From the Editor

Anthony A. Caldamone, M.D.

The SPU Research Initiative was started in 1999. In fulfilling its objectives, the SPU felt that providing start-up funds for young investigators to generate initial data that might lead to more substantial and sustainable funding would be an important accomplishment. This was voted on and approved by the members of the SPU. It is important to point out, however, that in spite of progressively decreasing external funding for educational programs of the SPU, the organization has continued to maintain this initiative as a priority. In addition some support has come from voluntary contributors from past presidents of the SPU. In that light, 21 projects have been funded over the years.

This program was initially directed by John Brock as head of the Research Committee of the SPU. John put in place an infrastructure for review of grant proposals by committee. Each proposal is reviewed and scored independently. Subsequent to John’s tenure, David Diamond and then John Park (currently) headed the Research Committee and have continued this essential mission of the SPU.

I have asked those who have been recipients of such a grant to provide the SPU membership with an update on their research projects. This serves as a feedback to us on the merits of the scientific research initiative of the SPU. Our hope is that the SPU will be able to continue these efforts in years to come as they will form the seed for the development of both clinical and basic science research in pediatric urology in the future.

2011-2012 Research Grants

Barry D. Duel, MD, Cedars-Sinai Medical Center
"Analysis of the Genitourinary Microbiome in Pediatric UTI"

His-Yan Wu, MD, Stanford University Medical Center
"Maturation of Bladder-External Urethral Sphincter Coordination"

2010-2011 Research Grants

Ahmed Haddad, MD, Hospital for Sick Children
"Establishing Biological Rationale for Preoperative Androgens in Hypospadias Surgery: Initial Human Studies in Vitro"

Stephen Zderic, MD, Hospital of Philadelphia
"Remembrances of Micturitions Past"

2009-2010 Research Grants

Dominic Frimberger, MD, University of Oklahoma Health Science Center
"Survival and Role Potential of Bone Marrow Stromal Cells in Bladder Regeneration"

Lynn Woo, MD, Vanderbilt University Medical Center
"Mesenchymal Stem Cell Recruitment, Function and Therapeutic Potential in the Bladder Following Outlet Obstruction"

2008-2009 Research Grants

Stacy T. Tanaka, MD / John C. Pope IV, MD
Vanderbilt Children’s Hospital
"The Role of Bone Marrow Derived Cells in Bladder Obstruction and Fibrosis"

Michael Hsieh, MD, PhD / James Versalovic, MD, PhD
Texas Children’s Hospital
"Probiotics-Mediated Suppression of Vaginal Biofilm Function and Pediatric Bacteriuria"

2007-2008 Research Grants

John Makari, M.D. / John Pope, MD
Vanderbilt University
"Investigation of Bladder Epithelial-Stromal Interaction Using a Bone Marrow Derived Mesenchymal Stem Cells and Embryonic Bladder Mesenchymal Tissue Recombination Model"

Patricio Gargollo, MD / Alan Retik, MD
Children’s Hospital of Boston
"The Transcriptional Regulation of Lower Urinary Tract Development"
Investigation of Bladder Epithelial-Stromal Interaction Using a Bone Marrow Derived Mesenchymal Stem Cell and Embryonic Bladder Mesenchymal Tissue Recombination Model

John H. Makari, MD, Connecticut Children's Medical Center, Division of Urology, Hartford, CT
John C. Pope, IV, MD, Division of Pediatric Urology, Vanderbilt University School of Medicine, Nashville, TN

The overall objective of this work was to enhance the understanding of (a) bladder tissue formation and (b) the mechanisms involved in TGF-b mediated bladder development. The main goal of the proposal was to characterize a new model for bladder development using human bone marrow mesenchymal stem cells (BMSC) and to study developmental defects in this model of bladder tissue formation. In order to address these goals, we sought to pursue two specific aims. All investigation was performed under approval of the Vanderbilt University Institutional Animal Care and Use Committee.

Specific Aim 1:
To characterize the ability of human bone marrow derived mesenchymal stem cells to undergo directed differentiation into urothelium and mature bladder tissues.

Characterization of human bone marrow mesenchymal stem cells
Initially, BMSC were isolated from bone marrow samples drawn from normal human subjects during bone marrow biopsy. Excess fresh bone marrow without patient identifiers was acquired from the Department of Pathology when it was deemed to be free of infectious or pathologic diagnosis. BMSC were isolated according to the study protocol. We confirmed that this cell line had characteristics consistent with BMSC by immunohistochemistry and PCR analyses. Specifically, the cells demonstrated Sca-1 positivity and α-SMA negativity by IHC. PCR demonstrated the absence of CD-34 and CD-45, compared with controls.

Tissue recombination grafts of human BMSC with mouse EBLM
We performed tissue recombination of 50,000 human BMSC and one mouse embryonic bladder mesenchymal (EBLM) shell and performed xenografting into a renal subcapsular location of athymic nude mice according to the study protocol. Grafts were harvested and stained according to the study protocol.

We found that in this tissue recombination model using human BMSC and mouse EBLM, xenografts displayed the normal histologic architecture of mature bladder structures. Human BMSC differentiated into mature urothelium, as evidenced by expression of uroplakin. Ku70 positivity by IHC demonstrates that the differentiated urothelium was produced by tissue of human origin.

We conclude that human bone marrow derived mesenchymal stem cells possess the ability to undergo directed differentiation into urothelium and mature bladder tissues.

Specific Aim 2:
To determine the role of bladder stromal cell TGF-b signaling modification/gene manipulation in bladder tissue formation using a TbRIIloxp/loxp-Col2a-Cre-ERTam transgenic mice model.

We conclude that bladder stromal cell TGF-b signaling modification/gene manipulation in bladder tissue formation using a TbRIIloxp/loxp-Col2a-Cre-ERTam transgenic mice model.

Xenografting human BMSC with Tgfb2lop/lop-Col2a-Cre-ER mouse EBLM recons.
The role of TGF-â in normal bladder development was investigated by performing the tissue recombinations/xenografts using the techniques described in the study protocol. All recombinations were performed with 50,000 human BMSC and one Tgfb2lop/lop-Col2a-Cre-ER E15 EBLM shell per graft. Untreated Tgfb2lop/lop-Col2a-Cre-ER EBLM shells served as controls (Group 1). Group 2 included Tgfb2lop/lop-Col2a-Cre-ER EBLM shells treated with 4-hydroxytamoxifen in vitro immediately after harvest, prior to recombination with human BMSC and subsequent xenografting. Groups 3-7 consisted of Tgfb2lop/lop-Col2a-Cre-ER EBLM shells recombined with human BMSC and then xenografted. These groups were used to investigate the temporal role of TGF-â signaling in normal bladder development. The nude mice hosts were treated with systemic 4-hydroxytamoxifen at days 7, 14, 21, 28, and 35, respectively, thereby knocking out stromal TbRII at various time points throughout bladder development. All host mice were euthanized on day 42.

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Representative images from each of these groups are shown below.

**Conclusions**

We have seen that this model does serve as an important method to study bladder development. This model does allow for manipulation of the TGF-α pathway at various points in bladder development.

We have seen that alteration of the TGF-α pathway markedly affects normal bladder development in this tissue recombination model. We are working to characterize the stromal-epithelial interactions that play important roles in normal bladder development and continue to investigate the specific role of TGF-α in bladder development. Further, we continue to investigate the temporal impact of the role of the TGF-α pathway in bladder development.

