ROLE OF *HPSE2* IN UROFACIAL SYNDROME AND OTHER NON-NEUROGENIC VOIDING DYSFUNCTIONS

by

Shorouq Hassan M Al Rebh

M.B.B.S (King Abdulaziz University) 2006

MOCK GRANT PROPOSAL

Submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL SCIENCE

in the

GRADUATE SCHOOL

of

HOOD COLLEGE

July 2015

Accepted:

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DEDICATION

I dedicated this work to my family. My husband, Jafar, for his love and continuous support and motivation. I could not have done this without you being in my life. To my two little angels, Mousa and Elias, for their uncoditional love and patience. To my parents, father-in-law, mother-in-law and grandma for their continuous prayers. And to all my sisters and brothers for being part of my life.

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to the faculty members of Biology Department at Hood College. In addition, I would like to thank my Reading Committee members Dr. Rachel Bagni, Assistant Professor of Biology and Director of the Biomedical Sciences Master's program; Dr. Ann L. Boyd, Professor of Biology; and Dr. Kristen Kindrachuk, Adjunct Professor. I would also like to thank Dr. Maria Cowles, Dean of the graduate school.

Mock Grant Application

Modeled after Department of Health and Human Services
Public Health Services
(based on Form PHS 398)

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Type	Activity	Version – HCBMS.011712
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*
Role of *HPSE2* in Urofacial syndrome and other non-neurogenic voiding dysfunctions

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR — **New Investigator** ☐ No ☒ Yes

3a. NAME (Last, first, middle)	3b. DEGREE(S)	3h. eRA Commons User Name
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4. HUMAN SUBJECTS RESEARCH	4a. Research Exempt	
☐ No ☒ Yes	N/A	
4b. Federal-Wide Assurance No.	4c. Clinical Trial	4d. NIH-defined Phase III Clinical Trial
N/A	☒ No ☐ Yes	☒ No ☐ Yes

5. VERTEBRATE ANIMALS ☐ No ☒ Yes — 5a. Animal Welfare Assurance No. N/A

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)

9. APPLICANT ORGANIZATION
Name: Hood College
Address: Department of Biology
Hood College
401 Rosemont Ave
Frederick, MD 21701

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
DUNS NO. N/A Cong. District N/A

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14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*
N/A

DATE
03/01/2015

PROJECT SUMMARY (See instructions):

Voiding dysfunctions account for 40% of pediatrics urological visits. They occur due to neurological problems where the nerve supply to the bladder is interrupted, or non-neurological problems where nerves are intact though the child is experiencing symptoms of neurological forms.

Some genetic diseases cause dysfunctional voiding in children known as Urofacial syndrome (UFS). Although UFS is rarely reported in the medical literature, it might be underdiagnosed either due to physicians' lack of knowledge or because it is presented in a dose-dependent manner. UFS is caused by mutation in *HPSE2* gene, which encodes for heparanase-2 protein. Heparanase-2 function is not well understood, and studies of its role in voiding dysfunction are ongoing.

Since UFS shares symptoms similar to those of other voiding dysfunctions, it can be a useful model to study the pathoetiology of voiding disorders, because it is caused by dysfunction of protein that might have roles in other voiding problems.

This project aims to link the genotype of heparanase-2 with the phenotype in children with non-neurological voiding dysfunction by testing them for the presence of dysfunctional heparanase-2. Identification of the heparanase-2 function in the bladder organogenesis will be based on a mouse-knockout model using morpholinos (class of antisense) to ablate heparanase-2 function.

RELEVANCE (See instructions):

This project will help understand part of the genetic pathophysiology of congenital urinary tract diseases, therefore, improving the management of children having voiding dysfunctions and identification of therapeutic targets that are relevant to patients having bladder dysfunction disorders with unknown etiology.

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

Project/Performance Site Primary Location			
Organizational Name: Department of Biology, Hood College			
DUNS:			
Street 1: 401 Rosemont Ave		Street 2:	
City: Frederick	County: Frederick		State: MD
Province:	Country: United States		Zip/Postal Code: 21702
Project/Performance Site Congressional Districts: 6th Congressional District			

Additional Project/Performance Site Location			
Organizational Name:			
DUNS:			
Street 1:		Street 2:	
City:	County:		State:
Province:	Country:		Zip/Postal Code:
Project/Performance Site Congressional Districts:			

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

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eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

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King Abdulaziz University, Jeddah, Saudi Arabia	M.B.B.S	2006	Medicine and Surgery
Hood College, Frederick, MD	M.S.	2015 (anticipated)	Biomedical Science

A. Positions and Honors

2012-2013 Postdoctoral Research Fellow, Johns Hopkins University, Baltimore, MD

B. Selected peer-reviewed publications (in chronological order)

Firdaus W, Borbiev T, AlRebh S, Dada T, Johnston M, Reeves R, Burd I. Fetal inflammation-induced IL-1 beta is not essential for fetal brain injury. Reproductive Sciences. 2013 Mar; 20(3): 314a

Burd I, Al-Rebh S, AlShammary M, Kannan S, Borbiev T, Johnston M, Blakemore K. Maternal mesenchymal stem cell administration blocks the adverse effects of intrauterine inflammation on offspring neuroprogramming. American Journal of Obstetrics & Gynecology. 2014 Jan; 210(1): S233–S234

Dada T, Rosenzweig JM, Al Shammary M, Firdaus W, Al Rebh S, Borbiev T, Tekes A, Zhang J, Alqahtani E, Mori S, Pletnikov MV, Johnston MV, Burd I. Mouse model of intrauterine inflammation: sex-specific differences in long-term neurologic and immune sequelae. Brain, Behavior and Immunity. 2014 May; 38(1): 142-150. Cited in PubMed; PMID: 24486323

Burd I, Lei J, Firdaus W, Rosenzweig J, Alrebh S, Bakhshwin A, Borbiev T, Fatemi A, Blakemore K, Johnston MV. Murine Model: maternal administration of stem cells for prevention of prematurity. American Journal of Obstetrics and Gynecology. 2014 Dec; PMID: 25555657

C. Research Support

None

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

This project will be performed in the laboratories of Hood College Biology Department in the Hodson Science and Technology Center. This facility houses all equipment and space necessary for the execution of this project with the exception of the items to be purchased with the funding from this grant and the clinical part.

Clinical:

This study will be conducted at multiple research hospitals across the United States which has pediatrics urology clinics.

Animal:

All animal work will be conducted at Hood College in Hodson Science and Technology Center. Animals will be housed and treated in accordance with NIH's Office of Laboratory Animal Welfare (OLAW) guidelines and recommendations.

Computer:

This facility has computers available with all accessories necessary for routine use. Internet connection is available to facilitate the use of NCBI's BLAST.

Office:

All usual and customary office supplies necessary for the reporting and publication of the experiments are available.

Other:

N/A

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following major equipment is available on site:

- Centrifuges- for the collection of cell lysates.
- PCR machines – for RT-PCR
- Incubators, water bath, refrigerators.
- Confocal Microscope
- Protein Gel box and imager- For SDS-PAGE analysis
- Pipetters
- Freezers (-20°C and -80°C)- For storage of samples.

The following consumables will be purchased with grant funding:

- Protein and DNA Ladders
- Petri Dishes
- Pipette Tips (various sizes)
- Various sizes of tubes
- Nitrogen, Oxygen, and Carbon Dioxide tanks
- PBS
- Microinjector
- RNeasy Mini Kit
- RNA-to-cDNA Kit
- Custom rabbit anti-heparanase-2 antibody
- Cryostat machine
- Slides and coverslips
- Triton X-100
- Primers for *HPSE2*, *HPSE1*, *LRIG2*
- Alexa Flour secondary antibodies and DAPI

SPECIFIC AIMS

Fuller understanding of *HPSE2* gene and its role should help development of a treatment for one of the leading causes of renal failure in children and young adults. The overall goal of this project is to discover the role of *HPSE2* gene and its protein product heparanase-2 in UFS.

