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System Level Changes in Gene Expression in Maturing Bladder Mucosa

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Abstract

Purpose—Bladder problems clinically present early in life as birth defects that often lead to kidney failure and late in life as overactive bladder, incontinence and related disorders. We investigated the transcriptome of mouse bladder mucosa at juvenile and adult stages by microarray to identify the pathways associated with normal, healthy growth and maturation. We hypothesized that understanding these pathways could be key to achieving bladder regeneration or reawakening normal function in the elderly population.

Materials and Methods—RNA was isolated from the mucosa at 3, 6, 20 and 30 weeks postnatally. Affymetrix® Mouse 430 v2 arrays were used to profile the expression of approximately 45,000 genes. The software program Statistical Analysis of Microarrays was used to identify genes that significantly changed during the time course.

Results—No genes were significantly up-regulated during maturation. However, 66 well annotated genes demonstrated a statistically significant downward trend, of which 10 of 10 were confirmed by quantitative polymerase chain reaction. The main functions affected by age were transcription, regulation of cellular processes, neurogenesis, blood vessel development and cell differentiation. Notable genes included collagens, *Mmp2*, *SPARC* and several transcription factors, including *Crebbp*, *Runx1*, *Klf9*, *Mef2c*, *Nrp1*, *Pex1* and *Tcf4*. These molecules were indirectly regulated by inferred *Tgfb1* and *Egf* growth factors. Analysis of gene promoter regions for overrepresented upstream transcription factor binding sites identified specificity protein 1 and epidermal growth factor receptor-specific transcription factor as potentially major transcriptional regulators driving maturation related changes.

Conclusions—These findings identify a coherent set of genes that appear to be down-regulated during urothelial maturation. These genes may represent an attractive target for bladder regeneration or for treating age related loss of function.

Keywords

urinary bladder; urothelium; gene expression; aging; mice

Bladder problems are most prevalent early and then late in life with trauma, cancer, interstitial cystitis and a few other diseases taking a toll along the way. Early in life the problems are mostly congenital, including neurogenic bladder and the bladder epispadias-exstrophy complex. Although these malformations are relatively rare, high rates of perioperative morbidity, long-term complications and the need for surgical revision after surgical treatment mean that these disorders are expensive and lead to long-term loss of quality of life in patients and families.¹ The prevalence of bladder dysfunction in the form of decreasing bladder sensation, contractility and ability to postpone voiding increases with age in each gender, predisposing these individuals to various forms of incontinence and adverse consequences on quality of life.^{2,3} In 1995 incontinence was estimated to have cost in excess of \$26 billion to treat incontinence related morbidity in the elderly population.⁴

If the causes of bladder problems are to be understood and therapies improved, a key piece of the picture is surely to understand the underlying molecular mechanisms responsible for normal bladder development and regeneration. Most studies involved in understanding the molecular basis for the functional changes seen with age have focused on neural and detrusor physiology, and morphology changes in the aged population.⁵⁻⁷ Importantly some of these studies suggest that interventions directed at this molecular pathology can improve age related functional changes and restore voiding parameters.

Despite these studies there is a general paucity of knowledge on age related molecular and morphological changes in the urothelium. Although historically the urothelium has been viewed as a relatively static component of the lower urinary tract, several studies showed that it is a dynamic entity key to regulating bladder function.⁸⁻¹² Thus, the urothelium forms a key element in the system since urothelial changes alter the environment of the underlying tissue and likely result in the increasing prevalence of bladder dysfunction.

We used a mouse model to investigate molecular level changes that develop normally in the mucosa of a maturing bladder. By comparing gene expression in immature mice with growing bladders with that in mature mice when the bladder has attained full size we identified the pathways responsible for normal growth. Reawakening these pathways in abnormal bladders could potentially provide novel therapy for dysfunctional bladders as well as potential biomarkers that could be used to characterize the wide spectrum of bladder disorders.

