developing kidneys of *Hoxa 11*/*Hoxd 11* mutant mice. This work suggests that the *Integrin α8* gene may be a major effector of *Hoxa 11*/*Hoxd 11* function in the developing kidney.

The *Hoxa 11* and *Hoxd 11* genes have an important function in kidney development. *Hoxa 11* is expressed in the metanephric mesenchyme before ureteric bud invasion and continues in the condensing mesenchyme around the ureteric bud tips (1). As the condensing mesenchyme further differentiates and some cells undergo a mesenchymal-to-epithelial conversion, *Hoxa 11* expression is lost, suggesting a role for *Hoxa 11* early in the process of nephrogenesis. *Hoxd 11* expression in the developing kidney is similar (1). Gene-targeting studies show that the paralogous *Hoxa 11* and *Hoxd 11* genes are functionally redundant (2). Although the single knockout of each gene has no detectable renal phenotype, the *Hoxa 11*/*Hoxd 11* double knockout results in renal agenesis or the formation of a small kidney rudiment that contains very few nephrons (2). The ureteric bud of the double mutant shows a dramatic reduction in branching (1), consistent with the reduced number of nephrons formed, which suggests that the mutant mesenchyme, where *Hoxa 11* and *Hoxd 11* are expressed, does not properly signal the bud to branch.

To understand better the mechanisms of *Hox* gene function it is necessary to identify their downstream effectors. Despite considerable effort, relatively little progress has been made in this area. One strategy used with success has been immunoprecipitation of target genes that have been fixed *in vivo* to the transcription factor of interest (3–6). Sequence scanning of promoters for potential Hox protein-binding sites has identified the *β-amyloid* (7), *cytotactin* (8), and *neural cell adhesion* (9) genes as candidate downstream targets. Cell-line studies have often used a candidate target-gene approach, for example, showing that *Hoxb 7* up-regulates *bFGF* expression in melanoma cells (10). By using a combination of strategies the *p53* and the *progesterone receptor* genes were identified as targets of *Hoxa 5* (11, 12).

In this report we describe the results of a screen for the downstream targets of *Hoxa 11*. A cell-line strategy was used (13–15). Differential display, Gene Discovery Arrays (GDA), and Affymetrix GENECHIP probe arrays were used to identify genes that were up- or down-regulated in response to *Hoxa 11* expression. In both cell systems used, the *Integrin α8* gene showed the greatest fold change in expression, which is of particular note because the kidney phenotypes resulting from the *Hoxa 11*/*Hoxd 11* and *Integrin α8* mutations are strikingly similar (2, 16), suggesting that *Integrin α8* may be a key effector of *Hoxa 11* function in kidney development. The role of *Hoxa 11* in regulating *Integrin α8* expression *in vivo* was confirmed by *in situ* hybridizations. *Hoxa 11*/*Hoxd 11* mutant kidneys showed dramatic regional reduction in *Integrin α8* expression.

Methods

Isolation and Establishment of Kidney Cell Lines. *Hoxa 11-SV40 Large Tag* transgenic mice were bred with *Hoxa 11+/− Hoxd 11+/−* double-heterozygous mice. Triple-heterozygous mice (*Hoxa 11+/−*, *Hoxd 11+/−*, *Hoxa 11-SV40 Large Tag+*) were bred to produce embryos of the desired genotype. Kidney tissue was carefully dissected from E18.5 embryos and dissociated with trypsin, and the cells were plated on 100-mm tissue-culture dishes. Genotype was determined by PCR. The cells were cultured in DMEM (GIBCO/BRL/Invitrogen) with 10% FBS (GIBCO/BRL/Invitrogen) at 37°C in 5% CO_2. Media were changed every 2–3 days, and cells were split when confluent. After 10 or more passages, clones were established by dilution cloning. The *mK10* cell line has subsequently been grown for more than 20 additional passages, suggesting these cells are immortalized. *HEK293* Tet-Off cells were purchased from CLONTECH and maintained according to the manufacturer's protocol.