The specific aims of this study are:

1. To analyze *HPSE2* gene mutation and subsequent protein dysfunction in children having any voiding dysfunction disorder of unknown etiology. e.g. subclinical neurological bladder, occult neuropathic bladder, Hinman syndrome (non-neurogenic neurogenic bladder), and dysfunctional elimination.
2. To study the biological role of the normal heparanase-2 protein (product of *HPSE2* gene).
3. To determine the impact of total loss of function of heparanase-2 or protein deficiency on the human body especially the urinary tract.

BACKGROUND AND SIGNIFICANCE

Voiding Dysfunction

Dysfunctional voiding in children encompasses a broad spectrum of clinical entities, accounting for approximately 40% of pediatric urologist visits. It is estimated that 15% of school-age children experience daytime wetting. This prevalence includes variability in urinary incontinence from a few times per week to multiple episodes daily (Tu, Yang et al. 2014).

Voiding dysfunction refers to an abnormality in either the storage or emptying phase of urination. Due to high pressure in the bladder, the patients usually develop vesicoureteral reflux (backflow of urine from the bladder to the kidneys), along with hydronephrosis (distention of the renal pelvis and calyces). If left untreated, patients may die from kidney failure. Based on the definitions provided by the International Children's Continence Society, dysfunctional voiding is divided into neurogenic or non-neurogenic voiding disorders (Tu, Yang et al. 2014).

Neurogenic voiding dysfunction is associated with neurological diseases. The most common cause of neurogenic bladder dysfunction in children is neuro spinal dysraphism [abnormal fusion of the midline embryonic neural, vertebral and mesenchymal structures (Radiology Reference Articles 2015)], commonly an open back lesion, meningocele. Additionally, an occult or closed dysraphic state is now diagnosed with more frequency using neonatal spinal ultrasound, and magnetic resonance imaging (MRI) to visualize any lower midline spinal cutaneous or gluteal cleft malformation (Tu, Yang et al. 2014).

Other causes of neurogenic voiding dysfunction involving the spine including spina bifida, transverse myelitis, sacral agenesis, tethered spinal cord associated with imperforated anus, cloacal malformations or spinal cord trauma. Central nervous system abnormalities as well might cause neurogenic voiding dysfunction. e.g. spastic diplegia. Cognitive conditions such as attention deficit hyperactivity disorder or attention deficit disorder are also contributing possibilities (Tu, Yang et al. 2014).

In contrast, non-neurogenic voiding dysfunctions encompass functional problems as manifested by the absence of apparent neurological or obstructive abnormalities as in Urofacial syndrome (UFS) (Tu, Yang et al. 2014). In this type of voiding dysfunction, all the nerve supply to the bladder and lower urinary tract appear normal in (MRI).

Urofacial (Ochoa) Syndrome

UFS is also called Ochoa syndrome. It is a rare autosomal recessive disorder, and over 100 patients have been diagnosed. It is characterized by the abnormal facial architecture such as distortion of facial expression (grimacing as if in pain or sadness when they try to smile or laugh), along with urinary tract infection (UTI), enuresis, vesicoureteral reflux and hydronephrosis without any underlying neurological problems or urinary obstruction. Some patients also express nocturnal lagophthalmos (inability to close the eyelids during sleep) (Tu, Yang et al. 2014).

UFS patients appear to share voiding features with other voiding dysfunctions' patients in the general population, therefore, it could be an excellent model for dissecting the underlying pathoetiology of voiding dysfunction disorders in general (Tu, Yang et al. 2014).

Diagnosis and Treatment of Urofacial Syndrome

Early diagnosis and appropriate management could significantly improve the prognosis in patients with UFS (Ozcakar, Burgu et al. 2010). Diagnosis early in childhood improves long-term renal prognosis as patients progressively develop renal scarring, chronic kidney disease (CKD) and arterial hypertension if left untreated. Patients diagnosed at school age or later show more severe kidney impairment, that is, end-stage renal disease (Bacchetta and Cochat 2010; Stamatiou, Tyritzis et al. 2011)

Therefore, the aim of early treatment is the restoration of balanced bladder emptying and prevention of upper urinary tract deterioration. Achieving a low-pressure and adequate bladder capacity, reduces the chance of upper tract dilation and associated reflux, thus, reduces progressive renal scarring. Significant residual urine may cause recurrent febrile urinary tract infections. Therefore, clean intermittent catheterization (CIC) is useful in UFS patients (Aydogdu, Burgu et al. 2010).

Current therapeutic strategies consist of antibiotic prophylaxis, CIC, anticholinergics, and intravesical botulinum toxin injection. Efficacy of the treatment varies among individuals with UFS. Although CIC is a primary tool against recurrent UTIs, CIC and persistent VUR is a risky combination for triggering febrile UTIs. Such patients may require anti-reflux surgery (Aydogdu, Burgu et al. 2010). Urinary diversion or bladder augmentation is the standard of care to protect renal function (Stamatiou, Tyritzis et al. 2011).

History and Genetics of Urofacial Syndrome

Back in the early 1960s, Dr. Bernardo Ochoa studied a group of children in an isolated mountain area of Colombia, South America, who presented to the clinic with

urinary problems (dysuria, incontinence, frequency, urgency, enuresis, recurrent urinary tract infections or vesicoureteral reflux) but without apparent neurological or obstructive lesions. In addition, these children had a particular "inverted" facial expression when they smiled or laughed. This disease was not described in the medical literature at that time, which attracted researchers to study how facial expression links to voiding dysfunction. In 1979, "Ochoa syndrome" proposed by Dr. Rafael Elejalde was based on genetic analysis of three unrelated families, proposed the condition to have autosomal recessive inheritance. This recessive disease was thought to be locally restricted to a highly inbred population in Colombia, but several case reports published showed that UFS occurs worldwide (Mahmood, Beetz et al. 2012). Given that patients with similar clinical presentations were noted in other countries, the disease became known as Urofacial Syndrome (UFS). In 1997, the causative gene was localized to chromosome 10 (10q23-q24) by homozygosity mapping and DNA pooling (linkage-disequilibrium) strategies (Tu, Yang et al. 2014).

First gene associated with UFS is *HPSE2*. A second gene, *LRIG2,* was identified later. Most individuals that have been genetically studied with UFS carry biallelic null mutations of *HPSE2* or, less commonly, *LRIG2*. *HPSE2* gene (NG_023416.1); which is located at 10q24.2 encodes heparanase-2 protein (AAI12357.1) that binds heparan sulphate proteoglycans (HSPGs) and inhibit heparanase-1 enzymatic activity. *LRIG2* gene (NC_000001.11); which is located at 1p13.2 encodes for leucine-rich repeats and immunoglobulin-like domains-2 protein (LRIG2) (NP_055628.1). LRIG2 modulates receptor tyrosine kinase growth factor signaling which may contribute to tissue differentiation. Heparanase-2 and LRIG2 proteins are expressed in autonomic nerves growing into fetal bladders (Woolf, Stuart et al. 2014).

The collective evidence supports the hypothesis that UFS genes encode proteins involved in one pathway that facilitate neural growth and function within the bladder. This molecular pathway may also have relevance to the pathogenesis of other lower urinary tract diseases, including Hinman–Allen syndrome, or non-neurogenic bladder and among a subset of individuals who have primary vesicoureteric reflux accompanied by bladder dysfunction (Woolf, Stuart et al. 2014). Studies to dissect the biological functions of *HPSE2* and *LRIG2* in urinary abnormalities are ongoing (Tu, Yang et al. 2014).

