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A catalogue of gene expression in the developing kidney

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A catalogue of gene expression in the developing kidney.

Background. Although many genes with important function in kidney morphogenesis have been described, it is clear that many more remain to be discovered. Microarrays allow a more global analysis of the genetic basis of kidney organogenesis.

Methods. In this study, Affymetrix U74Av2 microarrays, with over 12,000 genes represented, were used in conjunction with robust target microamplification techniques to define the gene expression profiles of the developing mouse kidney.

Results. Microdissected murine ureteric bud and metanephric mesenchyme as well as total kidneys at embryonic day E11.5, E12.5, E13.5, E16.5, and adult were examined. This work identified, for example, 3847 genes expressed in the E12.5 kidney. Stringent comparison of the E12.5 versus adult recognized 428 genes with significantly elevated expression in the embryonic kidney. These genes fell into several functional categories, including transcription factor, growth factor, signal transduction, cell cycle, and others. In contrast, surprisingly few differences were found in the gene expression profiles of the ureteric bud and metanephric mesenchyme, with many of the differences clearly associated with the more epithelial character of the bud. In situ hybridizations were used to confirm and extend microarray-predicted expression patterns in the developing kidney. For three genes, *Cdrap*, *Tgfbi*, and *Col15a1*, we observed strikingly similar expression in the developing kidneys and lungs, which both undergo branching morphogenesis.

Conclusion. The results provide a gene discovery function, identifying large numbers of genes not previously associated with kidney development. This study extends developing kidney microarray analysis to the powerful genetic system of the mouse and establishes a baseline for future examination of the many available mutants. This work creates a catalogue of the gene expression states of the developing mouse kidney and its microdissected subcomponents.

The kidney provides a powerful model system for study of the principles of organogenesis. The developing kidney employs many developmental mechanisms, including budding, reciprocal inductive tissue interactions, stem cell growth and differentiation, cell polarization, mesenchyme to epithelia transformation, branching morphogenesis, angiogenesis, apoptosis, fusion (nephrons to collecting ducts), proximal-distal segmentation (along the length of the nephron), and the differentiation of several interesting cell types. Furthermore, nephrogenesis proceeds readily in culture [1], providing an important experimental advantage.

Significant advances have been made in understanding the genetic basis of kidney organogenesis. The uninduced metanephric mesenchyme produces glial-derived neurotrophic factor (GDNF), which interacts with the ret receptor on the ureteric bud to promote outgrowth. Mice with mutation of either the *GDNF* or *ret,* or its coreceptor the *GFRa1* gene, have severe failure of kidney development [2, 3]. There is also strong evidence implicating pleiotrophin as a mesenchyme-synthesized inducer of the ureteric bud and branching morphogenesis [4]. The initial signaling from the bud to the metanephric mesenchyme is less well understood, but Wnt6, synthesized by the bud and able to induce tubulogenesis in vitro, is a candidate [5].

Kidney organ culture and cell line studies have implicated a number of genes in kidney development, including *midkine* [6], *phosphatidylinositol 3 kinase* (*PI3K*) [7], *transforming growth factor-β1* (*TGF-β1*) [8], *galectin-3* [9], *bone morphogenetic protein-4* (*BMP-4*) [10], *vascular endothelial growth factor* (*VEGF*) [11], *hepatocyte growth factor* (*HGF*) [12], and others.

Gene-targeting studies have shown that *Lim1* [13], *Hoxa 11/Hoxd 11* [14–16], *Eya1* [17], *WT-1* [18], *Sall1* [19], α_3 β_1 *integrin* [20], α_8 β_1 *integrin* [21], *Emx-2* [22], *FOXd1* (*BF-2)* [23], *RARa*, and *RARb* [24] are all essential for kidney development. BMP-7 is essential for continued kidney growth [25, 26], and Wnt4, synthesized in the mesenchyme, is required for development past the aggregate stage of nephrogenesis [27]. Cadherin-6 promotes mesenchyme to epithelia conversion and nephron formation [28]. N-Myc promotes cell proliferation [29]. LIF appears an important signal from the later ureteric

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bud to the differentiating nephrons [30]. Despite this impressive progress, it is clear that we have only begun to understand the genetic basis of kidney formation.

Microarrays offer the opportunity to determine global definitions of the gene expression states of developing organs. As microarrays approach comprehensive coverage of the genome, it is becoming possible to assay expression levels of every gene. As target amplification procedures become more powerful, it is becoming possible to perform microarray analysis with ever-decreasing amounts of starting RNA. The ultimate goal is to define the complete gene expression patterns of individual cell types as they progress through kidney organogenesis.

Microarrays have been used to generate gene expression profiles of cell lines representing specific stages of kidney development [31]. The mK3 and mK4 cells correspond to early metanephric mesenchyme and later metanephric mesenchyme, undergoing epithelial transformation. Microarrays identified thousands of genes expressed in these two cell types and thereby implicated them in early kidney development. Comparison of the mK3 and mK4 gene expression profiles found a large number of genes differently expressed, likely reflecting changes in gene expression during epithelial transformation [31].

In another study, microarrays were used to examine kidney development in the rat [32]. Cluster analysis identified five groups of genes with interesting expression patterns. The entire set of microarray data, for 8741 genes, has been made available (*http://organogenesis.ucsd.edu/*). This work represents an important step in the use of microarrays to perform a global analysis of gene expression states in the developing kidney.

In this report,we extend these previous studies by performing a microarray analysis of early kidney development in the mouse. Affymetrix murine U74Av2 gene chip probe arrays with over 12,000 genes represented were used. Robust microamplification techniques were used to allow study of extremely small samples. Gene expression profiles were determined for microdissected embryonic day E11.5 ureteric buds and metanephric mesenchyme as well as E11.5, E12.5, E13.5, E16.5, and adult total kidneys. The results identified extensive sets of genes, of multiple functional categories, expressed in developmental timing and compartment specific patterns. In situ hybridizations were used to corroborate microarray expression data and to further define the expression patterns of selected genes during kidney development. This work creates a catalogue of the gene expression states of the total developing mouse kidney and selected subcomponents. This provides a baseline for the analysis of the many mouse mutants available with altered kidney development.

METHODS

Dissection of early metanephric and adult tissues and RNA isolation

E11.5 and E12.5 kidneys were dissected from wild-type CD-1 mice, pooled, and frozen at −80°C. Ureteric buds and metanephric mesenchyme were microdissected from E11.5 metanephric kidneys following mild trypsinization, pooled separately, and then frozen at −80°C. E13.5, E16.5, and adult kidneys were isolated and frozen at −80°C. All dissections were performed in phosphate-buffered saline (PBS).

Total RNA was prepared from ureteric bud, metanephric mesenchyme, and early kidney samples using Stratagene Absolutely RNA Nanoprep kit (La Jolla, CA, USA) for small samples. Adult kidney and P1 whole mouse total RNA was prepared using RNAzol (Tel-Test, Friendwood, TX, USA).

