

# REVIEW ARTICLE: THE MOLECULAR ERA OF BLADDER RESEARCH. TRANSGENIC MICE AS EXPERIMENTAL TOOLS IN THE STUDY OF OUTLET OBSTRUCTION

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## ABSTRACT

**Purpose:** To review the crucial role of transgenic mice as experimental tools in the study of outlet obstruction.

**Materials and Methods:** We reviewed the literature for studies that have used mice as models for outlet obstruction.

**Results:** The combination of genetic manipulations and cellular physiology defines state-of-the-art experiments that explore the reciprocal mesenchymal-epithelial interactions that regulate bladder cell mechanisms.

**Conclusions:** The use of transgenic mice in bladder research has provided important data with respect to the molecular signals that drive bladder development, homeostasis, and the response to injury.

KEY WORDS: mouse, bladder, obstruction, fibroblast growth factor, SPARC

Bladder dysfunction in children is common and covers a broad spectrum from urinary incontinence and urinary tract infection to neurogenic bladder and dysfunction. In their most severe forms, these complications can lead to end stage renal disease and renal transplantation. Disease entities that severely affect bladder function include: 1) neurogenic bladder dysfunction related to spinal cord malformation, 2) bladder dysfunction related to posterior urethral valves or other urethral obstruction (the most common cause of renal failure in young boys) and 3) bladder exstrophy. In all these conditions the bladder exhibits abnormal physiology and embryology, leading to significant changes in bladder function.

An understanding of the molecular signals that drive bladder development, homeostasis, and the response to injury would be of tremendous benefit to the clinician. Under normal conditions, the process by which mesenchymal cells of the bladder wall differentiate into smooth muscle cells and fibroblasts is under the direct influence of the bladder epithelium. Undifferentiated epithelial cells are believed to evolve into an urothelium through signals that originate in the mesenchyme. This reciprocal phenomenon is referred to as *cross-talk*. Under pathological conditions that lead to abnormal bladder compliance and function, similar types of cell-cell interactions between mesenchyme and epithelium are also involved. The fibroblast growth factor (FGF) receptor (FGFR) signal transduction complex is a major candidate to mediate these processes. Very little is known about how this complex is regulated in the bladder, however. It is believed that in response to injury FGF-7 is synthesized in the stroma, travels across the basement membrane, binds to FGFR (present on the basolateral face of transitional epithelium), and triggers the classical pathway of intracellular kinase signaling cascades during basal conditions that include homeostasis. These events are representative of a highly important mode of paracrine regulation that controls the proliferation of urothelial cells.<sup>1</sup> The turnover rate of normal

urothelium is among the slowest of mammalian epithelia and occurs at a rate of about once a year.<sup>2</sup> Yet, the regenerative capacity of the urothelium is known to be outstanding, especially during the first 48 hours after initiation of outlet obstruction in animal models. A determination of the factors involved in the process of normal bladder development at a molecular level could ultimately lead to methods to alter the course of abnormal bladder development.

## DEVELOPMENT OF THE MOUSE BLADDER

The bladder originates embryologically from the cloaca—the caudal part of the hindgut. The cloaca is the common cavity into which the intestinal, urinary, and generative canals open in birds, reptiles, amphibians, many fishes, and certain mammals, including mice and humans. The importance of the cloaca in bladder development is illustrated by exstrophy, a human bladder defect that results from abnormal development of the cloacal membrane. A midline closure defect develops, causing a failure of fusion of the entire lower anterior abdominal wall, including the symphysis pubis, lower urinary tract, and external genitalia. In the mouse mutation known as “Danforth’s short-tail,” homozygotes often lack the urethra and bladder, a developmental effect that can be traced to a structurally abnormal notochord, abnormal vertebrae, and a reduction of the cloaca and tail gut.<sup>3,4</sup>

*Staging criteria for embryos.* The development of the mouse embryo has typically been classified according to morphological landmarks visible at the level of the light microscope resolution.<sup>5,6</sup> Different mouse strains develop at different rates and often display variances in the relative rates of organ development. It is therefore important to realize that embryos of the same gestational age may differ in their stage of development. We refer the reader to a standard anatomical nomenclature database that contains data from crosses between F1 hybrid (C57BL x CBA) mice.<sup>7,8</sup> The C57BL is the most widely used of all inbred strains and C57BL/6 alone accounts for >14% of inbred strains reported in the literature.<sup>9</sup> The CBA strain is also widely distributed and is used as a general-purpose strain.

*Identification of bladder components from staged embryos.* Kaufman (1992) has meticulously described the embryology

Accepted for publication January 19, 2000.

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Supported by grants from NIDDK P50-DK-47659–01, the University of Washington Royalty Research Fund, and the Children's Hospital Foundation.

and development of the mouse and we refer the reader to his Atlas<sup>10</sup> for definitive and detailed information.

*8.5 to 9.5 days post coitum (dpc).* The first features of a developing urogenital system are an uncanalized pronephric duct and a nephric duct. Mice, like other rodents, exhibit a unique series of events whereby an inversion of the germ layers at the primitive streak/early somite stage takes place. The mouse embryo completes this inversion, or "turning" process, during this period, achieves the "fetal" position, and surrounds itself with extra-embryonic membranes.

*10 to 10.25 dpc.* The hindgut and its diverticulum are evident. The cloaca is present and partitioned from the exterior by the cloacal membrane. The mesonephric duct (common excretory duct) is found proximal to the site of entry into the cloaca. These ducts are now patent along their lengths but do not yet drain into the cloaca.

*11 dpc.* Abundant mesonephric vesicles are present and will eventually drain through tubules into mesonephric ducts. The latter will establish contact with the cloaca at the position where the urogenital sinus will ultimately form. The ducts do not drain into the sinus until 14 dpc and because no well-defined mesonephric glomeruli have been observed in mice, Kaufman (1992) has suggested that the mesonephros does not function as an excretory organ in mice.

*11 to 11.5 dpc.* The urogenital sinus is detectable as a developing entity, the ureteric bud can be seen in sagittal sections, and the gonad is at the "indifferent" stage.