MATERIALS AND METHODS

RNA Isolation

We used wild-type FVB/N strain mice that had served as untreated controls in a cancer development study approved by the M. D. Anderson Cancer Center institutional animal care and use committee (ALS). The mucosa was teased away from the muscularis. Total RNA was isolated by homogenization of snap frozen mucosa using TRI Reagent®, followed by standard organic extraction, precipitation and purification on RNeasy® RNA purification columns. Purity and yield were determined by ultraviolet absorbance over a range of 220 to 320 nm. RNA concentration was determined by the SmartSpec™ Plus Spectrophotometer.

DNA Microarrays

Probe synthesis and array hybridization were done using established Affymetrix methods. Briefly, 2 µg purified mucosal RNA were reverse transcribed into cDNA using a T7 promoter-(deoxythymidine)24 primer. After second strand synthesis biotin labeled cRNA was generated from the double strand template using T7 RNA polymerase. The quality of the cRNA probe was verified by running an aliquot on agarose gel. Exactly 20 µg labeled cRNA were hybridized to the Affymetrix GeneChip® Mouse Expression Set 430 chip for 16

hours at 45°C in 300 ml premixed hybridization solution containing labeled hybridization control prokaryotic genes (bioB, bioC, bioD and cre). Replicate spots for each control gene are present on the chip. Chips were washed in the GeneChip Fluidics Station automatic washer and scanned on the GeneArray® fluorometric scanner.

qPCR Confirmation of Expression

RNA was isolated from a completely different set of bladders from mice at ages 6 and 30 weeks. RNA was pooled from 5, 6-week and 3, 30-week samples, and reverse transcribed, as described. Concentrations of 10 messages were then determined using the equivalent of 100 ng total RNA in duplicate using primers. All primers were selected so that the products would have T_m in the range of 80°C to 84°C. All primers were tested with standard mouse qPCR reference mouse total RNA (Stratagene®) to show a single product and that no product was obtained in the absence of template.

Analysis

Data—Affymetrix.CEL files were preprocessed with the *affy* Bioconductor¹³ package using the *rma* function with quantile normalization. The total number of chips was calculated as 4 time points (3, 6, 20 and 30 weeks) × 2 replicate bladder mucosa RNA samples, except for the one at 6 weeks, which only had a single sample. SAM¹⁴ was used to identify genes that significantly changed during the mouse life span. Due to 1 missing replicate at 6 weeks sample labels for SAM time course analysis were set as 1Time3Start 1Time6 1Time20 1Time30End 1Time3Start 1Time20 1Time30End. One class time course analysis was performed with default settings and the time summary method, slope. This analysis looks for genes with a slope of gene expression during the time course that statistically significantly differs from the slope of most genes. The δ parameter was set to 0.6 to achieve an FDR of less than 10%.

Bioinformatics—Significantly overrepresented gene ontologies were identified using DAVID (<http://david.abcc.ncifcrf.gov/>).¹⁵ The gene list was submitted to DAVID and gene functional classification was performed against the Affymetrix Mouse430_2 array background.

Biologically relevant networks were assembled from clusters and groups of common genes using IPA (<http://www.ingenuity.com/>). Common TREs shared by genes in each ontological cluster were analyzed using the Web based Promoter Analysis and Interaction Network Toolset (<http://www.dbi.tju.edu/dbi/tools/paint/>)¹⁶ against the whole list of genes in the microarray with default settings. The TRANSFAC PRO database was used and Affymetrix Mouse430_2 array was set as background. FDR was set to 0.3.

RESULTS

SAM identified 66 unique genes, all of which showed a downward trend with age. Three of these 66 genes (*SPARC*, *Col1A2* and *Tmem176a*) were present as duplicate spots on the array and the 2 spots were independently identified as differentially expressed. With the parameters used the expected median number of false-positive findings was 6.84 with an 8.55% false discovery rate. Table 1 shows gene ontology. Of the 66 genes 26 were involved with transcriptional regulation. These genes were known transcriptional regulators or zinc-ion binding proteins, which indicates a transcriptional regulator of unknown function. The second major ontology was the extracellular matrix. Included were genes coding for the 2 α 1 collagen chains along with collagens 4 α 1 and 6 α 3, and the matricellular gene *SPARC*. Also in this ontological cluster were *Mmp2* and *Tgf β i*, which codes for a multifunctional protein that responds to transforming growth factor- β 1 and regulates cellular proliferation

