Novel Regulators of Kidney Development from the Tips of the Ureteric Bud

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Mammalian nephrogenesis depends on the interaction between the ureteric bud and the metanephric mesenchyme. As the ureteric bud undergoes branching and segmentation, the stalks differentiate into the collecting system of the mature kidney, while the tip cells interact with the adjacent cells of the metanephric mesenchyme, inducing their conversion into nephrons. This induction is mediated by secreted factors. For identifying novel mediators, the tips of the ureteric tree were isolated and microarray analyses were performed using manually refined, multistep gene ontology annotations. For identifying conserved factors, two databases were developed, one from mouse E12.5 and one from rat E13.5 ureteric buds. The overlap of mouse and rat data sets yielded 20 different transcripts that were enriched in the ureteric bud compared with metanephric mesenchyme and predicted to code for secreted proteins. Real-time reverse transcriptase–PCR and in situ hybridization confirmed these identifications. One of the genes that was highly specific to the ureteric bud tip was cytokine-like factor 1 (CLF-1). Recombinant CLF-1 in complex with its physiologic ligand, cardiotrophin-like cytokine (CLC), triggered phosphorylation of signal transducer and activator of transcription 3 in mesenchyme, a pathway characteristic of mesenchymal-to-epithelial conversion. Indeed, when applied to isolated rat metanephric mesenchyme, CLF-1/CLC (3 nM) induced mature nephron structures expressing glomerular and tubular markers. These results underline the power of this first comprehensive gene expression analysis of the ureteric bud tip to identify bioactive molecules.


Development of the mammalian kidney depends on the interaction of the ureteric bud with the metanephric mesenchyme. The initial outgrowth of the ureteric bud from the nephric duct is induced by growth factors from the metanephric mesenchyme. Specifically, glial cell line–derived neurotrophic factor (GDNF) derived from mesenchymal cells acts on the receptor tyrosine kinase Ret on ureteric bud cells. As the ureteric bud undergoes branching morphogenesis, the tubule undergoes segmentation into the structures of the renal collecting system and distinct tip zones. Only the tip cells maintain expression of Ret, distinguishing a highly dynamic and proliferative tip population from the maturing epithelia of the stalk (1,2). These tip cells are in close proximity to competent mesenchymal cells, where they control multiple processes, including epithelial-to-mesenchymal conversion and formation of the nephron (3).

The ureteric bud–derived factors that interact with the metanephric mesenchyme have a variety of effects. We and others have shown that the IL-6 family cytokine leukemia inhibitory factor (LIF) and lipocalin 2 (also called neutrophil gelatinase associated lipocalin) induce formation of glomeruli and tubules in rat metanephric mesenchymes (4–6). In contrast, Wnt-6, also a ureteric bud factor, has been shown to induce tubules but not glomeruli (7). The ureteric bud also secretes antiapoptotic and growth-promoting factors, such as TGF-α (8) and basic fibroblast growth factor (bFGF) (9,10). Although these ureteric bud–derived factors are sufficient to trigger mesenchymal responses, genetic deletion studies indicate that some of these signals are highly redundant in vivo as evidenced by mild or absent kidney defects in mutant animals (4,6,11,12). Therefore, additional ureteric bud factors may be present and even dominant in mediating the interaction of the ureteric bud with the metanephric mesenchyme. Localization to the tip of the ureteric bud should be a characteristic feature of such inductive factors.

The recent advent of whole genome gene expression analysis using microarray technology provides a unique tool to identify developmentally regulated factors on a large scale. In the field of kidney development, four groups have carried out microarray analysis on embryonic kidney, using mouse (13,14), rat (15,16), and human (17) material. These founding papers have provided novel data from intact developing kidneys and from the early ureteric bud before branching and segmentation into tips and stalks. In this study, we used microarray technology to identify transcriptional profiles of the tips of the ureteric tree from mouse E12.5 kidneys and compared this profile with genes
expressed in ureteric bud stalks and metanephric mesenchymes. To limit the analysis to factors that are conserved between species, we performed a similar analysis on rat embryonic kidney, combined the two data sets, and annotated gene ontology. We have identified a set of 20 conserved, secreted molecules that are specific to the ureteric bud and confirmed the results by independent methods. Furthermore, we present evidence that one of the factors that are highly specific to the ureteric bud tip, cytokine-like factor 1 (CLF-1), induces signal transducer and activator of transcription 3 (STAT3)-dependent signaling in the metanephric mesenchyme and promotes epithelial-to-mesenchymal conversion.