*12.5 to 13 dpc.* The metanephros is now comprised of a recognizable outer cortical zone (with primitive vesicles) and an inner medullary region (with tubules of varying degrees of differentiation). Downward growth of the urorectal septum begins to divide the cloacal region into the urogenital sinus anteriorly and the hindgut posteriorly.

*14 to 14.5 dpc.* Primitive glomeruli are drained by copious collecting tubules which, after joining together at the hilum to form a primitive renal pelvis, empty into the ureter which travels to and opens into the urogenital sinus. At the point of contact with the mesonephritic duct, the sinus begins to differentiate into an upper portion (vesicourethral canal), which subsequently evolves into the vesical part of the urogenital sinus and ultimately the bladder. The embryo is 9 to 10 mm. in length from crown to rump after fixation. The urogenital sinus will soon be separated from the rectum. Although the mesonephris is regressing, the mesonephritic duct remains to drain the testis.

*14 to 15 dpc.* The vesical part of the urogenital sinus has developed a thick, muscular wall and a lumen becomes obvious.

*15.5 dpc.* The urogenital sinus lumen exhibits an endodermal lining. The male embryo displays ejaculatory ducts, a canilized phallic urethra, and a prostatic region of the urethra.

*15.5 to 16 dpc.* Abundant glomeruli are differentiating and migrating to the outer one-third of the kidney. By 17 dpc, glomeruli have completed their localization to the cortical region of the kidney. Major and minor calices begin to appear and will eventually function to drain into the renal pelvis by 18 dpc.

*16.5 dpc.* The vesical part of the urogenital sinus exhibits a thickening muscular wall, a recognizable lamina propria, and a distinct endodermal lining of the lumen. The prostatic urethra is clearly evident in sagittal sections.

*17.5 to 18 dpc.* Kaufman (1992) describes the ureters as fairly easy to follow as they descend and open into the lumen of the prominent bladder at the lateral angles of the trigone. Detrusor muscles are comprised of 3 layers of non-striated muscle fibers: an internal non-striated layer, a middle layer of circulatory arranged fibers, and an external non-striated layer. The mucosal membrane exhibits properties that can be classified as transitional epithelium and the submucosa appears to be able to fold when the bladder is empty.

*Summary.* It is generally accepted that an obstructive phenomenon occurs at the ureterovesical junction and in the ureter at 37 to 49 dpc and at 37 to 45 dpc in humans, respectively, and at 15 to 17 dpc and at 13.5 to 17 dpc in rats, respectively.<sup>11</sup> This phenomenon protects the kidney from high cloacal pressure when urine is not yet formed. During these events, layers of undifferentiated mesenchymal cells surround the ureter and eventually disappear to allow the ureter to expand and to recanalize.<sup>11</sup> It is becoming increasingly clear that cellular programs of cell death, or apoptosis, are involved in the disappearance of these cells and that interference with the timing of these programs has profound influences on the development of the urinary tract and bladder.<sup>12</sup>

#### MOUSE MODELS IN BLADDER RESEARCH.

*Advantages and limitations.* There are 3 outstanding reasons to use mice in bladder research. 1) There is a wealth of information in the literature that includes >60,000 and >27,000 articles indexed under keywords "mouse AND development" and "mouse AND bladder", respectively. Since this article cannot cover all published articles in this field, we have selected key studies that we feel warrant consideration. 2) The period of gestation is 21 days, a very short period of time when compared with the average 266 days for humans. 3) Powerful transgenic tools exist to manipulate the mouse genome.

Of course, the use of mice in bladder research is not without its challenges. 1) Cystometrical measurements have clearly shown that bladder compliance is influenced by bladder filling rates and circadian variations.<sup>13</sup> 2) Numerous studies have shown that rodents tend to void more at night than during the day. 3) The laboratory environment can also influence and inhibit voiding in these animals. Therefore, to minimize the influence of the laboratory environment in the inhibition of voiding, studies that measure voiding should be performed under nocturnal conditions, after the animals have been acclimated for several days. 4) When mice are housed together in groups they develop social ranking. The dominant male will designate his territory by urinary marking patterns and the submissive males will hold their urine and void in one gush within the territorial boundaries set by the dominant male.<sup>14</sup> Dominant and submissive males produce the same amount of urine, however. 5) Catherization of the urethra of male mice has been described as very difficult.<sup>15</sup> 6) Mice older than 10 months are also prone to developing submucosal mesenchymal tumors of the bladder.<sup>16</sup> Since the histogenesis of these tumors is likely to originate from primitive mesenchymal cells of the submucosa (lamina propria), it may be prudent to use young mice to avoid this concern all together.

The molecular response of the bladder to outlet obstruction via urethral ligation is a variable event despite the reports from various laboratories that an enlarged bladder is consistently observed in mice, rats, rabbits, and guinea pigs. Although the same genes seem to be affected, the extent to which the expression of these genes affected can vary. Much of what we know about these responses are derived from either northern or RT-PCR analysis of RNA extracted from bladder compartments at selected times after obstruction.<sup>15,17-21</sup> It is important to recognize that such analyses are a function of the steady-state levels of these mRNAs because these macromolecules are constantly being synthesized and degraded. However, the overall anatomical response appears to be constant from animal model to animal model and many of these events mirror the overall response in humans.<sup>21-23</sup> These events include urothelial proliferation within 24 to 48 hours<sup>2,24-26</sup> and subsequent hypertrophy of the bladder wall. Carefully controlled studies that measure the relative steady-state levels of *both* mRNA and protein provide valuable information since mRNA level increase does

not necessarily imply an increase in the corresponding protein.

The various laboratories that have published their animal studies of outlet obstruction recognize that the model is not perfect because of variable bladder response, namely genetic changes, contraction, and bladder contractile function. Considerable efforts have been made to minimize this variability at the level of method of ligation: nylon alone;<sup>15</sup> catheterization (or placement of indwelling rod) of urethra prior to ligation in rats<sup>21</sup> and in rabbits;<sup>19,22</sup> and the placement of jeweler's rings around the urethra of immature guinea pigs.<sup>17</sup>

*Wild-type, transgenic, and mutant mouse studies of bladder obstruction.* There are only a handful of studies where the molecular response to outlet obstruction has been studied in mice. After urethral ligation, the bladders of mice (like those of rats, rabbits, and guinea pigs) become larger with time. Presented below is a brief summary of studies that illustrate the power of mouse genetics that have advanced our knowledge of the basic biology of the bladder.

