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A genetic female mouse model with congenital genitourinary anomalies and adult stages of urinary incontinence

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AIMS: To characterize the urinary incontinence observed in adult *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ female mice and identify the defects underlying the condition.

METHODS: *Gli2*$^{+/-}$ and *Gli3*$^{\Delta 699/+}$ mice were crossed to generate: wild-type, mutant Gli2 (*Gli2*$^{+/-}$), mutant Gli3 (*Gli3*$^{\Delta 699/+}$), and double mutant (*Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$) female mice, verified via Polymerase Chain Reactions. Bladder functional studies including cystometrogram (CMG), leak point pressure (LPP), and voiding testing were performed on adult female mice. Female bladders and urethras were also analyzed via ink injection and histological assays.

RESULTS: CMG tracing showed no signal corresponding to the filling of the *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ bladders. LPP were significantly reduced in *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ mice compared to wild-type mice. CMG studies revealed a decrease in peak micturition pressure values in *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ mice compared with all other groups. No significant differences between mutant and wild-type mice were detected in urinary output. Histological analyses revealed *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ mice exhibited a widened urethra and a decrease in smooth muscle layer thickness in the bladder outlet and urethra, with increased mucosal folding.

CONCLUSIONS: *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ adult female mice display persistent urinary incontinence due to the malformation of the bladder outlet and urethra. This presents a consistent and reliable genetic mouse model for female urinary incontinence and alludes to the key role of genetic factors involved in the condition.

KEYWORDS

female urinary incontinence, Gli transcription factors, mouse model, smooth muscle development

1 | INTRODUCTION

Urinary incontinence is a prevalent issue in females.[1–4] To date, most animal models which aim to recapitulate the clinical conditions of urinary incontinence have been generated via inducing injury to various parts of the urinary system, such as vaginal distention and urethral cauterization.[4,5] Although such injuries have been able to help tremendously with understanding the processes involved in similar clinical cases, there has not yet been a genetic model which recapitulates this condition.[3,4] This is critical, because a genetic animal model can help uncover potential genetic factors involved in human urinary incontinence, as well as provide a much more practical means of studying the disorder since it would not require an elaborate procedure where injury is introduced to individual mice in a labor intensive procedure.

The hedgehog (Hh) pathway has been widely studied, and has been identified to play a key role in many early developmental processes such as urogenital sinus and hindgut formation.[6–10] Two transcriptional regulators of the pathway, Gli2 and Gli3, which most commonly act as activator and repressor respectively, often control pathway activity.[6,9]

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Previously, it has been identified that severely reduced Hh pathway activity via reduced Gli2/Gli3 dosages leads to severe hindgut and urogenital sinus malformations.[6,10] Although these severe malformations have revealed critical information about the genetic factors involved in these developmental processes, they have mainly failed in providing relevant animal models due to the lethality of these severe developmental defects.[4,10–12]

Previously, we have generated a compound mutant ($Gli2^{+/-}$; $Gli3^{\Delta 699/+}$) with a loss of Gli2 activator, and a gain of a C-terminally truncated Gli3 that renders the transcription factor a constitutive repressor, $Gli3^{\Delta 699}$,[11] which carries urogenital sinus malformations into its adult stages of life. These mutant mice display several urogenital malformations in both males and females; however here, we will focus on a specifically relevant phenotype found in adult $Gli2^{+/-}$; $Gli3^{\Delta 699/+}$ female mice, which is their urinary incontinence. Functional, anatomical, and histological studies were conducted on these adult female mice to evaluate and characterize their urinary incontinence.

2 | MATERIALS AND METHODS

All procedures were conducted in accordance with Case Western Reserve University and The Hospital for Sick Children Institutional Animal Care and Use Committee policies. Only female CD1 mice (12-14 weeks) were used in this study and were fed a standard diet with free access to water.

2.1 | Mutant generation and mating

Gli2 mutant mice carry a targeted deletion of DNA-binding zinc-finger motifs of the gene.[6] $Gli3^{\Delta 699/+}$ mutant mice carry a targeted deletion 3′ of the zinc finger motif.[11] Crosses between $Gli2^{+/-}$ and $Gli3^{\Delta 699/+}$ were used to generate $Gli2^{+/-}$, $Gli3^{\Delta 699/+}$, and $Gli2^{+/-}$;$Gli3^{\Delta 699/+}$ mice. All mice were of CD-1 background. Genotypes and sexual identities of the mice were determined by Polymerase Chain Reaction (PCR) analysis of ear notches (Postnatal 21 days upon weaning) using *Gli2*, $Gli3^{\Delta 699}$, and *Sry* primers.

