Molecular Characterization of the Genital Organizer: Gene Expression Profile of the Mouse Urethral Plate Epithelium

Brooke A. Armfield, Ashley W. Seifert,* Zhengui Zheng,† Emily M. Merton, Jason R. Rock,‡ Maria-Cecilia Lopez, Henry V. Baker and Martin J. Cohn§

From the Departments of Molecular Genetics and Microbiology (BAA, ZZ, EMM, JRR, MCL, HYB, MJC) and Biology (AWS, MJC) and Howard Hughes Medical Institute (MJC), University of Florida Genetics Institute, University of Florida, Gainesville, Florida

Purpose: Lower urinary tract malformations are among the most common congenital anomalies in humans. Molecular genetic studies of mouse external genital development have begun to identify mechanisms that pattern the genital tubercle and orchestrate urethral tubulogenesis. The urethral plate epithelium is an endodermal signaling region that has an essential role in external genital development. However, little is known about the molecular identity of this cell population or the genes that regulate its activity.

Materials and Methods: We used microarray analysis to characterize differences in gene expression between urethral plate epithelium and surrounding tissue in mouse genital tubercles. In situ hybridizations were performed to map gene expression patterns and ToppCluster (https://toppcluster.cchmc.org/) was used to analyze gene associations.

Results: A total of 84 genes were enriched at least 20-fold in urethral plate epithelium relative to surrounding tissue. The majority of these genes were expressed throughout the urethral plate in males and females at embryonic day 12.5 when the urethral plate is known to signal. Functional analysis using ToppCluster revealed genetic pathways with known functions in other organ systems but unknown roles in external genital development. Additionally, a 3-dimensional molecular atlas of genes enriched in urethral plate epithelium was generated and deposited at the GUDMAP (GenitoUrinary Development Molecular Anatomy Project) website (http://gudmap.org/).

Conclusions: We identified dozens of genes previously unknown to be expressed in urethral plate epithelium at a crucial developmental period. It provides a novel panel of genes for analysis in animal models and in humans with external genital anomalies.

Key Words: urethra, genitalia, gene expression, microarray analysis, congenital abnormalities

External genitalia are complex organs that perform excretory and reproductive functions. Congenital malformations of the penis are among the most common human birth defects with hypospadias (incomplete formation of the urethral tube characterized by an ectopic or oversized meatus, or multiple meatuses) affecting approximately 1/250 live male births.1 Although the causes of hypospadias remain poorly understood, progress in mouse developmental genetics has identified a small number of genes with essential roles in urethral tubulogenesis.2–7

Abbreviations and Acronyms
E = embryonic day
FACS = fluorescence activated cell sorting
FoxA = Forkhead box A
GFP = green fluorescent protein
Shh = Sonic hedgehog

Accepted for publication April 22, 2016. No direct or indirect commercial incentive associated with publishing this article.

The corresponding author certifies that, when applicable, a statement(s) has been included in the manuscript documenting institutional review board, ethics committee or ethical review board study approval; principles of Helsinki Declaration were followed in lieu of formal ethics committee approval; institutional animal care and use committee approval; all human subjects provided written informed consent with guarantees of confidentiality; IRB approved protocol number; animal approved project number.

Supported by National Institutes of Health Grants U01 DK094523 and R01 ES017099 (MJC) and F32 HD070721-03 (BAA).
* Current address: Department of Biology, University of Kentucky, 211 TH Morgan Building, Lexington, Kentucky 40506.
† Current address: Department of Physiology, School of Medicine, Southern Illinois University, MC 6512, Carbondale, Illinois 62901.
‡ Current address: Department of Anatomy, University of California-San Francisco, 513 Par- nassus Ave., Box 0452, San Francisco, California 94143.
§ Correspondence: Department of Molecular Genetics and Microbiology, Genetics Institute, University of Florida, P.O. Box 103610, Gainesville, Florida 32610 (telephone: 352-273-8101; FAX: 352-273-8264; e-mail: MCohn@ufl.edu).