Ongoing experiments for investigation of Specific Aim 2 include: completion of additional time points/groups as stated in the project proposal, completion of the immunohistochemical staining for all of these groups as stated in the project proposal and further validation of our findings to improve the ability to objectively quantify our findings.
Dialogues in Pediatric Urology

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2007-2008 Society for Pediatric Urology Research Grant

Six1 and Eya1 are Critical Regulators of Peri-cloacal Mesenchymal Progenitors During Genitourinary Tract Development

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The evolutionarily conserved Six1–Eya1 transcription complex is central to mammalian organogenesis, and deletion of these genes in mice results in developmental anomalies of multiple organs that recapitulate human branchio-oto-renal (BOR) and DiGeorge syndromes. Herein, we report that both Six1 and Eya1 are strongly expressed in the peri-cloacal mesenchyme (PCM) surrounding the cloaca, the terminal end of hindgut dilation. Six1 and Eya1 are absent from the intra-cloacal mesenchyme (ICM), a cell mass that divides the cloaca into dorsal hindgut and ventral urogenital sinus. Deletion of either or both Six1 and Eya1 genes results in a spectrum of genitourinary tract defects including persistent cloaca — hypoplastic perineum tissue between external urogenital and anorectal tracts; hypospadias — ectopic ventral positioning of the urethral orifice; and hypoplastic genitalia. Analyses of critical signaling molecules indicate normal expression of Shh in the cloaca and cloaca-derived endodermal epithelia. Using a Cre/loxP genetic fate mapping strategy, we demonstrate that Six1-positive PCM progenitors give rise to the most caudal structures of the body plan including the urogenital and anorectal complex, and the perineum region. Thus, Six1 and Eya1 are key regulators of both upper and lower urinary tract morphogenesis. Results from this study uncover essential roles of the PCM progenitors during genitourinary tract formation.

Introduction

Understanding morphogenesis remains a major challenge in developmental biology. At the most caudal end of a developing mammal, the embryonic cloaca undergoes morphological changes to form two separate structures: the dorsal anorectal and the ven-

Fig. 1. Spatiotemporal expression patterns of Six1 and Eya1 during genitourinary tract development. (A–H) whole mount in situ hybridization using Six1 (A–D) and Eya1 (E–H) specific probes revealed their broad expression pattern in peri-cloacal mesenchyme (PCM) cells surrounding cloaca and metanephric mesenchyme (MM) at e10.5 (A, B, E and F) and genital mesenchymal cells at e13.5 (C, D, G and H). (I–L) Schematic representation of Six1 and Eya1 expression patterns in PCM (orange), MM (green) and intra-cloacal mesenchyme (ICM, pink) at e10.5 (I, J) and genital mesenchyme (orange) at e13.5 (K, L). Cloaca membrane (CM) is red. (M–T) Section in situ hybridization of e12 and e11.75 serial sagittal sections showed Six1 (M–P) and Eya1 (Q–T) expression in the PCM but not ICM cells. C, cloaca; dis, distal; dor, dorsal; dPCM, dorsal PCM; GT, genital tubercle; HG, hindgut; IC, intraembryonic coelom; int, intermediate; iPCM, intermediate PCM; PC, phallic cloaca; pro, proximal; SO, somite; TG, tail gut; US, urogenital sinus; ven, ventral; vPCM, ventral PCM.

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tral urogenital tracts. Caudal cloaca endodermal epithelial cells make direct contact with ectodermal epithelia to form the cloacal membrane (CM), where there is no intervening intra-embryonic mesoderm; a similar situation is observed at the rostral extremity of the gut tube in the oropharyngeal membrane. Mesodermal progenitors from the caudal part of the primitive streak pass around the side of the CM to form the peri-cloacal mesenchyme (PCM). The intra-cloacal mesenchyme (ICM) forms a cell mass that is known as the urorectal septum. Asymmetric growth and patterning of the cloacal mesoderm result in the division of the cloacal cavity and formation of the genital tubercle (GT). Malformations of the urogenital and anorectal structures are among the most common forms of congenital human birth anomalies. However, the molecular embryology of these caudal structures remains poorly understood.

Patterning of the cloacal mesoderm and morphogenesis of caudal structures likely depends on the coordinated actions of intrinsic transcriptional regulators and extrinsic signaling molecules. Recent studies have identified key extrinsic signals including sonic hedgehog (Shh) ([Haraguchi et al., 2001], [Haraguchi et al., 2007], [Lin et al., 2009], [Miyagawa et al., 2009a], [Perriton et al., 2002], [Petiot et al., 2005], [Seifert et al., 2009a] and [Seifert et al., 2009c]), fibroblast growth factors (Fgfs) ([Haraguchi et al., 2000], [Petiot et al., 2005] and [Yucel et al., 2004]), bone morphogenetic proteins (Bmps) ([Morgan et al., 2003], [Suzuki et al., 2003] and [Wu et al., 2009]), Wnts ([Lin et al., 2008], [Miyagawa et al., 2009a], [Nakata et al., 2009] and [Yamaguchi et al., 1999]), and ephrins (Dravis et al., 2004). However, our understanding of the intrinsic transcriptional mechanisms underlying these key developmental processes remains limited ([Lin et al., 2008], [Mo et al., 2001], [Morgan et al., 2003] and [Scott et al., 2005]). We, as well as others, have shown previously that Six1 and Eya1 transcription factors are critical regulators of mammalian organogenesis ([Li et al., 2003], [Oliver et al., 1995], [Xu et al., 1997] and [Xu et al., 2003]). Mouse deletions of Six1 and Eya1 recapitulate the most common features found in human branchio-oto-renal syndrome (BOR) and DiGeorge/22q11 deletion/velo-cardio-facial syndromes ([Guo et al., 2011], [Li et al., 2003], [Ruf et al., 2004] and [Xu et al., 1999]). Here, we report functional characterization of the Six1 and Eya1 transcription factors, and genetic fate mapping of Six1-positive progenitor cells during genitourinary tract development. Results from these studies show that 1) Six1 and Eya1 transcription factors are critical intrinsic regulators of PCMs; 2) cells of the ICM do not express either Six1 or Eya1, suggesting that ICM is molecularly distinct from PCMs; 3) deletion of Six1 and/or Eya1 causes a spectrum of genitourinary tract defects including persistent cloaca, hypospadias and hypoplastic genitalia; and 4) the Six1-positive PCMs but not ICM is the major source of progenitors of caudal structures of the body plan including the urogenital and anorectal complexes, and the perineum region. Together, our findings identify an essential set of transcription regulators in the PCM progenitor cells and begin to shed light on cloacal morphogenesis and human congenital urogenital/anorectal anomalies.

Results

Six1 and Eya1 are expressed in the PCM progenitor cells

The renal and genitourinary systems are descendents of the inter-mediate mesoderm. The juxtaposition and intimate relationship of the renal and genitourinary mesoderm with the cloacal endoderm suggests that they are part of a common developmental entity. Therefore, the molecular programs underlying development of these structures might be integrated. We and others have shown that Six1 and Eya1 are critical regulators of early stages of renal development ([Li et al., 2003], [Xu et al., 1999] and [Xu et al., 2003]). To investigate the possibility that Six1 and Eya1 might also be involved in the formation of the genital system, we first examined whether Six1 and Eya1 are expressed in mesodermal progenitor cells surrounding the cloaca (Fig. 1). As reported previously ([Li et al., 2003], [Oliver et al., 1995] and [Xu et al., 1997]), both Six1 and Eya1 were expressed in the intermediate mesoderm progenitors, including the metanephric mesenchyme (MM), at embryonic day 10.5 (e10.5). Six1 was also expressed in the somites of the paraxial mesoderm. In addition, both Six1 and Eya1 were expressed in the PCM cells surrounding the cloaca at e10.5 and in the developing GT at e13.5 (Figs. 1A–L).