2.2 | Catheter insertion and conscious cystometrographic (CMG) studies

A separate cohort of 18 mice (4 wild-type, 5 $Gli2^{+/-}$, 4 $Gli3^{\Delta 699/+}$, and 5 $Gli2^{+/-}$; $Gli3^{\Delta 699/+}$) were used for this study. Under anesthesia (2% isoflurane, 2 L/min flow), the animal's abdomen was shaved and prepped with alternating wipes of betadine and alcohol (3×). A small lower abdominal incision was made and bladder was delivered through this incision. A circular purse string suture (6-0 monofilament, non-absorbable) was placed on the bladder wall near dome of bladder. A small incision was made in the dome of the bladder wall, and the catheter (PE-10 tubing with a flared tip) was implanted. The purse string suture was tightened around the catheter. The distal end of the tubing was sealed, tunneled subcutaneously using a trocar and externalized at the back of the neck, out of reach of the animal. The catheter was plugged until used and the abdomen was closed with 5-0 monofilament absorbable suture and skin was closed separately with 5-0 monofilament non-absorbable suture. Two days after bladder catheterization, the conscious animal was placed in a specially modified metabolic cage (Catamount Research and Development, CAT-CYT-M) and the catheter was attached via a stopcock to both a pressure transducer and a flow pump. The bladder was emptied using a syringe, and then filled via the catheter with 0.9% saline at 1 mL/h while bladder pressure was recorded. The animal was awake and able to void and/or leak urine (saline) through the urethra during the study. Urine was collected on an analytical balance directly underneath each cage. After an initial stabilization period of about 30 min, 6-8 reproducible micturition cycles were recorded. Although the number of micturition cycles differed among animals, the means were calculated to analyze CMG parameters.[13]

2.3 | Leak point pressure (LPP) testing

A separate cohort of 27 (5 wild type, 7 $Gli2^{+/-}$, 10 $Gli3^{\Delta 699/+}$, and 5 $Gli2^{+/-}$; $Gli3^{\Delta 699/+}$) mice was used for this study. LPP testing was performed as described by Damaser et al.[14] Investigators performing LPP were blinded to the genotype of each animal. For unconscious LPP testing, urethane was chosen over less toxic methods of anesthesia, like ketamine/xylazine, because it relaxes the muscles of the lower urinary tract better and gives more physiological results.[15] While under urethane anesthesia (1.2 g/kg), the animal's abdomen was shaved and prepped with alternating wipes of betadine and alcohol (3×). A midline longitudinal abdominal incision was made 0.5 cm above the urethral meatus, and the bladder was exposed. A 26 g IV catheter was placed into the dome of the bladder. The animals were placed supine at the level of zero pressure and the bladder was emptied manually. Then the distal end of the tubing was connected to a syringe pump (Kent Scientific Corp., Torrington, Connecticut) to deliver 0.9% saline solution at 1 mL/h during leak point pressure testing. Pressure signals were amplified and digitalized for computer data collection at 10 samples per second. Peak bladder pressure was calculated for each LPP measurement at half bladder capacity by slowly and manually increasing pressure on side walls of bladder until a leak occurred, upon which external pressure was immediately withdrawn.[14] As described previously, half bladder capacity was defined as half the volume infused that resulted in a spontaneous leak.[14] In the case of the $Gli2^{+/-}$;$Gli3^{\Delta 699/+}$ mice, bladder was filled for a few seconds since animals displayed constant

spontaneous leakage. LPP was performed 9-10 times per animal. The bladder was emptied and refilled between LPP measurements.

2.4 | Functional voiding studies

Twenty-four hour micturition and drinking habit of 37 mice (7 wild type, 4 *Gli2*$^{+/-}$, 13 *Gli3*$^{\Delta 699/+}$, and 13 *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$) was measured by placing the mice individually in computer-controlled metabolic cages (Catamount Research & Development, St. Albans, Vermont). Urine was collected in a plastic tray placed on an analytical balance directly underneath each cage. Balances were connected to a data acquisition software program designed by the vendor. During the testing, mice were given free access to special milk and water. Testing room was maintained on a 12-h light/dark cycle similar to their normal housing conditions. Twenty-four hours before 24-h micturition testing, solid food was removed from cages and mice were be given Lactaid-brand lactose-free milk, which reduces feces output and prevents food droplets from falling onto balance.[16] Once testing period was over, animals were returned to regular solid food diet. Total urine volume was measured.