*Inherited urinary obstruction.* Mouse strains often exhibit enlarged bladders due to outlet obstruction via inherited genetic processes. For example, males heterozygous for the th7 haplotype, a sterile phenotype derived from a duplication of the t-complex on mouse chromosome 17, exhibit a strong propensity for acute urinary obstruction.<sup>27</sup> Lyon suggests that the obstruction is due to an unidentified abnormality of the accessory sex glands.

*Fbn1<Tsk> - "Tight Skin".* Mice carrying the "tight skin" (Tsk) mutation have thickened skin and visceral fibrosis.<sup>28</sup> A tandem duplication within the fibrillin 1 gene<sup>29</sup> causes an accumulation of extracellular matrix molecules. Although bladder collagen content and concentration was found to be 70% greater in 5 to 6 months tight-skin mice than age-matched controls, bladder mass, protein content and concentration were similar.<sup>30</sup> Overall bladder function was approximately normal. This mutation illustrates the result of an oversized Tsk fibrillin-1 gene product that is polymerized and is stably incorporated into a discrete population of beaded microfibrils with altered molecular organization.<sup>31</sup>

*Estrogenization.* Neonatally estrogenized mice have infravesical obstruction which leads to permanently altered voiding patterns.<sup>32</sup> Estrogen receptors were predominant in the nuclei of cells of the periurethral stroma, the preprostatic urethra, the bladder neck and base, but not in control mice.<sup>32</sup> Another study indicates that neonatal estrogenization affects both basal and estrogen stimulated c-fos mRNA levels in the prostate of mature mice, supporting the hypothesis that estrogen-induced morphological changes in mouse prostate may involve altered c-fos expression.<sup>33</sup>

*NOS.* Nitric oxide (NO) participates in diverse physiological processes ranging from neurotransmission to muscle relaxation (reviewed in Tiritilli, 1998).<sup>34</sup> NO is a gas with no known storage mechanism. It exhibits a biological half-life of several seconds, is extremely labile, and diffuses freely across membranes. NO can be either beneficial or detrimental depending on the cellular context. NO synthase (nNOS) must therefore be tightly regulated. One level of regulation involves synthesis of numerous nNOS mRNA transcripts by alternative splicing.<sup>35</sup>

NO is synthesized from L-arginine by NO-synthase whose activity may be regulated by intracellular calcium concentration and modulated by pharmacological compounds such as acetylcholine, 5-hydroxytryptamine, bradykinin and ADP, as well as the sheer forces produced by blood flow.<sup>34</sup> NO stimulates soluble guanylate cyclase in smooth muscles. Its action is mediated by increased intracellular cGMP which provokes smooth muscle relaxation. A body of evidence suggests that alterations in nitric oxide production may lead to impotence, urinary obstruction, or ejaculatory problems.<sup>36</sup> Therapeutic intervention in levels of nitric oxide or its mechanism of action may restore or produce desired functional effects.

The mechanisms of nitric oxide in the genitourinary tract may be enormous clinical value in the future. Mice that contain a disrupted gene for neuronal nitric oxide synthase (nNOS) exhibit gastric<sup>37</sup> and bladder outlet obstructions.<sup>36,38</sup> These studies describe an exciting in vivo model that does not require surgically-created outlet obstructions. nNOS null mice exhibit hypertrophic dilated bladders and dysfunctional urinary outlets which do not relax in response to electrical field stimulation or exposure to L-arginine,<sup>36</sup> the amino acid substrate for nNOS. In this study, the mice also urinated more frequently, permitting the authors to describe this study as a helpful model of idiopathic voiding disorders of humans.<sup>36</sup> However, in a separate study using the same NOS<sup>-</sup> animals, the increased voiding pattern was clearly linked to increased fluid intake.<sup>38</sup> These two studies illustrate the need for careful controls when mice are used.

A separate study reports on the role of inducible nitric oxide synthase (iNOS) in mice as a function of partial infravesical obstruction.<sup>39</sup> Wild-type mice were subjected to obstruction for 1, 3, and 5 weeks. Subsequent measurements included cystometric evaluations, bladder strip stimulation and relaxation studies, and reverse-transcription and polymerase chain reaction and Western analysis for iNOS. The physiologic changes associated with obstruction were comparable to other animal models and included larger bladder and abnormal cystometric curves. The enhanced expression of iNOS observed at 1 and 3 weeks after obstruction suggests as a mechanism by which the bladder responds to improve oxygenation during obstruction-induced ischemia.

*COX-2.* There are two distinct isoforms of cyclooxygenase (COX) which convert arachidonic acid to prostanoids. COX-1 is constitutively expressed in many tissues and is responsible for producing prostanoids that control normal physiologic functions. In contrast, COX-2 is the inducible isoform responsible for prostanoids synthesis in response to a variety of stimuli in different tissues and for mediation of inflammation and pain in certain diseases. Significant research and resources have been devoted to elucidating its molecular and physiological mode of action. COX-2 has been detected in the bladder during fetal development and re-expressed in response to outlet obstruction induced by urethral ligation.<sup>15</sup> This report utilizes reverse transcription and polymerase chain reaction (RT-PCR) methods to quantify the dramatic increase in the relative steady-state levels of COX-2. A follow-up report demonstrates that COX-2 expression is expressed predominantly in bladder smooth muscle cells in response to mechanical stretch after outlet obstruction.<sup>40</sup> Stretch-activated COX-2 expression thus may participate in bladder smooth muscle cell proliferation and thereby might play a role in wall thickening after obstruction.<sup>40</sup>