**Materials and Methods**

**Dissection of Ureteric Buds and Metanephric Mesenchymes**

Metanephric mesenchyme and ureteric buds were dissected from E13.5 rats or E12.5 mice. At this stage of development, the ureteric buds had already initiated branching morphogenesis, the rat demonstrating the initial stage of branching (T-shaped tubule), whereas the mouse demonstrated a second and third round of cleavage (four or more tips attached to one stalk). We isolated these structures using DNase (100 U/ml; Boehringer Mannheim, Mannheim, Germany) and trypsin (1 mg/ml; Sigma, St. Louis, MO) for 10 min at 37°C in 1-15 medium, followed by mechanical disruption with minuten pins (Fine Science Tools, Foster, CA). Cross-contamination between ureteric buds and metanephric mesenchymes was ruled out by visual inspection and staining with Dolichos biflorus lectin, which selectively stains the ureteric bud at this stage (18). For organ culture, rat metanephric mesenchymes were placed on filters (Corning Transwell; collagen-coated, 0.4-μm pore size) and grown in DMEM/F12 with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), dexamethasone (5 μg/ml), prostataglandin (5 μg/ml), T3 (5 ng/ml) (Sigma), and combinations of cytokines (R&D Systems, Minneapolis, MN).

**Preparation of Total RNA**

Total RNA from ureteric buds and metanephric mesenchymes was extracted using the RNaseasy mini kit (Qiagen, Valencia, CA) with on-column DNase digestion according to the manufacturer’s instructions. An Agilent Bioanalyzer was used to confirm RNA integrity.

**Probe Labeling for Microarray Experiments**

One microgram of total RNA from ureteric buds and metanephric mesenchymes was used for cDNA synthesis. Briefly, RNA was reverse-transcribed with T7 promoter-containing oligo-dT24 primer and Superscript II (Invitrogen, San Diego, CA). Double-stranded cDNA was purified using GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA), providing a template for in vitro transcription of cRNA with a synthetic biotinylated nucleotide analog. cRNA was purified using Agilent Bioanalyzer, providing a template for transcriptome analysis (RMA) using the Affymetrix microarray *.cel image file and GeneTraffic (Lobion Informatics, La Jolla, CA) software. RMA is composed of three steps: Background correction, quantile normalization, and robust probe set summary (19). This approach has similar accuracy but greater precision than earlier Affymetrix algorithms (e.g., MAS5) (20). Antilogs of RMA values were computed for each gene and averaged for replicates. The selection criterion for enrichment in a specific compartment was set at two-fold overexpression independent of absolute signal intensity. We have made the gene expression data available in the National Center for Biotechnology Information’s Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), accessible through GEO Series accession number GSE1983.

**Real-Time Reverse Transcriptase–PCR**

To authenticate our identifications, we synthesized cDNA using Omniscript Reverse Transcriptase (Qiagen) on total RNA from intact ureteric bud, ureteric bud tips, stalks, and metanephric mesenchymes for conventional and real-time PCR. Primers were designed to both mouse and rat sequences using Primer3 (21), and primer dimer formation and nonspecific amplicons were excluded for each primer set (Supplemental Table 1). Specific fragments were verified by sequencing. The real-time PCR reaction contained iQ SYBR green super mix (Bio-Rad, Hercules, CA), 200 nM of each primer, and 0.2 μl of template in a 25-μl reaction volume. Amplification was carried out using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with incubation times of 2 min at 95°C, followed by 50 cycles of 95°C for 30 s and 60°C for 30 s. Specificity of the amplification was checked by melting-curve analysis and agarose gel electrophoresis. Relative levels of mRNA expression were calculated according to either the standard curve method or the ΔΔCt method (22). Individual expression values were normalized by comparison with β-actin mRNA expression.