2.5 | Ink injection assay and histological analysis

At postnatal 4 months and under aseptic conditions, 12 wild-type and 8 *Gli2*$^{+/-}$;*Gli3*$^{\Delta 699/+}$ mice were sacrificed via CO_2 euthanasia. Abdominal incisions were made and the abdomen was entered and bladder/urethra structure was centralized. Using a 22 gauge needle, bladder was injected with buffer (blue) and ink (black) to assess bladder size and urethral diameter. Photographic pictures were obtained to record data. For histology, a midline suprapubic abdominal incision was made and the abdomen was entered. Bladder and urethra were dissected out and fixed in 4% paraformaldehyde overnight at 4°C. They were dehydrated, processed, and embedded in paraffin wax before sectioning at 5 μm. Slides were then dewaxed, rehydrated, and stained hematoxylin and eosin (H&E).

3 | RESULTS

3.1 | Functional studies

CMG tracing highlighted the significant difference in *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ mice compared with all other groups in that these mice were the only mice which did not show the steep upward pattern indicative of bladder filling (Fig. 1). This rather straight line CMG data indicates that these double mutant bladders do not fill and empty on a cyclical basis, something that was not observed in wild type, *Gli2*$^{+/-}$, or *Gli3*$^{\Delta 699/+}$ mice (Fig. 1), thus highlighting the urinary incontinence in these mice and their inability to store urine. Cystometrography studies also revealed that *Gli2*$^{+/-}$;*Gli3*$^{\Delta 699/+}$ mice displayed significantly decreased peak micturition pressure and leak point pressure values compared to wild-type mice (Table 1), functionally supporting the urinary incontinence of these mice. Compared with wild-type group, there was no significant difference in total urine production, measured by 24 h voided volume, summarized in Table 2.

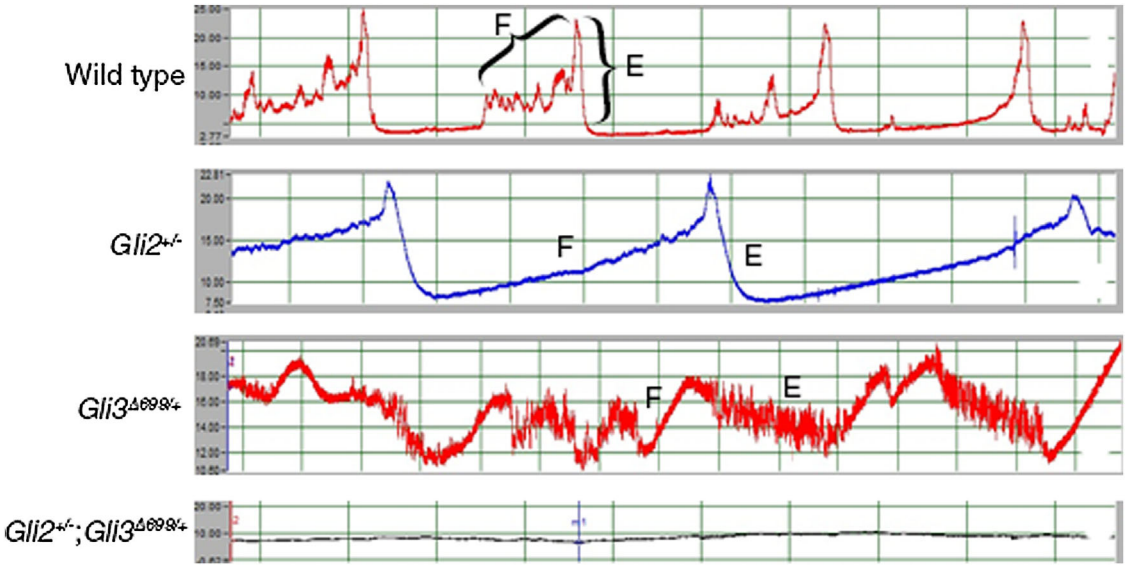

FIGURE 1 CMG tracing highlighting bladder filling and emptying phases. Wild type, *Gli2*$^{+/-}$, and *Gli3*$^{\Delta 699/+}$ mice CMG tracing readings display a pattern of filling (indicated via "F"), and emptying (indicated via "E") of the bladder. *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ mice display a straight line CMG pattern, indicating no filling of the bladder

TABLE 1 Peak micturition and leak point pressure testing of *Gli* mutants in comparison with wild-type mice