*Epidermal growth factor (EGF) receptor-null mouse.* The epidermal growth factor (EGF) family consists of a large number of structurally-related polypeptides that activate one or more of the four receptors of the family. The role of EGF ligands in bladder development and regeneration was studied with bladders from mice harboring a targeted disruption of the EGF-receptor (EGFR) gene.<sup>41</sup> Overall bladder size for EGFR-null mice were less massive than corresponding wild-type mice, but otherwise exhibited no distinguishable difference by light microscopy. Since EGFR-null mice die within several days after birth, their bladders were maintained under the renal capsule or in a bladder detrusor pocket of adult athymic nude mice. After 30 days, both EGFR-null and wild-type bladders survived and grew well under the renal capsule of rodent (mice or rats) hosts. Histological analysis revealed no differences. The combined data from this study demonstrated that bladder growth and regeneration was not impaired in the EGFR-null mice, suggesting that signaling through the EGFR pathway is not necessary for normal bladder and development or bladder regeneration after injury. A

possibility is that an unidentified factor was able to diffuse from the host to the bladder graft.

*Elastin-transgenic mouse.* The most abundant component of elastic fibers in the extracellular matrix of the lamina propria is the protein elastin, one of the most hydrophobic proteins known to science. In addition to providing elasticity to tissues, recent literature clearly indicates that the presence of elastin in extracellular spaces has very complex implications that involve many other molecules. A recent study describes the biosynthetic, histological, and physiologic consequences of expression of a rat tropoelastin variant protein in transgenic mouse bladder.<sup>39</sup> Bladders of transgenic animals were found to be more compliant than bladders of non-transgenic littermates. In non-transgenic mice, experimentally-induced outlet obstruction induced the typical increase in bladder volume seen in other animal models. In striking contrast, bladder volumes and compliance were unchanged in the transgenic animals. This study demonstrates the importance of elastic fibers to bladder compliancy and that the response of the bladder to injury is dependent on elastin synthesis.

*Angiotensin type 2 receptor (ATR2)-null mouse.* When urine outflow is mechanically hindered, the phenomenon of ureteral pressure-sensitive activation of renin-angiotensin is triggered.<sup>42,43</sup> Mice that lack a functional angiotensin type 2 receptor gene display congenital anomalies of the kidney and urinary tract.<sup>12</sup> Establishment of this anomaly is preceded by delayed apoptosis of undifferentiated mesenchymal cells. It seems very likely that two key ontogenic events have profound influences on the development of a functional urinary tract and bladder in mice: ureteral budding and expansion of the ureter. The phenotype of these mice impressively mimic a similar anomaly in humans where a nucleotide transition perturbs AGTR2 splicing efficiency.

*Organ cultures.* Organ cultures of intact 16 and 18-day fetal mouse bladder explants have proven useful to study development in a defined medium. For example, as the fetal bladder matures, there is an increase in the ratio of muscle protein to collagen<sup>44</sup> at the time when urine production begins.<sup>45</sup> In response to ligation of the ureters and the urethra, an enhanced packing of collagen fiber bundles within the luminal edge of the lamina propria compared with unligated bladder explants over a 4 days period.<sup>44</sup>

Explant cultures of adult mouse bladder mucosa were propagated on transparent porous membranes (uncoated or coated with extracellular matrix substrates) and used in an in vitro model for regenerating urothelium over a 22-day period.<sup>46</sup> Exposure to epidermal growth factor (EGF) and FGF-1 and culture of laminin resulted in significant expansion of the urothelium. The EGF phenomenon is attributed to increased proliferative activity and an increase in cell

number whereas FGF-1 activity is described as the consequence of increased cellularity and migration.

#### ROLE OF FGF IN DEVELOPMENT, HOMEOSTASIS, AND INJURY

*Fibroblast growth factor (FGF) family of proteins.* The FGF receptor (FGFR) signal transduction complex is comprised of 3 components:<sup>47</sup> regulatory polypeptides (FGF), transmembrane tyrosine kinases (FGFR TK), and heparan sulfate proteoglycans (FGFRHS).

The FGF system is bi-functional: 1) it mediates communication among cells to maintain homeostasis within tissues and 2) it responds to a wide variety of external biological stimuli. Thus, the system monitors both the external environment of tissues at the level of the organism and the local extracellular environment at the tissue and cell level. It is unlikely that external environmental conditions associated with health and disease do not have an impact on the FGF system within tissues.<sup>47</sup> A major challenge is the dissection of the combinatorial specificity and control of function of the three components of the FGF family in our understanding of basic bladder biology.

Each FGF polypeptide is encoded by a distinct gene.<sup>47</sup> For consistency, we use the nomenclature listed in the first column of table 1. Because FGF-1 and FGF-2 were the first to be cloned and characterized, they have been the most widely studied. FGF-1 and FGF-2 exhibit the widest expression among diverse cells and tissues. All FGF polypeptides exhibit a specific affinity for heparin and heparan sulfate that aids in purification and characterization from native and recombinant sources.

We have generated a database that contains all available data for the DNA and amino acid sequences of FGF-1 through FGF-20. This database has been compiled through an extensive literature search and by sequence-comparison analyses. An especially helpful source was the National Library of Medicine (ncbi.nlm.nih.org), a NIH-sponsored web site that has compiled an impressive amount of DNA, protein, and structural information. Based on our prior experience and training in sequence-comparison algorithms, we have developed an appreciation for complexity of the various variants and isoforms that comprise the FGF family of polypeptides. A comparison of the amino acid sequence of FGF-7 with the entries of our FGF database is shown in fig. 1. It is clear that FGF-7 and FGF-10 are closely related to each other (40% identity). Humans contain several FGF7-like sequences (FGF7-II, FGF7-III, and FGF7-IV) that are not present in mice.<sup>48</sup>

**FGF-1** stimulates the proliferation of a wide variety of

TABLE 1. FGF proteins in the bladder

	Acronym	aa <sup>1,2</sup>	Role/Function in the Bladder
FGF-1	aFGF <sup>3</sup> , HBGF <sup>5</sup> -1	155	↑ urothelial expansion/proliferation; TCC progression
FGF-2	bFGF <sup>4</sup> , HBGF-2	155	Autocrine factor in endothelial cells
FGF-3	int-2, HBGF-3	245	Cell line
FGF-4	hst-1/kFGF	206	
FGF-5		267	Cell line
FGF-6	hst-2	210	No reports
FGF-7	KGF <sup>6</sup> , HBGF-7	194	Mesenchymal-epithelial paracrine factor, see text
FGF-8	AIGF <sup>7</sup>	215	No detection in mouse in vivo; in cell lines <sup>97</sup>
FGF-9	GAF <sup>8</sup>	208	No reports
FGF-10		215	Similar to FGF-7, KO mice lack lungs, no reports in bladder
FGF-11 through FGF-20			No reports

<sup>1</sup> # amino acids of principal, mature protein from tissue; only FGF-3 thru -8, and FGF-10 contain secretory signal peptides in the nascent primary structure.