Mutations in *HPSE2* vary in patients with UFS. Genetic analysis of a Pakistani family with three UFS affected siblings identified a missense mutation in *HPSE2*, which causes the children to the develop UFS, suggesting that functional alterations (expression levels or functional activity) in *HPSE2* may also cause UFS. Those data also support the notion that patients with UFS could be under diagnosed in the general population, particularly for those patients without typical distorted facial expression (Tu, Yang et al. 2014).

Variation in mutations in UFS patients may correlate with symptom variance. For example, UFS patients may have voiding disorder but they don't have the abnormal facial expression, and these patients were found to be carrying one defective *HPSE2* allele (UFS carriers). In addition, many parents of UFS patients or sibling carriers experienced dysfunctional voiding similar to that of UFS patients. This suggests that *HPSE2* may contribute to the disease phenotype in a dose-dependent manner, in which complete loss of *HPSE2* function correlates with both facial (distorted facial expression and/or nocturnal lagophthalmos) and urinary abnormalities, while reduced *HPSE2* function leads to development of voiding disorders without distorted facial expression. Based on these

observations, the disease frequency for UFS in the general population maybe underdiagnosed (Tu, Yang et al. 2014). The focus of this project is to study the role of *HPSE2* in the developing fetal bladder and its role in the development of non-neurogenic voiding dysfunctions.

Significance

Lower urinary tract (LUT) (i.e. ureter, bladder and urethra) and renal abnormalities are considered the most common cause of end-stage renal disease in children and may account for a significant number of young adults requiring renal replacement therapy (Woolf, Stuart et al. 2014). There is enough evidence to suggest that patients with Urofacial syndrome and other voiding dysfunctions in the form of subclinical neurogenic bladder, occult neuropathic bladder, non-neurogenic neurogenic bladder, and dysfunctional elimination may have the same congenital disorder (Aydogdu, Burgu et al. 2010).

The pathogenesis of voiding dysfunction disorders is unclear and may be clarified by understanding the basis of UFS (Daly, Urquhart et al. 2010). UFS patients share similar voiding problems as those patients with other voiding dysfunctions in the general population, therefore, it could be an excellent model for dissecting the underlying pathoetiology of voiding disorders such as frequency, urgency, enuresis, dysuria or incontinence (Tu, Yang et al. 2014). If the pathogeneses of such conditions were better understood, more efficient and fundamental therapies could emerge instead of treatments that do not target the primary cause of the disease (Woolf, Stuart et al. 2014).

PRELIMINARY REPORT / PROGRESS REPORT

Research to determine the biological function of heparanase-2 protein (product of *HPSE2* gene) is in progress. To my knowledge, the study published by Roberts, N. A., et al. on April 2014 was the first study to demonstrate the *in vivo* developmental functions of heparanase-2 in a model organism (specifically *Xenopus tropicalis*).The data showed that heparanase-2 is required for functional peripheral neural development and that it modulates growth factor signaling during embryogenesis (Roberts, Woolf et al. 2014).

Xenopus tropicalis Heparanase-2

Using bioinformatics (tblastn) and human heparanase-2 amino acid sequence, Roberts, Woolf et al. 2014 searched *Xenopus tropicalis* genome project database for heparanase-2 orthologue. They found that heparanase-2 is extensively conserved among species (human, mouse and frog). They identified a domain in Xenopus heparanase-2 with homology to glycosyl hydrolase domain of heparanase-1 in the human gene. Mutants with substitution of essential amino acid residues required for breakdown of heparan sulphate proteoglycans (HSPGs) in Xenopus heparanase-2 express no or little enzymatic activity, just like human heparanase-2 (Roberts, Woolf et al. 2014).

Heparanase-2 Expression During Embryonic Development

To identify the relationship between Urofacial syndrome and embryonic development gene expression, Roberts, Woolf et al. 2014 used reverse transcription-polymerase chain reaction (RT-PCR) on RNA extracted from Xenopus whole embryos at different embryonic developmental stages (1 to 45), and from urinary bladder of an adult frog, to examine the expression of *HPSE2*, *HPSE1*, and *LRIG2*. In maternal expression

(Stage 1); *HPSE1*, *LRIG2*, but not *HPSE2* transcripts were detected. At Stages 15 and 20 (neurulation stage when formation and development of the neural tube take place), *HPSE2* and *HPSE1* were expressed, whereas *LRIG2* transcripts were not. At Stages 30 and 40 (when motor nerves originating from the truncal neural tube form functional units with the adjacent somite-derived myotomes, and when the visceral morphogenesis take place), transcripts for *HPSE2*, *HPSE1*, and *LRIG2* were prominently detectable. The expression of the three transcripts were maintained until the developing embryo reached Stage 45 and were detected in the urinary bladder of the adult frog as well. The results are shown in Figure 1. (Roberts, Woolf et al. 2014).

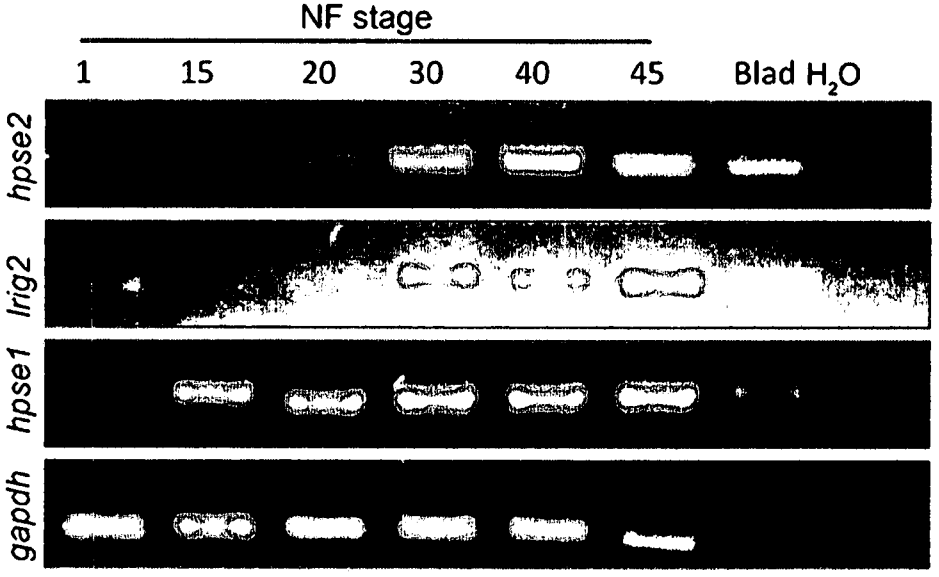

Figure 1. Expression of Urofacial syndrome genes using RT-PCR at different embryonic developmental stages and adult bladder. *HPSE2* was absent at Stage 1 but detected from Stages 15 to 45. *LRIG2* was expressed at Stage 1, then weakly detected at Stages 15 and 20, after which transcript levels became prominent. *HPSE1* was detected at all stages analyzed. All three transcripts were present in adult urinary bladder (Blad). *gapdh* was included as a cDNA loading control. In the H2O column, no cDNA was used (Roberts, Woolf et al. 2014).

Immunohistochemistry (IHC) was used to identify the anatomical locations where HPSE2 and LRIG2 are located in the development of Xenopus embryo (Roberts, Woolf et al. 2014). A custom-designed antibody against heparanase-2 and bright field IHC in Stage 30 embryo sections, showed heparanase-2 in the neural tube in a ventrolateral pattern, and the notochord and flanking myotomes (Figure 2A) (Roberts, Woolf et al. 2014). LRIG2 expression followed a similar pattern at Stage 30 and also present in the dorsal part of the neural tube and less prominent in the notochord. At Stage 40, immunofluorescence of heparanase-2 was still prominent in the developing skeletal muscles, but less prominent in the neural tube than that at Stage 30 embryo. At Stage 42 (a time point after the archenteron has undergone coiling), heparanase-2 immunofluorescence was detected in the apical zone/luminal surface of the gut epithelium (Roberts, Woolf et al. 2014).