Mesenchymal cells surrounding cloaca can be defined by their geometric positions along body axes. The dorsal PCM (dPCM), which is also known as tail gut mesenchyme, caps the most caudal end of the cloacal cavity adjacent to the dorsal cloaca membrane (dCM). The intermediate PCM (iPCM) forms bilateral genital fields along the sides of cloaca. The ventral PCM (vPCM) cells form the infraumbilical ventral wall at this stage. To determine the spatiotemporal expression pattern of Six1 and Eya1 during cloaca morphogenesis, we performed section in situ hybridization experiments on serial sagittal sections at e12 and e11.75 (Figs. 1M–T). At this stage, genital protrusion becomes apparent as a consequence of the rapid increase of the number of iPCM and vPCM cells. As expected from the whole mount in situ results at e10.5, Six1 and Eya1 were continuously expressed in the PCM at e11.5 (Figs. 1M–T). Six1 appeared to have stronger expression in the dPCM than the vPCM (Figs. 1M–P). However, neither Six1 nor Eya1 was detected in the ICM at any stage analyzed.

At e13.5, asymmetric growth of PCM cells along the dorsoventral axis has successfully relocated the CM to the ventral side of the GT, and the dorsoventral axis of CM is now elongated into the proximodistal axis of GT. The dorsoventral axis of PCM along CM is reversed to become the ventrodorsal axis of GT. Both Six1 and Eya1 were continuously expressed in a sub-population of the GT mesenchyme (Figs. 1C, D, G and H).

Six1 and Eya1 mutants exhibit urogenital and anorectal defects

To determine the potential roles of Six1 and Eya1 transcription factors in the formation of the caudal structures, we examined e17.5 and newborn single and compound Six1;Eya1 mouse gene-deletion mutants (Fig. 2). Less than 30% of Six1−/− mice had hypospadias phenotype, where urinary meatus (UM) was displaced at the ventral and proximal region of the genitalia (data not shown). Loss of one copy of Eya1 increased penetrance of the Six1−/− hypospadias phenotype to 100% (Fig. 2B). All Eya1−/− mutants had severe hypospadias phenotype and hypoplastic genitalia (Fig. 2C). Additional loss of one or both copies of Six1 gene increased severity of genital phenotype of Eya1 null mutants (Figs. 2D and E), an observation that is consistent with synergistic relationship between Six1 and Eya1 during renal and cardiac development ([Guo et al., 2011], [Li et al., 2003] and [Sajithlal et al., 2005]). The perineum, which separates the base of genitalia and anus, was hypoplastic in the Eya1 mutant and was completely absent from the Six1+/−;Eya1−/− and double null mutants (Figs. 2C, D and E). The anogenital distance was reduced by 50% in Eya1−/− mutants and almost nonexistent in the Six1−/−;Eya1−/− and double null mutants (Figs. 2C, D and E, bracket). Thus, while Eya1 seems to play predominant roles, Six1 and Eya1 synergistically regulate lower urinary tract development.

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To confirm these gross observations, we performed histological analysis of sagittal sections of both male and female mutants (Figs. 2F–O). At e17.5, a dense population of stromal cells was clearly visible in the perineum of both male and female control embryos (Figs. 2F and K, black asterisks). This tissue, however, was hypoplastic in the mutants, compared to controls (Figs. 2L, M, N and O). Together, these findings demonstrate the essential roles of Six1 and Eya1 transcription complex in the PCM and GT mesenchyme and therefore growth of genitalia (Seifert et al., 2009) (data not shown). However, the mutant urethral plate labeled by Shh appeared to be shorter than that of controls, which was consistent with the grossly smaller genitalia of the mutants at later developmental stages (Figs. 2A–D).

Unlike Shh, Bmp4 is expressed in the PCM and GT mesenchymal cells during genitourinary tract development ([Lin et al., 2008], [Lin et al., 2009] and [Suzuki et al., 2003]), which is similar to the expression pattern of Six1 and Eya1. Bmp4 is regulated by the activities of both Shh and canonical Wnt/β-catenin signaling pathways ([Lin et al., 2008], [Lin et al., 2009] and [Miyagawa et al., 2009a]). Exogenous Bmp4 suppresses cell proliferation and promotes apoptosis in GT organ culture (Suzuki et al., 2003). Conditional deletion of Bmp receptor 1a (Bmpr1a) in the surface ectoderm of GT results in increased expression of Fgf8 in the distal urethral plate endoderm and confers an overall growth advantage to the genitalia (Suzuki et al., 2003). Thus, Bmp4 is a critical component of the signaling network controlling mesenchymal cell proliferation and survival during GT development. We have reported previously that Bmp4 was ectopically up-regulated and Fgf8 was down regulated in the Six1/Eya1 compound mutants during cardiovascular development (Guo et al., 2011) (data not shown). In the Eya1 mutants, Bmp4 expression in the GT mesenchyme was increased at e11.5 (Figs. 4G and H). Consistently, Bmp4 expression was expanded in mandible component of the first pharyngeal arch and significantly increased in the second pharyngeal arch (Figs. 4I and J). At late stages of GT development (e14.0 and e14.5 Figs. 4K–N), mutants were significantly smaller than the wild type controls. Expression of Bmp4 was maintained in the distal GT region and mesenchymal cells surrounding

Fig. 2. Genitourinary tract phenotypes of Six1 and Eya1 mutants. (A–E) Gross defects of urogenital and anorectal complex of newborn male pups. Brackets indicate anogenital distance, which is reduced in the Six1−/−;Eya1−/− (C), Six1+/−;Eya1−/− (D) and Six1−/−;Eya1−/− (E) mutants. (F–O) H&E histological analyses of newborn female pups (F–J) and male pups (K–O). Urogenital and anorectal systems were separated by perineum (black asterisk), which was hypoplastic (G, H, L and M) or completely absent (J, N and O). The severe Six1−/−;Eya1−/− mutant phenotype (I, J, N and O) resembles persistent cloaca defect, in which the urinary meatus (UM), vagina (V) and anus (A) share a common opening. Brackets indicate anal channel. R, rectum.
urethral plate (Figs. 4K–N). Real time quantitative PCR (rt-qPCR) analyses, however, did not detect any significant increases of \textit{Bmp4} expression in the micro-dissected \textit{Eya1}−/− and \textit{Six1}−/−;\textit{Eya1}−/− GTs at these stages (data not shown). Since expression of \textit{Grem1}, a \textit{Bmp} antagonist, is \textit{Six1}-dependent during renal development (Nie et al., 2011), we examined its expression level in the micro-dissected e13.5 GT tissue (Fig. 4O). We found that \textit{Grem1} was significantly lower in \textit{Eya1}−/− and \textit{Six1}+/−;\textit{Eya1}−/− mutants based on quantitative rt-qPCR analyses (Fig. 4O and data not shown). Expression of \textit{Fgf8}, which is inversely correlated with \textit{Bmp} signaling during genital development (Suzuki et al., 2003), did not show any statistically significant difference (data not shown). However, a downstream effector of \textit{Fgf} signaling, dual specificity protein phosphatase 6 (\textit{Dusp6}) (Seifert et al., 2009b), was dramatically reduced in \textit{Eya1}−/− and \textit{Six1}+/−;\textit{Eya1}−/− mutants (p < 0.0001) (Fig. 4O and data not shown). Collectively, \textit{Eya1} and \textit{Six1} mutants had augmented \textit{Bmp} but attenuated \textit{Fgf} signaling activities.

Inactivation of \textit{Hoxa13} in mice leads to hypospadias and reduced AR expression (Morgan et al., 2003), and AR is essential for GT development and masculinization ( [Miyagawa et al., 2009b] and [Yucel et al., 2004]). No significant difference was observed for \textit{Hoxa13} and \textit{AR} expression in \textit{Eya1} null mutants based on rt-qPCR analyses of microdissected external GT tissues (Fig. 4O and data not shown).