RNA amplification and target RNA isolation

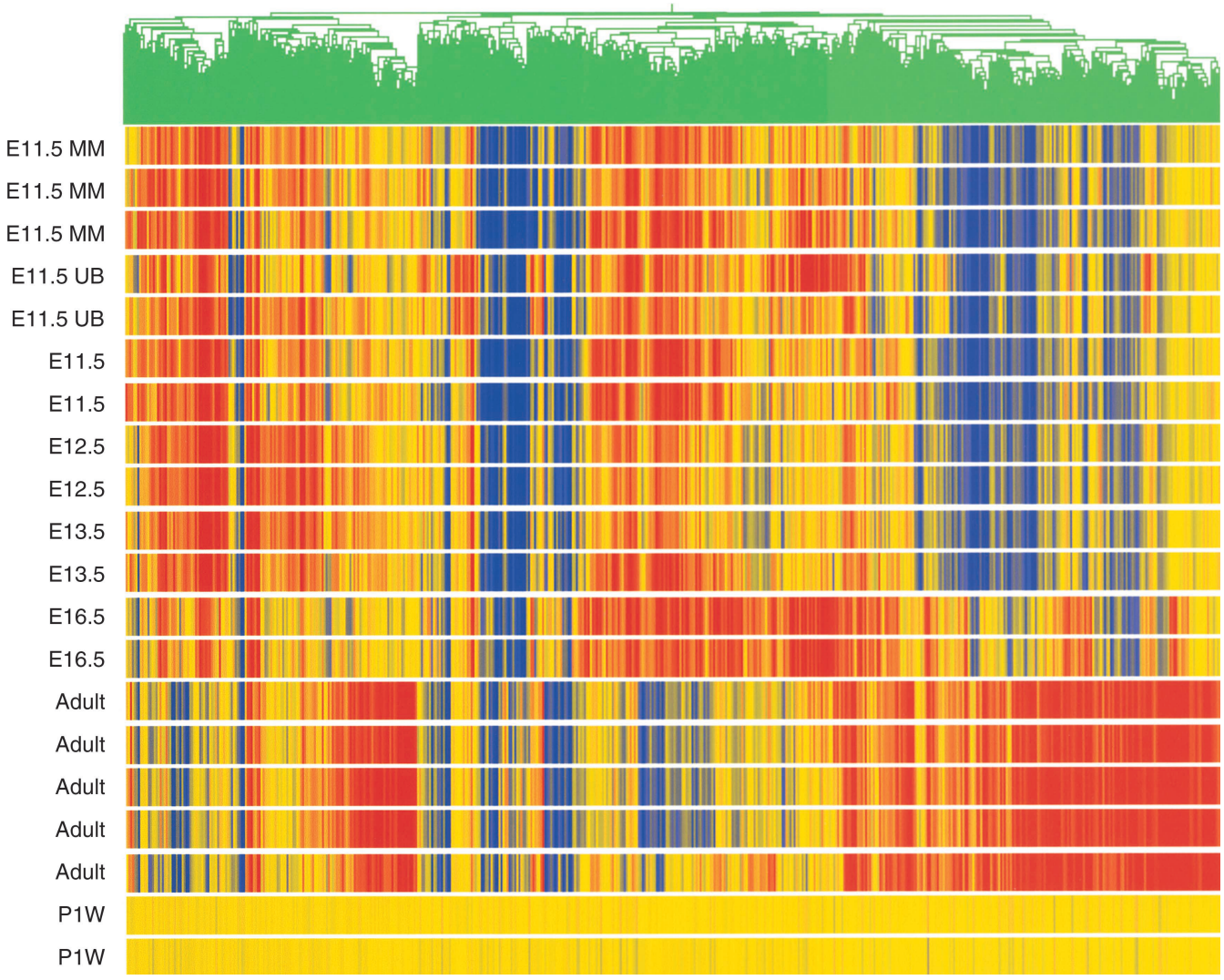
Total RNA was linearly amplified by using a previously described procedure [33] with 30 ng (ureteric bud, metanephric mesenchyme, and two adult kidney replicates) or 100 ng (remaining samples, including two adult kidney replicates). Briefly, total RNA was reverse transcribed into cDNA using a T7 promoter-dT primer [5′-GGCCAGTGAATTGTAATACGACTCACTATA GGGAGGCGG-$(T)_{24}$], amplified through an in vitro transcription reaction using T7 RNA polymerase, and the products then reverse transcribed into cDNA again, using random hexamer primers. A final in vitro transcription reaction using the Bioarray High Yield RNA transcript labeling kit (Enzo Life Sciences, Farmingdale, NY, USA) was performed producing biotinylated cRNA for microarray hybridization.

Microarray analysis of each developmental stage/tissue of the developing kidney and adult kidney was performed in biologic duplicate by obtaining tissues from different mice. In addition, technical replicates of the same adult kidney total RNA sample were performed using both 30 ng and 100 ng amounts of total RNA. Technical duplicates were also performed from the same P1 whole mouse total RNA preparation. The same amplification procedure was used for all samples.

Gene expression profile analysis

Amplified, biotinylated cRNA samples were hybridized to Affymetrix murine U74AV2 microarrays, according to standard procedures as described by Affymetrix (Santa Clara, CA, USA).

Both Microarrary Suite 5.0 (Affymetrix) and GeneSpring 4.2.1 and 5.1 (Silicon Genetics, Inc., Redwood City, CA, USA) software were used for data analysis. For cluster analysis, data were normalized using per chip scaling in RMA [Affymetrix package in R that has been



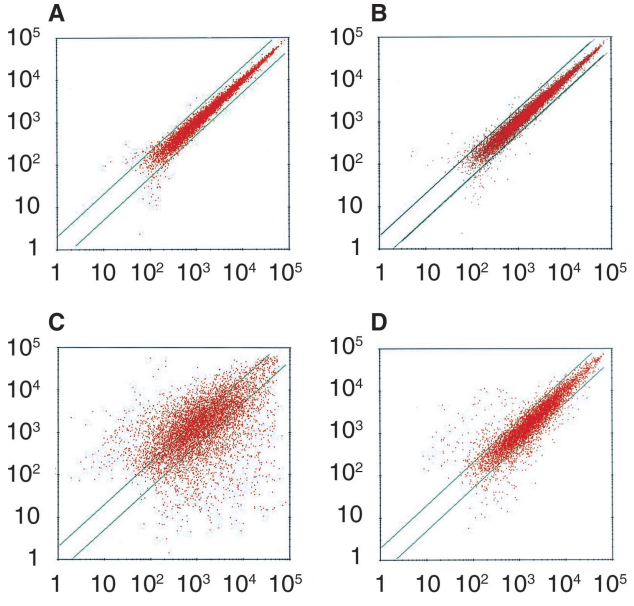


Fig. 1. Scattergram comparisons of microarray hybridizations. (*A*) Technical replicate. Two 30 ng aliquots from a single adult kidney RNA sample were processed in parallel and hybridized to two U74Av2 microarrays. The resulting scattergram shows less than 1% of genes with more than a twofold difference in hybridization signal. (*B*) Biologic replicate. RNA samples from two separately dissected E12.5 kidneys were processed in parallel. The resulting scattergram shows more variation than for the technical replicate, but again with well below 1% of genes giving over a twofold difference in hybridization signal. (*C*) Comparison of E12.5 and adult kidney microarray hybridization patterns. A high level of biologic variation is observed. (*D*) Comparison of E11.5 ureteric bud and metanephric mesenchyme. Scattergrams show comparisons of hybridization signals. Only genes with sufficient signal to be called present by MAS5 for at least one of the two samples being compared are shown. The scattergrams show comparisons of raw hybridization signals of each probe set. For (A), for example, this results in 101 probe sets called over twofold different in expression level on the two microarrays. The MAS5 comparison, however, performs a more sophisticated analysis of the probe set hybridization data and identified only 43 genes with over a twofold difference. For both methods twofold outliers are below 1% of total.

Fig. 2. Hierarchical cluster analysis of genes expressed in the early developing and adult kidney. (*A*) Cluster analysis used several analysis of variance (ANOVA)-based statistical criteria, which identified genes strongly expressed in developing or adult kidney. Genes with similar expression profiles were clustered using hierarchical tree algorithm applied to the log relative gene expression values using Pearson correlation. Rows represent the different tissues examined. Abbreviations are: MM, metanephric mesenchyme; UB, ureteric bud; W, whole mouse; all others, total kidney. Red represents high expression, blue represents low expression, and yellow is intermediate, normalized to P1 whole mouse. A more complete set of comparisons is available at *http://genet.chmcc.org* (login is Nephrome, password is reviewer, click login, not login as guest, click continue on U74Av2_mouse).

developed by the Bioconductor Consortium (*http://bioconductor.org*)] followed by transformation of log2 signal back to linear signal that was then normalized to the day 1 whole mouse reference. All group to group comparisons (metanephric mesenchyme:ureteric bud, E11.5:E12.5, E11.5:E13.5, E11.5:E16.5, E12.5:E13.5, E12.5:E16.5, E13.5:E16.5, and adult kidney:whole mouse for genes with relative expression more than twofold higher in adult kidney) were performed using Welch *t* test analysis of variance (ANOVA) with $P < 0.05$ without multiple testing rate correction because most samples were collected and analyzed with two replicates. Genes that passed the ANOVA were then ranked by average fold difference with cutoffs used that were appropriate for each comparison (>∼twofold for each group), pooled, and converted back to log expression ratios, which were then subjected to the hierarchical tree clustering algorithm with the Pearson distance metric as implemented in GeneSpring 5.1 (Silicon Genetics, Inc.). All Affymetrix.cel files, RMA results, gene lists, and gene trees are available at the following website: *http://genet.chmcc.org* (login is Nephrome, password is reviewer, click login, not login as guest, click continue on U74Av2_mouse).

RNA tissue section in situ hybridization

E12.5, E13.5, E15.5, and E17.5 embryos were obtained from CD-1 mice. Embryos and tissues underwent fixation and embedding as previously described [15]. Four micron sections, including the developing kidney, were made. In situ hybridizations to RNA in tissue sections were performed using a ^{33}P-uridine triphosphate (UTP)-labeled riboprobe generated from cDNA clones. The following probes were made from the corresponding cDNA clone: *Birc5*, 373242; *Capn5*, 733856; *Cdrap*, 427314; *Col15a1*, 406344; *Mns1*, 480118; *Nr2f1*, 420535; *Rab6kifl*, 764732; *Smoh*, 439010; and *Tgfbi*, 734101. All cDNA clones were obtained from the Incyte GEM1 mouse library (University of Cincinnati Genomics and Microarray Laboratory). Slides were then dipped in Kodak NTB-2 nuclear track emulsion and exposed for 4 weeks at 4°C. After exposure, slides were developed using Kodak D-19 developer at 15°C. Slides were stained with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI).

RESULTS

The goal of this work was to establish a reference data set of gene expression states during normal early mouse kidney development. This data set could facilitate the identification of new genes and pathways involved in kidney development and provide a standard for microarray studies of the many mouse mutants available with abnormal kidney development.