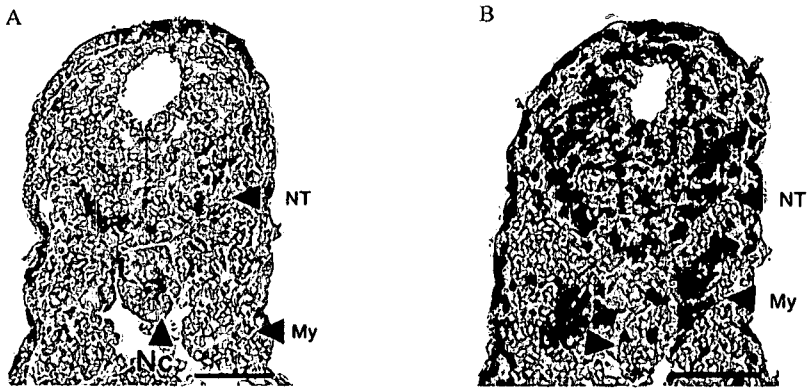

Figure 2. Immunohistochemistry of Urofacial syndrome proteins showing the proteins' localization during development. (A) Stage 30 embryo: Transverse section through the trunk with the dorsal surface uppermost. Prominent heparanase-2 immunofluorescence is detected in the ventrolateral regions of the neural tube (NT), in the notochord (Nc) and in the flanking skeletal muscle myotomes (My). (B) Adjacent section to (A), with the same orientation, showing the immunostaining for LRIG2 localization. The pattern is similar to that of heparanase-2, extending to the dorsal half of the neural tube (Roberts, Woolf et al. 2014).

These expression data show the presence of both heparanase-2 and LRIG2 in the developing neural tube and skeletal muscles of the Xenopus embryo, which support the

hypothesis that one neuromuscular developmental functional pathway is mediated by these two proteins (Roberts, Woolf et al. 2014).

Data from both RT-PCR and IHC collectively demonstrated that there is potential regulatory role for heparanase-2 on heparanase-1 activity from neurulation stages within the neural tube. Roberts, Woolf et al. 2014 demonstrated this by localizing heparanase-2 to the ventrolateral neural tube at Stage 30 and the heparanase-1 in the neural tube, somite and gut at Stage 39. The more prominent early expression of *HPSE1* in contrast to *HPSE2* suggests that heparanase-1 has independent functions in the early embryo. This is supported by the fact that heparanase-1 knockdown in Xenopus is lethal between Stages 17 and 28 (Bertolesi, Michaiel et al. 2008), a developmental period which corresponds to neural tube formation. Roberts, Woolf et al. 2014 reported that heparanase-2 knockdown resulted in lethality much later in development (Stage 41) (Roberts, Woolf et al. 2014).

Dynamic Developmental Expression of Heparanase-2 in the Neural Tube

To refine the anatomical localization of heparanase-2, Roberts, Woolf et al. 2014 used co-immunofluorescence between Stages 30 and 42 with antibodies against specific cell types. At Stage 30, heparanase-2 immunofluorescence staining in the ventrolateral neural tube overlapped with that of acetylated α-tubulin (a cytoskeletal neuronal protein) (Figure 3A-C) suggesting that heparanase-2 was located in the neural cell bodies. At Stage 42, the neural tube signal of heparanase-2 was undetectable (Figure 3D and G). At Stages 30 and 42, heparanase-2 immunolocalized in cells positive for 12/101 (it is the name of an antibody against skeletal muscle myofibers) (Figure 3A and D-F) (Roberts, Woolf et al. 2014).

In humans, *HPSE2* mutations can be associated with kidney disease causing renal failure. Therefore, Roberts, Woolf et al. 2014 performed colocalization of heparanase-2 with the kidney epithelial marker Na^+/K^+-ATPase at Stage 42 (a time when a pronephros has developed on each side of the embryo, each with a duct terminating in the cloaca). They found that there was no significant IHC staining for heparanase-2 in the pronephric tubule at Stage 42 (Figure 3G and H, arrows). They also noticed similar lack of signal at Stages 32 and 40. At Stage 42, heparanase-2 immunofluorescence was detected in the gut epithelium (Figure 3G-I arrowheads) (Roberts, Woolf et al. 2014).