**Discussion**

In this report, we provide initial functional evidence that the evolutionarily conserved \textit{Six1} and \textit{Eya1} transcription factors are essential for normal development of the genitourinary tract. We show that \textit{Six1} and \textit{Eya1} are critical intrinsic regulators of the PCM progenitors. The restricted expression patterns of \textit{Six1} and \textit{Eya1} in the PCM suggest that cells of the ICM are molecularly distinct from those in the PCM. The genetic fate mapping studies demonstrate for the first time that the PCM is the major source of progenitors of the genitourinary tract, and that PCM cells are also required for complete separation of cloaca into urogenital and anorectal tracts.

The embryological theory of mammalian cloaca morphogenesis is an ongoing debate. Theories of cloaca transformation are largely based on histological and 3-dimensional reconstruction analyses of normal and abnormal embryos ( [Hynes and Fraher, 2004], [Kluth et al., 1995], [Paidas et al., 1999], [Penington and Hutson, 2003]; [Rathke, 1832]).

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Along the rostrocaudal axis, the rostral-localized ICM expands rapidly between e10.5 and e12.5. On the other hand, the CM at the caudal cloaca lacks intra-embryonic mesoderm and eventually breaks open to form the anal and urinary orifices. This asymmetry leads to formation of the dorsal anorectal tract and ventral urogenital tract. Our observation that ICM lacks expression of Six1 and Eya1 is consistent with the idea that ICM is molecularly distinct from the PCM. This does not imply, however, that the ICM is an anatomically distinct structure from the PCM. In addition to the potential role of ICM in cloaca morphogenesis, the persistent cloaca phenotype of Six1 and Eya1 compound mutants suggests that a complete separation of urogenital and anorectal tracts also depends on the PCM. This finding is consistent with the genetic fate mapping data, which demonstrated that the Six1-positive PCM contributes to the genitourinary tract, including the perineum stromal tissue between the anus and the base of the genitalia.

Along the dorsoventral axis, proliferation and expansion of the vPCM and IPCM lead to the external protrusion and formation of the GT. This morphogenetic event coincides with increased levels of apoptosis of the dPCM and the tail gut, which are likely important for the ventral shift of cloaca and the CM ([Qi et al., 2000a], [Qi et al., 2000b] and [Sasaki et al., 2004]), and therefore patterning along the rostrocaudal axis. Previous studies using chick embryos demonstrated that the tail bud-derived mesenchyme promotes urinary tract segmentation during renal development (Brenner-Anantharam et al., 2007). Interestingly, Dil-labeled tail bud mesenchyme contributes extensively to mesenchyme surrounding the chick cloaca. Therefore, it is tempting to speculate that dPCM is important for both upper and lower urinary tract development. The dPCM is apparently expanded in the Sd mutants ([Kluth et al., 1995] and [Nakata et al., 2009]), Skt knockout (Suda et al., 2011), as well as retinoid-induced teratogenic mouse mutants ([Liu et al., 2003] and [Nakata et al., 2009]). This dorsoventral growth/patterning defect is accompanied by shortening of the dorsal CM. In these mutants, the ICM or urorectal septum fails to reach the surface and therefore is unable to divide the cloaca completely. Consequently, the dorsoventral patterning defect is coupled with abnormal development of the rostrocaudal axis in these mutants. We speculate that both CM and dPCM are involved in patterning the cloaca along these axes.

Our model predicts that genes asymmetrically expressed in the PCM might be important for cloaca and GT morphogenesis. Interestingly, Six1 appeared to be highly enriched in the dPCM (Fig. 1 and Fig. 5), which suggests that Six1–Eya1 transcription complex is directly involved in asymmetric patterning of PCM. In a preliminary gene expression array analysis, we have identified a putative Six1 target gene, which is expressed only in the dPCM but not iPCM or vPCM (unpublished data). Six1 and Eya1 functionally interact with Shh signaling pathway and both Six1 and Eya1 are down regulated in Shh mutants (unpublished preliminary observations). Although Shh is ubiquitously expressed in cloacal endoderm epithelial cells and their derivatives, Shh signaling activity seems to be asymmetrically localized based on a Gli-reporter activity ([Haraguchi et al., 2007] and [Lin et al., 2009]). Integration of Shh and canonical Wnt/β-catenin signaling pathways is critical for growth and patterning of cloaca and genitalia ([Lin et al., 2008], [Lin et al., 2009] and [Miyagawa et al., 2009a]). Therefore, in addition to evidence presented here suggests that Six1–Eya1 transcription complex might be a converging point of multiple signaling pathways during lower urinary tract development. Independent of regulating gene expression, Eya1 controls cell polarity, cell fate and Notch signaling in lung development (El-Hashash et al., 2011); and regulates actin cytoskeleton, cell shape and mobility through its intrinsic phosphatase activity (Pandey et al., 2010). Future studies will be key to understand how Six1–Eya1 complex regulates growth and morphogenesis of anorectal and urogenital complex.
Probiotics-Mediated Suppression of Vaginal Biofilm Function and Pediatric Bacteriuria

Children and adults with urinary tract infections (UTI) account for more than 1.1 million physician visits annually. In girls and women, intestinally-derived uropathogens gain access to the urinary tract via the vagina. Healthy females harbor commensal organisms such as *Lactobacillus* species which dominate the vaginal and peri-urethral microbiota. *Lactobacillus* may grow readily in the vagina because of estrogen-stimulated epithelial production of glycogen, a bacterial food source. The relationships between estrogen, glycogen metabolism, the vaginal epithelium, and lactobacilli may explain in part why prepubertal girls and post-menopausal women are prone to UTI. Many *Lactobacillus* species have been investigated for their properties as probiotics, which are “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. One mechanism by which probiotic *Lactobacillus* species may prevent UTI is through formation of vaginal epithelial biofilms which inhibit uropathogen-associated biofilm development. Specifically, competitive biofilm inhibition may occur via production of anti-adhesive factors, microbicides, occupation of available vaginal epithelial binding sites, and consumption of limited nutrients. An intriguing final possibility is that *Lactobacillus* biofilms may favorably immunomodulate the host epithelium to help it resist infection by uropathogens.

To test the hypothesis that *Lactobacillus* biofilms immunomodulate human vaginal epithelium, we first established a probiotic *Lactobacillus*-genitourinary epithelial co-culture system. VK2/E6E7 is a human vaginal epithelial cell line that was established through *in vitro* immortalization of normal premenopausal vaginal tissue using a retroviral vector expressing the human papillomavirus 16/E6E7 gene. Near-confluent VK2/E6E7 cells were co-cultured with stationary phase broth cultures of *Lactobacillus reuteri* ATCC 55730, a purported human-derived probiotic strain. An *L. reuteri* 16S rRNA-specific FISH probe and DAPI counterstaining were used to detect metabolically active *L. reuteri* cells (Figure 1). Parallel cultures were subjected to hematoxylin and periodic acid-Schiff staining (Figure 2). Both techniques revealed viable *L. reuteri* biofilms in close association with vaginal epithelial cells.

We then quantitatively confirmed both epithelial and bacterial cell viability in short-term co-cultures. VK2/E6E7 cells were co-cultured with stationary phase broth cultures of *L. reuteri* ATCC 55730. Six hours later, bacterial aliquots were drawn and OD_{600} was determined by absorbance spectrophotometry (Figure 3, right panel). Although the OD_{600} was lower for wells containing bacteria co-cultured with vaginal epithelial cells ($p=0.009$), the absolute values were similar and the decreased optical density likely reflects adherence of some bacterial cells to VK2/E6E7 cells. In parallel, VK2/E6E7 cells were trypsinized and live cells were counted using trypan blue staining (Figure 3, left panel). VK2/E6E7 cell viability was not changed by co-culture with probiotic bacteria ($p>0.05$). These experiments demonstrated that both vaginal epithelial cells and bacteria remained viable in co-culture conditions.