and differentiation. Another 12 genes were associated with the regulation of cellular processes, including genes associated with neurogenesis, angiogenesis, cell differentiation and sex differentiation or sex steroid regulated processes. Protein modification, particularly glycosylation, also comprised a significant set of gene ontologies. Although they were too small to determine statistical significance, we also noted *Bach1* and *Dchr24*, 2 genes involved with oxidative stress. Ten of these genes were confirmed by independent assays of qPCR of RNA obtained from a completely different set of mouse bladder urothelial samples. Results showed the relative change in expression of 9 genes with significant expression changes on microarray and 1 (glyceraldehyde-3-phosphate dehydrogenase) that was expected to be constant. Figure 1 shows that the correlation between the 2 measurements was greater than 90% with a mean $\pm$ SD slope of 0.93 ± 0.10 , which was not statistically different from the ideal of 1.0. The independent confirmation of all 10 genes supports that the stringent statistical criteria selected few false-positive results.

The existence of upstream promoter sequences is independent of any a priori knowledge other than the sequence of DNA. The finding of overrepresented promoter binding sequences or TREs in a cluster of genes with similar expression profiles may suggest that a common regulatory motif is responsible for the similarity in expression profiles. Figure 2 shows the identification of overrepresented TREs in SAM identified genes by Entrez identification number and name. Of the 66 SAM identified genes 39 had specificity protein 1 and/or epidermal growth factor receptor-specific transcription factor promoter sequences in the upstream region.

Pathway Analysis

Canonical pathways represent well studied, well understood networks. Finding the membership of differentially expressed genes in canonical pathways can provide insight into the biological functions that are represented. With a stringent gene selection approach only a few members of pathways may be identified as differentially expressed when in fact the expression of most or all genes in the pathway or network are changing in concert to a lesser degree. Thus, the genes identified by rigorous statistical criteria can be considered beacons for an entire pathway. Table 2 lists all canonical pathways that have statistically significant overrepresentation ($p < 0.01$) among the genes identified by the 2 methods. Many of these genes were previously identified in studies of hepatic fibrosis. Examination of the expression of all genes in the network showed that for the most part all followed the same downward trajectory.

Pathway analysis tools such as IPA can also construct networks that describe interactions among genes that were reported in the literature. Figure 3 shows the network formed from the 66 genes of interest. Only a single gene (*1439180_AT*) was completely unannotated and only 5 lacked sufficient information to be included in networks. Given that a significant fraction of the genome is poorly annotated, the probability of drawing 65 annotated genes is vanishingly small ($p = 3.3 \times 10^{-5}$). This indicates that the processes are well studied and represent central or core networks. Figure 3 shows the genes of interest in yellow and those inferred by the software in white. The expression of all of these genes was evaluated to ensure that all inferred genes were expressed above background. The 66 genes of interest formed a coherent network with the key hub genes *TP53*, *Tgfb1*, *Egf*, *Cebpb* and *Pparγ*.

DISCUSSION

The mouse bladder continues to grow until about the time of sexual maturity, which is attained at ages 6 to 8 weeks. The first 2 time points included the equivalent of childhood and the later 2 included young adulthood through the human equivalent of middle age. Of interest is that only down-regulated genes were identified. To be sure genes could be

identified that showed increased expression at the later time points. However, none of them showed the kind of systematic change needed to pass the stringent statistical tests of the SAM algorithm. Also, such genes did not show the kind of coherent network seen with those identified by the SAM approach (data not shown).