See Editorial on page 983.
Nonetheless, the details of the gene regulatory networks involved in genital development remain largely uncharacterized.\textsuperscript{3,8}

In the mouse external genital development begins at E10.5 with the initiation of paired genital swellings on either side of the cloacal membrane.\textsuperscript{3,9} These swelling merge medially to form a single genital tubercle and endoderm from the ventral side of the cloaca extends with the tubercle to form the bilaminar urethral plate. In the mouse the lumen of the penile urethra develops when the 2 sides of the urethral plate epithelium delaminate from proximal to distal.\textsuperscript{10} The urethral plate separates from the ventral surface ectoderm by apoptosis and the adjacent mesenchyme fills the space previously occupied by the ventral urethral plate to internalize the urethral tube.\textsuperscript{9,11,12}

The human urethra also forms from cloacal endoderm but urethral tubulogenesis differs from that of the mouse. Morphogenesis of the human urethral tube resembles neurulation (closure of the neural tube) in that the lateral edges of the urethral plate are brought together medially to transform the plate into a tube.\textsuperscript{13,14} Although the processes involved in urethral lumen formation in mice and humans are becoming clearer, the underlying mechanisms are poorly understood and few candidate genes are available for screening patients with external genital defects.

In addition to giving rise to the lining of the urethra, the urethral plate epithelium functions as a signaling center to control outgrowth and patterning of the genital tubercle.\textsuperscript{3,15} The signaling activity of the urethral plate epithelium is mediated by a secreted signaling molecule encoded by the \textit{Shh} gene. Shh signals to surrounding mesenchymal tissue, where it regulates the molecular pattern of the genital tubercle and controls outgrowth and urethral tubulogenesis via control of cell cycle kinetics.\textsuperscript{11} Early deletion of \textit{Shh} causes agenesis of the genital tubercle.\textsuperscript{2,3} At later stages \textit{Shh} is required for urethral tube formation and sexual differentiation of the phallus.\textsuperscript{16–18}

A specialized transitional epithelium known as urothelium lines the bladder and functions as a barrier to contain urine. Urothelial development has been well characterized.\textsuperscript{19} However, the molecular control of cell type differentiation in the urethra is not well understood. Progress in identifying the mechanisms of urethral cell signaling and differentiation has been hindered by the paucity of molecular markers for urethral epithelial cells.

To better characterize the transcriptome of early urethral epithelium, we used FACS to purify genetically labeled urethral plate epithelial cells from mouse genital tubercles for microarray analysis at E12.5, when these cells have been shown to have signaling activity.\textsuperscript{3} We report the identification of 84 genes that are enriched in the urethral plate endoderm of the developing mouse genital tubercle.

**MATERIALS AND METHODS**

**Mice**

\textit{Shh} is expressed in endodermal cells that give rise to the urethral plate epithelium of the genital tubercle.\textsuperscript{2,3,10} This enabled us to label urethral plate epithelial cells using a GFP-Cre fusion cassette knocked into the \textit{Shh} locus (\textit{ShhGFPCre}).\textsuperscript{20} Endodermally derived cells were genetically labeled by crossing males heterozygous for \textit{ShhGFPCre} with females homozygous for the \textit{R26ReYFP} allele. \textit{ShhGFPCre/+;R26ReYFP} embryos develop normally, and all \textit{Shh} expressing cells and their descendants are positive for eYFP.\textsuperscript{10,20} This resulted in labeling the entire urethral plate epithelium (fig. 1, A and B). The morning that a vaginal plug was detected was considered E0.5. Pregnant dams were sacrificed by cervical dislocation followed by thoracotomy. Embryos were harvested in ice-cold phosphate buffered saline.

For cell purification experiments, embryos were harvested at embryonic day E12.5 and viewed under a stereomicroscope with epifluorescence to detect GFP/eYFP activity. In all embryos confirmed by polymerase chain reaction to carry the \textit{ShhGFPCre} allele and at least 1 copy of \textit{R26ReYFP}, we detected bright yellow-green fluorescence throughout the urethral plate epithelium of the genital tubercle.

![Image](image.png)

**Figure 1.** A, whole mount \textit{ShhGFPCre;R26ReYFP} mouse at E12.5. Reduced from ×10. Inset, high magnification of genital tubercle (GT). Reduced from ×63. B, schematic of sagittal section through E12.5 mouse genital tubercle shows epithelial tissue expressing \textit{Shh} (blue areas) and dissection plane (dashed red line).\textsuperscript{10} URS, urorectal septum. cm, cloacal membrane. C, cell distribution by fluorescence emitted during FACS.
The genital tubercle was dissected at the base of the genital tubercle and placed in 0.05% trypsin at 37°C for 5 minutes. A single cell suspension was achieved by trituration using a glass pasteur pipette. The cells were then centrifuged at 1,800 rpm for 2 minutes and resuspended in 0.5 μM EDTA (ethylenediaminetetraacetic acid) containing 0.5% bovine serum albumin in phosphate buffered saline for sorting.