Next, we established a quantitative assay for longer-term co-cul-

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**Figure 1.** *L. reuteri*-specific FISH and DAPI staining of probiotic *L. reuteri*-VK2/E6E7 co-cultures. The fluorescent bacillus in the center of the field is a metabolically active *L. reuteri* cell surrounded by vaginal epithelial cells.

**Figure 2.** Periodic acid-Schiff staining and hematoxylin counterstaining of *L. reuteri*-VK2/E6E7 co-cultures. Multiple lactobacilli can be seen in association with vaginal epithelial cells. Magnification 100x.

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ture biomass using crystal violet. VK2/E6E7 cells were co-cultured with stationary phase broth cultures of *L. reuteri* ATCC 55730. Sixteen hours later, crystal violet staining and spectrophotometry (OD<sub>540</sub>) were performed to measure combined biofilm and epithelial biomass (Figure 4). Co-culture of VK2/E6E7 cells with *L. reuteri* ATCC 55730 (“VK2+55730”) resulted in stable biomass relative to single cultures of VK2/E6E7 cells or *L. reuteri* ATCC 55730 alone (“VK2+No E” and “55730+No E”) [p>0.05]. Moreover, addition of physiological concentrations of estradiol (1 mM) to single VK2/E6E7 or *L. reuteri* ATCC 55730 cultures (“VK2+E” and “55730+E”) or VK2/E6E7-*L. reuteri* 55730 co-cultures (“VK2+55730+E”) did not alter overall biomass (p>0.05).

Hence, vaginal epithelial cells and probiotic lactobacilli can be successfully co-cultured with evidence of good viability and bacterial biofilm formation.

Next, we sought to determine the effects of estrogen on glycogen production by VK2/E6E7 cells, which have been shown to express and transduce signals through estrogen receptors. VK2/E6E7 cells were cultured for 1-3 days in media containing titrated amounts of estradiol (0-1000 nM). Epithelial cell cultures containing 1000 nM estradiol had higher numbers of PAS-positive staining for intracytoplasmic glycogen than cultures with no estradiol (Figures 5 and 6, p<0.05 for comparisons of 1000 nM versus no estradiol on matching days). Therefore, the VK2/E6E7 vaginal epithelial cell line responds to estradiol by producing glycogen, a potential food source for lactobacilli.

Our next goal was to delineate how exposing vaginal epithelial cells to probiotic lactobacilli in vitro would alter expression of immune response genes. VK2/E6E7 cells were grown to near-confluence either with or without 1 mM of 17-betaestradiol. On the second day, stationary phase cultures of *L. reuteri* ATCC 55730 or *L.
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Table 1. Probiotic lactobacilli and estrogen interactively regulate immune activation-related genes expressed by vaginal epithelial cells. Red or green font indicates relative upregulation or downregulation based on comparative gene expression, respectively. Font size for gene names is proportional to magnitude of upregulation or downregulation (from 2- to 14-fold). Darker red or green colors denote gene expression comparisons with lower p values (from p=0.0002 to p=0.05).

Table 1. Probiotic lactobacilli and estrogen interactively regulate immune activation-related genes expressed by vaginal epithelial cells. Red or green font indicates relative upregulation or downregulation based on comparative gene expression, respectively. Font size for gene names is proportional to magnitude of upregulation or downregulation (from 2- to 14-fold). Darker red or green colors denote gene expression comparisons with lower p values (from p=0.0002 to p=0.05).

reuteri PTA 6475 (another purported human-derived probiotic) were added. Six hours later, the epithelial cells were lysed and DNA-free RNA was isolated. High quality RNA was reverse transcribed to cDNA and subjected to PCR amplification using a thermocycler and qPCR arrays. Differential expression of 84 immune genes was compared between VK2/E6E7 cells cultured in the absence or presence of estradiol and with either no bacteria, L. reuteri PTA 6475, or L. reuteri ATCC 55730 (Table 1). Comparisons of differential gene expression revealed that L. reuteri 6475 and 55730 induce and suppress the expression of multiple immune response-related genes in VK2/E6E7 cells. Additionally, these responses are modulated by estradiol. For example, the antigen presentation molecule CD1d is significantly downregulated in VK2/E6E7 cells by L. reuteri 6475. Addition of estradiol to L. reuteri 6475-VK2/E6E7 co-cultures abolishes L. reuteri 6475-induced downregulation of CD1d. Interferon-related factor 1 (IRF1) expression is increased by L. reuteri 55730, and the interferon-g receptor (IFNGR1) is upregulated by both L. reuteri 55730 and estradiol. Therefore, vaginal epithelial cell expression of genes involved in immune responses is significantly affected by estradiol as well as probiotic lactobacilli in a strain-specific manner.

Summary

In summary, our data indicate that probiotic lactobacilli are potent, strain-specific immunomodulators of the genitourinary epithelium whose effects are molded by estrogen. Ongoing studies are further examining the interactions between probiotic biofilms, estrogen, genitourinary epithelium, and the immune system. Future work may translate these discoveries into novel probiotic-based approaches to preventing and treating pediatric UTI.

2008-2009 Society for Pediatric Urology Research Grant

The Role of Bone Marrow Derived Cells in Bladder Obstruction and Fibrosis

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Mesenchymal Stem Cell Recruitment, Function and Therapeutic Potential in the Bladder Following Outlet Obstruction

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In children with anatomical or functional bladder outlet obstruction, anticholinergic therapy and clean intermittent catheterization are often required to prevent bladder deterioration. Surgical augmentation cystoplasty, accompanied by risk of adverse metabolic consequences and need for continued catheterization, can become necessary if bladder capacity or compliance becomes too low. With Society for Pediatric Urology Research Grant support, we studied the recruitment of bone marrow derived mesenchymal stem cells (MSC) to the bladder after outlet obstruction and the role of MSC in preserving bladder function.

Partial bladder outlet obstruction in mice causes histologic and urodynamic changes in the bladder. Chemokines alter recruitment of MSC to sites of tissue injury. We observed an upregulation of the chemokines CCL2 and CXCL12 which are associated with fibrosis in other organ systems following bladder outlet obstruction. We hypothesized that bladder outlet obstruction injury would recruit MSC to the bladder. To study MSC recruitment, we used chimeric mice whose bone marrow cells were labeled with green fluorescent protein (GFP). The bone marrow of wild type C57Bl/6 mice was reconstituted using cells from transgenic mice ubiquitously expressing GFP. The chimeric mice then underwent partial bladder outlet obstruction. MSC were consistently present in the urothelial and stromal layers of obstructed mice from 1 to 12 weeks after obstruction.

We then hypothesized that intravenous administration of additional exogenous MSC would modulate the bladder’s response to bladder outlet obstruction injury. Administered MSC were labeled with GFP (continued on next page)
and given to wild type C57Bl/6 mice that had undergone partial bladder outlet obstruction. We found that the majority of obstructed mice that were administered exogenous MSC had improved compliance curves on urodynamics compared to obstructed controls. Additionally, we found an association between the number of MSC recruited to the bladder, improved compliance and decreased collagen deposition between bladder muscle bundles.

Because we found the MSC at different locations throughout the bladder wall, we hypothesized that MSC may affect the injured bladder by release of paracrine factors. We found clusters of cells with activated EGF receptors around recruited MSC after obstruction. We also found that obstructed mice that received MSCs had less local bladder hypoxia and increased blood vessel density/vascular perfusion compared to obstructed controls.

