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Comprehensive microarray analysis of Hoxa11/Hoxd11 mutant kidney development

Kristopher Schwab [a], Heather A. Hartman [b], Hung-Chi Liang [a], Bruce J. Aronow [c], Larry T. Patterson [b], S. Steven Potter [a,*]

[a] Division of Developmental Biology, Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229, USA
[b] Division of Nephrology, Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229, USA
[c] Division of Bioinformatics, Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229, USA

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Abstract

The Hox11 paralogous genes play critical roles in kidney development. They are expressed in the early metanephric mesenchyme and are required for the induction of ureteric bud formation and its subsequent branching morphogenesis. They are also required for the normal nephrogenesis response of the metanephric mesenchyme to inductive signals from the ureteric bud. In this report, we use microarrays to perform a comprehensive gene expression analysis of the Hoxa11/Hoxd11 mutant kidney phenotype. We examined E11.5, E12.5, E13.5 and E16.5 developmental time points. A novel high throughput strategy for validation of microarray data is described, using additional biological replicates and an independent microarray platform. The results identified 13 genes with greater than 3-fold change in expression in early mutant kidneys, including Hoxa11s, GATA6, TGFbeta2, chemokine ligand 12, angiotensin receptor like 1, cytochrome P450, cadherin5, and Lymphocyte antigen 6 complex, Iroquois 3, EST A930038C07Rik, Meox2, Prkcn, and Slc40a1. Of interest, many of these genes, and others showing lower fold expression changes, have been connected to processes that make sense in terms of the mutant phenotype, including TGFbeta signaling, iron transport, protein kinase C function, growth arrest and GDNF regulation. These results identify the multiple molecular pathways downstream of Hox11 function in the developing kidney.

Keywords: Kidney development; Microarrays; Hox genes; Laser capture microdissection; Metanephric mesenchyme; Ureteric bud; Morphogenesis

Introduction

The kidney is an excellent model system for studying the principles of organogenesis. The developing kidney exhibits many interesting processes, including establishment of an early metanephric field by tissue interactions, budding, reciprocal inductive interactions between ureteric bud (UB) and metanephric mesenchyme (MM), stem cell growth and differentiation, and conversion of mesenchyme into epithelia. In addition, there is branching morphogenesis, apoptosis, fusion (nephrons to collecting ducts), and proximal–distal segmentation along the length of the nephron [for review, see (Davies and Bard, 1998)]. Metanephric, or adult, kidney development begins in the mouse at around embryonic day 11.0 (E11.0) as the UB grows from the nephric duct and invades the MM. The UB induces the cells of the MM to condense, epithelialize, and form renal vesicles, each of which develops into a functional nephron containing a glomerulus, proximal tubule, loop of Henle and distal tubule. Of particular importance, kidney morphogenesis can be readily studied in organ culture (Saxen and Lehtonen, 1987).

We have made significant advances in understanding the genetic regulation of kidney organogenesis (Bouchard, 2004; Yu et al., 2004). Hox genes play important roles in this process. These transcription factor-encoding genes often occupy high level positions in the genetic hierarchy of development. It is interesting that a total of 27 Hox genes show specific domains of expression in the developing kidney (Patterson and Potter, 2004). Mutations in the Hoxa11, Hoxc11 and Hoxd11 closely

* Corresponding author. Fax: +1 513 636 4317.
E-mail address: steve.potter@cchmc.org (S.S. Potter).

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related paralogous genes reveal that they have redundant functions in several aspects of kidney development (Davis et al., 1995; Patterson et al., 2001; Wellik et al., 2002). These three genes are expressed in the early MM, and the combined mutation of all three gives a loss of GDNF synthesis and failure of the UB to form (Wellik et al., 2002). A hypomorphic mutant allele combination, with mutations in Hoxa11 and Hoxd11 but not Hoxc11, results in a MM that does not properly drive branching morphogenesis of the UB, and in turn does not respond correctly to UB signals (Patterson et al., 2001). These results indicate that the Hox11 paralogs play crucial roles in several stages of kidney development. There are still important gaps, however, in our understanding of the downstream genetic pathways regulated by these Hox genes.

The microarray is a useful tool for gaining deeper insight into the genetic program of kidney development. Microarrays can provide an important gene discovery function, identifying all genes expressed in the developing kidney and cataloging changes that occur over time. In addition, they allow an impartial global view of altered gene expression profiles in mutant developing kidneys. Instead of looking at just a few selected marker genes by in situ hybridization, it is now possible to conduct an unbiased and universal analysis of gene expression patterns in mutants.

In this paper, we extend the previous microarray studies of normal kidney development, and then use this wild type baseline to analyze the altered gene expression patterns of the Hoxa11/Hoxd11 mutant kidney. We used Affymetrix MOE430 oligonucleotide microarrays to examine gene expression profiles of the complete normal kidney at E12.5, E13.5, E16.5 and adult. In addition, we determined the gene expression patterns of the E11.5 MM and UB, using both laser capture microdissection and manual microdissection to isolate tissues, thereby identifying over 1500 genes with strong differential expression. These results serve to identify the gene expression networks and signaling pathways active in these kidney primordia. Finally, we performed an extensive microarray dissection of the altered gene expression patterns present in Hoxa11/Hoxd11 double mutant kidneys. Several developmental time points were examined, including E11.5, E12.5, E13.5 and E16.5. To allow a more robust microarray analysis of the mutant differences we combined independent data from the Affymetrix and Illumina microarray platforms. The results identify a battery of downstream genes that provide deeper insight into the molecular mechanisms of Hox11 function in kidney development.