Groups	Peak micturition pressure (cmH2O)	Leak point pressure (cmH2O)
Wild type ($n = 4$; $n = 5$)	20.24 ± 6.45	18.64 ± 3.4
Gli2$^{+/-}$ ($n = 5$; $n = 7$)	14.20 ± 5.83*	18.27 ± 3.4
Gli3$^{\Delta 699/+}$ ($n = 4$; $n = 10$)	17.19 ± 4.45	23.47 ± 2.7
Gli2$^{+/-}$; *Gli3*$^{\Delta 699/+}$ ($n = 5$; $n = 5$)	4.28 ± 2.40**	6.66 ± 1.6*

Data presented as mean ±SEM
*Indicates a significant difference with wild-type mice ($P < 0.05$).
**Indicates a significant difference with wild-type mice ($P < 0.001$).

3.2 | Bladder and urethral structure

Ink injection assays indicated a widened bladder outlet and urethra in *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ female mice (Fig. 2), outlined specifically for wild-type and *Gli2*$^{+/-}$;*Gli3*$^{\Delta 699/+}$ mice in Fig. 2E and F in white dotted line, and the separation of white arrows indicating widened bladder outlet and urethra. In order to analyze this more deeply, bladders were fixed, sectioned, and stained histologically. Histological analysis findings supported the functional and ink injection assays (Fig. 3). Histological analysis showed a significantly compromised and widened bladder outlet and urethra, indicated by double black arrows in Fig. 3. *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ bladders' mucosa showed transitional epithelial hyperplasia. *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ female mice showed a decrease in circular smooth muscle layer thickness in bladder outlet and urethra zone. Other significant changes in urethra included significantly increased mucosal folding, and reduced luminal space.

4 | DISCUSSION

Established models of urinary incontinence have helped develop our understanding of the human condition, however, all these models to date have been stress/injury induced, and as such do not take into account the genetic component that could be involved.[3–5] Additionally, due to requiring a procedure done to each mouse in order to induce urinary incontinence, they are labor intensive, and could offer varying results based on the skillset of the experimenter.[3–5] In this study, we report a genetic mouse model of female urinary incontinence that overcomes both of these limitations.

TABLE 2 Urinary output of *Gli* mutant mice

Groups	24 h voided volume (ml)
Wild type ($n = 7$)	22.15 ± 6
Gli2$^{+/-}$ ($n = 4$)	20.44 ± 2
Gli3$^{\Delta 699/+}$ ($n = 13$)	21.25 ± 2
Gli2$^{+/-}$; *Gli3*$^{\Delta 699/+}$ ($n = 13$)	26.50 ± 5

Data presented as mean ± SEM.
No significant difference was found between any groups.

Previously, the importance of Shh signaling to urogenital development had been elucidated.[6–12,17] Transcription mediators of the pathway, Gli2 and Gli3, have been shown to be heavily involved in proper urogenital sinus formation.[6–12,17] As such, the development of an animal model which recapitulates clinical phenotypes of urogenital malformation via genetic manipulations of these regulatory factors comes of no surprise. However, the challenge so far has been in modulating the activity of the Shh pathway precisely low enough to elicit malformations, yet high enough to promise viability. Previous models which have reduced Shh pathway activity, either via Shh or Gli2 knockout, have proven to be embryonically lethal.[6,14] As such, they have failed at providing relevant animal models which recapitulate conditions often encountered in the clinic, such as urinary incontinence. Here, we have generated a compound genetic mutant, *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$, that is viable well into adulthood while displaying urogenital malformations including urinary incontinence in its adult females.

When assessed by functional voiding studies, no change in total urine production was observed. This suggests similar urine production across all genetic conditions analyzed, shifting the focus of consistently wet female mice to a problem in urine excretion rather than urine production. After performing LPP tests, urethral leak point pressure values were found to be much lower in *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ group as compared with wild-type group as well as single knockout groups (*Gli3*$^{\Delta 699/+}$ and *Gli2*$^{+/-}$). Additionally CMG studies revealed that *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ mice had a very low peak micturition pressure. This, combined with LPP findings suggests that detrusor over activity is not contributing as the cause of incontinence and it can be solely attributed to bladder outlet and urethra defect.