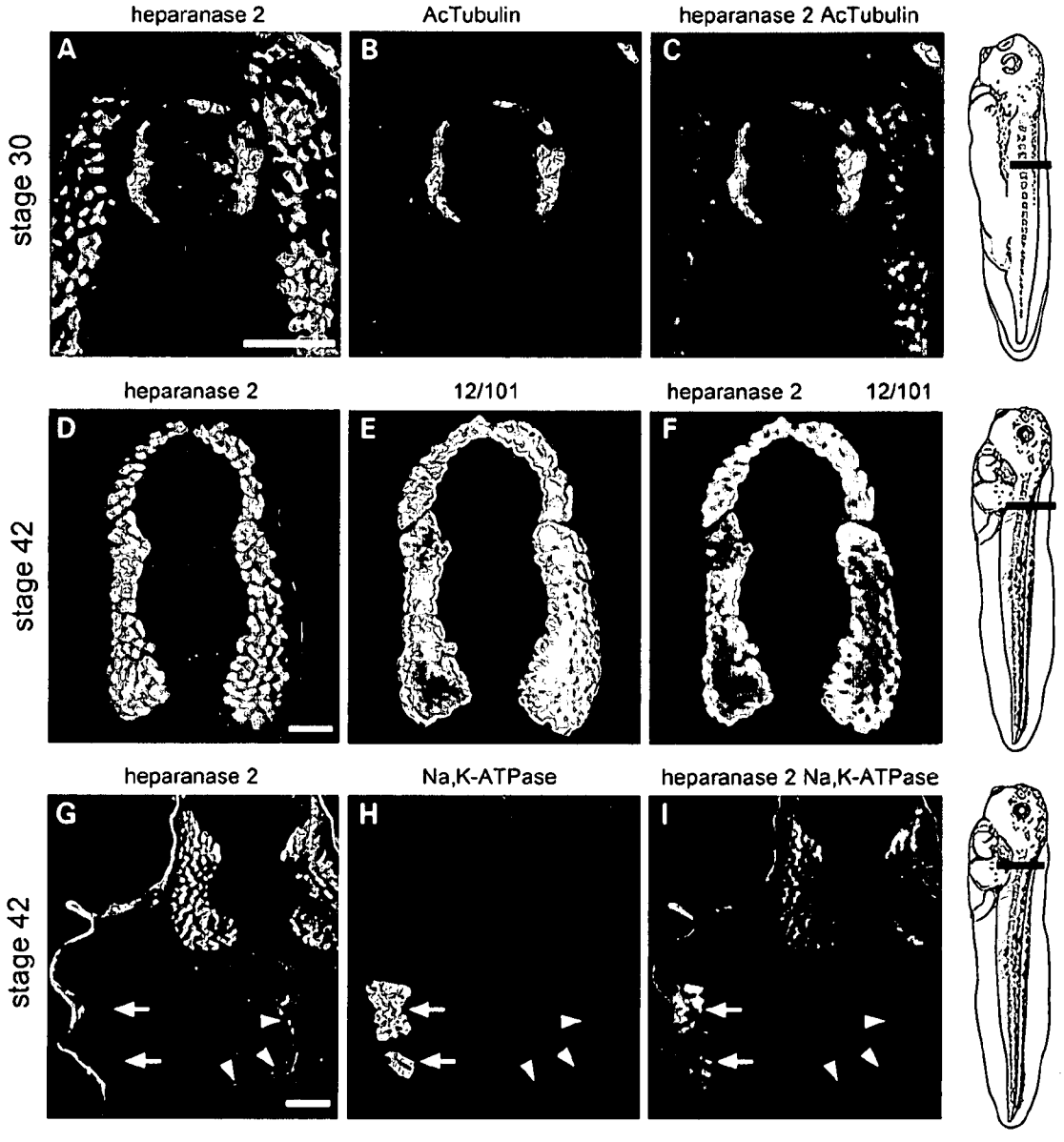

Figure 3. Tissue-specific localization of heparanase-2. Transverse sections through the trunk of Xenopus embryos, as indicated in the diagrams shown on the right, imaged by immunofluorescence, with the neural tube outlined by blue dashed circles. In merged images, heparanase-2 localization is indicated in red while other proteins are indicated in green. (A–C) Heparanase-2 colocalized with acetylated a-tubulin (AcTubulin) in the lateral zones of the Stage 30 neural tube. The flanking myotomes were also positive for heparanase-2. (D–F) At Stage 42, heparanase-2 was absent in the neural tube but myotomes, co-immunostained with the muscle-marker antibody 12/101 (it is the name of an antibody). (G–I) At Stage 42, pronephric tubule did not display a specific IHC signal for heparanase-2; note that here, the weak signal is background autofluorescence. The two arrows indicate a proximal tubule which in H&I is seen to be reactive with Na^+/K^+-ATPase antibody .The set of three arrowheads in (G–I) demonstrate specific heparanase-2 immunostaining in the apical zone of epithelia lining the gut lumen (Roberts, Woolf et al. 2014).

Morpholino-Induced Knockdown of Heparanase-2

To determine the role of heparanase-2 during embryonic development, Roberts, Woolf et al. 2014, used morpholino (MO)-induced knockdown experiments. They designed three MOs; (MO1) to target the splice acceptor of exon 2, (MO2) to target the splice donor of exon 2, and (ATG MO) to target the ATG start codon (Figure 4, red blocks). They injected single-cell embryos with increasing doses of MO1 (1-5 ng) and cultured it to Stage 36 when they analyzed *HPSE2* messenger RNA (mRNA) from whole embryos by RT-PCR using primers flanking exon 2 (Figure 4) (Roberts, Woolf et al. 2014).

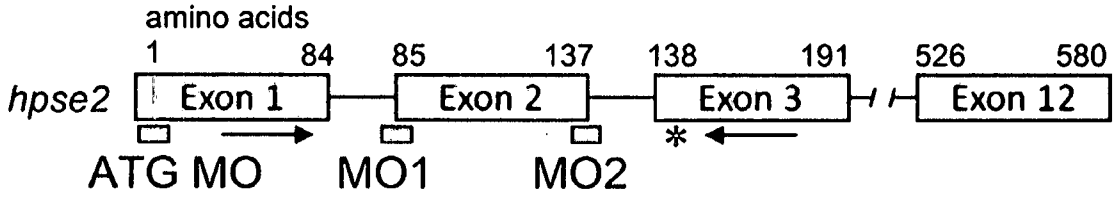

Figure 4. Morpholino-knockdown of heparanase-2. Schematic diagram of *X. tropicalis HPSE2* gene, showing: exons (blue blocks); ATG MO and splice MO targets (red blocks) at the splice acceptor (MO1) and splice donor (MO2) sites of exon 2; PCR primers flanking exon 2 (black arrows); and the premature stop codon in exon 3 (red asterisk) generated by MO1 (Roberts, Woolf et al. 2014).

Experiments showed that in embryos injected with standard control (CTL) morpholino (CTL MO), a band of a size predicted for wild-type *HPSE2* mRNA was amplified (Roberts, Woolf et al. 2014). When they increase the dose of MO1, there was an amplification of a smaller band, consistent with the size predicted after exclusion of exon 2 from *HPSE2* (exon 2Δ in Figure 5). After injecting 5 ng of MO1, only the shorter band was amplified. The PCR products were sequenced revealing the longer PCR product consistent with wild-type *HPSE2*, while the smaller band/amplicon corresponded to

HPSE2 lacking exon 2, with the resultant mRNA containing a novel, in-frame premature stop codon in exon 3 (Figure 5) (Roberts, Woolf et al. 2014).

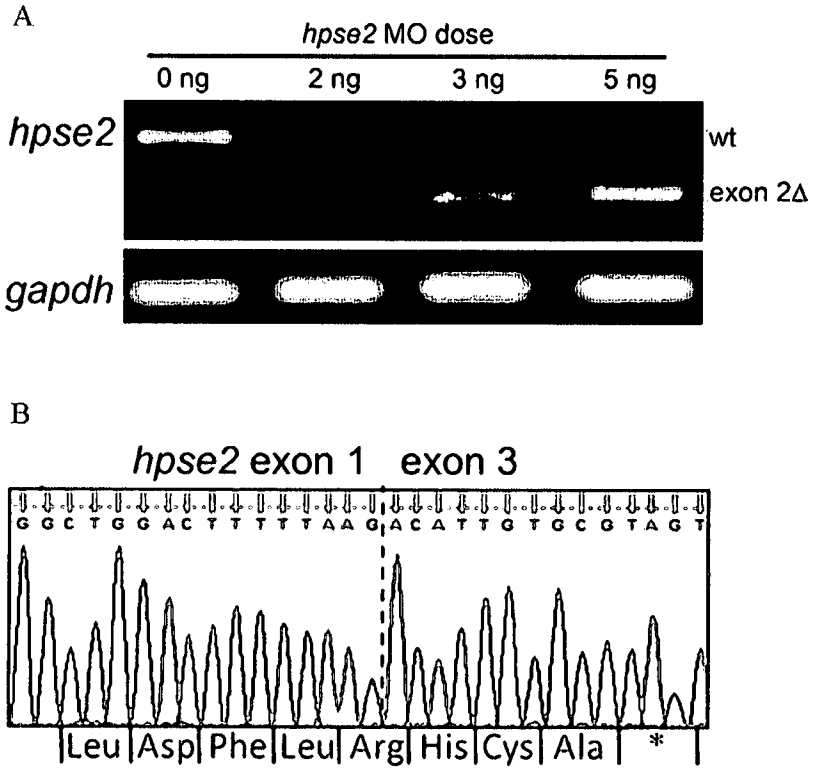

Figure 5. Morpholino-induced knockdown of heparanase-2. (A) Administration of increasing doses of MO1 caused missplicing of *HPSE2*, with 5 ng abolishing the expression of wild-type (wt) *HPSE2* mRNA in favor of a shorter RNA (exon 2Δ). (B) Sanger sequencing of the shorter PCR product confirmed the absence of exon 2 (indicated by the dotted vertical line). A novel, in-frame pre-mature stop codon was generated in exon 3 (asterisk) (Roberts, Woolf et al. 2014).

IHC on Stage 40 embryos was used to identify the effect of MO1 on heparanase-2 protein (Roberts, Woolf et al. 2014). Injection of 5 ng of MO1 caused a near-complete loss of heparanase-2 immunofluorescence signal (Figure 6A). Incubation of these 5 ng MO1-injected embryos to tadpole stages, revealed three main phenotypes; hypomotility, gut defect, and tail defect (Figure 6B). The hypomotility phenotype, appeared in about two-thirds of the MO1-knockdown embryos. CTL MO-injected embryos hatched normally at

Stages 26-28, showing the appropriate physical reflexes (the hatching reflex) to facilitate this process, and thereafter responded to touching of either the head or tail by swimming away (the escape reflex). In contrast, the 5 ng MO1-injected embryos hatched late, at Stage 35, with embryos continuing to elongate within their enveloping embryonic membrane before they hatched. Morphants lacked both the hatching and escape reflexes. These defects were also noted at a lower MO1 dose of 2.5 ng which did not generate the gut or tail defect. Blood continues to circulate in embryos with these skeletal muscle motility defects, indicating intact heart muscle function (Roberts, Woolf et al. 2014).