We refer to the endodermal cell population in the dissected genital tubercle as urethral plate epithelium, although proximally (at the base of the genital tubercle) the urogenital and anorectal sinuses have not completely separated. Therefore, this cell population included a small proportion of endodermal cells that line the cloacal membrane and the urorectal septum (fig. 1, A and B).

**FACS and RNA Preparation**

Cells that were GFP/eYFP positive (urethral) and negative (rest of the genital tubercle) were purified by FACS using a FACS Vantage SE Turbo Sort flow cytometer (BD® Biosciences) (fig. 1, C). Re-analysis of the recovered sample confirmed greater than 94% enrichment in GFP/eYFP positive cells. Three cell sorts were performed to produce 3 independent pools of GFP/eYFP positive (urethral epithelial) and GFP/eYFP negative (outside the urethra) cells. Total RNA was then extracted from each FACS sorted population using the RNeasy® Mini Kit according to the manufacturer protocol. Sample integrity was assessed with an Agilent® 2100 Bioanalyzer to compare the relative amounts of 18S and 28S rRNA, and verify that each sample yielded at least 0.1 μg RNA.

**Microarray and Gene Function Analysis**

For each sample 50 ng total RNA were used as the template in the Two-Cycle cDNA Synthesis kit according to the Eukaryotic Target Preparation Manual (Affymetrix®). After cleanup biotin labeled cRNA was synthesized using the GeneChip® IVT Labeling Kit. For each sample 15 μg labeled cRNA were fragmented and hybridized to a Mouse Genome 430 2.0 GeneChip at 45°C for 16 hours. The GeneChips were washed and stained with a GeneChip Fluidics Station 450 according to the Expression Analysis Technical Manual (Affymetrix).

A GeneChip Scanner 3000 was used to collect data into GCOS (GeneChip Operating Software) using default parameters with global scaling as the normalization protocol. The trimmed mean target intensity was arbitrarily set at 500 for each GeneChip. Expression data from all 6 chips were collated into a single class comparison analysis using BRB ArrayTools (http://brb.nci.nih.gov/BRB-ArrayTools/) to identify genes expressed at elevated levels in the urethral epithelium. To identify cell specific markers of urethral plate epithelium that are effectively absent from the adjacent mesenchyme we set a differential expression threshold of 20-fold, which allowed for a presence/absence call. The complete gene expression data set is available on the GUDMAP website (http://www.gudmap.org/About/Projects/Gudmap2/Cohn.html).

Gene enrichment analysis was performed with ToppCluster, which classifies genes by known molecular functions, biological processes, pathways, co-expression and mouse phenotypes. Co-expression analysis was performed by comparing our data to previously reported transcriptomic data on the GUDMAP website. Cytoscape (http://www.cytoscape.org/) was used to annotate the figures.

**Probe Construction and In Situ Hybridization**

Probes were made from I.M.A.G.E. clone plasmids (Open Biosystems, Lafayette, Colorado) containing the known full-length cDNA clones or by polymerase chain reaction amplification from cDNA. Sense control and antisense probes were synthesized in transcription reactions containing Dig-UTP. In situ hybridization was done on E12.5 embryos as described previously3 with certain adjustments, that is embryos were digested in 10 μg/ml proteinase K for 10 minutes, probe concentration was 0.1 to 0.5 μg/ml and 7 KTBT washes of 1 hour each were performed before the color reaction.

**RESULTS**

Using a minimum threshold of 20-fold as described we identified 89 genes that are differentially expressed between urethral and surrounding tissue, of which 84 were expressed at least 20-fold higher in the urethral endoderm (http://www.gudmap.org/Docs/Armfield_et_al_2016/Supplementary_Table1.xls). The supplementary table (http://jurology.com/) shows the complete data set. Only 5 genes were expressed at least 20-fold higher in the surrounding mesenchyme/ectoderm than in the urethral plate epithelium.

The screen was designed to identify novel markers of the urethral plate epithelium by identifying genes with enriched expression in GFP/eYFP positive cells. Thus, we expected that Shh would be detected at higher levels in GFP/eYFP positive cells. We found that Shh transcripts were greater than 20 times higher in GFP/eYFP positive cells than in unlabeled mesenchyme and ectoderm, consistent with our objective to purify urethral epithelial cells (supplementary table, http://jurology.com/).

We next performed in situ hybridizations to validate that the genes recovered from our array screen were detectable in the urethral endoderm and to map the spatial domains in the embryo. Expression domains were mapped for a subset of 54 genes, of which 45 showed strong expression in the urethral plate. These domains were homogenous throughout the developing urethral plate epithelium and they lacked any obvious regionalization along the developing urethra. Figure 2 shows select genes. The complete in situ hybridization data set can be accessed at http://www.gudmap.org/gudmap/pages/focus_in situ_browse.html?batchId=368.