To investigate whether this attribution was correct, we investigated bladder and urethral structure via ink injection assays and histology. Ink injection assays revealed a significantly wider urethral exit from the bladder compared to wild-type mice. Histological evaluation of wild-type and mutant bladders revealed that the urethra is significantly wider in *Gli2*$^{+/-}$;*Gli3*$^{\Delta 699/+}$ mice, and mice show less developed smooth muscle around the bladder outlet and urethra as compared to controls. We believe this leads to the inability of these double mutant mice to hold urine in their bladders efficiently, resulting in their urinary incontinence.

Human conditions of urinary incontinence are often stress induced whereby the loss of urine is caused by an increase of intra-abdominal pressure such as lifting something heavy, coughing, or sneezing. Often, the physiological phenomenon that results in this condition arises from the stretching and weakening of the pelvic floor muscles during pregnancy. Aside from pelvic floor muscles, additional physiological

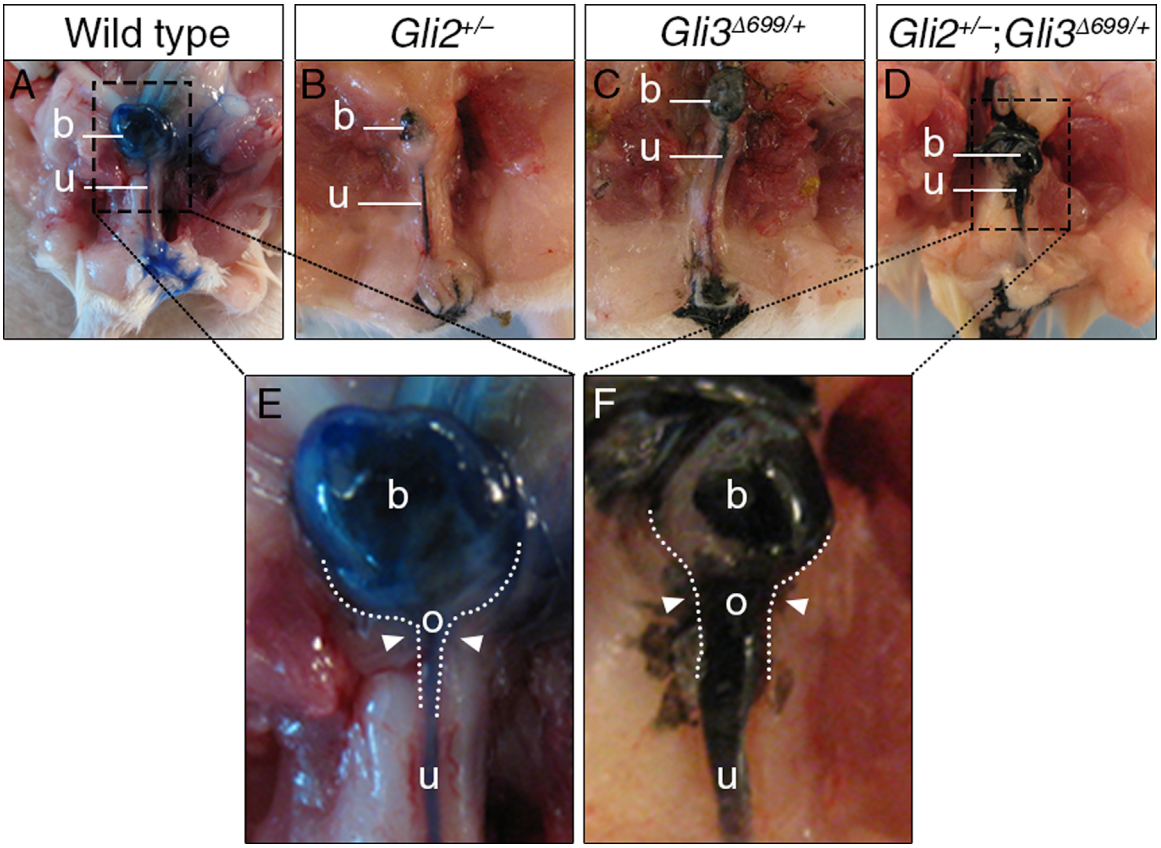

FIGURE 2 Dye/ink injection assays revealing bladder/urethral flow path. (A-D) Ink/dye injection assays highlighting flow from the bladder to the external urethral orifice. Close up image of the bladder neck in (E) wild-type mice and (F) *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ mutant mice, highlighting the difference in anatomical structure. White dotted lines outline bladder outlet and urethral zone; white arrows indicate area and width of urethra; b, bladder; o, bladder outlet; u, urethra

phenotypes have been associated with birth induced urinary incontinence such as the thinning urethral smooth muscles.[1,3,4] In our model, the *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ double mutant female mice were simply not able to withhold urine as adults, and did not need an increase in abdominal pressure to expel urine involuntarily, due to the developmental defects in the bladder neck and urethra, rather than the weakening of the pelvic floor muscles. Although the pathophysiological phenomenon is