The second phenotype is the gut defect. Approximately two-thirds of MO1-injected embryos had a gut that lacked normal coiling; instead, the organ remained as an ovoid mass (Figure 6B and C), as normally found in the undifferentiated archenteron. This phenotype was associated with a protruding proctodeum (Figure 6C) but the shape of the cloaca appeared normal on gross examination. In addition, imaging of pronephric tubules and ducts did not show any gross anatomical anomalies nor were embryos oedematous (Roberts, Woolf et al. 2014).

The third phenotype is the tail defect. Each embryo with gut defect also showed dorsal kinking of its tail (Figure 6B and C). The gut and tail defects were found in < 5% of CTL MO-injected embryos, and CTL MO embryos had no motility defects (Figure 6B) (Roberts, Woolf et al. 2014).

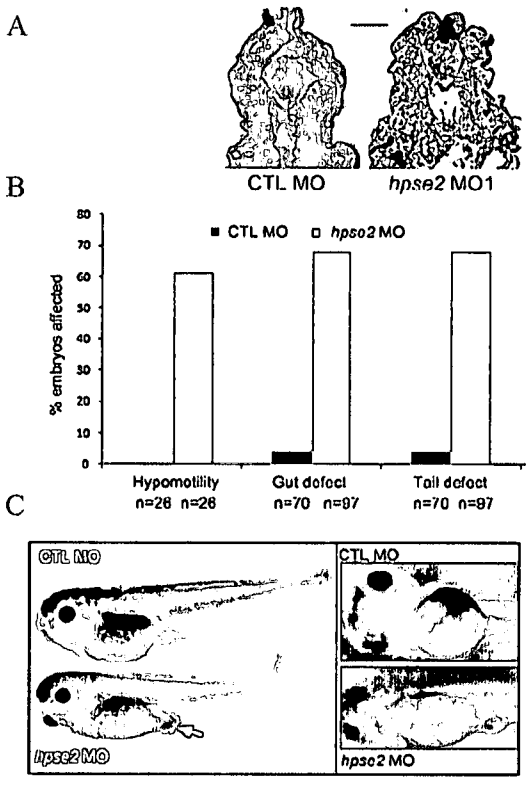

Figure 6. Morpholino-knockdown of heparanase-2. (A) Administration of 5 ng MO1 led to near-complete loss of heparanase-2 neural tube and myotome immunoreactivity, as demonstrated in this Stage 40 embryo; CTL MO on left and MO1on right. (B) Frequency (%) of the hypomotility, lack of gut looping (gut defect) and tail curvature (tail defect) phenotypes associated with administration of CTL MO or MO1, with total numbers of embryos assessed indicated by 'n'. (C) The upper two images depict CTL MO-administered embryos and the lower two images show effects of MO1. Left-hand section depicts embryos viewed from the side; note the protruding proctodeum (white arrow) and tail curvature in the morphant. The two frames on the right depict the embryos viewed from their ventral aspects; note that gut coiling is present in the control embryo but not in the morphant (Roberts, Woolf et al. 2014).

The incidence of each of the three phenotypes (i.e. motility, gut, and tail defects) in the 5 ng MO1 morphants versus CTL MO-injected embryos was highly significant ($P < 0.001$). Eleven percent of CTL MO-injected embryos and 16% of those injected with MO1 showed non-specific effects (P=0.38). Injection of 5 ng of MO1 was lethal beyond Stage 41, possibly due to complete failure of gut formation. Roberts, Woolf et al. 2014 were able to replicate the three phenotypes upon injection of either MO2 which targets the exon 2

splice donor site, or the ATG MO which target the start codon, which support the specificity of the MO1 effects (Roberts, Woolf et al. 2014).

Knockdown of Heparanase-2 Changes Peripheral Nerve and Myotome Morphology

Because of lack of motility phenotype in the majority of MO1-administered embryos, Roberts, Woolf et al. 2014, performed confocal microscopy on whole-mount embryos probed with acetylated α-tubulin antibody to identify motor neurons originating from the neural tube. They found that in MO1 morphants at Stages 36, 38 and 41, motor neurons were present and their spacing along the anterior–posterior axis were similar to that found in CTL MO-injected embryos (Figure 7A-F). However, the morphants' axons within the nerve trunk appeared less compactly bundled than those in the controls. In addition, the morphants showed that the paths of individual nerves meandered compared with the more linear organization in stage-matched CTL MO-injected embryos (e.g. compare A with B, C with D, and E with F in Fig. 7). For each embryo, up to six nerves originating from the neural tube were scored as being 'normal' or 'abnormal'. The abnormality was defined by having one or more of the following defects: (i) perpendicular projection from the neural tube, (ii) non-linear path, (iii) lack of coherent axonal bundling and/or (iv) abnormally splayed nerve termini. In Stage 36 embryos, they found that one of 22 nerves imaged in the controls had abnormal morphology and 12 out of 14 nerves imaged in morphants were abnormal ($P < 0.0001$). In Stage 36 embryos, two out of 22 nerves imaged in CTL MO animals were abnormal and 26 out of 30 nerves imaged in morphants were abnormal ($P < 0.0001$). In Stage 40 embryos, three out of 17 nerves imaged in CTL MO animals were abnormal and of 18 nerves imaged in morphants, all were abnormal ($P < 0.0001$) (Roberts, Woolf et al. 2014).

Roberts, Woolf et al. 2014 also measured the axonal length (distance between exit from neural tube to detectable termini and axonal path length) which revealed a tendency for nerves in the morphants to be shorter than in the control ones, although this did not have statistical significance (Fig. 7I and J). At Stage 41, using the myofiber-specific 12/101 antibody, they found a lack of clear separation of skeletal muscle fibers at somite boundaries in morphant versus CTL MO-administered embryos (Fig. 7G and H), suggesting mildly dysmorphic myotome formation. These results demonstrated that heparanase-2 is required for motor neuron development and/or function. (Roberts, Woolf et al. 2014).

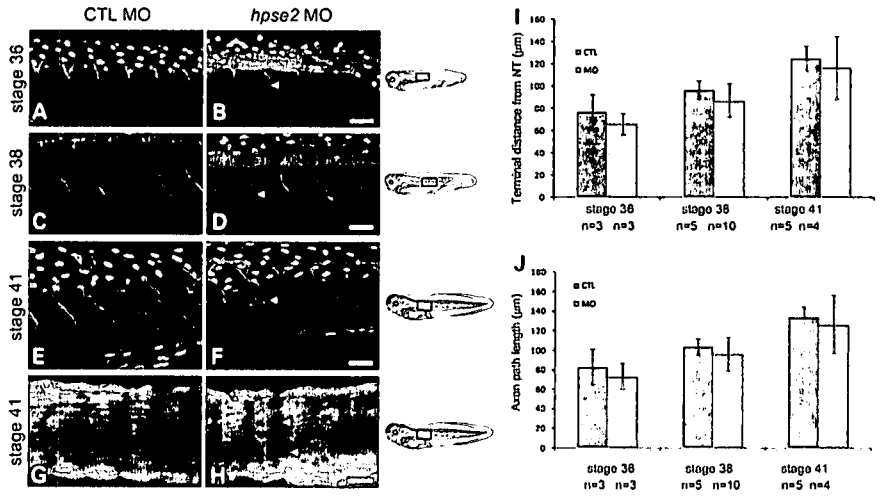

Figure 7. Visualization of motor neurons in parasagittal imaging plane. (A–F) whole-mounts immunostained with antibody to acetylated α-tubulin. This labels axons and also multiciliated round organs in the skin, the latter appearing as white ovals. (G) and (H) were probed with the muscle antibody 12/101. (A), (C), (E) and (G) are from CTL MO-injected embryos while (B), (D), (F) and (H) are from MO1-injected embryos. Scale bars are 50 μm. (A–F) across the top of each frame, a longitudinal section of the neural tube is evident, with the anterior to the left. In morphants, at Stages 36, 38 and 41, neurons which had originated from the neural tube were regularly spaced but their axons lacked compact bundling and coherent directional extension seen in controls. The irregular topography of certain morphant nerves is indicated by arrowheads in (B), (D) and (F). (G and H) Morphants showed lack of clear separation of skeletal muscle blocks at somitic boundaries (arrowheads in H). (I and J) Nerve lengths (means ± SD), as assesed by determining the shortest distance between where they exited the neural tube and their overt termini (I) or by tracing individual nerves (J). The average length of 4–6 nerves per embryo was used to generate a value for each embryo, with 3–10 embryos in each experimental group, as indicated (Roberts, Woolf et al. 2014).