Reproducibility of the target amplification procedure

Powerful amplification techniques were necessary to analyze the small samples provided by the early embryonic mouse kidneys and their microdissected components. Several protocols were tried and the one described by Baugh et al [33], using two rounds of in vitro transcription, gave excellent results. Technical replicates were performed to measure reproducibility of the resulting microarray data. Total RNA was prepared from a single adult kidney and multiple small aliquots were removed and used in parallel for target amplification and hybridization to Affymetrix murine U74Av2 microarrays. Figure 1A shows a scattergram comparing the raw hybridization signals from two samples of 30 ng of total RNA. Each point represents a single gene, with the hybridization levels from the two microarrays shown on the two axes. A single line at 45° would designate perfect reproducibility. The two lines flanking the center line indicate points of twofold difference in signal intensity in the two microarray hybridizations. Figure 1A only shows genes with sufficient hybridization signal to be called expressed (*P*) by Microarray Suite 5.0 (MAS5). We observed a high level of technical reproducibility for these genes with well below 1% (43/12488) called more than a twofold different in expression by MAS5. Genes with very low levels of expression, near noise, and called absent or not-expressed by MAS5, gave more variation, but in performing analyses, we used expression level cutoffs to exclude these genes.

Biologic duplicates

In this study all kidney samples, embryonic and adult, were examined in biologic duplicate, using RNA from separate biologic samples. Figure 1B illustrates the reproducibility of biologic duplicates for E12.5 kidneys. In this case, the biologic noise was added to the technical

Table 1. Elevated expression in the E12.5 embryonic kidney

Name	Description	Fold change[a]	Name	Description	Fold change[a]
		Cell adhesion and extracellular matrix			
Bysl	Bystin-like	4.0	Emu2	Emu2 gene	12
Cdh2	Cadherin 2	26	Gja1	Gap junction channel protein alpha 1	20
Cldn6	Claudin 6	48	Gpc3	Glypican 3	21
Col5a2	Procollagen, type V, alpha 2	5.7	Mfap2	Microfibrillar-associated protein 2	34
Cspg2	Chondroitin sulfate proteoglycan 2	150	Ncam1	Neural cell adhesion molecule 1	8.6
Cspg6	Chondroitin sulfate proteoglycan 6	14	Tgfbi	Transforming growth factor beta induced	7.0
Efs	Embryonal Fyn-associated substrate	4.6			
		Growth factor and growth factor related			
Fstl	Follistatin-like	6.1	Mdk	Midkine	4.9
Gdnf	Glial cell line-derived neurotrophic factor	9.8	Sfrp2	Secreted frizzled-related sequence protein 2	7.5
Hdgfrp2	Hepatoma-derived growth factor, related protein 2	6.1			
		Kinases and phosphatases			
EST	RIKEN cDNA 2310076D10 gene	20	Stk18	Serine/threonine kinase 18	21
Akap8	A kinase (PRKA) anchor protein 8	5.7	Stk5	Serine/threonine kinase 5	16
Cks1	CDC28 protein kinase 1	30	Stk6	Serine/threonine kinase 6	28
Fbln1	Fibulin 1	10			
		Receptors			
Crlf1	Cytokine receptor-like factor 1	11	Nr2f1	Nuclear receptor subfamily 2, group F, member 1	56
Ephb4	Eph receptor B4	13	Unc5h3	Unc5 homolog (*C. elegans*) 3	9.8
Fzd2	Frizzled homolog 2 (*Drosophila*)	4.9			
		Signaling			
Arhu	Ras homolog gene family, member U	6.1	Racgap1	Rac GTPase-activating protein 1	12
Crmp1	Collapsin response mediator protein 1	11	Ranbp1	RAN binding protein 1	5.7
Ect2	Ect2 oncogene	7.0	Rasl2-9	RAS-like, family 2, locus 9	3.7
Lag	Leukemia-associated gene	5.3	Rcn	Reticulocalbin	7.5
Lsp1	Lymphocyte specific 1	4.9, 8.0[b]	Trip13	Thyroid hormone receptor interactor 13	39
Mlp	MARCKS-like protein	9.8	Wsb1	WD-40-repeat-containing protein with SOCS box 2	6.5

(*Continued*)

Table 1. Continued

Name	Description	Fold change[a]	Name	Description	Fold change[a]
		Splicing			
EST	RIKEN cDNA 4930512K19 gene	5.7	Sfrs2	Splicing factor, arginine/serine-rich 2 (SC-35)	4.8
Igfbp2	Insulin-like growth factor binding protein 2	11	Sfrs3	Splicing factor, arginine/serine-rich 3 (SRp20)	6.6
Lamr1	Laminin receptor 1 (67 kD, ribosomal protein SA)	9.2	Sfrs9	Splicing factor, arginine/serine rich 9 (25 kD)	5.7
Rnps1	Ribonucleic acid binding protein S1	5.7	Silg41	Silica-induced gene 41	5.3
Sfrs10	Splicing factor, arginine/serine-rich 10	5.7	U2af1	U2 small nuclear ribonucleoprotein auxiliary factor (U2AF)	4.0
		Transcription			
Baf53a	BRG1/brm-associated factor 53A	7.5	Meox2	Mesenchyme homeobox 2	720
Cbfb	Core binding factor beta	4.9	Mrgx	MORF-related gene X	6.1, 8.0[b]
Cbx2	Chromobox homolog 2	6.1	Ndn	Necdin	14
Cnbp	Cellular nucleic acid binding protein	3.7	Nfyb	Nuclear transcription factor-Y beta	5.7
Dnmt1	DNA methyltransferase 1	6.1	Nmyc1	Neuroblastoma myc-related oncogene 1	21
Eed	Embryonic ectoderm development	7.0	Pbx3	Pre B-cell leukemia transcription factor 3	4.3
Erh	Enhancer of rudimentary homolog	5.7	Pttg1	Pituitary tumor-transforming 1	6.5
Ezh2	Enhancer of zeste homolog 2	39	Setdb1	SET domain, bifurcated 1	3.7
Foxc2	Forkhead box C2	7.0, 11[b]	Six2	Sine oculis-related homeobox 2	18
Foxd1	Forkhead box D1	29.9	Smarce1	SWI/SNF related, subfamily e, member 1	9.2
Gtf2e2	General transcription factor IIE, 2	6.5	Snai2	Snail homolog 2 (*Drosophila*)	9.2
Gtf2h1	General transcription factor II H, 1	16	Sox11	SRY-box containing gene 11	32
Hdac1	Histone deacetylase 1	5.3	Taf1a	TBP-associated factor, RNA polymerase I, A	9.2
Hdac2	Histone deacetylase 2	9.2	Taf9	TAF9 RNA polymerase II, TBP-associated factor	6.0, 6.5[b]
Hmga2	High mobility group AT-hook 2	34	Tcf12	Transcription factor 12	6.5
Hmgb2	High mobility group box 2	32	Tead2	TEA domain family member 2	24
Hmgb3	High mobility group box 3	6.1	Tgif	TG interacting factor	4.3
Hmgn2	High mobility group nucleosomal binding 2	5.7	Trim27	Tripartite motif protein 27	3.7
Hoxa11	Homeo box A11	6.1	Trp53bp1	Transformation related protein 53 binding protein 1	7.5
Hoxd11	Homeo box D11	7.5	Zfhx1a	Zinc finger homeobox 1a	4.3
Ilf3	Interleukin enhancer binding factor 3	4.3	Zfp105	Zinc finger protein 105	6.5
Lama4	Laminin, alpha 4	6.1	Zfp61	Zinc finger protein 61	7.0
Maged1	Melanoma antigen, family D, 1	4.6	Zipro1	Zinc finger proliferation 1	5.7
Mcmd4	Mini chromosome maintenance deficient 4	17			

[a] Fold change denotes the average fold change. Signal log ratio (SLR) for each probe set was generated using comparison files from Affymetrix MAS 5.0 and converted into fold change according to MAS 5.0 documentation.
[b] Genes represented by two probe sets on the microarrays and the corresponding fold change for each

noise, and as expected, more variation was observed than for technical replicates. Nevertheless, in biologic replicates, there were still well below 1% of genes showing more than a twofold difference in gene expression.

This modest noise, of less than 1%, is almost entirely eliminated by performing experiments in biologic duplicate. The 1% of the approximately 12,000 genes on a microarray is 120 genes, a significant number. But the noise is largely random, so only 1% of these 120 genes, or approximately one gene, would again show noise-related variation on the duplicate.

The use of biologic duplicates provides a minimum of two times two, or four, comparisons between two sample types. By screening out genes with low expression levels and genes with less than threefold change in expression, we have previously observed over 95% of genes identified as differently expressed in such comparisons are validated by Northern blot, reverse transcription-polymerase chain reaction (RT-PCR), and/or Western blot [31].