**In Situ Hybridization**

*In situ* hybridization analysis was performed with digoxigenin-labeled riboprobes (23). cDNA encoding fragments for mouse ectodin (Sostdc1; Genebank BC021458, position 56 to 715), mouse CLF-1 (NM_018827, position 195 to 1072), mouse chemokine (CXC motif) ligand 14 (CXCL14; NM_019568, position 335 to 721), mouse stem cell factor (X95381, position 36 to 926) and mouse Cyr61 (NM_010516, position 136 to 1427) were amplified by PCR and cloned into pBluescript KS(+) by directional cloning using linkers. The resulting plasmids were linearized with either NotI (Sostdc1, Cyr61, stem cell factor) or SacI (CXCL14, CLF-1), and T3 polymerase was used to produce riboprobes, which were subsequently fragmented by alkaline hydrolysis. The Ret probe was described previously (23).

**ImmunobLOTS and Immunofluorescence**

Phospho-STAT3 was analyzed by immunoblot (BD Biosciences Pharmingen, San Diego, CA). Frozen sections or whole-mount metanephric mesenchymes were stained with antibodies to endostatin (Collagen XVIII; a gift of V. Sukhatme, Beth Israel-Deaconess, Boston, MA), podocalyxin (R&D), and Pax-2 (Santa Cruz Biotechnology, Santa Cruz, CA).

**Results**

**Ureteric Bud Tips and Stalks**

To identify novel factors with highly restricted expression in tip and stalk segments of the ureteric bud, we developed a novel dissection approach in mouse embryos. Whereas conventional microdissection protocols separate the ureteric bud from...
the metanephric mesenchyme at E11.5, when the ureteric bud has just entered the metanephric mesenchyme and is T-shaped (Figure 1, A and B), we isolated branched ureteric buds from older mouse E12.5 metanephric kidneys that had already produced multiple tips attached to one stalk (Figure 1C). This allowed us to isolate three tissues, ureteric bud tips, ureteric bud stalks, and metanephric mesenchyme and to analyze gene expression in each of these structures (Figure 1D). To characterize these pools of RNA, we tested three established molecular markers of the ureteric bud tip, Ret (24), Ros1 (25), and Wnt-11 (26). Using real-time reverse transcriptase–PCR (RT-PCR), we found that these markers were highly specific to our pool of dissected tips (Figure 1E), confirming the quality of the dissection. Importantly, mouse E12.5 ureteric bud tips were more highly enriched (five- to 10-fold) in these markers compared with mouse E11.5 ureteric bud, although these structures appeared morphologically similar. This indicates that the ureteric bud undergoes functional segmentation between primary (E11.5) and secondary (E12.5) branching events.