Research supported by the Society for Pediatric Urology has led to two published manuscripts.1,2 and several presentations at national meetings. Additionally, using our data from our supported research, we have been able to compete successfully for continued funding of our work with the bladder and MSC.

Our subsequent research has focused on a better understanding of the mechanisms of MSC recruitment to the bladder after obstruction injury. The MSC in the previous experiments were a heterogeneous population of cells. We have been using cell surface protein markers to better characterize and select the administered MSC. We have also been studying the temporal changes in the bladder chemokine profile after obstruction injury and how this might affect MSC recruitment. Finally, we have ongoing experiments both with neutralizing antibodies and transgenic mice to selectively block specific chemokine interactions and to alter MSC recruitment. MSC recruitment to the bladder after bladder outlet obstruction is associated with preserved function. Elucidation of how MSC are recruited to preserve bladder function may potentially present additional therapeutic targets in our patients at high risk for bladder deterioration.

REFERENCES

Survival and Role Potential of Bone Marrow Stromal Cells in Bladder Regeneration

**Introduction**

Bone marrow stromal cells (BMSCs) have been shown to be a multipotent progenitor cell with broad differentiation capabilities. In bladder regeneration, BMSCs have been shown to improve regeneration in several small animal models after being seeded onto bioscaffolds (1). In previous studies, human embryoid stem cells were stained with a fluorescence label in vitro before transplantation for later in vivo detection. Histological studies demonstrated fluorescence labeled cells in the bladder wall four weeks after transplantation (2). However, the underlying mechanisms of bladder regeneration using cell seeded scaffolds have not been fully investigated. Possibilities for the improved regeneration include in vivo differentiation of BMSCs to cell types composing the bladder wall, or support of regeneration through the production of growth factors or chemokines. In the past genetically altered Sprague-Dawley rats have been introduced that express green fluorescent protein (GFP) throughout their entire cell lines as long as they live (“Green rats”) (3). Bone marrow cells from these green rats consistently express GFP and have shown to remain stable even after transplantation into wild-type white Sprague-Dawley rats (SD) (4). This project was designed to determine whether Green rat BMSCs survive or undergo in vivo differentiation following bladder augmentation of regular white SD rats.

**Methods and Results**

Adult BMSCs were harvested from the femur and tibia of green fluorescence protein (GFP) transgenic SD rats strain [SD-Tag(GFP)Bal]. The collected cells were plated on four 60 mm tissue culture dishes in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The BMSC cultures were incubated at 37°C for 48 hours for adherence, the floating hematopoetic cells were removed. Cell culture media was changed every two days and cell passages were performed at approximately 90-95% confluence. All adherent cells showed GFP expression. We have found that BMSCs are relatively easy to isolate and replicate at a rapid rate. At the third passage, cells were trypsinized, harvested, and seeded on SIS at the concentration of 1x10^6 cells/cm^2 in DMEM plus 10% FBS. The cell composite SIS was incubated at 37°C for another 7 days, removed from the frame, and cut to the size of 1x1 cm^2 for bladder augmentation in rats. Sections of the SIS were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin (H&E) as well as Masson’s trichrome. Ingrowth of BMSCs in the SIS was demonstrated with fluorescence microscopy and these stains.

To perform bladder augmentation, adult female SD rats were anesthetized. Following partial cystectomy to remove a 1 cm² segment of the apical bladder, the 1 cm² section of BMSC-composite SIS was sutured to the native bladder edges using 6-0 polyglactin 910 (Ethicon) in a water-tight fashion. The four corners of the suture line were marked with 6-0 nylon (Ethicon) suture to locate the grafts. Augmented bladders were harvested at 14, 28 and 56 days after augmentation.

At harvest, the bladders are inflated with 4% paraformaldehyde and fixed for 16 hours, and then sectioned in half vertically. One half of the bladder was embedded in optimal cutting temperature (O.C.T.) compound and frozen at -80°C. Frozen sections were cut at 5 µm on a cryostate at -20°C and mounted on charged microscope slides. Frozen sections were visually inspected under a fluorescent microscope to (continued on next page)
determine the presence and location of GFP expressing cells. Inspection of the area near the SIS is limited by autofluorescence of the SIS itself under fluorescence microscopy. At days 14 and 28, there were GFP-expressing cells present in the graft areas, suggesting that seeded BMSCs survived during the early stages of regeneration process. In addition, a limited number of GFP-positive cells appeared to have migrated into the surrounding submucosal tissues. At day 56, neither the smooth muscle bundles nor the urothelium show any significant GFP fluorescence.

The second half of the bladder was embedded in paraffin and sectioned at 5 μm, mounted on glass slides, baked at 60 °C and stored at room temperature. Sections were stained with H&E as well as Masson’s trichrome per standard protocols. Histological characterization of these tissue sections did not exhibit enhancement in regeneration at days 14, 28 and 56 as compared to regenerated bladders augmented with unseeded SIS.

### Discussion and Future Plans

The preliminary results suggested that the BMSCs may not directly incorporate into the regenerated bladder wall. A larger number of animals will be performed to confirm our current observations. In order to further evaluate the survival of the BMSCs, earlier time points will be included and tissue sections will be stained for cell death markers including terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and activated caspase-3 as well as cell proliferation markers such as Ki-67 along with GFP staining.

It is possible that seeded BMSCs may improve regeneration by improving vascularization of the graft tissue (5). An attempt will be made to quantify number of blood vessels in regenerated bladder using immunohistochemical staining for CD31; and results will be compared with bladders augmented with unseeded SIS.

In the future, differentiation of BMSCs to bladder smooth muscle cells (SMCs) will be characterized in vitro. Several studies have suggested that BMSCs are capable of differentiation to SMC lineage (6). To accomplish this goal, molecular techniques as well as functional assays will be applied to evaluate the gene expression profiles and contractile capabilities of BMSC after in vitro induction for SMC differentiation.

### REFERENCES

The impact of social rank on voiding function was first observed by Desjardins in 1972 when he observed that male social subordinates developed urinary retention when placed in contact with dominant males. This finding was referenced in the classic work of Dr Frank Hinman, *The Art and Science of Piddling*, which was appropriate given his seminal contributions to voiding dysfunction. In the course of performing surgical outlet obstruction, we noted that large bladders were occasionally seen in the absence of any procedure, and that this observation was made in mice with bite marks and other signs of aggression. Given our use of the NFAT-luciferase reporter strain of mice for the experiments in outlet obstruction, we were able to measure increased luciferase activity in these large bladders and realized that the calcineurin pathway must have contributed to this remodeling.

In order to effectively use this murine model, it was necessary to develop a behavioral stress protocol in detail so as to achieve reproducibility. The critical elements in this model are as follows. 1) The aggressor mice must be screened. Retired C57B16 breeder males are placed in a cage with a tubally ligated female. Screening is accomplished by removing the female and inserting into the cage a 6 week old male Swiss Webster mouse and then measuring the time needed for the retired breeder to begin to bully the younger male. This protocol works best if the aggressor attacks in under 1 minute. These encounters must be closely supervised, and the mice are separated if biting takes place. 2) The cage must be divided in half using a wire mesh barrier. Even the most dominant male will not attack if the cage size is too big; aggression will only manifest if there is a perceived threat to scarce resources. 3) The wire barrier is also critical to the behavioral protocol because once the subordinate mice are stressed by a 1 hour (or less, if biting occurs) direct exposure, the mice are then separated by the barrier. This allows for visual, auditory, and olfactory (this is probably the most important sense in the murine world) exposure which serves to reinforce the fear.