Modification of Cell Lines. The plasmid pcDNA 3.1/Hygro (Invitrogen) was used to drive the expression of full-length *Hoxa 11* cDNA in *mK10* cells. For the 293 Tet-Off cells, a FLAG epitope tagged *Hoxa 11* cDNA was cloned into the pBI Tet expression vector (CLONTECH) and introduced by transfection. One day after calcium chloride transfection, cells were subjected to Hygromycin selection and colonies picked after sufficient growth. FLAG epitope tagging was introduced with PCR by using the following primers: 5′-CCCCAAGCTTCCACCATGGCTCCAAAGAAGAAGCGTAAGGTAGACTACAAGGACGACGATGACAAGGATTTTGATGAG-3′ and 5′-AGGAAGCTTAACCACGGAGATCTG-3′. The FLAG

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Abbreviations: GDA, Gene Discovery Array; RT, reverse transcription.

†Present address: Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138.

§To whom reprint requests should be addressed. E-mail: steve.potter@chmcc.org.

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epitope was detected with the M5 monoclonal antibody (Sigma). Doxycycline (2 μg/ml) was added to the media for 2 days with media changes every 12 h to regulate *Hoxa 11* expression in the 293 Tet-Off cells.

Gene Expression Profile Differences. Differential display was performed by using GeneHunter (Nashville, TN) reagents and protocols. The first five sets of randomly designed hexamers were used on each sample and done in triplicate. For GDAs (Genome Systems/Incyte Pharmaceuticals, Palo Alto, CA), probe labeled from cell line mRNA with a poly(dT) oligonucleotide in a reverse transcription reaction with [^{33}P]UTP was used to probe the arrays according to the manufacturer's protocol. Affymetrix GENECHIP probe arrays were probed with biotinylated cRNA prepared according to the manufacturer (Affymetrix, Santa Clara, CA). The Human 6800 gene chip set was used in these studies.

Northern Blots and Reverse Transcription (RT)-PCR. Total RNA was isolated from trypsinized cells by using RNAzol (Tel-Test, Friendswood, TX). Poly(A)$^+$ was selected with the Oligotex mRNA isolation kit (Qiagen, Carlsbad, CA). Reverse transcription was performed with Superscript II (GIBCO/BRL/Invitrogen) and a random hexamer mix according to manufacturer's directions. After reverse transcription, PCR used *Taq* polymerase (Qiagen). Semiquantitative RT-PCR was done by adding 1 μCi of [^{32}P]dATP to each 50-μl PCR. The linear amplification phase was determined for each primer pair by determining incorporation at 18, 20, 22, 24, and 26 cycles. Gels were exposed to phosphoimager screens, and plots of intensity vs. cycle number were generated. The lowest cycle number possible was selected for the comparison analysis. All gene expression was normalized to *GAPDH*. Probes used for Northern blots were made by RT-PCR. Primers used: *Hoxa 11 RT* 5′-AAAACCTCGCTTCCTCCGACTACC-3′ and 5′-CGCAATGTGGCTTGACCTTGTC-3′; *GCMa* 5′-GAACCTGACGACTCTGATTCTGAAG-3′ and 5′-TCACAGTTGGGACAGCGTTTCC-3′; *Integrin α8* 5′-AGCTACTTCGGCTACTCACTGGAC-3′ and 5′-TCCTCCCACTATAAGGTCTCCATTC-3′; *PDGF-A* 5′-GTGCTTTATTGCCAGTGTGCG-3′ and 5′-AAGACATTCCTGCTTCCTGCG-3′; *GAPDH* 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′; *IAP* 5′-TTGGAGCCATTCTTTTCGTCC-3′ and 5′-AAGGGTCTCATAGGTGACAACCAG-3′; *ZFP192* 5′-CTGTGCTTCTTCCCATCTCCT-3′ and 5′-AATAACGAGTGAGGTGCTGAGGC-3′; *NrCAM* 5′-CTCAAAATCTTGTGCTGTCCCC-3′ and 5′-GCAGTTCCCTGTTGTCCTTCAG-3′.

***In Situ* Hybridization.** For whole-mount *in situ* hybridizations the genitourinary block was isolated from E13.5 embryos. Whole mount and section *in situ* hybridizations were performed according to established protocols (17, 18).