Gene expression state of the E12.5 kidney

The set of genes expressed in the embryonic kidney defines its potential for growth and development. Microarrays, by providing a more comprehensive and unbiased view, can promote the discovery of new genes not previously implicated in developmental processes. For example, 5542 genes were called expressed in both biologic duplicates of the E12.5 kidney by MAS5, with the detection *P* value set at the default 0.04. Errors in absence-presence calls occur most often for genes expressed at a low level, near noise. Excluding genes with expression signal below 100 [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal, for example, was 5237 and 5149 for the replicates], MAS5 called 3914 genes expressed. Further screening of these genes by selection with a detection *P* value of less than or equal to 0.03 found 3847 genes expressed in the E12.5 developing kidney. This list of genes active during early kidney development provides an important foundation for further study of kidney morphogenesis. This gene collection is too extensive for detailed discussion, but the data set is fully provided at a supplementary Web site. All Affymetrix. cel files, RMA results, gene lists, and gene trees are available at *http://genet.chmcc.org* (login is Nephrome, password is reviewer, click login, not login as guest, click continue on U74Av2_mouse).

Genes with elevated expression levels in the E12.5 kidney

Among the genes expressed in the developing kidney are housekeeping genes and others of relatively little developmental consequence, many of which will be expressed in both the embryonic and adult kidney. Of particular interest from a development perspective are the genes comparatively more active in the early developing kidney, which might play important roles in the many processes of kidney formation. A scattergram presentation of the many differences in the gene expression patterns of the E12.5 and adult kidneys is shown in Figure 1C. We performed a stringent screen of the microarray data to identify genes with elevated expression levels in the E12.5 embryonic kidney when compared to adult. The E12.5 microarray analysis was performed in biologic duplicate, and the adult kidney analysis was performed in biologic duplicate, with one sample analyzed in technical quadruplicate, giving a total of five adult microarrays, and allowing a total of ten crosswise comparisons, between the two embryonic and five adult microarray hybridizations. The microarray analysis software MAS5 found 1858 genes with consistently increased expression levels in the embryonic kidneys versus adult in all ten comparisons. Further screening of these genes required that all ten comparisons show greater than threefold (signal log ratio of 1.7) higher expression in embryonic kidney, and that the expression signal in embryonic kidney be greater than 150, to further reduce possible artifacts due to low signal to noise ratio.

This rigorous screen identified 428 genes with significantly elevated expression in the embryonic kidney. Approximately 10% of these genes are involved in the regulation of transcription (Table 1). Some of these (*Taf1a*, *Gtf2e2*, *Gtf2h1*, and *Taf9*) can be grouped as general transcription factors. Others play an important role in regulating gene expression through their effects on chromatin configuration. These include the histone deacetylases genes *Hdac1* and *Hdac2*, the *SWI/SNF*-related *Smarce1*, BRG/brm-associated factor *53A*, the HMG box genes *Hmgb2* and *Hmga2*, the chromobox homolog *Cbx2*, the histone methylase gene silencer *Setdb1*, and the Polycomb group genes *eed* and *EZH2*, which form a complex together [34], and inactivate *Hox* genes through histone methylation [35].

Another group of genes were found that likely encode transcription factors based on motif, protein interactions, or strong homologies to known transcription factors. These include the zinc finger encoding genes *Zfp105* and *Zfp61*, the zinc finger homeobox gene *Zfhx1a*, the HMG gene *Hmgn2*, and others such as *Trp53bp1*, *Cbfb8*, *Mcmd4*, *Cnbp6*, and *Trim27*.

The E12.5 kidney also expressed 19 better-characterized transcription factor genes at elevated levels. Several of these have been previously implicated in kidney development. The only *Hox* genes identified by these strict screening criteria were *Hoxa 11* and *Hoxd 11*, which give absent or rudimentary kidneys when both are mutated [14, 15]. *Pbx3*, encoding a homeodomain protein that interacts with Hox proteins was also identified. *Sox11* was previously associated with mesenchyme-epithelia transformation in kidney development by a differential display study [36]. The *Six2* gene was previously shown

to be down-regulated in compound *Hoxa 11*, *Hoxd 11*, *Hoxc 11* triple mutant kidneys [16]. The *Six*, *Eya*, and *Dach* genes form a regulatory network in several developing systems, and *EYA1* mutant mice have absent kidneys [17]. *Tcf12* encodes a basic helix loop helix transcription factor. The forkhead *FOXd1* (*BF-2*) gene, with important function in stromal cells [23], was expressed. The *FOXc2* gene, also encoding a forkhead transcription factor, was also expressed at elevated levels at E12.5. *FOXc2* has been implicated in the repression of GDNF signaling, with *FOXc1* mutants showing urinary tract duplications and the *FOXc1* and *FOXc2* genes showing considerable functional redundancy in both cardiovascular and kidney development [37]. *N-Myc* was also identified, and mutation of *N-Myc* gives poorly developed kidneys [29]. The mesenchyme homeobox gene *Meox2* is of interest. The *Meox* genes, also called *Mox*, have been shown to interact with PAX proteins [38], which are known to play a major role in kidney development. In addition, the *Meox* genes have been implicated in mesenchyme-epithelia interactions [39], again important in kidney formation. Other transcription factor genes identified include *Zipro*, a zinc finger proliferation gene, *Pttg1*, a pituitary tumor transforming gene, *Maged1*, Tea Domain gene *TEAD2*, the zinc finger *Snail2*, the interleukin binding factor *Ilf3*, the MORF-related *Mrgx*, and *ERH* (enhancer of rudimentary homolog).

It is clear from the microarray data that a major function of the early kidney is simply to grow. Thirty five cell cycle–associated genes were found elevated in expression in the E12.5 kidney, consistent with a high rate of cell division. An additional 90 genes with high expression in the developing kidney were involved in intermediary metabolism, ribosome biogenesis, DNA synthesis, and other processes connected with rapidly dividing cells. A similar conclusion was drawn in a previous microarray study of rat kidney development [32].

Twenty one genes with roles in signaling were expressed at elevated levels in the mouse embryonic kidney. Several of these are involved in guanosine triphosphatase (GTPase)-mediated signaling, including RAN binding protein 1 (*Ranbp1*), Rac GTPase activating protein *Racgap1*, the oncogene *ect2*, and the ras homologs *Arhu* and Ras-like family 2 locus 9 (*Rasl2-9*). Other genes of interest were *2310076D10*, involved in mitogen-activated protein kinase (MAPK) inactivation, and the *Wsb1* gene, with a SOCS (suppressor of cytokine signaling) box.

Growth factor–related genes detected by this stringent screen in the early kidney included *GDNF*, *midkine*, secreted frizzled-related protein 2 (*Sfrp2*), follistatin-like protein (*FSTL*), and hepatoma-derived growth factor–related protein2 (*Hdgfrp2*). Although kidney development functions for *GDNF* and *midkine* have been extensively investigated [2, 6], the possible roles of *Sfrp2*, *Hdgfrp2* and follistatin-like protein in kidney formation are largely unexplored.

Several interesting extracellular matrix (ECM)–encoding genes were expressed at elevated levels in the developing kidney. Chondroitin sulfate proteoglycan 6 (also called Bamacan) has been previously associated with mesangial cells and tubulogenesis. Fibulin 1 and laminin alpha 4 were also expressed, as was collagen type V alpha 2. *Glypican 3* mutations have been linked to kidney malformations in both mice and humans [40, 41], likely due to altered growth factor signaling [42]. Other ECM genes of interest include *microfibrillar-associated protein 2* and *Emu2*. The *Emu2* gene is often expressed in mesenchymal cells undergoing mesenchymal-epithelial interactions [43].

Many other genes of interest were found expressed at elevated levels in the early embryonic kidney. These included the protease inhibitors *Serpinf1* and *Serpinh1*. In addition, six receptor encoding genes were found, including the nuclear receptor *Nr2f1* (*COUP-TF1*), the ephrin receptor *Ephb4*, the frizzled receptor *Fzd2*, the *laminin receptor 1*, the netrin receptor *Unc5h3*, and the cytokine receptor-like factor *Crlf1*. Several of these receptors can be linked to known processes in kidney development. For example, it is known that the ureteric bud cell line upregulates the expression of the membrane-bound ephrins when undergoing branching morphogenesis in culture [44]. There were also 125 expressed sequence tags (ESTs) of currently unknown function that showed high expression levels in the developing kidney.

It is important to emphasize that this list of 428 genes does not represent a complete compilation of developmentally important genes expressed in the early developing kidney. It excludes, for example, many *Hox* genes expressed in both the developing and adult kidney. The high stringency of the screen also eliminated some genes of known importance in kidney development. Other genes were not represented on the microarrays. This study does, however, implicate a large number of interesting new genes in the process of kidney formation.