The findings show a coherent picture identifying matrix deposition and remodeling, transcriptional regulation, cell-surface events and growth as significant processes in bladder urothelial maturation. The collagens represent the deposition of matrix. The protein encoded by *SPARC* is associated with matrix remodeling that is permissive of urothelial proliferation^{17,18} and may also function as a collagen chaperone.¹⁹ Analysis of the upstream promoters suggested that urothelial function during this period is regulated by 2 main transcription factors, specificity protein 1 and epidermal growth factor receptor-specific transcription factor. These general transcription factors regulate a large number of genes. However, the largest functional cluster identified by DAVID consisted of genes with transcriptional regulatory functions that undoubtedly further modify this process. Other canonical pathways that seem to be involved include retinoic acid receptor signaling, oxidative stress management, the Wnt/ β -catenin developmental pathway, PPAR γ signaling and α -adrenergic signaling. The set of genes could be merged into a single coherent network with several hub genes. Hub genes in these networks usually identify key regulatory functions and may not be differentially expressed. However, they are inferred as key to the network since genes regulated by them are differentially expressed. Figure 3 shows a set of key hub genes that involve events occurring at the cell surface and the extracellular matrix. Included are the collagens along with *Tgf β i*, the encoded protein of which is an arginyl-glycyl-aspartyl containing protein that binds to the collagens. *Egf*, which is well known to have an important role in bladder growth,^{20,21} is also inferred from the number of epidermal growth factor responsive genes in the nucleus, as is *Mmp2*, which is activated by *SPARC*. Together this suggests that the entire set of genes is involved with bladder growth, matrix deposition and remodeling. Also of interest is *Ctnf*, which may be involved in recently described signaling between the urothelium and neurons.²²

A number of hub genes were identified as involving transcriptional regulation in the nucleus. *TP53*, which regulates the cell cycle, *Ppar γ* , which regulates differentiation, and *Cebpb* are inferred genes. Although PPAR γ protein is generally thought to regulate adipocyte differentiation, it has been implicated in urothelial cell differentiation²³ and animals treated with PPAR γ inhibitors experience urothelial changes.²⁴ Thus, it appears to be a member of the group of genes regulating differentiation and proliferation in the urothelium. The identification of leptin receptor and insulin receptor substrate suggests that glucose homeostasis may also be involved. CEBPB interacts with the promoter region of the *Ppar γ* gene and regulates the expression of differentiation related genes.²⁵ Several genes of interest identified by the SAM algorithm are also key hubs in the nucleus, notably *Crebbp* and *Runx1*. The *Crebbp* gene has a crucial role in embryonic development and growth control.²⁶ Thus, it seems to be an element in the growing bladder. *Runx1* expression is regulated by transforming growth factor- β 1 and epidermal growth factor but the gene has a complex set of functions, including modulating mesenchymal-epithelial interactions through fibroblast growth factor signaling²⁷ in palate development. Our evidence suggests that it also has a role in the final production of the adult bladder.

Several other transcriptional regulatory genes with fewer connections were also identified. *Nr2f2* regulates retinoic acid and steroid hormone signaling and may be involved in cell migration. The *Gsk3B* gene product is involved in energy metabolism, neuronal cell development and body pattern formation.²⁸ Also apparently part of the proliferation related complex is the transcriptional regulatory gene *Nrip1* and *Tcf4*, which is responsive to the Wnt/ β -catenin pathway. The major process was labeled hepatic fibrosis but in fact

undoubtedly represents bladder growth. Pathways are often named by the tissue or process in which they were first studied and the same genes can be involved in completely different processes.

CONCLUSIONS

In this study of the expression of the entire genome we identified 66 genes as significantly differentially expressed during final maturation of the adult mouse bladder. The main picture that emerged is of down-regulation of the processes involved in urothelial growth and differentiation, including synthesis and remodeling of the extracellular matrix, and regulation of gene expression. The findings are hypothesis generating in the sense that several pathways are implicated as involved with the processes. Understanding how this process of growth is regulated could lead to its reawakening for bladder repair or correction of congenital bladder problems. Urothelium seems to be the master controller of bladder growth²⁹ since it specifies smooth muscle growth. However, at a mechanistic level little is known about most bladder problems, including incontinence, overactive bladder, interstitial cystitis, hypertrophy and other disorders.³⁰ Thus, the key to treating bladder disorders is to dissect complex changes into comorbid and disease related processes, and then manipulate the processes associated with homeostasis. We suggest that the processes identified in this study could potentially provide targets to restore damaged bladders to normal function.

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Abbreviations and Acronyms

DAVID	Database for Annotation, Visualization and Integrated Discovery
FDR	false discovery rate
IPA	ingenuity pathways analysis
PPARγ	peroxisome proliferator activated receptor γ
qPCR	quantitative polymerase chain reaction
SAM	significance analysis of microarrays
TRE	transcription regulatory element

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Figure 1.