Methods

Breeding and genotyping Hoxa11/Hoxd11 mutant mice

Hoxa11 and Hoxd11 mutant mice were previously described (Davis et al., 1995; Patterson et al., 2001; Small and Potter, 1993). The colony was maintained on a mixed genetic background of four strains of mice (129, C57, C3H and CF1). Hoxa $11^{+/-}$, Hoxd$11^{+/-}$ double heterozygous female mice have uterine defects that severely limit reproductive capacity (Hsieh-Li et al., 1995). We therefore isolated zygotes from double heterozygote crosses, with superovulated females, and transferred them to pseudo-pregnant surrogate wild type CD-1 females (Nagy, 2003). Noon of the day when the vaginal plug was observed was considered embryonic day 0.5 (E0.5). All mice and embryos were genotyped as previously described (Patterson et al., 2001). This study focuses on Hoxa11, Hoxd11 double homozygous mutant mice.

Tissue dissections

Wild type tissues from E12.5 and older were obtained from outbred CD-1 mice. Whole embryonic kidneys and urogenital ridges were dissected in ice-cold PBS then either frozen at −80°C, or quick-frozen in Tissue-Tek® OCT compound (Sakura, Torrence, CA) using liquid nitrogen cooled 2-methylbutane. MM and UBs, up to T-shaped stage, were isolated by treating dissected E11.5 kidneys with 0.5 mg/ml collagenase B (Roche, Indianapolis, IN), in D-MEM (Invitrogen, Carlsbad, CA) for 30 min at 37°C and carefully dissecting the mesenchyme from the UB, followed by storage at −80°C.

Laser-capture microdissection

E11.5 whole embryos were frozen in OCT. For later time points the kidneys were removed and frozen in OCT. Serial sections (7μ) were made using a Microm HM 550 cryostat (Richard-Allan Scientific, Kalamazoo, MI), collected on Fisher Superfrost plus precleaned slides (Hampton, NH), and stored at −80°C. Alternate sections were hematoxylin and eosin stained and used to help identify the UB and MM. For LCM, the remaining sections were air dried at room temperature for 3 min, acetone fixed for 2 min, rinsed in ice cold 1/10 PBS for 3 min and then dehydrated in 75%, 95%, 100%, 100% ethanol, followed by two 5-min rinses with xylene. Laser capture microdissection was performed using the Arcturus Pixcell II system, according to Arcturus protocols (Mountain View, California).

RNA isolation and target RNA amplification

Total RNA from wild type and mutant whole kidneys was prepared using the Stratagene Absolutely RNA Nanoprep Kit (La Jolla, CA) and amplified as previously described (Schwab et al., 2003). Target RNA was then hybridized to both the MOE430A and MOE430B Genechips (Affymetrix, Santa Clara, CA). Microarray analysis of each stage was performed in biological duplicate using either 30 ng or 100 ng of starting total RNA.

Each microarray hybridization represented a biological replicate, using an independent biological sample. We pooled 3–9 wild type UB for each sample, and 2–4 MM for each sample. Mutant E11.5 MM was not pooled.

LCM RNA was prepared using the RNeasy Micro Kit (Qiagen, Valencia, CA), with 30 ng poly-inosine carrier (Epicentre, Madison, WI) added to the RLT buffer. Target RNA was prepared using the TargetAmp™ 2-Round aRNA Amplification Kit 1.0 (Epicentre, Madison, WI), and hybridized to Affymetrix MOE430_v2 microarrays.

To validate the Affymetrix results, total RNA was isolated from HoxA11/D11 null and normal E13.5 whole kidneys or E11.5 MM, amplified using the Epicentre TargetAmp™ 2-Round aRNA Amplification Kit (Madison, WI) and hybridized to Sentrix MouseRef-8 Beadchip microarrays (Illumina, San Diego, CA) containing over 24,000 probes.

Gene expression profile analysis

Affymetrix raw data in the CEL file format was normalized using RMA Express 0.2 (Bolstad et al., 2003) and analyzed using Genespring 7.0. Illumina raw signal data was imported into the Affymetrix MOE430 genome on basis of gene symbol for analysis. Wild type whole developing kidney samples were normalized to adult samples. Normal E11.5 MM samples were normalized to E11.5 UB. HoxA11/D11 null samples were normalized to the corresponding wild type control. Hierarchical clusters were generated using the Pearson Correlation Function. All microarray data are available from Signet (http://cypher.cchmc.org:1104/servlet/GeNet, login as "Guest, select MOE430 genome, data contained in "SPotter/Schwab et al. 2005 folders") allowing interactive analysis of the data, through other public databases (GEO, GUDMAP), and will be provided upon request.