Results

Strategy. To better understand the developmental function of *Hoxa 11* it is necessary to identify the downstream target genes it regulates. To this end we used a cell-line strategy similar to those described (13–15, 19). In essence, the gene expression patterns of otherwise identical cells with and without *Hoxa 11* expression were compared. The *Hoxa 11* responsive genes were altered in expression level.

Cell Lines. The use of developmentally appropriate cell lines promotes the identification of legitimate downstream gene targets. Such cells have the correct cofactor combination required for proper target binding. We are particularly interested in the function of the *Hoxa 11* gene in kidney development. *Hoxa 11* is expressed in the metanephric mesenchyme of the developing kidney before induction by the ureteric bud and during the condensation of these cells to form the epithelia of the nephron (1). The *Hoxa 11* gene is turned off during the later stages of epithelia differentiation.

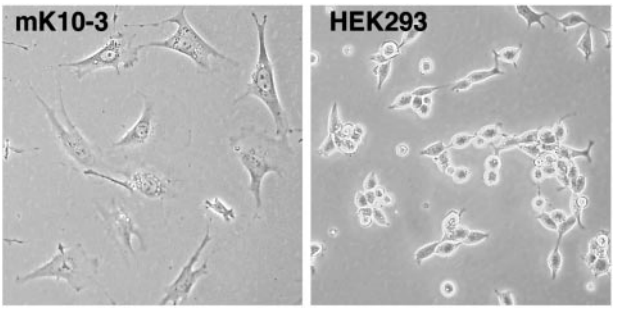

Fig. 1. Morphology of *mK10* and *HEK293* cells. The *mK10* cells, double-homozygous mutant for *Hoxa 11* and *Hoxd 11*, have a flattened, often spindle-shaped appearance, typical of fibroblasts. The *HEK293* cells are more rounded, often appearing polygonal at confluence, which suggests an epithelial character.

Two cell lines were used. The Human Embryonic Kidney 293 (*HEK293*) cells represent a late stage of kidney epithelia development. No *Hoxa 11* transcripts were detected in these cells by RT-PCR or Northern blot (data not shown). *HEK293* cells are used extensively for study of channel and transporter proteins (20–22).

The other cell line used, *mK10*, was derived specifically for these studies. The *Hoxa 11* promoter was used to drive restricted expression of SV40 large T antigen (Tag) in transgenic mice, to immortalize and developmentally arrest early kidney cells. The transgene DNA construct included 5.1 kb upstream of the *Hoxa 11* transcription start site, 3.8 kb downstream, and the intact *Hoxa 11* intron. Ten *Hoxa 11-SV40* transgenic founder mice were made, and one was mated with *Hoxa 11*, *Hoxd 11* double-heterozygous mutants to make *Hoxa 11*$^{+/-}$ *Hoxd 11*$^{+/-}$, *Hoxa 11-SV40* transgenics. These mice were then mated, and fetuses collected at E18.5. The *mK10* kidney cell line was made from a single *Hoxa 11*$^{-/-}$ *Hoxd 11*$^{-/-}$ *Hoxa11-SV40 Tag* fetus. After ten passages dilution cloning was used to derive the *mK10* cells, which have now been passaged more than 20 times, suggesting they represent an immortalized cell line. The morphologies of the *HEK293* and *mK10* cells are shown in Fig. 1.

Making Cell Lines With and Without *Hoxa 11* Expression. *HEK293* cells with inducible *Hoxa 11* expression were made by using the Tet-Off system developed by Bujard and colleagues (23). An amino-terminal FLAG-tagged *Hoxa 11* cDNA construct was cloned into the Tet-responsive vector (CLONTECH) and transfected into modified *HEK293* cells that express the tet-Transactivator. Hygromycin-resistant clones were picked and expression levels of FLAG-Hoxa 11 in the presence and absence of doxycycline determined by Western blot (Fig. 2). A single

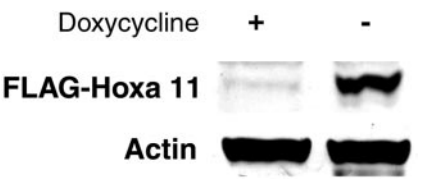

Fig. 2. Induction of *Hoxa 11* expression in *HEK293* cells. Western blot detection of Hoxa11-FLAG fusion protein in cells not induced (+doxycycline) and induced (−doxycycline). Actin served as a loading control.