Gene expression relative change at 6 weeks and 6 months on microarray vs qPCR. Results are shown as fold change $\log_2$, ie difference of 2.0 corresponds to 4-fold expression change.

Figure 2.
TREs shared by genes of interest. Red bars indicate FDR 0.3, corrected p < 0.1.

Figure 3.

Combined network of genes of interest plotted by cellular site. IPA was used to assess known interactions, which was not restricted by cell type due to relative paucity of functional genomic data on urothelium, among focus gene set of 66 genes identified by SAM. Inferred genes are often hubs where processes are controlled, often by hub genes. Each gene identifier was mapped to corresponding gene object in Ingenuity Pathways Knowledge Base. Genes were not weighted by expression level and biological networks were built on this assumption. Resulting networks were merged into 1 network, further filtered by removing molecules not related to mouse (Tool Built/Keep/Species—Mouse, Relaxed filter). Network was further manually curated to remove genes inferred by Ingenuity that were expressed below background noise threshold. Yellow fill indicates focus genes. White fill indicates genes inferred by IPA from literature references.

Table 1

Ontology of genes identified by SAM as being differentially expressed during development

	p Value	Genes	
		No.	Names
Zinc ion binding (transcriptional regulators)	0.00034	19	<i>2610507B11Rik, Adam23, Akap13, Brd1, Chd4, Cntf, Crebbp, Fut11, Klf9, Limd1, Lpp, Mmp2, Nr2f2, Nsd1, Rest, Rnf170, Trim2, Zcchc14, Zfp592</i>
Transcription regulation	0.036	15	<i>Bach1, Chd4, Cntf, Crebbp, Id4, Klf9, Mef2c, Nr2f2, Nrip1, Nsd1, Pbx1, Rest, Runx1, Tcf4, Zfp592</i>
Proteinaceous extracellular matrix	0.0017	7	<i>Col1a1, Col1a2, Col4a1, Col6a3, Mmp2, Sparc, Tgfb1</i>
Pos/neg cellular process regulation	0.001	12	<i>Bex1, Cntf, Dhcr24, Gsk3b, Id4, Nrip1, Nsd1, Rdx, Rest, Socs2, Spnb2, Tcf4</i>
Neurogenesis	0.015	6	<i>Bex1, Cntf, Id4, Nr2f2, Runx1, Socs2</i>
Blood vessel development	0.077	4	<i>Mef2c, Mmp2, Nr2f2, Runx1</i>
Differentiation:			
Cell	0.092	12	<i>Bex1, Cntf, Dhcr24, Dpysl2, Gsk3b, Id4, Mef2c, Nr2f2, Pbx1, Ptgs1, Runx1, Socs2</i>
Sex	0.054	3	<i>Dhcr24, Nrip1, Pbx1</i>
Protein modification process	0.058	12	<i>B3galnt1, Cntf, Crebbp, Dhcr24, Fut11, Fut9, Gsk3b, Nsd1, Podxl, Socs2, Spnb2, Ugcgl1</i>
Protein amino acid glycosylation	0.0053	4	<i>B3galnt1, Fut11, Fut9, Ugcgl1</i>

Table 2

Ingenuity Canonical Pathways	p Value	Molecules
Hepatic fibrosis/hepatic stellate cell activation	0.0006	COL1A2, COL1A1, LEPR, MMP2
JAK/STAT signaling	0.0014	IRS1, CREBBP, SOCS2
RAR activation	0.0016	NSD1, NR2F2, CREBBP, NRIP1
NRF2 mediated oxidative stress response	0.0018	CREBBP, GSK3B, DNAJB14, BACH1
α -Adrenergic receptor signaling	0.0046	IRS1, CREBBP, MEF2C, GSK3B
Oct4 role in mammalian embryonic stem cell pluripotency	0.0087	REST, NR2F2
Wnt/ β -catenin signaling	0.0132	TCF4, CREBBP, GSK3B
PPAR signaling	0.0295	CREBBP, NRIP1