Once this protocol was refined over the course of a 1 year period, we subjected a population of 5 week old Swiss Webster mice to social stress over a 4 week time frame and were able to demonstrate the following findings. 1) Stressed mice void less frequently and with higher voided volumes. 2) These mice do not void with an elevated voiding pressure; rather they appear to suppress their voiding reflex as noted by their higher volume at micturition. These findings would suggest that social stress contributes to the abnormal voiding pattern. We were also interested in understanding whether the “memory” of the social stress contributed to the abnormal voiding pattern. A major benefit to our team is the availability of these two model systems. The rat offers the benefits of a larger bladder in which cystometry is more readily performed and a larger brain stem allows for neural recording from Barrington’s nucleus. The mouse offers a more cost effective model for long term observational studies and the benefits of numerous genetically altered strains. The following studies we describe were carried out using our murine model and were supported in part by a generous grant from the Society for Pediatric Urology.

We hypothesized that disruption of memory in the amygdala and hippocampus would mitigate the effects of social stress and prevent the voiding dysfunction. To study this we relied upon the inducible transgenic mice developed at UCLA by Alcino Silva for the study of memory circuits. The concept behind these mice was established by Eric Kandel who showed that memory is consolidated in the amygdala and hippocampus by the action of the transcription factor CREB (the cyclic AMP responsive element binding protein). Kandel also showed that the â-Calmodulin-II–Kinase (âCamKII) promoter was capable of driving gene expression to the forebrain with the amygdala and hippocampal regions being especially rich in expression. The Silva mice contain a mutation of the CREB protein that allows it to function as an inhibitor of this transcription factor (hence it is referred to as CREB). Silva developed these mice by inserting DNA coding for the CREB in front of the âCamKII promoter and put these under the control of a
ligand binding domain that was responsive to tamoxifen. In extensive testing using an afferent conditioning protocol, freeze time was reduced when the mice were primed with tamoxifen in advance of the shock that was paired with the bell tone. However no studies have been done to consider how inhibition of this pathway could affect a neural visceral remodeling process.

For these experiments, Wild Type (WT) and heterozygous transgenic (Tg) αCamKII -CREB +/- mice were subjected to the murine social stress protocol for 1 week. In one set of experiments, half of these mice were administered tamoxifen by intraperitoneal injection, the other half received peanut oil vehicle as a control. The average number of voids were clearly abnormal in the stressed group when compared to their controls in all categories except the Tg tamoxifen group (see figure 1). The differences between the stressed and control mice were highly significant (P < 0.05) for all categories except for the group that were administered the tamoxifen. The Tg mice who were stressed but received tamoxifen voided with a normal pattern and could not be distinguished from their non stressed Tg and WT controls. At 1 week with a sample size of 6 mice per group, the bladder mass was unchanged in the stress Tg tamoxifen group and increased in the Tg oil group though this difference did not quite achieve significance (P = 0.1). This is not surprising given how long we know it takes for the bladder hypertrophy to develop. This data shows that social stress induced voiding dysfunction is prevented by activation of the αCamKII -CREB transgene. We also have shown that the effect is not a function of the tamoxifen treatment alone, since stressed WT mice administered tamoxifen still develop the abnormal voiding pattern (data not shown).

We also used a part of our SPU funding to carry out a long term study of the effects of reversal. The benefit of the murine model is evident in such an experiment. A limitation of these bio behavioral studies is that they require single cage housing. As such a 1 year analysis of the long term impact of social stress on the voiding phenotype becomes very expensive (even in the mouse this trial will cost nearly $15,000 to complete). Larger species would be even more costly.

Following a 1 month period of the social stress protocol, 21 WT Swiss Webster mice were separated into individual cages. Voiding patterns were measured upon completion of the social stress protocol and at 3 month intervals thereafter. As of this time, the data at 9 months reveals a significant drop in average voids per 12 hour cycle. The stressed mice continue to void 3 times per session, far less frequently than their age matched non stressed controls who continue to void an average of 7 times. These differences remain highly significant. One confounding factor in these long term studies is the higher death rate in the stressed cohort; 45% of these mice have died. Half of these deaths are attributed to a dilated urinary tract, the other half may reflect the effects of stress on the cardiovascular or nervous systems. In contrast there have been no deaths in the group of 10 age matched control mice.

These findings would suggest that the shift in the voiding phenotype that results from social stress is not reversible even after a prolonged period free from stress. The long term data is troublesome as it suggests there is truth to the epidemiologic studies which hint that the dysfunctional voiders of today are the overactive bladder patients of tomorrow. The data from the αCamKII-CREB, +/- mice would suggest that the social stress is processed in the forebrain by mechanisms that are dependent (in part) on the transcription factor CREB. It remains to be seen if this mechanism would be reversible and which anatomic portions of the amygdala, hippocampus, and or forebrain mediate this effect. Our current ongoing work seeks to understand how these stress induced changes in the forebrain lead to the observed shifts in Barrington’s nucleus that have been noted in the rat model. Given the time and expense involved in treating dysfunctional voiding, pediatric urologists deserve a better understanding of the pathways that regulate the voiding phenotype in response to a variety of stressors. From such basic observations we hope to identify the targets for the next generation of treatments which will benefit patients across their lifespan.

REFERENCES
Maturation of the Bladder – External Sphincter Coordination

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The external urethral sphincter (EUS) is the neglected stepchild in the treatment of neurogenic bladder and dysfunctional voiding. That neglect might be justified, given how difficult it is to control, compared to bladder overactivity. Once we learned that CIC could bypass the EUS, and botulinum toxin could temporarily turn it off, there was not much need to think about the mechanisms behind EUS control. Yet, the most dangerous consequences of neurogenic bladder (autonomic dysreflexia, high pressure storage) and the pathophysiology of dysfunctional voiding are due to abnormal EUS function. My interest in the normal maturation of bladder-EUS coordination began with the clinical finding that voiding pressures fall dramatically in children as they approach 18 months of age, more so in boys than girls.1 Understanding what goes right during maturation might suggest ways to fix what goes wrong in disease. There are 2 key findings which need mechanistic explanation:

1. Spinal cord regulation of the EUS occurs at lumbar levels (L3-L4), not just at Onuf’s nucleus (L6-S1 in the rat) and the pontine micturition center.2

2. Male EUS function is markedly different from female EUS function.3

The first finding is based on spinal cord transection experiments in adult rats. In the normal rat, EUS activity (bursting) increases during voiding, the opposite of what occurs in humans. It is believed that this bursting activity helps to pull urine through the urethra of rodents, especially male rodents.3 Spinal cord transection above L3-L4 temporarily interrupts bursting activity, but bursting re-emerges 5 weeks after spinal cord injury. This led to the hypothesis that the lumbar spinal cord possesses an independent coordination center, which takes over when the pontine micturition center is removed from the circuit.2

The second finding was noted when cystometry was compared in male and female rats. Male rats exhibit many more non-voiding contractions, where the bladder pressure rises without EUS bursting,3 as illustrated below in the upper panels. These are reminiscent of what is seen in patients in dysfunctional voiding, where failure of the EUS to relax during voiding contraction leads to interrupted and staccato voiding. When a serotonergic agonist (8-OH-DPAT) is given (lower panels), the duration of EUS bursting increases, the bladder capacity increases, and in the male, non-voiding contractions are converted into voiding contractions.