<sup>2</sup> SSP, secretory signal peptide present at the N-terminus

<sup>3</sup> aFGF, acidic fibroblast growth factor;

<sup>4</sup> bFGF, basic fibroblast growth factor;

<sup>5</sup> HBGF, heparin-binding growth factor;

<sup>6</sup> KGF, keratinocyte growth factor;

<sup>7</sup> AIGF, androgen-induced growth factor;

<sup>8</sup> GAF, glial activating factor.

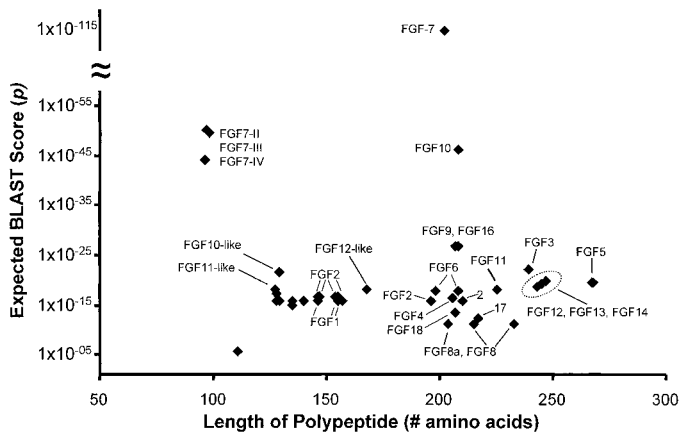


FIG. 1. FGF-7 and FGF-10 are closely related members of FGF-family of polypeptide growth factors. Amino acids sequence of precursor form of human FGF-7 (accession P21781) was used to query nonredundant protein databases with sequence comparison algorithm of BLASTP version 2.0.7.<sup>98</sup> Data are expressed as probability of random sequence alignment as a function of amino acid sequence length. Expected BLAST score of  $1 \times 10^{-115}$  is for 100% sequence identity match between FGF-7 with itself. These scores reflect *probability* that alignment will occur by chance. All data points presented indicate scores that are statistically significant.

cells including mesenchymal, neuroectodermal and endothelial cells. In a mouse organ culture that mimicked the differentiation and multilayering of normal urothelium, FGF-1 was found to stimulate expansion of the urothelium via increased cellularity and migration. A synergism was observed with laminin in the spreading of the expanded cultures.<sup>46</sup> FGF is immunohistochemically detected in tumors of the urothelium.<sup>49,50</sup>

**FGF-2** (and its variants that arise from CUG start codons or from alternative splicing) are a major mitogen for cells of various embryological origin. They are important for development, tissue regeneration, cancer, and inflammatory diseases. Its detection is highest in activated tissues. In certain cancers such as melanomas, FGF-2 is an autocrine growth factor. Neutralization of its activity leads to cessation of growth. The gene for FGF-2 is bidirectionally transcribed as an antisense transcript and encodes a functional nuclear protein with nucleoside diphosphate hydrolyzing activity.<sup>51</sup> The collective evidence points to a role for FGF-2 in bladder hypertrophy, tumor progression, and invasiveness.<sup>19,52</sup>

**FGF-7** (also known as keratinocyte growth factor [KGF]), is synthesized and secreted by stromal cells in epithelial organs thereby functioning as a paracrine mediator of epithelial cell proliferation. Unlike other FGF polypeptides that are active upon a variety of cell types, FGF-7 is specific for epithelial cells. The mRNAs encoding FGF-7 and the KGF receptor are constitutively expressed in the bladder.<sup>53</sup> FGF-7 elicits mesenchymal-epithelial signaling through a common splice variant of FGFR2 (IIIb) that is expressed in epithelial cells of developing and adult animals, including humans.<sup>54,55</sup> In contrast, bladder FGF-7 protein synthesis is restricted to stromal cells. FGF-7 synthesis in the urothelium has not been reported.

A panel of growth factors (including FGF-7) were studied by RNase protection assays in a rat model of partial outlet obstruction.<sup>21</sup> In this report, the mRNA expression for FGF-7 and receptor was unchanged with respect to control rats at 1, 2, and 4 weeks after obstruction. There was no mention of any analysis prior to 1 week, which indicates to us that this study missed the 48-hour window during which FGF-7 levels would have risen to stimulate urothelial cell proliferation and then gradually declined to basal levels by 1 week. A followup study demonstrated that at 12 hours after injury FGF-7 mRNA levels in the anterior wounded bladder half

and posterior non-wounded bladder half were 8 and 6 times higher, compared with unoperated control bladders.<sup>1</sup> Administration of recombinant human FGF-7 to neonatal mice by subcutaneous injection resulted in the marked stimulation of urothelial proliferation when compared with age matched control animals as judged by [<sup>3</sup>H]-thymidine labeling.<sup>1</sup>

Two studies that elicited overexpression of FGF-7 in systemic circulation warrant consideration. 1) Injection of recombinant FGF-7 to rats and monkeys induced a dramatic increase in epithelial cell proliferation as soon as 1 day after injection.<sup>53</sup> This increase was not quantified. 2) Expression of FGF-7 was directed to hepatocytes during the later period of mouse gestation using a human apolipoprotein E (ApoE) gene promoter and its associated liver-specific enhancer. In organs that expressed the KGF receptor there were distinct morphological abnormalities—the most striking being marked hyperplasia and cystic dilation of the cortical and medullary kidney collecting duct system, a phenotype resembling infantile polycystic kidney disease in humans. Prominent and variable hyperplasia was observed in biliary epithelium and in bladder/ureter urothelium, respectively.<sup>56</sup> The collected evidence is consistent with the function of FGF-7 in mesenchymal-epithelial signaling required for normal embryonic growth and development. The phenotypic changes observed in the developing ApoE:FGF-7 transgenic mouse embryo are similar to those observed in adult rats following administration of recombinant FGF-7.