**Microarray Analysis Identifies Molecules Highly Specific to Ureteric Bud Tips, Stalks, and Metanephric Mesenchyme**

To obtain genome-wide transcriptional profiles of microdissected ureteric bud tips, ureteric bud stalks, and metanephric mesenchyme, we used the Mouse Genome 430 2.0 Array (Affymetrix), which contains >45,000 probe sets that interrogate a large proportion of the transcribed mouse genome. To avoid multiple amplification steps, we microdissected 1000 ureteric bud tips, 350 ureteric bud stalks, and 300 metanephric mesenchymes to obtain sufficient RNA (>1 μg of total RNA). Scatter plots (Figure 2A) of log-transformed expression values from ureteric bud tips, ureteric bud stalks, and metanephric mesenchyme showed distinct expression profiles, which differed from the plot of identity (Figure 2A, top left). Hierarchical cluster analysis using metanephric mesenchyme as a baseline revealed a strong consistency between replicates and distinct patterns of gene expression among the three compartments (Figure 2B). It is interesting that a great number of transcripts are specific to the ureteric bud tip, whereas only few genes are
Although many types of comparisons are possible between these data sets, we selected genes that were expressed more than two-fold in either tip or stalk compared with metanephric mesenchyme. A two-fold threshold was a clear discriminator of genes with excess expression in a specific compartment (Figure 2). This yielded 1335 probe sets (Supplemental Table 2). By comparing the ureteric bud tip and the stalk databases, we then ranked these genes in a gradual transition from tip-specific molecules to stalk-specific molecules. We also defined genes specific for metanephric mesenchyme by identifying transcripts expressed more than two-fold in mesenchyme compared with both tip and stalk compartments. This yielded 151 probe sets (Supplemental Table 3). In each comparison, we found a few genes with established localizations, verifying our isolations. As predicted, ureteric bud tip markers Ret, Ros1, and Wnt-11 were among the ureteric bud tip–specific transcripts (Supplemental Table 2). Conversely, transcripts that are known to be expressed in stalks, such as cadherin 16 and sonic hedgehog, were represented as stalk transcripts. The metanephric mesenchyme specific subset (Supplemental Table 3) contained many genes that are known to localize to this compartment, e.g., Eya1 (27), WT-1 (28), Sall1 (29), and GDNF (30,31). However, the vast majority of genes that we identified as ureteric bud tip specific, stalk specific, or metanephric mesenchyme specific have never been examined in the context of kidney development.

Comparison of Mouse and Rat Metanephros Identifies Conserved Molecules

Because of the quantity of microarray data, efficient filtering methods for the selection of highly relevant molecules are needed. One approach is to identify molecules that are conserved in a particular compartment across species. We therefore extended the microarray analysis by microdissecting T-shaped ureteric buds and metanephric mesenchyme from rat E13.5 embryos (Figure 1B) and performing microarray analysis using the Rat Genome U34 Chip Set (Affymetrix), which contains >24,000 probe sets (Figure 2A). Again, we identified transcripts that were enriched more than two-fold in ureteric bud compared with metanephric mesenchyme, which generated a list of 561 probe sets. Next, we identified the mouse
orthologs using the HomoloGene web-based interface (National Center for Biotechnology Information), which yielded 442 orthologous probe sets but at the time of manuscript preparation (December 2004) could not identify the remaining 119 orthologs (21% of our genes). This is mostly related to the fact that annotations for rat genes are currently limited. A total of 193 of the 442 orthologous probe sets were also enriched more than two-fold in either tip or stalk compared with metanephric mesenchyme on the mouse microarrays. The complete list of the corresponding genes is shown in Figure 3 with a transition from tip-specific transcripts (green) to stalk-specific transcripts (blue). Hence, we were able to generate a list of molecules that were ureteric bud specific and conserved in rat and mouse.

**A Set of Conserved, Secreted Molecules Specific to the Ureteric Bud**

For the ureteric bud–specific, conserved probe sets, we performed a detailed analysis to identify secreted molecules. Initially, we reviewed the published literature and gene ontology databases such as Mouse Genome Informatics or Gene Ontology Consortium for each molecule. When gene ontology annotation with respect to cellular localization was insufficient from these sources, we identified corresponding clones in the RIKEN database (32) and assayed their presence in the mouse secretion with respect to cellular localization was insufficient from these sources, we identified corresponding clones in the RIKEN database (32) and assayed their presence in the mouse secretome (33), a computationally derived database of putatively secreted molecules based on structural prediction models. With the use of these sources, the predicted subcellular localization of the corresponding protein of 164 (85%) of 193 probe sets could be determined, 29 (18%) of which were predicted to be secreted (26 based on published evidence, three based on presence in the secretome database). These probe sets corresponded to 20 different genes. Table 1 indicates their expression patterns in rat ureteric bud, mouse ureteric bud tip, and mouse ureteric bud stalk (compared with metanephric mesenchyme) with tip-specific molecules at the top of the table. Of these genes, only three had been reported to be expressed in the ureteric bud (Wnt-6, BMP-7, and collagen XVIII), one (CLF-1) has been independently identified in a screen for genes induced by GDNF in the ureteric bud (B.L. and F.C., unpublished observations), and we extended this list with 16 additional ureteric bud–specific, secreted factors that are conserved between rat and mouse, including the kit ligand stem cell factor and the Wnt/BMP-signaling modulator ectodin. Two cytokines, CXCL14 and CLF-1, were highly enriched in the ureteric bud tips (Tables 1 and 2), suggesting that these factors may be involved in the interaction between ureteric bud tips and the adjacent metanephric mesenchyme.