We found that neonatal rats which undergo bladder reduction (BR) at 1 week of life exhibit voiding before 3 weeks of life, which is when the mature voiding reflex takes over for the neonatal perigenital-bladder reflex.4 This suggested that bladder reduction might be changing EUS function, so that bursting was occurring at an earlier age. Since previous studies had shown that serotonergic agonists enhance EUS bursting,2 we compared the effects of bladder reduction and serotonergic agonists. We found that there were no differences in cystometric parameters between BR and sham animals at 2, 3, and 4 weeks of life, and that serotonergic agonists did not change the intravesical pressure, contraction duration, or bladder capacity. However, EUS function was different in 3 week old animals. BR animals exhibited bursting amplitudes that were twice as large as shams, and administration of 8-OH-DPAT could bring the EUS bursting amplitude in shams up to that of BR animals. In addition, 8-OH-DPAT increased the EUS bursting duration of sham animals to match those of BR animals, but there was no further increase in bursting duration in BR animals when 8-OH-DPAT was given. This suggested that BR might be enhancing EUS function via a serotonergic mechanism.

Currently, we are working on creating a functional map of lumbar spinal cord response to electrical stimulation, to determine any other sites which may respond to cause EUS emptying. It will be important to determine if the lumbar coordination center acts as an on/off switch

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which responds to electrical stimulation, or if electrical stimulation only modulates the decision to cause bladder contraction and EUS emptying. We envision that a combination of pharmaceutical treatment (such as serotonergic agonists) and electrical stimulation could relieve patients of the need to perform CIC. Proper management of the bladder would still be required to maintain continence, since converting multiple non-voiding contractions into multiple voiding contractions would not help get patients out of diapers. Pairing up new bladder and EUS treatments might be the way forward in managing neurogenic bladder and dysfunctional voiding.

References


Analysis of the Genitourinary Microbiome in Pediatric Urinary Tract Infections: Microbiome Analysis in Pediatric UTI

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Urinary tract infections (UTI) account for over one million pediatric visits annually in the U.S.

1 Even with the best available treatment, these infections can cause serious complications, including hypertension and renal failure. In economic terms, the cost of pediatric UTI is enormous, in excess of $180 million per year for outpatient care alone. Unfortunately, the pathogenesis of UTI is not well understood and mechanisms of recurrence remain to be defined. High prevalence, severe long-term complications and the lack of effective preventive therapy prompt urgent, high priority investigation of UTI pathology.

The genitourinary mucosa is the major interface with the resident microflora, which plays a significant role in mucosal homeostasis and associated pathologies. While this mucosal interface can be attacked by several aggressive bacterial and viral pathogens, the resident microbiome, primarily composed of lactobacilli in adult women

2 plays a protective role by “crowding out” most opportunistic pathogens and establishing an acidic non-accommodating local environment. The genitourinary microbiome in pediatric UTI is not well understood, and investigators have only begun to apply modern molecular microbiology methods to the study of local microbiome composition and its dynamics in adults. We will use a culture-independent 16S rRNA sequencing approach to define the composition and diversity of the vaginal microbiome in prepubertal girls. This approach allows us to identify organisms which would not grow under ordinary culture conditions. Microbiomai “signatures” may provide candidate biomarkers indicative of infection, susceptibility to infection, elevated mucosal or systemic inflammatory activity, and allow prediction response to antibiotic or other intervention. We suspect that unique vaginal microbial composition and diversity profiles define differences between the recurrent and non-recurrent UTI independent of active infection. Our ultimate goal is to define a microbiome “signature” for UTI susceptibility, and develop non-antibiotic interventions for prevention and treatment.

It is a clinical fact that urinary tract infections are ascending, resulting from initial invasion of the urethra and bladder by organisms residing on the perineum, and subsequent adherence of these organi-
isms to the lining of the urinary tract. This may explain the lower rate of UTI in boys; the longer male urethra presents a physical barrier to bacterial invasion. By contrast, children with Dysfunctional Elimination Syndrome have higher rates of infections likely caused by incomplete voiding. However, anatomy and voiding function are not the only explanation for UTI. For instance, certain strains of E. coli express virulence factors which enhance adherence to the mucosal lining of the bladder and urethra, and that such aggressive microbial colonists are more prevalent in children with UTI.

Comprehensive microbiological analysis has yet to be applied to the study of UTI in children, but initial evidence indicates that the bacterial composition of the genitourinary and perineal compartments plays a critical role. In one study, female infants with UTI were shown to have decreased vaginal lactobacillus counts, based on cultures. A second report has further shown that organisms isolated from the vaginal microflora of infants have antimicrobial activity against urinary tract pathogens (5). However, these were culture-based studies targeting specific lactobacillus strains, which represent only a part of the organisms residing in the vaginal and perineal compartments. Specifically, we only find those organisms which can be grown in clinical cultures, while we still lack knowledge of the detailed composition and dynamics of the pediatric vaginal and perineal microflora prior to, or at onset of UTI, during treatment, and following treatment in recurrent cases.

Hypothesis and Goals of the Present Study

First, we postulated that the microflora of the pediatric genitourinary and perineal compartments is more complex than previous approaches have been able to demonstrate. Second, we expect that the composition of the microflora is dynamic and changes in response to such factors as pathogen exposure and antibiotic therapy. Since the resident microflora are protective against UTI, we hypothesize that UTI will be associated with perturbations in the protective homeostatic vaginal and perineal microbiome.

Our study is currently accruing patient specimens rapidly, and the analysis should be ready within the next six months. Due to the nature of pyrosequencing technology, sequences are run in batches to reduce costs. The DNA sequences from each sample have a unique “barcode” segment attached prior to sequencing. During subsequent analysis, these sequences are then recovered and tracked back to the individual specimens. Computer analysis of the sequence information then allows us to identify individual species from the specimens, and identify any differences between patient groups.

Future Directions and Long Term Goals

1. Evaluation of Children with Dysfunctional Elimination Syndrome

Dysfunctional Elimination Syndrome (DES) is a spectrum of disorders where neurologically intact children exhibit incomplete bladder emptying, bladder instability, and functional bowel problems. Given the associated chronic constipation, we hypothesize that microbiome alterations exist, and may play a role in DES patients with UTI vs. those who present primarily with symptoms such as incontinence. Treatment of the constipation, especially with probiotics, may result in a decreased incidence of recurrent UTI via re-establishment of normal protective flora.

2. Microbiome Analysis of Children with Vesicoureteral Reflux

Vesicoureteral reflux (VUR) affects up to 5% of children, and up to 50% of those who have had a culture-proven UTI. Of children with VUR, some will have no further problems but others will go on to have recurrent UTI and resultant renal scarring. Differentiating patients likely to develop recurrent UTI and associated complications is a focus of active research in pediatric urology. While past studies focused primarily on host factors such as age, sex and grade of reflux, very few investigators have examined host-pathogen interactions. It is possible that children with clinically significant reflux have some difference in their microflora or immune response accounting for their recurrent infections and renal damage. We plan to serially follow children with vesicoureteral reflux to see if there are any differences in their microflora vs. normal children. It will be most important to see whether the standard treatment, daily prophylactic antibiotics, results in adverse alterations in the microbiome. There are some studies showing no difference in breakthrough infections in children with low-grade reflux vs. those not on prophylaxis, and we currently have no explanation for this finding. In addition, we plan to study whether endoscopic or surgical treatment of VUR results in any alteration of the microflora independent of antibiotic treatment.

3. Effect of Probiotic Treatment on Microflora

There is currently great interest in treatment of children with recurrent UTI with probiotic supplements. These are live bacteria, usually lactobacillus species, which, in theory, can displace some of the potential pathogens in the large intestine, resulting in decreased risk of urinary tract colonization. To date, studies have shown little, if any benefit from these supplements. We plan to investigate whether probiotic treatment alters the genital, stool and perineal microflora. Specifically, we would like to evaluate children with a history of UTI before, during and after treatment with a standardized probiotic supplement, and correlate perineal, rectal and stool microbiome profile with the likelihood of recurrent urinary tract infection. Parallel studies could also be carried out with other alternative treatments such as cranberry extract.

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