Table 1. Confirmed gene expression differences

Screen/cells used	Unigene cluster	Description	Fold change
Differential display: *mK10 & Pcmv-Hoxa 11*	Mm.930	Cathepsin L	−2.0
	Mm.14860	Annexin A1	−2.0
GDA: *mK10 & Pcmv-Hoxa 11*	Mm.112139	Expressed sequence tags	−1.8
	Mm.8068	Est2 repressor factor	−1.4
Affymetrix GeneChip: *Tet inducible Hoxa* 11/HEK 293	Hs.91296	Integrin α8	+23.0
	Hs.37040	PDGF-A	−2.0
	Hs.28346	GCMa	+1.6
	Hs.7912	hBRAVO/NrCAM	+2.0
	GB Z25521	IAP/CD47	+3.0
	Hs.57679	Zinc finger protein 192	+2.2

+, Increased expression with *Hoxa 11* expression.

clone showing greater than 10-fold induction in the absence of doxycycline was selected for subsequent studies.

The *mK10* cells were transfected with *pCMV-Hoxa 11*, which drives expression of *Hoxa 11* from the *CMV* promoter. Among the resulting hygromycin-resistant clones, the *mK10/pCMV-Hoxa 11* clone with the highest level of *Hoxa 11* expression, was selected for comparison studies.

Screens for *Hoxa 11* Responsive Genes. Differential display, GDA, and Affymetrix GENECHIP probe arrays were used to compare gene expression profiles of cells, with and without *Hoxa 11* expression, to search for *Hoxa 11* downstream targets.

RNAs from *mK10* and *mK10/pCMV-Hoxa11* cells were examined by differential display. Each PCR was done with three independent RNA isolations. More than 100 differences were identified and tested by reverse Northern (RNA labeled, PCR products blotted), which identified 33 clones apparently differently expressed. Repeated Northern analysis, however, confirmed only two genes as reproducibly differently expressed. GAPDH was used as a loading control, with phosphoimager quantitation. Both genes were down-regulated approximately 2-fold by *Hoxa 11* expression. *Cathepsin L* is a widely expressed cysteine protease found in lysosomes that is sometimes secreted by expressing cells (24). *Cathepsin L* has been implicated in tumor invasion (25), trophoblast invasion during implantation (26, 27), and endochondral bone formation (28). *Annexin AI* (*Lipocortin I*) is a member of the calcium–phospholipid-binding family of proteins thought to be involved in signal transduction (29). *Annexin AI* is expressed in restricted epithelial kidney structures in the adult including Bowman's capsule, macula densa, and collecting ducts (30).

The double-mutant cell line was also used in a screen with GDAs, with more than 18,000 cDNAs blotted onto a nylon filter. Duplicate arrays were probed with ^{33}P-labeled cDNA made by reverse transcription of each mRNA sample. Analysis of the phosphoimage files by Genome Systems (Incyte Pharmaceuticals, Palo Alto, CA) listed 705 cDNAs altered at least 2-fold between the *mK10* and *mK10/pCMV-Hoxa 11* RNA samples (420 up, 285 down). Each blotted cDNA was examined manually to eliminate artifacts caused by blotting errors, and forty of the most promising cDNAs were selected for confirmation by Northern blot analysis. Expression levels were again normalized to *GAPDH* expression by using a phosphoimager. Two clones showed a reliable, although small, expression difference (Table 1). One clone represented an expressed sequence tag cluster designated Mm.112139 with weak homology to *EHD1*, an EH domain-containing protein expressed in the developing limb buds, testis, and kidney (31). EH domain proteins are implicated in signaling and ligand-induced endocytosis (32). The *Est2 repressor factor* (*ERF*) gene is an ets-domain transcription factor expressed widely in the adult (33) that acts as an antagonist to other ets-domain transcriptional activators (34).