Genes differentially expressed between the E11.5 metanephric mesenchyme and ureteric bud

Gene expression profiles were determined for isolated ureteric bud and metanephric mesenchyme of the early developing kidney. These two elements were separated by microdissection from E11.5 kidneys following mild trypsinization. The similarity of the gene expression patterns of these two compartments that give rise to the kidney is shown in Figure 1D. Using a parametric t test ($P < 0.05$) we identified only 78 genes that were reproducibly differentially expressed between the E11.5 metanephric mesenchyme and ureteric bud (Table 2). This relatively short list of consistent differences indicates that the gene expression states of the metanephric mes-

Table 2. A partial list of enriched genes and corresponding fold change from comparison of E11.5 metanephric mesenchyme and ureteric bud

E11.5 ureteric bud			E11.5 metanephric mesenchyme		
Name	Description	Fold change[a]	Name	Description	Fold change[a]
	Cell adhesion and extracellular matrix			Cell adhesion and extracellular matrix	
Cdh	Cadherin 16	16		None	
Cldn3	Claudin 3	9.8, 18.4[b]			
Cldn4	Claudin 4	16.3			
Cldn7	Claudin 7	29.9			
Cldn8	Claudin 8	4.5			
Emb	Embigin	4.7			
Lamc2	Laminin, gamma 2	10.6			
Ppl	Periplakin	2.5			
	Cell cycle			Cell cycle	
Cdkn1a	Cdk inhibitor 1a (p21)	2.8, 4.9[b]	Cnn2	Calponin 2	3.5
	Protein modifiers and enzymes			Protein modifiers and enzymes	
Capn5	Calpain 5	2.1	Alas2	Aminolevulinic acid synthase 2	3.6
Hpn	Hepsin	3.4	Lyzs	Lysozyme	5.6
Tmprss2	Transmembrane protease, ser 2	6.5			
	Signal transduction			Signal transduction	
Calb1	Calbindin-28k	19	Fes	Feline sarcoma oncogene	2.6
Calcr	Calcitonin receptor	28.3	Klra7	Killer cell lectin-like receptor, A7	2.1
Gfra1	Gdnf receptor alpha 1	3.2	Sgk	Serum/glucocorticoid regulated kinase	3
Itk	Il2-inducible t-cell kinase	3.3	Tek	Endothelial receptor tyrosine kinase	3.1
Ros	Ros1 proto-oncogene	11.9, 16.0[b]	Tie1	Tyrosine kinase receptor 1	9.4
Tacstd2	Tumor-associated calcium signal transducer 2	11.9			
Tgfa	Tgf alpha	3.5			
Tnfrsf19	Tnf receptor 19	4.2			
Vsnl1	Visinin-like 1	5.5			
Wnt6	Wnt 6	9			
	Transcription			Transcription	
En2	Engrailed 2	2.2	C1d	Nuclear DNA binding protein	2.6
Pea3	Polyoma virus enhancer activator 3	2.9	Foxc1	Forkhead box C1	3.8
Tcf2	Transcription factor 2	16.3	Hoxa10	Homeo box A10	3
			Sox2	SRY-box containing gene 2	2.8
			Sox18	SRY-box containing gene 18	6.7
			Wt1	Wilms tumor homolog	9
	Transport			Transport	
	None		Hba-a1	Hemoglobin alpha, adult 1	4.1
			Hbb-b1	Hemoglobin beta, adult 1	4.8
			Vamp5	Vesicle-associated membrane protein 5	6.1

[a] Fold change denotes the average fold change. Signal log ratio (SLR) for each probe set was generated using comparison files from Affymetrix MAS 5.0 and converted into fold change according to MAS 5.0 documentation.

[b] Genes represented by two probe sets and the corresponding fold change for each

enchyme and ureteric bud are surprisingly similar, perhaps reflecting their common intermediate mesoderm origin. We identified 53 genes with increased expression in the ureteric bud, and 25 genes with increased expression in the metanephric mesenchyme.

Ureteric bud–enriched genes

The list of ureteric bud–enriched genes includes many previously shown to be important in metanephric kidney development, or specifically associated with epithelial structures. Ten of the 53 genes more highly expressed in the ureteric bud function in forming cell-cell adhesions or ECM (Table 2). *Claudin* genes *3*, *4*, *7*, and *8* were enriched in the ureteric bud. Claudins are integral membrane proteins that function in forming tight junctions between epithelial cells. Cartilage-derived retinoic acid–sensitive protein is an ECM protein containing a SH3 domain that is repressed in the presence of retinoic acid and functions in cell culture as a tumor growth inhibitor [45, 46]. Cadherin 16 was first identified as a kidney-specific cadherin and is expressed in the epithelia of the developing kidney [47, 48]. Laminin gamma 2 is a subunit of the laminin-5 complex and is found within the basal lamina of the ureteric bud [49]. In vitro studies have shown that ureteric bud branching is decreased when laminin-5 is blocked through use of antibodies to itself and its receptors, the α_3 and α_6 integrins [50].

Genes with elevated expression in the bud also include growth factors and signal transducers. Two identified growth factors, TGF-α and WNT6, were previously implicated in kidney development. TGF-α is expressed in the ureteric bud tips and ducts and is able to induce metanephric kidney growth in vitro, while growth is inhibited by TGF-α antibodies [51, 52]. WNT6 is expressed in ureteric bud and can induce nephrogenesis in vitro [5].

Notable signaling transducers detected in the ureteric

bud include GFRA1, FGFr3, and ROS1. GFRA1 is co-receptor for RET, which is also expressed in the ureteric bud. Mutation of *Gfra1* gives a similar phenotype to the *GDNF* and *Ret* knockout mice, in which there is an absent kidney due to loss of ureteric bud outgrowth [53]. FGFr3, a fibroblast growth factor (FGF) receptor, may function in early signaling within the metanephric kidney, as expression of a secreted dominant-negative FGF receptor in transgenic mice results in kidney agenesis [54].

Several genes involved in calcium-mediated signaling showed elevated expression in the ureteric bud. This is consistent with the observed response of Madin-Darby canine kidney (MDCK) cells, which quickly become polarized and form tight junctions and desmosomes following calcium level elevation [55]. VSN11 is a calcium-dependent regulator of cellular signaling [56]. The calcitonin receptor is known to regulate Ca^{++} excretion in the kidney and might also play a role in organogenesis [57]. Calbindin-28K, a calcium-binding protein, was previously shown to be expressed in the ureteric bud [58], and TACSTD2, a tumor-associated calcium signal transducer, also showed elevated expression the ureteric bud.

Only four ureteric bud elevated genes, including *Tcf2*, *Pea3*, and *En-2*, encoded known transcription factors. *Tcf2* is expressed in the forming collecting ducts in the developing kidney as well as in the liver [59]. Tissue-specific targeting in the liver shows that this gene is essential for bile duct morphogenesis [60], suggesting a possible role in branching morphogenesis in the kidney. Recently, *Pea3* has been implicated in muscle innervation. Of interest, *Pea3* is downstream of GDNF in motor neurons [61]. *En-2*, an engrailed homolog, has been found to regulate boundaries in the developing brain and has not been previously implicated in kidney development [62].

Other genes identified encoded proteases and protein modifiers such as CAPN5, hepsin, and transmembrane protease 2. Also, a cell cycle regulator, *Cdkn1a*, was identified. Other genes with elevated expression in the ureteric bud were involved in metabolism, the cytoskeleton, or of unknown function, including ESTs.

Metanephric mesenchyme–enriched genes

We identified 25 genes more highly expressed in the metanephric mesenchyme than in the ureteric bud. Six of these encoded the transcription factors WT-1, FOXc1, SOX2, SOX18, HOXA 10, and C1D. The *WT-1* gene has previously been shown to be expressed in the metanephric mesenchyme and is required for early kidney development [18]. FOXc1, a forkhead/winged helix transcription factor, as discussed previously, is an apparent regulator of GDNF signaling. *Sox2* and *Sox18* encode transcription factors containing HMG boxes. For *Hoxa 10*, we have confirmed restricted expression in the metanephric mesenchyme (Patterson, unpublished observations, 2003). C1D is a DNA high-affinity binding protein that serves as a nuclear receptor corepressor [63].

Genes encoding four signal transduction proteins, TEK, TIE1, SGK, and FES, were enriched in the metanephric mesenchyme. TEK and TIE1, receptor tyrosine kinases, function in vascular endothelium development and mutations in these genes cause endothelial defects [64, 65]. Of particular interest, TIE-deficient cells were unable to contribute to adult kidney epithelium in chimeras [65]. *Sgk1* encodes a serum/glucocorticoid-regulated kinase. Targeted mutation of *sgk1* gives an impaired sodium retention phenotype [66]. The protein kinase FES has been reported expressed in epithelia cells, hematopoietic cells, and vascular endothelial cells [67]. Transgenic studies indicate a role for FES in angiogenesis [68]. Targeted mutation of the *fes* gene revealed function in cardiovascular development and myeloid cell proliferation [69]. It is interesting that the E11.5 mesenchyme already expressed so many genes (*Sox18*, *tek*, *tie*, and *fes*) associated with vasculogenesis.