To confirm the accuracy of the microarray, we performed real-time RT-PCR on freshly dissected rat and mouse ureteric buds and metanephric mesenchymes (a typical amplification graph is shown in Figure 4A). As predicted by the microarray, all mRNA were overexpressed in ureteric bud of both species (Figure 4B). There was a positive correlation between the overexpression in ureteric bud (log2 ratio) predicted by microarray and the actual overexpression as determined by real-time RT-PCR (correlation coefficient 0.37), and a strong correlation was present between real-time RT-PCR data in the two species (correlation coefficient 0.66). In general, fold differences of expression levels tended to be higher by RT-PCR compared with microarray.

To confirm localization of message in the predicted compartments of the kidney, we performed in situ hybridization for selected molecules (Figure 5). As predicted from the microarray data (compare with Table 1), the secreted molecules CLF-1 and CXCL14 showed an expression pattern indistinguishable from the ureteric bud tip marker Ret. Conversely, molecules less specific to the ureteric bud tip showed a wider expression pattern in ureteric bud tips and stalks by in situ hybridization (ectodin, stem cell factor) or by immunofluorescence (collagen XVIII). In the case of ectodin, expression was preserved in adult collecting ducts but also appeared in the developing and adult loop of Henle. Cyr61 was detected in ureteric bud and metanephric mesenchyme, reflecting the moderate overexpression in the ureteric bud (approximately two-fold) according to microarray and real-time RT-PCR. Highest expression for this molecule was found in cells associated with the maturing glomeruli.

To extend the confirmation of the microarray data and to compare gene expression in ureteric and lung buds, another epithelial tubule that interacts with an adjacent mesenchymal compartment, we assayed the presence of the 20 conserved, secreted ureteric bud genes in a public in situ hybridization...
database (GenePaint; Max-Planck Institute, Hannover, Germany; www.genepaint.org). Three of these genes, collagen XVIII α1, laminin α1, and Wnt-6, were detectable in the lung bud. Stem cell factor was absent from the lung bud but expressed in the lung mesenchyme, an inverted pattern compared with the kidney. Eighteen other ureteric bud–specific molecules were examined by GenePaint, and 10 of these were also expressed in the lung bud, including E-cadherin, P-cadherin, tumor-associated calcium signal transducer 1, villin 2, transcription factor 2, and FGF receptor 4. Conversely, eight ureteric bud–specific molecules were not present in the lung bud, including LIM homeobox protein 1, calbindin 1, and GATA binding protein 3.

Ureteric Bud Tip Molecule CLF-1 Induces Epithelial Conversion in Metanephric Mesenchyme

The tips of the ureteric bud induce epithelialization in the metanephric mesenchyme. From our set of conserved, secreted molecules, the cytokines CLF-1 and CXCL14 are highly specific for the ureteric bud tip and hence are candidate mediators. CLF-1 is a secreted cytokine receptor, which has been shown to bind to cardiotrophin-like cytokine (CLC), an IL-6 family member (34). This composite cytokine induces downstream signal transduction events by binding to the ciliary neurotrophic factor (CNTF) receptor complex that consists of gp130, LIFRβ, and CNTF receptor (CNTFR). CXCL14 is a novel chemokine that has chemotactic and antiangiogenic properties (35,36) and whose receptor is unknown.