These results are significant because the initial response of the bladder to obstruction is proliferation of the urothelium and upregulation of FGF-7 expression.<sup>18</sup> Normal urothelial turnover is estimated to occur at a rate of once a year, one of the slowest turnover rates among mammalian epithelia.<sup>2</sup>

The original report for disruption of the gene encoding FGF-7 indicates that these mice (FGF-7-null) appeared developmentally normal and fertile.<sup>57</sup> Tissues examined included back skin, tongue, ear, fore-stomach, salivary glands, kidney, lung, spleen, liver, small intestine, and heart. The one feature observed in FGF-7-null mice that was not compensated for by other factors was a matted fur appearance and a defect in cells giving rise to the hair shaft.<sup>57</sup> The second report on FGF-7-null mice<sup>58</sup> demonstrates that FGF-7 modulates ureteric bud growth and nephron numbers in the developing kidney.

A recent report demonstrates that FGF-7 is internalized by receptor-mediated endocytosis and illustrates the involvement of clathrin-coated pits in this process.<sup>59</sup> The exogenous FGF-7 used was a chimeric protein obtained by fusion of FGF-7 to the HFc portion of immunoglobulin G<sup>60</sup> that facilitated tracking of the exogenous protein. No mention was made of nuclear translocation.

**FGF-10** is a potent mitogen for mouse epidermal keratinocytes. It is expressed during development and preferentially in the adult lung.<sup>61</sup> FGF-10 deficient mice lack limbs and lungs, indicating that FGF-10 is absolutely required for normal patterning and developing during early phases of limb bud initiation and pulmonary branching morphogenesis.<sup>62</sup> The sequence of FGF-10 is related to the sequence of FGF-7 and both proteins interact with the same high affinity receptor—the KGFR isoform of FGFR2.<sup>61</sup> Heparin inhibits the mitogenic activity of FGF-7 but stimulates that of FGF-10, presumably through a regulatory mechanism involving the extracellular matrix.<sup>61</sup> Whether FGF-10 is expressed in the bladder has not been reported.

*Regulation of FGF-receptor complex formation.* The 3 components of the FGF regulatory system originate and often preexist within tissues, suggesting that restriction of their activity and access to each other are major features of their regulation as well as overall synthesis and metabolism.<sup>47</sup> The participation of pericellular HS proteoglycan as an integral component of the FGFR complex has been studied in the laboratory with heparin and heparan sulfates. The

range of heparin-like agents that affect FGF activity is much more restricted than the range of agents that bind to the isolated factor. This point is illustrated by the observation that heparin inhibits the mitogenic activity of FGF-7 but stimulates that of FGF-10 presumably through a regulatory mechanism involving the extracellular matrix.<sup>61</sup>

Upregulation of FGF polypeptide in response to bladder obstruction is believed to occur in stromal cells. Newly synthesized FGF (of mesenchymal origin) would then target its receptor present on the basolateral face of epithelial cells. These interactions would likely be associated at sites where epithelial cells contact their basement membrane. In the lens, inducers of epithelial cell differentiation (for example, FGF-1 and FGF-2) can be transported from various sites across the basement membrane where they can bind to heparan sulfate proteoglycans<sup>63</sup> and to specific FGF receptors.<sup>64</sup> The mechanism by which these soluble inducers of differentiation cross the basement membrane and the extracellular matrix is unknown.

**Translational regulation of FGF expression.** FGF-2 expression is also controlled at the translational level.<sup>65</sup> The FGF-2 mRNA is the most complex system of translational regulation known. Ninety percent of the 6,744 nt long FGF-2 mRNA is composed of nontranslated regions with a GC-rich 5' leader of a 350 nt and an AU-rich 3' untranslated region (UTR) of about 6,000 nt.<sup>66</sup> FGF-2 mRNA can be translated by 2 alternative mechanisms depending on the availability of the cap binding protein eIF-4E: the classical cap-dependent scanning mechanism<sup>67,68</sup> or the internal ribosomal entry site (IRES) mechanism. The balance between these 2 mechanisms seems to control the use of the different initiation codons, and that this balance seems to be influenced by the long 3' UTR of the FGF-2 mRNA as well as the extent to which regulatory elements bind to FGF-2 mRNA. The identification of these FGF-2 mRNA binding factors will be an important piece of the puzzle. One additional layer of mRNA complexity is worth mentioning: the length of the 3' UTR is regulated according to cell density<sup>69</sup> through the use of alternative polyadenylation sites.<sup>66</sup>

The end result of these translational regulatory mechanisms is that larger forms of FGF are synthesized through alternative initiation codons (that is CUG instead of the canonical AUG). Table 2 summarizes the available information for FGF-2. This growth factor is the prototypic example of how alternative translational initiation codons can lead to a longer form of FGF that contains additional nuclear localization signals (NLS) (see below), and thus ultimately generates a more powerful FGF-2 polypeptide. For example, the 34 kDa FGF-2 isoform is involved in cell growth maintenance, prevents cell death in low-serum conditions, and appears to be a survival factor; however, it is found in various transformed and immortalized cell types but not in primary cells.<sup>70,71</sup>