Other genes of interest more highly expressed in the metanephric mesenchyme included *Vamp5*, which encodes an integral membrane protein that is enriched during in vitro myogenesis and is highly expressed in heart and skeletal muscle, but with lower levels also detected in the adult kidney and other organs by Northern blot [70]. Adult hemoglobin alpha and beta chains, lysozyme (Lyzs), an antimicrobial enzyme produced by macrophages, and aminolevulinic acid synthase 2 (Alas2), an erythroid-specific enzyme functioning in heme production, are enriched within the metanephric mesenchyme. The elevated expression of these blood-related genes could simply reflect a greater blood content for the metanephric mesenchyme compared to the ureteric bud. Alternatively, it could suggest a possible hematopoietic function for the metanephros, similar to that previously described for the mesonephros. Five EST genes, with unknown functions, were also enriched in the metanephric mesenchyme.

Genes with elevated expression at E16.5

The E16.5 kidney showed elevated expression of many genes when compared to E12.5 (Table 3). Of particular note, there were 32 aquaporin, ion channel, solute carrier, and other transport molecules actively expressed at E16.5, consistent with ongoing tubule maturation. A number of other genes with previously assigned kidney function were also identified, including nephronectin (integrin ligand), polyductin (polycystic kidney disease), neuropilin (semaphorin receptor), uromodulin, glomerular epithelial protein 1, and renin. In addition, a large number of genes not previously associated with kidney development, from a variety of functional categories, were found (Table 3).

Table 3. Elevated expression in the E16.5 embryonic kidney, compared to E12.5

Name	Description	Fold change[a]	Name	Description	Fold change[a]
		Cell adhesion and ECM			
Alcam	Activated leukocyte cell adhesion molecule	5.8	Gpc4	Glypican 4	5.8
Cdh16	Cadherin 16	4.3	Igsf4	Immunoglobulin superfamily, 4	8.6
Cldn1	Claudin 1	7.7	Itga6	Integrin alpha 6	5.0
Cldn11	Claudin 11	5.5	Muc1	Mucin 1	17.4
Dcn	Decorin	8.0	Spp1	Secreted phosphoprotein 1	13.5
Dutt1	Roundabout homolog 1	4.7	Timp3	Tissue inhibitor of metalloproteinase 3	4.1
		Cytoskeletal			
Acta2	Actin, alpha 2	6.3	Myo6	Myosin VI	4.6
Ank3	Ankyrin 3	4.8	Scin	Scinderin	42.2
Enah	Enabled homolog	26.9	Sorbs1	Sorbin and SH3 domain 1	4.4
Kif5b	Kinesin family, 5B	7.0	Sprr2f	Small proline-rich protein 2F	20.7
Myh11	Myosin heavy chain 11	6.6	Vil	Villin	66.3
		Growth factor			
Cish2	Cytokine inducible SH2-containing 2	8.0	Kng	Kininogen	5.3
Ctgf	Connective tissue growth factor	4.2	Nrp	Neuropilin	7.5
Dab2	Disabled homolog 2	7.9	Ogn	Osteoglycin	10.7
Emp1	Epithelial membrane protein 1	4.4	Penk1	Spermatogenic-specific proenkephalin	16.0
Eps8	EGFR pathway substrate 8	10.9	Pkhd1	Polyductin	38.1
Fin15	FGF inducible 15	20.7	POEM	POEM; nephronectin	5.1
Gadd45	GADD45 protein	25.5	Pparbp	Ppar binding protein	10.9
Htk	Ephrin B2	4.4	Sdfr2	Stromal cell derived factor receptor 2	11.9
Igf1r	Insulin-like growth factor I receptor	5.3	Sfrp1	Secreted frizzled-related 1	6.3
Igfbp7	Insulin-like growth factor binding protein 7	8.1	Umod	Uromodulin	26.0
		Kinases and phosphatases			
Csnk1e	Casein kinase 1, epsilon	3.9	Pdpk1	3-PI dependent protein kinase 1	4.1
Emk	ELKL motif kinase	8.1	Ppp1cb	Protein phosphatase 1, beta	5.7
Glepp1	Glomerular epithelial protein 1 precursor	29.9	Snrk	SNF related kinase	6.4
Hipk2	Homeodomain interacting protein kinase 2	6.0	Mylk	Myosin, light polypeptide kinase	7.5
Mekk1	MAP kinase kinase kinase 1	10.4			
		Protease and protease inhibitors			
Cast	Calpastatin	8.0	Serpinf2	Ser or Cys proteinase inhibitor, F2	5.5
Cfi	Complement factor i	28.8	Mep1b	Meprin 1 beta	105.8
Ctsh	Cathepsin H	6.3	Kdap	Kidney-derived Asp protease-like	7.2
		Signaling			
ACE2	Angiotensin-converting related carboxypeptidase	3.8	Ndrl	N-myc downstream regulated-like	5.2
Admr	Adrenomedullin receptor	4.6	Pdzk1	PDZ domain containing 1	137.2
Apbb2	A4 precursor protein-binding, B2	4.4	Pkib	Protein kinase inhibitor beta	6.1
Calb3	Calbindin-D9K	19.0	Procr	Protein C receptor, endothelial	6.0
Calml4	Calmodulin-like 4	10.4	Rasd1	RAS, dexamethasone-induced 1	4.6
Cckar	Cholecystokinin type-A receptor	13.5	Ren1	Renin 1 structural	19.4
Dab2	Disabled homolog 2	6.5	RhoB	Ras homolog gene, AB	4.4
Ednrb	Endothelin receptor type B	84.4	Semcap2	SemaF cytoplasmic associated protein 2	14.9
Gna11	G nucleotide binding protein, alpha 11	10.7	Styx	Phospho Ser/Thr/Tyr interaction protein	73.5
Gna13	G nucleotide binding protein, alpha 13	78.8	Tia1	Cytotoxic granule-associated protein 1	4.6
Gnai1	G nucleotide binding protein, alpha inhibiting 1	4.8	Ube3a	Ubiquitin protein ligase E3A	7.9
Hrsp12	Heat-responsive protein 12	4.4	Itgp	Integrin-associated protein	10.9
Il13ra1	Interleukin 13 receptor, alpha 1	8.0			
		Transcription			
Cbx1	Chromobox homolog 1	7.5	PABII	Poly(A) binding protein II	43.7
Cited2	Cbp/p300-interacting transactivator 2	15.7	Nfib	Nuclear factor I/B	4.3
Crtr1	Transcription repressor CRTR-1	4.4	Nfix	Nuclear factor I/X	30.4
Ddx3	DEAD box 3	17.4	Nmi	N-myc (and STAT) interactor	8.4
Ddx36	DEAD/H box 36	4.5	Nrip1	Nuclear receptor interacting protein 1	28.8
Ddx6	DEAD box 5	5.8	Pcaf	p300/CBP-associated factor	6.7
Elf5	E74-like factor 5	5.1	Peg3	Zinc finger protein	6.6
Elk3	ETS oncogene member	8.3	Pou2f1	POU domain, 2-1	5.2
Etv1	Ets variant gene 1	4.2	Smarcc1	SWI/SNF related, regulator of chromatin, C1	6.2
Gata3	GATA binding protein 3	6.3	Sp1	Trans-acting transcription factor 1	18.1
Id4	Inhibitor of DNA binding 4	7.9	Tcf2	Transcription factor 2	4.0
Klf5	Kruppel-like factor 5	7.9	Terf2	Telomeric repeat binding factor 2	8.1
Mad4	Max dimerization protein 4	5.2	Zfp36l2	Zinc finger protein 36, C3H type-like 2	4.4
Mafg	V-maf, G	4.8	KF-1	KF-1, Zinc finger protein 103	7.9

(*Continued*)

Table 3. Continued

Name	Description	Fold change[a]	Name	Description	Fold change[a]
		Transport			
Abcg2	ATP-binding cassette, G2	4.2	Kcnj1	Potassium channel, J1	23.0
Anxa11	Annexin A11	9.5	Lamp2	Lysosomal membrane glycoprotein 1	18.4
Aqp1	Aquaporin 1	13.2	Mal	T-cell differentiation protein	6.6
Aqp2	Aquaporin 2	5.3	NKCC2	Kidney-specific Na-K-Cl cotransport B	81.6
Atp6v1a1	ATPase, H+ transporting, A1	8.3	Slc16a7	Solute carrier family 16, member 7	27.4
Atp7a	ATPase, Cu++ transporting, A	4.7	Slc17a1	Solute carrier family 17, member 1	6.3
Tm9sf3	Transmembrane protein 9, member 3	4.0	Slc22a1	Solute carrier family 22, member 1	4.5
Cav3	Caveolin 3	8.9	Slc22a2	Solute carrier family 22, member 2	17.8
Clcn3	Chloride channel 3	24.3	Slc22a6	Solute carrier family 22, member 6	20.7
Clcn4-2	Chloride channel 4-2	4.1	Slc27a2	Solute carrier family 27, member 2	4.4
Cubn	Cubilin	8.7	Slc2a2	Solute carrier family 2, member 2	10.6
Fabp3	Fatty acid binding protein 1	6.1	Slc34a1	Solute carrier family 34, member 1	7.1
Fmo1	Flavin-containing monooxygenase 1	6.5	Slc5a1	Solute carrier family 5, member 1	54.8
Folr1	Folate receptor 1	9.2	Snca	Synuclein, alpha	4.4
Fxyd2	FXYD domain ion transport regulator 2	32.6	Stx3	Syntaxin 3A	6.1
Gsr	Glutathione reductase 1	7.0	Itpr2	IP3 receptor type 2	39.4

[a]Fold change denotes the average fold change. Signal log ratio (SLR) for each probe set was generated using comparison files from Affymetrix MAS 5.0 and converted into fold change according to MAS 5.0 documentation.