To detect the bioactivity of these factors, we applied recombinant versions of CXCL14 and of the CLF-1/CLC complex to isolated metanephric mesenchyme from rat E1.3.5 embryos. When combined with bFGF (3 nM) and TGF-α (3 nM), which are necessary to prevent apoptosis of the isolated metanephric mesenchyme (8–10), CLF-1/CLC (3 nM) induced the formation of epithelial structures in the mesenchyme within 7 days of culture, whereas CXCL14 (500 nM) and control media lacked such activity (Figure 6A; n = 15). The epithelia acquired the morphology of S-shaped bodies with a tubular pole, positive for Pax-2, and a glomerular pole positive for podocalyxin (Figure 6B). The effect of CLF-1/CLC was similar to that of LIF, a potent inducer of nephrogenesis (4), but the overall growth of the mesenchyme was less pronounced (Figure 6A). Similar to the IL-6 family cytokines LIF and CNTF, CLF-1/CLC triggered phosphorylation of STAT3 in the metanephric mesenchyme (Figure 6C), indicating a common signal transduction pathway. These results identify CLF-1 as a novel secreted factor from the ureteric bud, which promotes nephrogenesis when complexed with an IL-6 family ligand.

**Table 1. Conserved, secreted molecules from the ureteric bud**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Description</th>
<th>Rat UB/MM</th>
<th>Rat Tip/MM</th>
<th>Mouse Stk/MM</th>
<th>Mouse Tip/Stk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cxcl14</td>
<td>Chemokine (CXC motif) ligand 14 (CXCL14)</td>
<td>5.8</td>
<td>25.9</td>
<td>4.4</td>
<td>5.9</td>
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<tr>
<td>Crfl1</td>
<td>Cytokine-like factor 1 (CLF-1)</td>
<td>17.8</td>
<td>30.3</td>
<td>6.2</td>
<td>4.9</td>
</tr>
<tr>
<td>Igbp2</td>
<td>Insulin-like growth factor binding protein 2</td>
<td>17.2</td>
<td>5.7</td>
<td>2.2</td>
<td>2.6</td>
</tr>
<tr>
<td>Wnt6</td>
<td>Wingless-related MMTV integration site 6 (Wnt-6)</td>
<td>5.1</td>
<td>4.6</td>
<td>1.8</td>
<td>2.6</td>
</tr>
<tr>
<td>Lama1</td>
<td>Laminin, α1</td>
<td>2.4</td>
<td>8.9</td>
<td>3.6</td>
<td>2.5</td>
</tr>
<tr>
<td>Wfde2</td>
<td>WAP four-disulfide core domain 2</td>
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<td>14.7</td>
<td>6.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Bmp7</td>
<td>Bone morphogenetic protein 7 (BMP-7)</td>
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<td>2.4</td>
<td>1.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Psek9</td>
<td>Proprotein convertase subtilisin/kexin type 9</td>
<td>4.0</td>
<td>2.6</td>
<td>1.5</td>
<td>1.8</td>
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<tr>
<td>Kitl</td>
<td>Kit ligand/stem cell factor</td>
<td>23.2</td>
<td>5.0</td>
<td>2.8</td>
<td>1.8</td>
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<tr>
<td>Sostdc1</td>
<td>Sclerostin domain containing 1/ectodin/wise</td>
<td>7.9</td>
<td>7.8</td>
<td>4.5</td>
<td>1.7</td>
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<tr>
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<td>6.0</td>
<td>3.5</td>
<td>1.7</td>
</tr>
<tr>
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<td>Laminin, α5</td>
<td>4.7</td>
<td>3.9</td>
<td>2.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Lmc1</td>
<td>Laminin, γ1</td>
<td>3.4</td>
<td>3.4</td>
<td>2.2</td>
<td>1.6</td>
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<tr>
<td>Prss8</td>
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<td>13.2</td>
<td>8.5</td>
<td>1.6</td>
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<td>Nme3</td>
<td>Expressed in nonmetastatic cells 3</td>
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<td>2.0</td>
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<td>1.5</td>
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<tr>
<td>Chtrc1</td>
<td>Collagen triple helix repeat containing 1</td>
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<td>3.6</td>
<td>2.6</td>
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<tr>
<td>Cyr61</td>
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<td>2.5</td>
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<td>1.3</td>
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<tr>
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<td>Agrin</td>
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<td>2.4</td>
<td>1.9</td>
<td>1.2</td>
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<td>2.9</td>
<td>2.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Rnas4</td>
<td>Ribonuclease, RNase A family 4</td>
<td>8.9</td>
<td>1.9</td>
<td>2.9</td>
<td>0.6</td>
</tr>
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</table>