Affymetrix GENECHIP probe arrays were used to compare gene expression profiles of *Hoxa 11* induced and uninduced *HEK293* cells. Of the approximately 6,800 genes screened, 309 were reported as 1.5-fold or greater differently expressed. Further analysis by Northern blot or quantitative RT-PCR confirmed expression differences for six genes (Fig. 3 and Table 1). *Integrin α8* is a cell-membrane protein that interacts with the extracellular matrix. Integrins regulate cell growth and differentiation, creating a link between the extracellular matrix environment and cell behavior (35). *Platelet-Derived Growth Factor A chain* (*PDGF-A*) is a well-characterized growth factor expressed in epithelial cells in many tissues including the kidney. There, *PDGF-A* expression is initiated in newly formed epithelial cells as they are formed from condensed mesenchymal cells around the ureteric bud tips, whereas the receptors for *PDGF* are expressed in mesenchymal cells (36). *Hoxa 11* is expressed in the condensing mesenchymal cells that are beginning the epithelialization process and turns off as the cells differentiate further (1). This observed pattern, with *Hoxa 11* expression decreasing as *PDGF* expression increases, is consistent with an *in vivo* role for *Hoxa 11* in repressing the *PDGF* gene. *GCM1* is a transcription factor gene cloned by homology to the *Drosophila Glial Cells Missing α* gene. Although responsible for neuronal cell-fate decisions in the fly, both known mammalian homologs are expressed in E16.5 kidneys and in the placenta, suggesting other roles in mammalian development (37). Gene knockout of *GCM1*

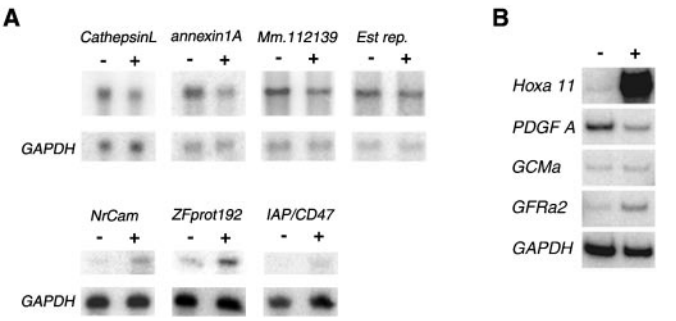

Fig. 3. Candidate *Hoxa 11* downstream targets. (*A*) Northern blots showing expression differences in mutant *mK10* cells (*Upper*) and *HEK293* cells (*Lower*) with and without *Hoxa 11* expression. + indicates with *Hoxa 11* expression. Blots were stripped and then reprobed with *GAPDH* as a loading control. (*B*) Semiquantitative RT-PCR confirmation of candidate target gene expression differences in *HEK293* cells. Observed differences for these genes were small but reproducible.

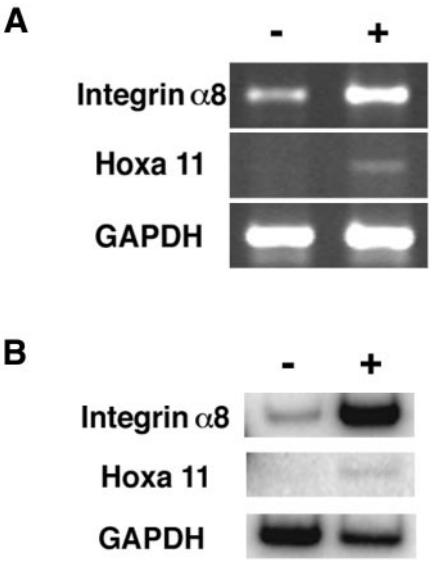

Fig. 4. Up-regulation of *Integrin α8* in *mK10* cells in response to *Hoxa 11* expression. (*A*) Nonradiolabel RT-PCR showed *Integrin α8* expression increased with expression of *Hoxa 11*. (*B*) Semiquantitative radiolabel RT-PCR further confirmed that *Hoxa 11* increased *Integrin α8* expression. Intensities were normalized to GAPDH. + marks lane with *Hoxa 11* expression.