**Nuclear translocation pathway of FGF.** NLS are defined as the sequences sufficient and necessary for nuclear localization of their respective proteins, functioning as entry signals through ligand-receptor-like interactions rather than through binding to chromatin or other nuclear components. Two broad classes of NLS have been described: 1) the SV40 large tumor-antigen (T-ag) NLS is active in many proteins from plants, yeast, and animals and contains a single stretch

of single amino acids (PKKKRKV<sup>132</sup> from T-ag);<sup>72</sup> 2) the "bipartite" class of NLS, prototyped by *Xenopus* nucleoplamin and found in many transcription factors, is comprised of two stretches of basic amino acids separated by a spacer of 10 to 12 residues in length (KRPAATKKAGQAKKKLKD<sup>170</sup> from nucleoplamin).<sup>73</sup>

FGF-1 and FGF-3 exhibit the classical "bipartite" NLS while FGF-1 and FGF-2 contain the "basic amino acid stretch" NLS. These motifs have been demonstrated to be functional by site-directed mutagenesis.<sup>74</sup> The emerging picture is that synergism between NLS is very important to translocation events. The major isoform of FGF-3 is distributed to the nucleus/nucleolus or to the secretory pathway. The competing effects of 2 NLS at the amino- and carboxy-termini are the principal regulators of localization. The import of FGF-3 in energy dependent requires binding to karyopherin  $\alpha$  and uses a nucleolar retention signal.<sup>74</sup>

**Recombinant forms of FGF and their biological activities.** Bacterial expression systems have been well-proven to be the method of choice for the generation recombinant preparations of biologically active FGF proteins.<sup>74-80</sup> Most, if not all, of the commercially available preparations of human FGF polypeptides have been expressed in *Escherichia coli* using pET plasmids under the control of the T7 promoter.<sup>81</sup> Isolation methods have exploited the high-affinity of all recombinant FGF (rFGF) protein for heparin.

FGF polypeptides possess multiple conserved cysteines. Some FGFs aggregate and form disulfide bonds between surface cysteines in solution, particularly at high concentrations and in the absence of heparin.<sup>47</sup> It appears that aggregation via disulfides correlate with loss of FGF activity.<sup>47</sup>

**Is SPARC a naturally-occurring inhibitor of FGF activity in the bladder? Localization of SPARC to the urothelial-stromal interface.** The extracellular matrix (ECM) provides the paracrine path for stromal FGF-7 to traverse across and ultimately bind to FGF-receptors present on the basolateral face of the urothelium. It is very likely that transport of FGF is mediated by proteins of the ECM. It is unclear if FGF travels across the stroma by diffusion or by an active system that requires energy. One class of extracellular proteins has been termed "matricellular proteins" and defined as proteins that function both inside and outside of cells in regulating cell-matrix interactions and in mediating the activities of peptide growth factors.<sup>82</sup> The prototypic matricellular protein—termed SPARC (secreted protein acidic and rich in cysteine)—has been shown to abrogate the mitogenic activity of FGF-2.<sup>83</sup> It is not known how SPARC influences the activity of FGF-2 nor is it known if SPARC influences the activity of other FGFs. SPARC has additional activities that are defined through specific domains: disruption of cell-matrix interactions via its interaction with vitronectin,<sup>84</sup> abrogation of cytokine activity,<sup>83,85,86</sup> and inhibition of proliferation via its regulation of the cell-cycle.<sup>87,88</sup> Mice that possess a targeted disruption of the *Sparc* gene develop cataract due to a defect in lens epithelial cell differentiation and fiber cell maturation,<sup>89</sup> an important discovery because it links matricellular proteins with epithelial development and homeostasis.

We have determined the distribution of SPARC in the bladder wall of wild-type mice. Distinct patches of SPARC immunoreactivity were found scattered throughout the submucosa (fig. 2, B) and were clearly evident at the interface

TABLE 2. Features of FGF-2 Polypeptides (Arnaud et al., 1999, and references within)

Initiation Codon	MW kDa	Cytosolic or Nuclear	In Vitro Cell Type	Mode of Action	Translational Regulation
AUG	18	Cytosolic	Primary cultures near confluence	Paracrine or autocrine	IRES <sup>1</sup>
CUG	22	Nuclear	Immortalized or transformed lines	Intracrine, induced by stress	IRES
CUG	22.5	Nuclear	Immortalized or transformed lines	Intracrine, induced by stress	IRES
CUG	24	Nuclear	Immortalized or transformed lines	Intracrine, induced by stress	IRES
CUG	34	Nuclear	Immortalized or transformed lines	Intracrine, induced by stress	CAP