*Expression ratios in rat and mouse microdissected tissues. UB, ureteric bud; Tip, ureteric bud tip; Stk, ureteric bud stalk; MM, metanephric mesenchyme.*

Discussion

Our current knowledge of the dynamic nature of the ureteric bud and the physiologic roles of the tip and stalk segments are derived from in vitro models of ureteric bud branching and analysis of physiologically relevant marker molecules. This study adds three principle findings to our understanding of the physiologic roles of segments of the ureteric bud: (1) The tips of...
the ureteric bud display a highly distinct gene expression profile, whereas only few genes are specific to the stalks; (2) a set of secreted factors from the ureteric bud are conserved in mouse and rat and expressed in a gradient from tip to stalk; (3) one of the tip-specific factors, CLF-1, induces cell conversion in rat metanephric mesenchyme when complexed with its physiologic ligand, an observation that is in agreement with the concept of a tip-derived inducer.

We have relied on a microdissection approach to analyze gene expression in ureteric bud tips, stalks, and metanephric mesenchyme. Microdissection has a striking advantage over FACS or laser capture microdissection in that it yields sufficient amounts of tissue to produce cRNA for hybridization to microarrays with a single round of amplification rather than the two-cycle protocols currently used for minute amounts of RNA (37). To analyze the accuracy of the microarray data, we used technical replicates, analysis of two different species at two developmental stages, real-time RT-PCR on independent samples, in situ hybridization (including results from the GenePaint in situ hybridization database), and interrogation of the published literature. We find that gene expression in each compartment of our dissection correlates closely with these independent data, confirming that the gene expression profiles accurately reflect the biology of the developing kidney.

To characterize the biologic role of the ureteric bud tips in relation to stalks and metanephric mesenchyme, we performed data mining to compare the compartments. Cluster analysis indicated that the ureteric bud tips displayed a highly specific subset of transcripts, whereas the stalk expressed few unique genes. When the analysis is limited to ureteric bud–specific transcripts, only 116 (8.7%) transcripts are stalk specific, whereas 274 (20.5%) are tip specific. Hence, in many ways, the stalks at this stage are characterized by a gradual loss of genes expressed in the tips, pointing toward their probable lineal relation. Conversely, these data underline the unique nature of the ureteric bud tips. It is interesting that several of the transcripts that are most highly enriched in ureteric bud tip com-

**Figure 5.** Localization of novel secreted molecules during metanephric development. Visualization of Ret, chemokine (CXC motif) ligand 14 (CXCL14), cytokine-like factor 1 (CLF-1), ectodin, stem cell factor (Kit), and Cyr61 by in situ hybridization and collagen XVIII α1 (Col18a1) by immunofluorescence staining on E14.5 to E16.5 mouse kidneys. (Left) Low-power, frozen sections. (Middle) high-power, frozen sections. (Right) Low-power, whole-mount staining.

**Figure 6.** CLF-1/cardiotrophin-like cytokine (CLC) induces nephrons in the MM. Isolated rat E13.5 MM were cultured in the presence of growth factors. (A) Phase contrast micrographs after 1 d (d1), 3 d (d3), and 8 d (d8). Note continued growth and appearance of tubules in the presence of CLF-1/CLC or leukemia inhibitory factor (LIF). (B) Mesenchyme treated with CLF-1/CLC for 8 d and subsequently stained for Pax-2 (green) and podocalyxin (Pdx; red). (Insert) High power. Note the appearance of distinct glomerular and tubular poles. (C) Immunoblot for phospho-STAT3 (pSTAT3) in mesenchymes that were treated with the indicated growth factors for 15 min. CON, control; bFGF, basic fibroblast growth factor; CXCL14, chemokine (CXC motif) ligand 14; CNTF, ciliary neurotrophic factor.
pared with stalk (Figure 3 and Supplementary Table 2) were also among the most highly induced transcripts found in a screen for targets of GDNF signaling in the E11.5 mouse ureteric bud (B.L. and F.C., unpublished observations). These include CLF-1, Catnd2, Arg2, Cxcr4, Ret, Wnt11, Ros1, and Myb. This finding suggests that GDNF signaling through Ret is an important mechanism for the establishment of tip-specific patterns of ureteric bud gene expression.