Cluster analysis of gene expression during kidney development

A more comprehensive view of the changes in gene expression that take place during kidney development can be provided by hierarchical cluster analysis (Fig. 2). Rows represent the different tissues examined, which included E11.5 metanephric mesenchyme, E11.5 ureteric bud, E11.5, E12.5, E13.5, E16.5, and adult total kidney, as well as P1 total mouse. Genes with similar expression patterns were clustered using hierarchical tree algorithm applied to the log relative gene expression values using Pearson correlation. Red represents high expression and blue represents low expression levels. The genes on the right showed higher expression in the adult kidney, and genes on the left had higher expression in the developing kidney. Figure 2 also provides graphic illustration of the similarities in gene expression patterns of the embryonic kidney at E11.5, E12.5, and E13.5. Compared with the striking embryo-adult differences, the changes in gene expression at these early developmental time points are subtle. The E16.5 kidney, in contrast, shows a distinctive gene expression profile when compared with either the embryonic or adult kidney, consistent with its intermediate state of differentiation.

In situ hybridization confirmation of microarray results

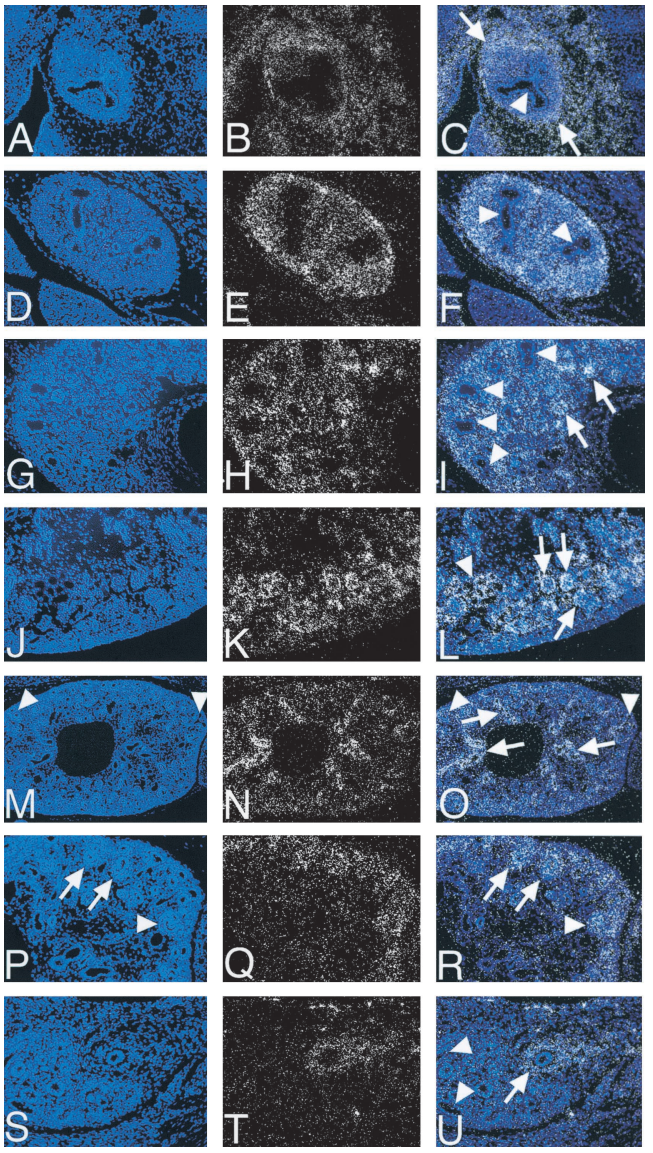
To confirm and extend the microarray results, we performed in situ hybridizations. The microarray data identified a large number of interesting genes, many of which had been previously implicated in kidney development, providing significant validation of the approach. For other genes there had been only indirect, or in many cases no previous association with kidney formation. To corroborate predictions of the microarray data and to better define the expression patterns of selected genes in the developing kidney, we performed in situ hybridizations.

The *Smoothened* (*Smoh*) gene was predicted by the microarray results to be expressed in the early developing kidney. In situ hybridizations showed a graded *Smoh* expression pattern in the E12.5 metanephric mesenchyme, with higher levels in prestromal regions more distant from the ureteric bud (Fig. 3 A to C). This is consistent with a recent study showing that SHH expression in the ureteric bud is an important regulator of proliferation and differentiation of the metanephric mesenchyme in the developing kidney [71].

Nr2f1 (*COUP-TFI*) expression was observed in the mesenchyme of the E13.5 kidney (Fig. 3 D to F). Similar to *Smoh*, expression was excluded from the bud and its flanking mesenchyme. At E15.5, *Nr2f1* expression remained off in the developing collecting ducts and was maintained in mesenchyme and forming tubules (Fig. 3 G to I). *Nr2f1* is an orphan member of the nuclear receptor family with no identified ligand [72]. It was previously shown to be highly expressed in developing neural tissue of the brain [73] and when knocked out in the mouse defects are observed in development of the peripheral nervous system ganglia [74].

At E17.5, *Birc5* (*survivin*) expression was observed in the developing tubules and glomeruli (Fig. 3 J to L). This gene functions to enhance proliferation and survival of cells.

Capn5 expression was limited to the epithelia of the collecting ducts at E15.5 (Fig. 3 M to O). This gene is a member of the calpain family, which encodes calcium-dependent intracellular proteases but lacks a calmodulin-like domain [75] and may act within signaling cascades [76].