Effects of Heparanase-2 Knockdown on Molecules Required for Neuromuscular Development

The data suggests that heparanase-2 is necessary for both normal embryonic motility and the shapes of motor neuron fascicles in developing Xenopus embryo, and that heparanase-2 protein is present in the ventrolateral neural tube where motor neurons originate. Therefore, Roberts, Woolf et al. 2014 analyzed the expression of key molecules required in the ventrolateral neural tube for motor neuron formation. Given that heparanase-2 was immunolocalized in myotomes, they also assayed markers of muscle differentiation and synaptic molecules. Semiquantitative RT-PCR used RNA from pools of CTL MO-injected embryos and heparanase-2 morphants. Figure 8 illustrates results from two stages, Stages 32 and 40; however, similar results were found in further experiments at Stage 39 and a Stage 40. At each Stage, both the control and the morphant cDNA were produced from a pool of three embryos. As expected, using exon 2 flanking primers, *HPSE2* transcripts were markedly reduced in morphants, with a shorter PCR product lacking exon 2. Expression levels of *HPSE1* transcripts were increased in morphants at both stages. Additionally, *LRIG2* transcripts appeared modestly upregulated in morphants. Morphants showed modest increases in levels of *fgf2*, *olig2* and *nkx6.1*, transcripts which encode molecules functional in motor nerve precursor cells. Levels of *myod1*, which encodes a skeletal muscle transcription factor, were increased in morphants at Stages 32 and 40. In contrast, levels of *myh11*, which encode a smooth muscle cytoskeletal protein, were reduced in heparanase-2 morphants at Stage 40 when *myh11* transcripts reach detectable levels in the controls. The expression of the synaptic markers *syn1* encoding synapsin, a synaptic vesicle protein and *chrnb2*, encoding a subunit of the

nicotinic acetylcholine receptor were similar in the heparanase-2 knockdown embryos compared with stage-matched CTL embryos (Figure 8) (Roberts, Woolf et al. 2014).

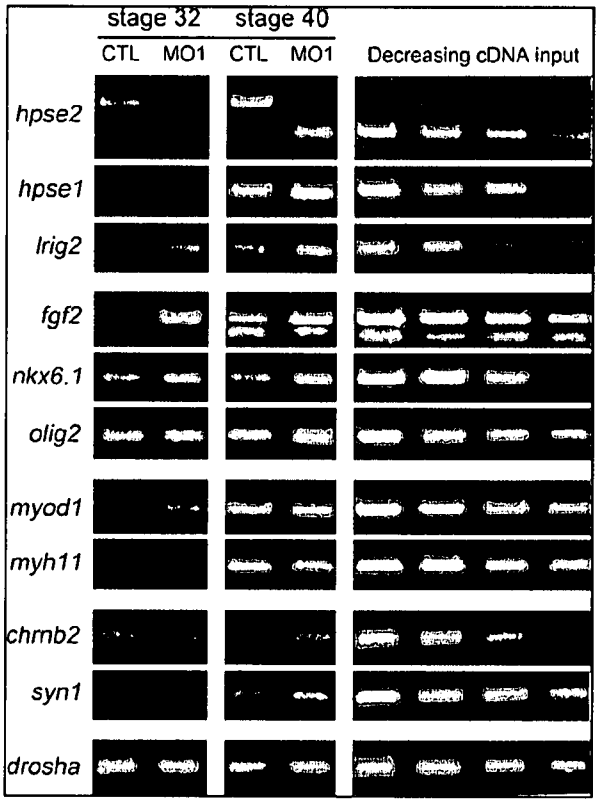

Figure 8. Expression analyses in heparanase-2 knockdown embryos. RNA from pools of Stages 32 and 40 control (CTL MO) and morphant (*HPSE2* MO1) embryos was subjected to RT-PCR, with serial dilutions of cDNA depicted on the right of each row. *drosha*, which encodes an RNase III enzyme, was used as a housekeeping control. Morphants had: downregulated wt *HPSE2* exon 2 and the appearance of the shorter exon 2Δ amplicon; increased levels of *HPSE1* and *LRIG2*; increased levels of *fgf2* and of the neuronal precursor markers *olig2* (at Stage 40) and *nkx6.1*; upregulated *myod1*, encoding a skeletal muscle transcription factor, and downregulated *myh11*, encoding a smooth muscle myosin (at Stage 40). Levels of *syn1* and *chrnb2*, encoding synaptic molecules, were similar in morphants and controls (Roberts, Woolf et al. 2014)

RESEACH DESIGN / METHODS

Aim 1: Measure the involvement of *HPSE2* gene mutation effect in non-neurogenic voiding dysfunction (Months 01-12)

This approach involves human subjects, specifically children. Thus, IRB approval of study will be obtained prior to recruitment and enrollment. All subjects will have proxy consent by their parents or legal guardians.

Having obtained informed consent from parents, we proposed to screen the children with voiding dysfunction of unknown etiology for the presence of dysfunctional heparanase-2 and compare them to those with neurogenic voiding dysfunction. These blood tests will be done on children between 0-18 years old who would come to the urology clinic with any form of voiding dysfunction. i.e., voiding dysfunction of unknown cause and neurogenic voiding dysfunction.

Detecting the presence of genetic mutation will be done using SNP-chromosomal microarray to look for loss of heterogeneity (LOH) in *HPSE2* gene loci at 10q24.2 which, if present, would be followed by *HPSE2* gene sequencing to confirm the diagnosis of Urofacial syndrome. Chromosomal microarray is a high-resolution whole-genome screening that can identify major chromosomal aneuploidy as well as submicroscopic abnormalities that are too small to be spotted by conventional karyotyping. It is considered to be a first-tier test in the genetic evaluation of infants and children with unexplained congenital anomalies (Committee 2013). Since chromosomal microarray is easy to do, sensitive and cost effective technique that involves only drawing blood samples from the

patients, it will be used as a screening test for all children who come to the urology clinic with voiding dysfunction.

Since we know that these children with non-neurogenic voiding dysfunction do not have a clear anatomical cause for their urinary tract problem, we are trying to find out if there is a genetic base and discover the role of *HPSE2*, as one of the possible affected genes. In addition, UFS is known to be an autosomal recessive disease, therefore, having two normal copies of *HPSE2* gene (AA), child will be normal. Having two affected copies of the gene (aa), the child will have UFS with voiding dysfunction and abnormal facial expression (though the severity of the symptoms varies widely). And having one normal and one affected copy of the gene (Aa), the child will be phenotypically normal or may have voiding dysfunction with normal facial expression. Therefore, this genotype: phenotype observation has to be clarified by testing more UFS patients since the disease is underdiagnosed because it is not well known, and the severity of illness varies from patient to patient.