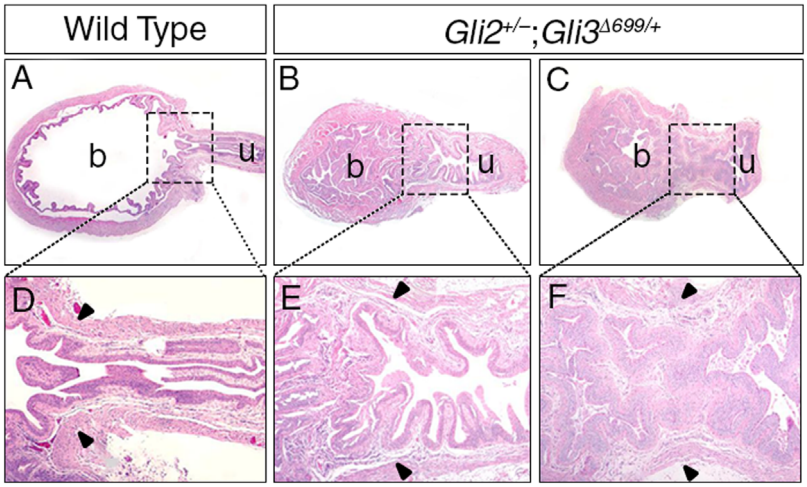

FIGURE 3 Histological evaluation of wild type and *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ female mice bladders. (A-C) low magnification images illustrating overall histology and structure of bladders and urethra in wild type and *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ female mice. (D-F) illustrating high magnification images of bladder outlet and urethra structure and width. Black arrows indicate bladder outlet and urethral position and size, as well as relative smooth muscle thickness; b, bladder; u, urethra

rather different, our mouse model and human clinical cases of urinary incontinence do share some similarities in that the bladder neck/urethral smooth muscles are compromised.

Previous epidemiological analyses has revealed that there likely exist genetic influences on urinary incontinence.[18,19] Here, we report that the compound genetic mutant *Gli2*$^{+/-}$; *Gli3*$^{\Delta 699/+}$ adult female mice display persistent urinary incontinence. Generating a model of urinary incontinence via genetic manipulations implies the involvement of genetic factor, and their potential involvement in human equivalent conditions. In addition to these translational implications, this mouse model can serve as a convenient model for female urinary incontinence, whereby individual mice to not have to be injured.[1,3,4] Additionally, it remains to be explored whether different methods of treatment such as surgical intervention or cell-based regenerative therapy targeting the urethral sphincter can improve the condition of these adult female mice.[3,20–22]

It has been previously identified that urothelial Shh signaling is responsible for bladder smooth muscle formation.[23–26] In addition, it has also been shown that Gli2 regulates bladder mesenchymal patterning, which is required for proper smooth muscle formation, and *Gli2*$^{-/-}$ mice lacked urethral tube formation, indicating a crucial role of proper Gli2 signaling for correct bladder/urethral development.[23,26] Specifically, it has been suggested that bladder mesenchyme (detrusor) development requires induction by a signal from bladder urothelium, and the Shh-Gli-Bmp4 signaling pathway is likely to be involved.[27] As such, by mildly reducing hedgehog signaling in *Gli2*$^{+/-}$;*Gli3*$^{\Delta 699/+}$ mice, we suspect to have impacted mesenchymal patterning within the bladder, and proper smooth muscle formation and maturation within the bladder and urethra, ultimately leading to the urinary incontinency observed in these double mutant mice. The functional and histological characteristics described in this work along with the existing evidence within the literature warrant further mechanistic investigation.

5 | CONCLUSION

Proper levels of Hh signaling are required for proper urogenital and hindgut development. Drastic reductions in Hh signaling yield severe and lethal urogenital sinus and hindgut malformations. By reducing Hh signaling mildly, we have elicited malformations that yield viable mice which live into adulthood, the female of which exhibit persistent urinary incontinence. In this work, we characterized this urinary incontinence of these mice functionally, and used histological analysis to highlight an underlying bladder neck defect which we believe is the cause of the underlying condition. We believe this presents a genetic mouse model for female urinary incontinence, which introduces potential genetic predisposition elements involved and help us better understand the human condition.

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