Two genes (Sez6l2 and Cab39l) showed no expression in the genital tubercle, although specific staining was seen in other areas of the embryo (eg the neural tube and the limb). Seven genes showed expression in the genital tubercle mesenchyme. For
example, the expression patterns of Mpeg1 and C1qa were detected in mesenchyme at the distal tip of the genital tubercle on either side of the distal urethral epithelium, and Ano9 and Dmd were observed surrounding the urethral plate (fig. 3, A and B). A few genes such as Aldoa, Smc2l1 and Pcdhl2 were expressed in urethral and mesenchymal cells (fig. 3, C).

Gonad differentiation in mice begins at E11.0. To determine whether early markers of sexual differentiation could be detected in the urethral plate at E12.5, we looked for sexually dimorphic expression patterns in staged matched male and female embryos that were genotyped for sex. Analysis of 54 genes by in situ hybridization revealed no obvious differences in expression patterns between male and female genital tubercles at E12.5 (figs. 2 and 3, and http://www.gudmap.org/gudmap/pages/focus_insitu_browse.html?batchId=368).

To predict functions and potential interactions among genes enriched in the urethral epithelium, we used ToppCluster to annotate gene ontology and connectivity (fig. 4 and http://www.gudmap.org/Docs/Armfield_et_al_2016/Supplementary_Table1.xls). Results of ToppCluster analysis revealed that many enriched genes have known biological processes in the development of other epithelial organs, such as epithelial tube branching in the lung, epithelial-mesenchymal signaling in the prostate gland, regulation of cell division and cell-cell adhesion (fig. 4). We found that structural molecules were the most represented functional class and FoxA genes were the most represented pathway (http://www.gudmap.org/Docs/Armfield_et_al_2016/Supplementary_Table1.xls).

Figure 2. In situ hybridization of select genes. Patterns showed little spatial regionalization along urethral plate and there were no obvious differences between male and female expression patterns.

Figure 3. In situ hybridization shows expression patterns of genes not found exclusively in urethral plate. A, genes expressed in mesenchyme surrounding distal urethral plate. B, genes expressed in mesenchyme surrounding midurethral plate. C, genes expressed throughout mesenchyme.
We also compared the list of genes enriched in the urethral epithelium of E12.5 mice to the set of genes previously found to be enriched in the urothelium of the developing bladder (E12.5, E13.5, E17.5 and E18.5, and postnatal day 2) and in the urethral epithelium (E13.5). We identified 31 genes unique to the E12.5 urethral epithelium (fig. 5).

DISCUSSION
To date, few genes (eg Shh and Fgf8) have been found to specifically label the urethral plate epithelium of the genital tubercle. A lack of genetic markers for these cells has hampered progress in understanding 1) how this signaling center regulates the patterning of the external genitalia, 2) how the urethral epithelium undergoes tubulogenesis and differentiation, and 3) how mutations and copy number variants in humans may relate to abnormal external genital development. The gene expression profiling experiment that we report identified 84 novel markers of the urethral plate epithelium at E12.5, the stage at which transplantation studies have shown polarizing activity.5 Based on this analysis, our data suggest that some genes identified in the urethral plate may be involved in its organizing function and some could have roles in the initiation of urethral tube development.

A total of 45 genes that the microarrays showed to be enriched in urethral plate epithelium were found by in situ hybridization to be selective markers of these cells (ie expression was undetectable in the adjacent mesenchyme). By producing a large set of novel markers of the early urethral plate epithelium, our analysis of differential gene expression between cell types in the E12.5 genital tubercle extends previous work that profiled gene expression in later stage, intact genital tubercles.22 We anticipate that mutations in some of these genes could disrupt external genital development. Thus, this panel may provide new candidate genes for use in genetic screening of patients with genitourinary anomalies.

Synthesis of sex hormones by the gonads is initiated at the developmental stage that we examined (E12.5). However, sexually dimorphic expression of genes located on sex chromosomes and autosomes can be regulated independently of hormones by genetic and epigenetic mechanisms.23,24 Therefore, we compared gene expression patterns in male and female genital tubercles by in situ hybridization. However, we found no obvious expression differences between the sexes at E12.5. These findings are consistent with previous reports that sexual differentiation of the genital tubercle does not begin until approximately E15 in the mouse.10

Notably, for some genes, including Ano9 and Dmd, the microarrays indicated that they were enriched in the urethral epithelium whereas in situ expression patterns revealed staining in the
mesenchyme as well as the epithelium (fig. 3). While we cannot offer a definitive explanation for this, they could represent type I errors in the array data set. More interesting is the expression pattern of C1qa and Mpeg, which localized to a pair of focal populations of cells on either side of the distal urethral epithelium. Delineation of these molecular subdomains of the distal genital tubercle provides a new opportunity to interrogate the identity and role of these cells, and highlights the ability of gene expression profiling to identify previously uncharacterized cell populations.