To limit our analysis to transcripts of biologic relevance, we compared two species and identified 20 different conserved transcripts specific to the ureteric bud coding for putatively secreted molecules. (This list is not exhaustive because of the limited number of sufficiently annotated probe sets on the current rat microarray, which make the identification of orthologous probe sets on the rat and mouse arrays impossible for a proportion of genes.) One of the molecules that are most highly enriched in the tips of the ureteric bud is the cytokine CLF-1, a chaperone required for efficient secretion of the IL-6 family member CLC (34). We show that the heterodimer of CLF-1 and CLC is an inducer of mesenchymal to epithelial conversion and nephrogenesis, suggesting that CLF acts as a cytokine agonist. Remarkably, though, we found only low levels of CLC mRNA in embryonic kidneys using real-time RT-PCR and our microarray database of embryonic kidneys (data not shown), suggesting the possibility that CLF-1 binds to another, unknown ligand in the embryonic kidney. The ligand might be another member of the IL-6 family, hence producing a third cytokine signal for the CNTFR complex. Importantly, a conserved FXXK motif of CLC, which is necessary for interacting with CLF-1, is also present in other IL-6 family cytokines, such as LIF and cardiotrophin 1 (38). One intriguing possibility is the interaction between CLF-1 and the known ureteric bud-derived inducer LIF (4). Alternatively, CLF-1 may be a co-factor for an unknown IL-6 cytokine or even for a different class of secreted proteins, although homologies are limited between CLC and other classes of molecules. These findings stress the physiologic relevance of the molecules identified by our analysis, confirm an important role of cytokine signaling in renal induction, but also underline our incomplete knowledge of the mechanisms by which the ureteric bud tip uniquely induces the metanephric mesenchyme.

Our analysis also identified many ureteric bud–secreted factors that were less specific to the ureteric bud tip. We hypothesize that these molecules may be involved in the interaction with metanephric mesenchyme but may also influence processes that are localized primarily to the stalk (e.g., localized branching or segmentation of the collecting duct) or to adjacent cells, such as medullary stromal cells, hemangioblasts, or the loop of Henle. One class is modulators of extracellular matrix: Four laminin family members, collagen XVIII α1 (39), and agrin (Agrn), as well as the extracellular protease prostasin (Prss8) and the secretory proprotein convertase subtilisin/kexin type 9 (Pcsk9). A second class is factors that modulate signaling, such as a novel putative BMP antagonist/Wnt signaling modulator, ectodin, also known as sclerostin domain containing 1 (Sostdc1) or Wise (40,41). Finally, our analysis has identified a factor that is likely to signal to cells outside the metanephric mesenchyme and hence may be paradigmatic of a new function for the ureteric bud. Stem cell factor, the ligand of the receptor tyrosine kinase c-kit and a proliferative and survival factor for hematopoietic stem cells, germ cells, and melanoblasts (42), is one of the conserved ureteric bud–specific molecules. Although this system had not previously been implicated in kidney development, our unpublished data indicate that the target cells for stem cell factor derive from the periaortic region, pointing toward a role for the stalk of the ureteric bud in signaling to novel types of progenitors. All of the molecules described above maintain a high expression level at later stages of development as demonstrated by in situ hybridization (Figure 5). These data underline the different functions of the ureteric bud tip and stalk in regulating different classes of cells.

We present here the first quantitative description of gene expression in different compartments of the ureteric bud. It is likely that these molecules are authentic regulators of nephrogenesis, because, although most of these transcripts have not been evaluated in the context of kidney development, they are conserved in the ureteric bud across species. In fact, from the limited data sets currently available from other organs, it seems that as many as one third of these molecules are found in the ureteric bud and not in other branching organs. This may reflect that in the kidney and in no other organ are epithelial cells born de novo from mesenchyme and in no other organ does fusion of induced epithelia with the original epithelial tree take place.

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