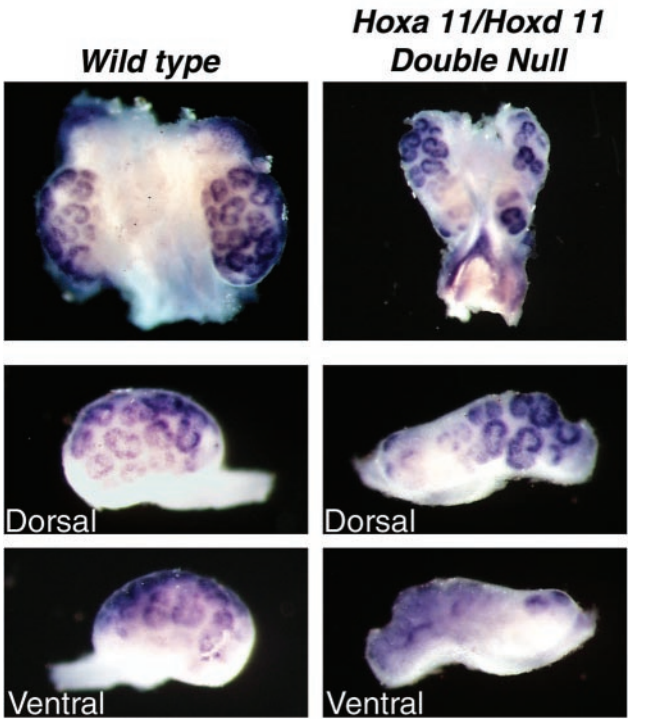

Fig. 5. Regional reduction of *Integrin α8* expression in *Hoxa 11/Hoxd 11* double-mutant kidneys. Whole-mount *in situ* hybridizations with E13.5 mutant and wild-type littermate kidneys. (*Top*) The more medial positioning of the mutant kidneys, with the posterior poles nearly touching. Part of the dorsal region of the mutant kidney, and the entire ventral region, showed reduced *Integrin α8* expression.

disrupted branching morphogenesis of trophoblast populations in the placenta, resulting in lethality at E10, before kidney formation (38). Neuronal cell adhesion molecule (*NrCAM*) plays an important role in the growth cone during neural development (39). Although no role for NrCAM in kidney development has been ascribed, other neural cell adhesion molecules have been described in the ureteric epithelium and ductal epithelial portions of the nephron (40). *Integrin-associated protein* (*IAP/CD47*) is a membrane protein important in the inflammatory response to infection (41). *Zinc finger protein 192* is a Kruppel family member expressed in the adult kidney (42).

The *Integrin α8* gene stood out as the most interesting of the ten candidate targets identified by the three screens for several reasons. First, this gene showed an approximately 20-fold up-regulation in response to *Hoxa 11* expression, whereas the other genes showed more modest changes. Second, the expression of *Integrin α8* closely matches that of *Hoxa 11* in the metanephric mesenchyme surrounding the branching ureteric bud, consistent with *Hoxa 11* being an important *in vivo* regulator of *Integrin α8* expression. Third, the kidney phenotype of the *Integrin α8* mutant mouse mirrors that of the *Hoxa 11* mutant, suggesting that *Integrin α8* may be a key mediator of *Hoxa 11* function in the developing kidney. Further studies therefore focused on the *Integrin α8* gene.

***Integrin* α8 Is Also Up-Regulated by *Hoxa 11* in *mK10* Cells.** The up-regulation of *Integrin α8* after *Hoxa 11* expression was seen in the *HEK293* cells by using the Affymetrix GENECHIP, but was not detected by Differential Display or GDA in the *mK10* cells. This lack of detection could have resulted from the incomplete nature of the Differential Display and GDA screens, or could have been the result of *Integrin α8* not responding to *Hoxa 11* in *mK10* cells. To distinguish these possibilities *Integrin α8* expression was first measured by standard RT-PCR in *mK10* cells, double mutant for *Hoxa 11* and *Hoxd 11*, and in *mK10/pCMV-Hoxa 11*cells, with *Hoxa 11* expression. Higher *Integrin α8* expression was seen in the *mK10/pCMV-Hoxa 11*cells (Fig. 4*A*), which was then confirmed by multiple repetitions of a semiquantitative RT-PCR assay monitoring label incorporation in the linear amplification phase (Fig. 4*B*). *Integrin α8* expression was up-regulated approximately 20-fold in the *Hoxa 11*-expressing cells.