Using ToppCluster we identified pathway interactions involving the FoxA family of transcription factors (FoxA1 and FoxA2) and Shh (http://www.gudmap.org/Docs/Armfield_et_al_2016/Supplementary_Table1.xls). Interestingly, FoxA1, FoxA2 and Shh were among the most enriched genes in the urethral epithelium (supplementary table, http://jurology.com/). FoxA proteins have been labeled pioneer transcription factors based on their ability to remodel the chromatin environment at binding sites and facilitate the recruitment of other transcription factors.25 FoxA1 and FoxA2 have important roles in differentiation and function of a number of different cell types, and deletions of the FoxA genes have been shown to cause malformations of the pancreas, vertebrae and liver.25,26 Moreover, FoxA1 and FoxA2 have been implicated in prostate and liver cancer, where they can interact directly with androgen receptor, modulate the expression of androgen receptor and estrogen receptor regulated genes, and regulate sexually dimorphic disease.27,28

Although to our knowledge the functions of FoxA1 and FoxA2 in the urethra are unknown, it is tempting to speculate that they may have roles in hormonally mediated sexual differentiation of the external genitalia. Furthermore, FoxA proteins can regulate Shh in other organ systems.29 Although Shh is required for external genital development,2,3,10,16 its regulation in the genital tubercle is not well understood. If FoxA1 and FoxA2 act upstream of Shh in the urethra, as they do in other organs, deletion of both genes in the urethral plate could recapitulate the phenotype of Shh−/− mutants.

Genes identified by ToppCluster as having structural functions include proteins with known roles in tissue maturation and maintenance, including Cldn3, Cldn6, Cldn7, Crygc, Dmd, Krt19, Krt6a, Krt7, Krt8, Upk1b and Wwc2 (fig. 2). Discovery of these factors in the urethral plate of the genital tubercle raises the

---

**Figure 5.** Genes found in E12.5 urethral plate epithelium vs genes reported to be in urogenital sinus epithelium during different developmental stages according to ToppCluster. Green boxes indicate genes reported only in E12.5 urethral plate epithelium. Blue boxes indicate genes reported in E12.5 urethral plate and other urogenital sinus epithelium.
possibility that lower urethral epithelial cells may share some properties with bladder urothelium.

Some genes enriched in the urethral endoderm at E12.5, including Upk1b, Cldn7, Krt8 and Sox2, continue to be expressed at later stages of lower genital development. This suggests that they might function at multiple stages, such as during early patterning of the genital tubercle and during later morphogenesis of the urethral tube (fig. 5). In addition, we identified 31 genes (Fgf4, Sox7, Gpr1, etc) that are enriched in the developing uretha but to our knowledge have not been reported to be enriched in other epithelial populations in the lower urinary tract (fig. 5). This raises the possibility that these genes may have specific roles in early development of the urethral epithelium. Future studies of these functions are needed to determine if and how they contribute to urethral development.

CONCLUSIONS

Taken together, the data presented identify dozens of new markers of the urethral plate epithelium, of which most have unknown functions in external genital development. The data contribute to a molecular atlas of gene expression in the genital tube (http://www.gudmap.org/gudmap/pages/focus_insitubrowse.html?batchId=368). Our analysis focused on a developmental stage (E12.5) when the urethral epithelium functions as a signaling center, organizing gene expression and patterning in the adjacent mesenchyme, but before tubulogenesis has been initiated. Previous studies have examined later embryonic stages when urethral tubulogenesis is under way at E13.5 to E17. Together these data sets provide a wealth of new tools for investigations of the molecular mechanisms of external genital development and dysmorphogenesis. We anticipate that the genes identified in this series will serve as useful molecular markers for urethral epithelial cell types, drive new research aimed at discovering developmental functions and provide new candidate genes for analysis in patients with structural defects of the external genitalia.

ACKNOWLEDGMENTS

Douglas Smith and Neal Benson assisted with FACS.

REFERENCES


29. Jeong Y and Epstein DJ: Distinct regulators of Shh transcription in the floor plate and notochord indicate separate origins for these tissues in the mouse node. Development 2003; 130: 3891.