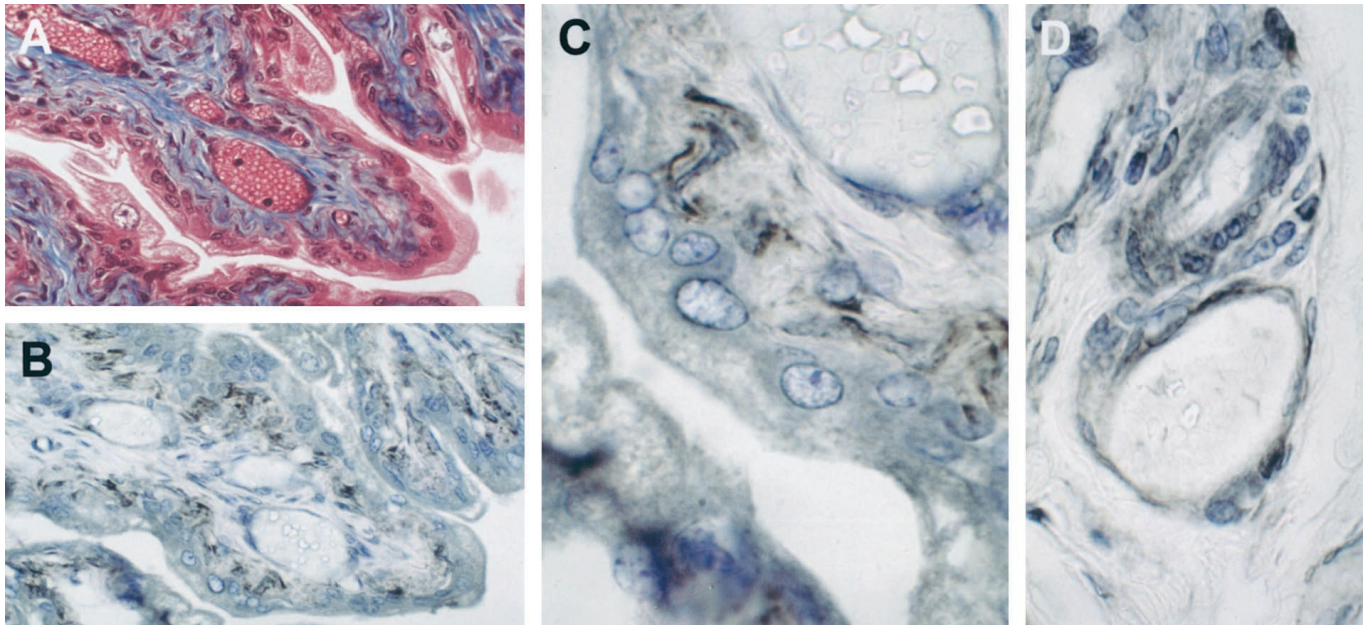


FIG. 2. Localization of SPARC to submucosa of bladder wall of wild-type mice. A, D, Masson's trichrome stain. Adjacent to bladder lumen is transitional epithelium (red). Collagen fibers of lamina propria are stained blue. Also present are blood vessels (red). B, C, E, immunohistochemical staining for SPARC (brown) via use of affinity-purified rabbit IgG raised against mouse SPARC. Counterstain is toluidine blue. Immunoreactivity is not apparent in superficial cells of transitional epithelium.

between the basolateral face of the transitional epithelium and the basement membrane (arrows in 2C). These patches of SPARC immunoreactivity are characteristic cell-matrix interactive sites and presumably indicate that SPARC is acting as a counter-adhesive factor to dismantle focal adhesion complexes.<sup>84,90</sup> This observation makes sense because of the well-known property of SPARC to influence cellular shape change<sup>91</sup> and because of the well-known property of transitional epithelium to change its shape. We have yet to determine the precise cell-type that synthesizes SPARC in the mouse urothelium. Our data indicate that the immunoreactive signal for SPARC is the least detectable in the superficial "balloon" cells and most detectable in the basal cells. This would indicate that the biosynthesis and secretion of SPARC might be polarized—a well-known property of epithelial tissue including the lens epithelium which also synthesizes SPARC.<sup>89</sup> In fig. 2, D, pronounced immunoreactivity was observed in endothelial and smooth muscle cells, a pattern consistent with that observed in human kidney aorta.<sup>84</sup>

#### OUTLOOK

*Current understanding of bladder development.* Recent work by investigators in basic science and clinical research demonstrates the complex and interdependent interaction of a variety of factors in bladder development. Mechanical processes such as cell stretch and pressure are intimately involved in the expression of growth factors which act to influence cell growth and cell signal transduction.<sup>92</sup> In vitro and in vivo studies suggest that mechanical stretch of urothelium results in the production of FGF-7 and vascular epidermal growth factor (VEGF) which act as potent mitogens.<sup>93</sup> These factors in turn influence and are influenced by neural development in utero and in the newborn period. The interplay between these processes affects cellular and extra-cellular matrix development of the bladder as well.

*Cross-talk between urothelium and mesenchyme.* Normal bladder development also relies on the interplay between urothelium and mesenchyme. This results in induction of smooth muscle function and differentiation by urothelium and vice-versa. The role of mesenchymal-epithelial interactions is not unique to bladder development. The gastro-

intestinal tract, skeletal system, and integument also rely on this interaction during organogenesis.<sup>21,94</sup> Through tissue recombination experiments investigators have shown that mesenchymal to smooth muscle differentiation in the bladder requires the presence of epithelium. Urothelial development is similarly influenced by the mesenchyme associated with it. Tissue recombination experiments using urogenital sinus mesenchyme and urothelium demonstrate prostatic ductal morphogenesis, epithelial androgen receptor expression, prostatic functional cytodifferentiation, and prostate specific secretory protein expression from normally planar bladder epithelium.

*Growth and mechanical factors.* Growth factors such as FGF-7, transforming growth factor  $\alpha$  or  $\beta$  (TGF $\alpha$  or TGF $\beta$ ), and epidermal growth factor (EGF) are likely to be some of the principle mediators of differentiation during development; the specific factors underlying this complex cell-cell signaling in bladder development remain to be elucidated, however.

Mechanical forces exerted on bladder smooth muscle and urothelium also produce changes in collagen type I and III synthesis in vitro.<sup>95</sup> These mechanical forces occur in utero with the onset of bladder cycling which begins as early as the fifth month of gestation in human embryogenesis.

From a structural standpoint, during development the normal bladder increases proportionately in weight and capacity in early and mid gestation and the rate accelerates in the last trimester. Bladder compliance increases significantly during gestation and has low intravesical pressure during early gestation that appears to be calcium-mediated tonic tension. Also, the bladder has poor contractile response to field stimulation in the early gestational period that appears to be a nitric oxide mediated phenomena.<sup>96</sup>

These studies highlight our increasing understanding that multiple factors acting interdependently include neuronal development, mechanical forces, cell signal transduction factors, and growth factors; other forces remain to be identified as significantly impacting bladder development during embryogenesis. These interactions promise to be complex.

*Value of transgenic mice in developmental biology.* Complex genitourinary anomalies influence bladder development

largely by producing urinary outlet obstruction or by creating urinary diversion. In humans, congenital anomalies such as posterior urethral valves cause antenatal urinary outlet obstruction whereas other conditions (such as bladder exstrophy) result in antenatal urinary diversion. Study of these congenital anomalies and their equivalent animal models helps us to understand the consequences of these conditions on bladder development and function.

Use of transgenic mice permits the greatest control of the environment in the study of bladder development. The ability to manipulate the genetic make-up of mice simplifies the task of determining the specific effect of a variable in this complex system. No other model affords investigators this opportunity.

The authors thank Dr. C. Plaire for a critical reading of the manuscript, Dr. H. Sage for the anti-SPARC immunoglobulins, and Mr. J. Rothmier for technical assistance.

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