Aim 2: Biological role of the normal heparanase-2 protein (Months 01-12)

Using bioinformatics (tblastn), we will look for heparanase-2 sequence homology between human (NP_001159716) and mouse (NP_001074726) species trying to find the conserved regions to figure out the role of that part of the sequence.

In addition, we will use the mouse model for several lab tests. Study of heparanase-2 protein assay will be adapted from Roberts, Woolf et al. 2014. using *X. tropicalis*. The mouse is better animal model to establish the role of *HPSE2* in the human because in the embryonic stages of the frog, the just-formed cloaca acts as a passive conduit for the excretory products as pronephric urine, therefore, it does not work as the human bladder

does (Roberts, Woolf et al. 2014). We choose to replicate their tests on the mouse because of its close genetic and physiological resemblances to humans at the known genetic loci, as well as the ease with which its genome can be manipulated and analyzed in addition to its relatively low cost of maintenance and its ability to quickly multiply, reproducing as often as every nine weeks (National Human Genome Research Institute 2015). In addition, the mouse could be used as a model for therapeutic options once we reach that stage before starting any clinical trials.

To establish the role of UFS and related genes (*HPSE2*, *HPSE1*, and *LRIG2*) during the embryonic development of the mouse, we will use reverse transcription-polymerase chain reaction (RT-PCR) from RNA extracted from the mouse embryos at different gestational ages and from adult mouse bladder. This will help identify the timing of expression during embryonic development and each genes' role in bladder and neuronal development. PCR amplification will use primer oligonucleotide: *HPSE2* f CTGAAGAATCCGGCAAAGAG r CTACGCACAATGTCGTCCTC; HPSE1 f TTTGGCGCAGGATCCTATAA r CCAGTAATCTGGTAAGGGTTCAA; and LRIG2 f AGCTCTTGGATCTAGACTTGTCAT r GCCCTTTAAAGACACCTTCAGC (Roberts, Woolf et al. 2014).

To specify the exact anatomical location of heparanase-2 during embryonic development, we will use immunohistochemistry (IHC) staining with custom-designed anti-heparanase-2 antibodies which will be made by immunizing with a peptide sequence that corresponds to a fragment of the full-length protein. The chosen sequence will be 10-20 amino acids in length which will provide optimal epitope diversity while still offering

higher specificity compared to immunizing with the full-length protein sequence (Pacific Immunology 2015).

Aim 3: The impact of loss of function of heparanase-2 on the human body especially the urinary tract (Months 01-12)

To create loss of function, we will use morpholino (MO)-induced knockdown approach. This can be done by injecting the embryos and the adult mouse with increasing doses of MO. Morpholinos have been proven to efficiently and specifically reduce translation of a target mRNA. Morpholino oligomers (oligos) as a class of antisense is a technology used to block access of other molecules to specific sequences of nucleic acids. Morpholinos block small (~25 base) regions of the base-pairing surfaces of ribonucleic acid (RNA) (Summerton 1999). We will use Gene Tools LLC (http://www.gene-tools.com/) to design MO that target splice acceptor of exon 2 of the mouse *HPSE2* mRNA, as well as standard control MO. *HPSE2* mRNA of the embryos will be analyzed by RT-PCR using primers flanking exon 2 (f TTAAGTTCCAA-GAGGTTAGTGAC r ATGAAACCATCTAGAAGGGCT) (Roberts, Woolf et al. 2014).

To determine the impact of injecting MO on heparanase-2 protein, IHC staining of sectioned embryos will be done using the custom-designed anti-heparanase-2 antibody. The MO-administered embryos will be allowed to complete gestation, and the newborns' phenotype will be observed to see the effect of loss of function of heparanase-2 protein. More tests will be done based on the results of the phenotypical observations of the mouse following protocols described by Roberts, Woolf et al. 2014.

HOOD COLLEGE

INFORMED CONSENT FORM

ROLE OF *HPSE2* IN UROFACIAL SYNDROME AND OTHER NON-NEUROGENIC VOIDING DYSFUNCTIONS

1. INTRODUCTION

Your child is being invited to participate in a research study. Before you decide whether or not your child should take part in the study, it is important that you and your child understand why the research is being done, and what it involves. Please read the information carefully and ask questions. This study is sponsored in affiliation with Hood College.

2. BACKGROUND

Renal tract abnormalities are highly variable with regard to symptoms. We are interested in the development of kidneys, bladder, ureters (the tubes from the kidneys to the bladder) and urethra (tube between the bladder and outside the body) in relationship to several human genes. We know that small inherited changes in individual genes can cause these types of problems. Genes are sections of our DNA that provide instructions to cells in our body and control development of special tissues and organs. We know that changes in some genes can cause renal tract problems. This study aims to find out more about which genes cause urinary tract problems and how we can use this information to help patients in the future.

3. WHY YOUR CHILD IS CHOSEN FOR THE STUDY

We are contacting you because your child is has urinary tract abnormality.

4. WHAT WILL HAPPEN TO YOUR CHILD IF YOU CHOOSE TO PARTICIPATE

If you decide to enroll your child in our study, we will test a small sample of blood to see if we can find a change in a gene that might be responsible for their renal tract problem(s). If genetic change is detected, we may use the same sample of their blood to do further testing. If no stored sample is available, your child's doctor will ask for a new blood sample to be taken (hopefully at the same time as other blood tests) and sent to us.

We will also ask your doctor to provide us with some detailed medical information about your child, including how their renal tract problem affects them and any other health issues they might have.

Your doctor might take a photograph of your child if required, or use a photo they have taken previously, and you can choose not to do so.

Please check: [ ] ok to use photo.

[ ] not ok to use photo.

5. RISKS/BENEFITS

For anyone having a blood sample drawn, there is a small risk of bruising or discomfort at the site where the blood is taken. This can be minimized by using local anesthetic spray. Where possible, the blood sample will be taken at the same time as other blood tests, to minimize discomfort to your child. A further potential risk of this study is that the genetic investigations may reveal an abnormality which is either unexpected or may have implications for other family members.

Your child will not likely benefit from this study directly, but we hope that the findings of the study will help us to understand the genetic causes of renal tract abnormalities, and improve treatment in the future.

6. CONFIDENTIALITY

All information will be kept strictly confidential and used for research purposes only. In any report that is published or presented, we will not include any information that will make it possible to identify your child. Only photographs specifically permitted by you will be used. Any information about your child will have their name and address removed so they cannot be recognized. No study information will be shown to anyone outside the research team, or individuals representing the research sponsor or regulatory authorities (for the purposes of monitoring, or auditing the study only).

7. VOLUNTARY NATURE OF THE RESEARCH

Your child's participation in this study is completely voluntary. Your decision whether or not your child will take part in the study will not affect your current or future relations with Hood College or any of its representatives. If you decide to participate in this study, you are free to withdraw from the study at any time without affecting those relationships. If you would like to withdraw from the study, please contact Shorouq Al Rebh at any point during the study.

Phone number: (312) 714-9573

Email: sha3@hood.edu

8. CONTACTS AND QUESTIONS

The researcher conducting this study is Shorouq Al Rebh. You may ask any questions you have right now, If you have any questions later, you may contact the researcher at (312) 714-9573 or email her at: sha3@hood.edu.

9. STATEMENT OF CONSENT

A copy of this form will be given to you to keep for your records. By signing this form, you give permission for your child for whom you serve as guardian to be enrolled in the study. The procedures of this study have been explained, and any questions have been addressed. You understand that participation is completely voluntary and that the subject may be withdrawn any time without penalty. The Chair of the Institutional Review Board (Dr. Joy Ernst) or the Chair of the sponsoring department of this research may be contacted at any time should questions arise.

Signature of Parent/Guardian

_____ Date _____

Name of person to be enrolled in the study _____

Signature of person obtaining consent

_____ Date _____

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