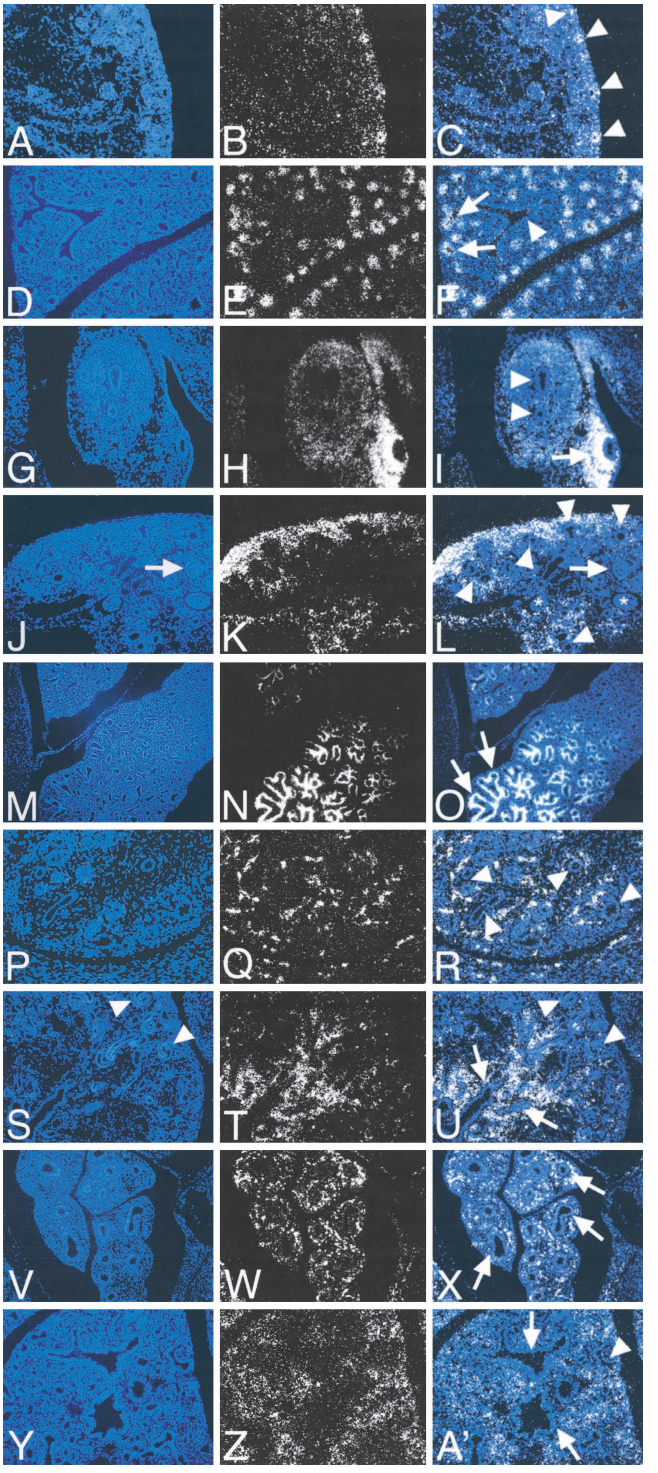


Fig. 3. In situ hybridization analysis of gene expression patterns in the developing kidney. (*A* to *C*) At E12.5, *Smoh* expression was found in the metanephric mesenchyme with the highest expression localized to the prestromal mesenchyme (arrows). Also, notice the absence of signal within the branching ureteric bud (arrowhead). (*D* to *F*) *Nr2f1* expression was diffuse throughout the metanephric mesenchyme in the E13.5 developing kidney, while expression was not found within the branching ureteric bud (arrowheads). (*G* to *I*) Later in the developing kidney, at E15.5, *Nr2f1* expression was maintained in the mesenchyme, and was found in the smaller tubular structures (arrows), but was absent in the mature ureteric stalks (arrowheads). (*J* to *L*) The *Birc5* gene was highly expressed in the tubular structures of the E17.5 kidney (arrowheads) and in developing glomeruli (arrows). (*M* to *O*) *Capn5* expression in the E15.5 kidney. High expression was restricted to epithelia of the large diameter ureteric stalks (arrows), while decreased expression is identified in the smaller ureteric tips (arrowheads) located at the periphery of the developing kidney. (*P* to *R*) *Mns1* expression in the E15.5 kidney was mainly localized to the early nephrogenic epithelia (arrows) within the cortex of the developing kidney and an S-shaped body (arrowhead). (*S* to *U*) *Rab6kifl* expression in the developing urogenital system at E12.5 was localized to mesenchyme surrounding the ureter (arrow) while absent from the mesenchyme surrounding the ureteric bud (arrowheads). (M to O) taken at 100×; all other images taken at 200×. Serial sagittal sections. (A, D, G, J, M, P, and S) show DAPI fluorescence. Dark-field imaging was used to view the hybridization signal (B, E, H, K, N, Q, and T). (C, F, I, L, O, R, and U) show overlays of dark-field image over the DAPI fluorescent image.

Fig. 4. Similar expression patterns within the developing lung and kidney suggesting roles in branching morphogenesis. (*A* to *C*) *Cdrap* showed high expression in the E17.5 kidney localized to the distal ends of the ureteric bud (arrowheads) at the periphery of the kidney. (*D* to *F*) A similar expression pattern for *Cdrap* was seen in the E15.5 lung with expression localized at the distal tips of the branching distal bronchi (arrows), while the proximal bronchi (arrowhead) lack *Cdrap* expression. (*G* to *I*) *Tgfbi* expression in the metanephric mesenchyme at E12.5 was diffuse with no detectable signal in the ureteric bud (arrowheads). There was high *Tgfbi* expression in the mesenchyme surrounding the Wolffian (mesonephric) duct (arrow). (*J* to *L*) The mesenchymal-specific expression pattern of *Tgfbi* was maintained in the E15.5 kidney within the nephrogenic mesenchyme surrounding the ureteric-derived duct (arrowheads), but decreased or absent expression was identified in mesenchymal-derived structures, including an S-shaped body (arrow) and immature glomeruli (asterisks). (*M* to *O*) A similar expression pattern was found in the lung at E15.5, with signal localized to the mesenchyme surrounding the bronchi (arrows). (*P* to *R*) Early in kidney development, at E13.5, *Col15a1* was highly expressed within patches of mesenchyme surrounding the ureteric epithelia (arrowheads). (*S* to *U*) Later, in the E15.5 metanephric kidney, *Col15a1* was highly expressed within the mesenchyme surrounding the ureteric bud–derived ducts (arrows), but absent in S-shaped bodies (arrowheads). (*V* to *X*) A similar expression pattern for *Col15a1* was found in the E13.5 lung, with high signal in patches of mesenchyme surrounding the bronchi (arrows). (*Y* to *A*') Later in development, the E15.5 lung showed a diffuse mesenchymal *Col15a1* expression pattern surrounding the proximal bronchi (arrows) and distal branching bronchi (arrowhead). Sections were stained with DAPI (A, D, G, J, M, P, S, V, and Y). Dark-field imaging was used to view the signal (B, E, H, K, N, Q, T, W, and Z). (C, F, I, L, O, R, U, X, and A') show overlays of dark-field image over the fluorescent image. All images were taken at 200×.

The *Mns1* gene (meiosis-specific nuclear structural protein 1) [77] showed expression in the early nephrogenic epithelia in the cortex (Fig. 3 P to R). The *Rab6kifl* gene showed restricted expression in the mesenchyme surrounding the ureter in the E12.5 kidney (Fig. 3 S to U). The *Rab6kifl* encoded protein is localized to the golgi apparatus and includes a kinesin motor domain as well as a Rab6 GTP interacting domain [78].

Although the focus of this study is on the developing kidney, the in situ hybridizations also defined expression domains in the other regions of the embryo. For some genes the expression patterns in the developing lung, which also undergoes extensive branching morphogenesis, were particularly striking.

The *Cdrap* (or *MIA*) gene was originally cloned as a secreted protein from human melanoma cell lines. It was reported expressed in cartilage primordia [46], as well as transiently in embryonic mammary buds, suggesting a possible role in embryonic events involving invasive growth [79]. Cdrap is a secreted ECM protein that adopts an SH3 domain-like fold in solution. The gene was recently knocked out. Malformations were detected in mutant mice in collagen fiber density, diameter and arrangement [80]. The mice are viable and have normal mammary gland development, but other tissues were not extensively examined. The microarray results suggested that *Cdrap* is expressed in the ureteric bud of the developing kidney. As predicted, *Cdrap* showed high expression in the distal ends of the branching ureteric bud of the E17.5 embryo (Fig. 4 A to C). Of interest, we also observed *Cdrap* expression in the distal tips of the branching bronchi of the lung (Fig. 4 D to F).

The *Tgfbi* gene was isolated as a gene that is induced by TGF-β1, and prevents cell adhesion [81]. It is mutated in corneal dystrophies [82]. The protein is a secreted, RGD-containing collagen-associated protein, and is thought to interact with the ECM. Consistent with the microarray results, this gene was expressed in the mesenchyme of the developing kidney at both E12.5 and E15.5 (Fig. 4 G to L). Similar expression was found in the developing lung at E15.5 (Fig. 4 M to O). These results support and expand earlier expression analysis of this gene [83].

There is evidence from cell culture studies that the collagen *Col15a1* gene may be modulated by cytokines [84]. The gene has been previously targeted causing muscular disease and defects detected in the cardiovascular system in mice [85]. In situ hybridizations confirmed the microarray predicted expression in the developing kidney, with distinct patches of expression observed in the mesenchyme of both the kidney and lung at E13.5 and E15.5 (Fig. 4 P to A'). This data support and expand a previous *Col15a1* expression analysis [86].

DISCUSSION

By coupling microarrays and robust target amplification techniques it is possible to perform global studies of the gene expression patterns of early developing organs and microdissected subcomponents. In this report, we present a catalogue of the gene expression states of the early developing mouse total kidney as well as the separated ureteric bud and metanephric mesenchyme compartments.

The 20 Affymetrix U74A microarrays used for this study generated over 240,000 gene expression level data points, which were analyzed by multiple crosswise comparisons as well as hierarchical cluster analysis. Hundreds of genes expressed at much higher levels in the developing kidney than the adult were found, while far fewer differences in gene expression patterns were found between the multiple early embryonic kidney samples. A surprisingly high fraction of the known genes to emerge from this study had been formerly implicated in kidney development or processes related to branching morphogenesis. This serves to authenticate the screen. In some cases, however, previously characterized genes had not been reported expressed in the developing kid-

ney. For selected genes, we performed in situ hybridizations to more precisely define spatiotemporal expression patterns.

The in situ hybridization results confirmed the microarray-predicted kidney development expression. Interesting similarities in expression patterns in the kidney and lung, which both undergo branching morphogenesis, were also observed for three genes, *Cdrap*, *Tgfbi*, and *Col15a1*. It is interesting to note, however, that for some of these genes, targeted mutant mice have been made and no kidney phenotype observed. This could reflect expression without kidney development function, functional redundancy, or the presence of a previously undetected kidney phenotype in the mutant mice. Even severely malformed kidneys can often provide sufficient function for survival. It would be interesting to begin to distinguish these possibilities by reexamination of these mutants.

Not all of the expected genes are actually identified in the comparisons. For example, over 30 *Hox* genes are expressed in the developing kidney, but most do not appear on the list of transcription factors expressed at elevated levels during development. There are several possible reasons for these absences. Some genes are simply not on the U74Av2 microarrays. Other genes are expressed at low, near noise, levels and are deliberately excluded to avoid artifacts. Additional genes are expressed at similar levels in both samples being compared, and are therefore not called different. Furthermore, the high stringency of the screening process eliminated some genes.

This work provides an extensive definition of gene expression states during mouse kidney development. The results suggest kidney development function for a large number of genes not previously implicated in this process. The complete data set is available (*http://genet.chmcc.org* Login, Nephrome with password "reviewer"). Since this work was performed with the standard commercial Affymetrix microarray platform, the resulting gene expression profiles can provide baselines for multiple future studies of kidney development in mouse mutants.

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