***In Vivo* Confirmation.** If *Hoxa 11* plays a critical role in the expression of *Integrin α8*, then we would predict the expression pattern to be altered in the *Hoxa 11/Hoxd 11* double mutants. To test this prediction, E13.5 kidneys were isolated from *Hoxa 11/Hoxd 11* double-mutant embryos and wild-type littermates and examined for *Integrin α8* expression by *in situ* hybridization. A dramatic, but localized reduction of *Integrin α8* expression was seen in the mutant kidneys (Fig. 5). Some regions showed near-normal *Integrin α8* expression, but other regions, and in particular the ventral side of the kidney, showed an almost complete loss of *Integrin α8* expression.

The altered expression of *Integrin α8* in *Hoxa 11/Hoxd 11* double-mutant kidneys was further confirmed by section *in situ* hybridization. The ventral renal mesenchyme adjacent to the ureteric bud showed a consistent reduction of *Integrin α8* expression (Fig. 6 *A* and *G*). This altered expression pattern was coincident with the observed severe reduction of branching morphogenesis in the ventral domain of the mutant kidney. The normal domain of *Hoxa 11* expression is outlined by *Foxd1* expression, a marker of stromal cells (Fig. 6*D*). In the poles of the kidney, where branching of the bud was more normal, we observed symmetric *Integrin α8* expression surrounding the bud (data not shown).

Discussion

To understand the genetic networks controlling kidney organogenesis it is necessary to identify the downstream targets of the transcription factors, such as *Hoxa 11*, which regulate this

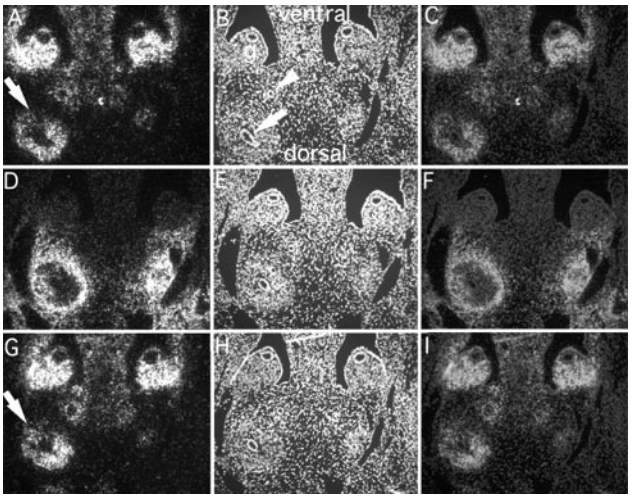

Fig. 6. Section *in situ* hybridizations further define the altered *Integrin α8* expression in *Hoxa 11/Hoxd 11* mutant kidneys. Serial transverse sections through E13.5 Hoxa 11/Hoxd 11 double-mutant embryos were hybridized with antisense riboprobes to *Integrin α8* (*A* and *C*, and *G* and *I*) or *Foxd1* (a marker of stromal cells) (*D* and *F*). *A*, *D*, and *G*, darkfield images; *B*, *E*, and *H*, 4′,6-diamidino-2-phenylindole fluorescent-stained images; *C*, *F*, and *I*, merged darkfield and fluorescent images. *B* shows the dorsoventral orientation of the embryo and the structures present. Arrow, ureteric bud; arrowhead, mesonephric duct; g, gonad. The arrows in *A* and *G* indicate the ventral renal mesenchyme, where *Integrin α8* expression is severely reduced compared with dorsal expression. For this double mutant the kidney shown on the right-hand side was extremely malformed, with an absence of detectable ureteric bud branching.

process. The cell-line-probe array strategy used in this study provides a relatively rapid screen that does not require target guessing or laborious testing of genes one at a time. As probe arrays provide more complete coverage of the genome these screens become more universal in nature. This strategy has been surprisingly successful, even when developmentally inappropriate cell lines have been used. For example, osteosarcoma cells with inducible *WT1* expression were used to identify *amphiregulin* as a downstream target of the Wilms tumor gene (15) in kidney development.

To optimize the screen for *Hoxa 11* targets in kidney development we used kidney cell lines. The *HEK293* cells seem to represent a late stage in kidney epithelia development, after *Hoxa 11* expression is turned off. The other cell line used, *mK10*, was designed to represent metanephric mesenchyme at an early stage of development, when *Hoxa 11* would usually be expressed. To remove *Hoxa 11* expression the cells were made from mice mutant for both *Hoxa 11* and the functionally redundant *Hoxd 11*. To make the *mK10* cell line we used *Hoxa 11* promoter-driven expression of SV40 Tag in transgenic mice, an approach with a long history of success. Surprisingly, SV40 Tag expression is able to not only immortalize cells, but to arrest them developmentally. For example, a 1.8-kb promoter for the *glycoprotein hormone α-subunit* was used to immortalize and developmentally arrest cells that expressed the α-subunit and responded to gonadotrophin-releasing hormone (43). A longer version of this promoter (5.5 kb), which expressed earlier, was used subsequently to establish more primitive α-subunit expressing cells, and the β-subunit leutinizing hormone promoter was used to make cell lines from later stages in the gonadotroph lineage (44). These and other cell lines generated in similar fashion have made significant contributions to our understanding of the genetic pathways of pituitary lineage-specific development (45–47). The *mK10* cell might also be developmentally arrested. The same *Hoxa 11-SV40 Tag* transgene was used to make the *mK3* kidney cell line from mice with wild-type *Hoxa 11* and *Hoxd 11* genes. The *mK3* cells are able to drive branching morphogenesis of the ureteric bud in organ coculture, showing they maintain early metanephric mesenchyme function (48).

Most of the candidate targets identified showed only a modest response to *Hoxa 11* expression, consistent with the results of similar studies (13–15). The biological significance of such 1.5- to 3-fold changes in expression level is uncertain. Nevertheless, at least two of these genes are of potential interest in kidney development. The growth factor *PDGF A* is expressed in newly formed epithelial cells derived from the condensing mesenchyme (35). *Hoxa 11* is expressed in the condensing mesenchyme until the cells begin to convert to epithelial cells. These complementary patterns are consistent with the observed repression of *PDGF A* by *Hoxa 11* in *HEK293* cells. Mutation of *PDGF A* results in defects in lung (49) and central nervous system (50) development, but no apparent kidney malformations. This could be the result of continued expression of functionally redundant *PDGF B* and *PDGF C* in the metanephric mesenchyme (51). The *GCM1* gene is of interest because its mutation gives a branching morphogenesis defect in the chorioallantoic placenta, resulting in embryo death at E10, before kidney development. Expression of *GCM1* has been detected in the E16.5 kidney by RT-PCR (37).

The *Integrin α8* gene showed a striking up-regulation in response to *Hoxa 11* expression in both the *HEK293* and *mK10* cells. The *Integrin α8* gene is usually expressed in the metanephric mesenchyme cells abutting the ureteric bud, where *Hoxa 11* is also expressed (1,16). Mutation of the *Integrin α8* gene gave a severe defect in the growth and branching of the ureteric bud (16), similar to that seen in the *Hoxa11/Hoxd 11* mutants (1). The *Integrin α8* gene therefore represented an excellent candidate effector of *Hoxa 11* in kidney development.

In vivo confirmation of the requirement of *Hoxa 11/Hoxd 11* function for normal *Integrin α8* expression was provided by *in situ* hybridizations showing reduction of *Integrin α8* expression in *Hoxa 11/Hoxd 11*mutant kidneys. Of interest, this reduction was regional, with the ventral domain of the kidney most severely effected. The relatively normal expression of *Integrin α8* in restricted areas may explain the frequent formation of a rudimentary kidney, rather than no kidney at all. At least seven Hox genes are expressed in the metanephric mesenchyme (48), some of which may provide localized functional redundancy with *Hoxa 11/Hoxd 11*, thereby explaining the limited continued expression of *Integrin α8*.

The integrins, as extracellular matrix receptors, are generally associated with receiving, not sending signals. Yet mutation of *Hoxa 11/Hoxd 11* and *Integrin α8*, which are expressed in the mesenchyme, give defects in the branching of the ureteric bud. These defects likely reflect the considerable crosstalk between the mesenchyme and bud during kidney development. Failure of the mesenchyme to receive a signal from the bud properly could then result in a deficiency of signaling from the mesenchyme to the bud.

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