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Sonic hedgehog regulation of human rhabdosphincter muscle: Potential implications for treatment of stress urinary incontinence

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Abstract

Aims: Rhabdosphincter (RS) muscle injury occurs during prostatectomy, and is a leading cause of stress urinary incontinence (SUI). Current SUI treatments engender significant side effects, which negatively impact patient quality of life. Thus an unmet need exists to develop novel RS regeneration methods. We have shown that Sonic hedgehog (SHH) is a critical regulator of penile smooth muscle, and we have developed novel peptide amphiphile nanofiber hydrogel delivery of SHH protein to the penis to regenerate smooth muscle after prostatectomy induced injury. If similar SHH signaling mechanisms regulate RS muscle homeostasis, this innovative technology may be adapted for RS regeneration post-prostatectomy. We examine the SHH pathway in human RS muscle.

Methods: Human RS obtained during radical cystoprostatectomy ($n=13$), underwent SHH pathway analysis. Primary cultures were established ($n=5$), and RS cells were treated with SHH protein, SHH inhibitor, or PBS (control). Immunohistochemical analysis for SHH pathway, skeletal muscle actin, and trichrome stain were performed. RS growth was quantified at 3 and 6 days.

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CONFLICTS OF INTEREST

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Results: SHH, it is receptors patched and smoothened, and transcriptional activators, GLI proteins, were identified in human RS muscle. At 3 and 6 days, RS cells increased 62% and 78% ($P = 0.0001$) with SHH treatment and decreased 40% ($P = 0.0001$) and 18% ($P = 0.039$) with SHH inhibition.

Conclusions: The SHH pathway was identified in human RS. RS growth increased with SHH treatment, indicating intervention may be possible to enhance RSregeneration, and impact SUI. Peptide amphiphile delivery of SHH may be applicable for RS regeneration and SUI prevention.

Keywords

muscle regeneration; rhabdosphincter; Sonic hedgehog; stress urinary incontinence

1 INTRODUCTION

Stress urinary incontinence (SUI) is an important public health concern, affecting both men and women, and with increasing incidence in the elderly. Iatrogenic injury to the rhabdosphincter (RS) muscle occurs during surgery for prostate or bladder cancer, and is a leading cause of SUI in men.¹ Seventy-two percent of patients develop SUI in the first week after prostatectomy and individuals who do not recover within 6 months, are unlikely to regain function without intervention (up to 44% prevalence).² While the majority of men undergoing radical prostatectomy will regain reasonable urinary control through pelvic floor strengthening exercises, many will suffer from bothersome SUI and require the use of incontinence pads and garments, which have significant detrimental impact on patient quality-of-life.³ Surgical treatments for SUI, include placement of urethral slings or artificial urinary sphincters, which have variable outcomes and significant side effects, including device failure, erosion of the urethra, transient pain and infection, and urinary retention, leading to a revision rate of 80% by 10–15 years.^{4–6} Thus there is a critical unmet need for alternative methods to preserve and/ or regenerate RS muscle.

Preservation of urethral length and limited dissection along the pelvic floor, which preserve RS muscle, may help mitigate adverse SUI effects after prostatectomy.^{7,8} The RS (external sphincter) is composed of specialized striated muscle and is found in the proximal urethra. The muscle extends without interruption from the bladder neck to the pelvic floor.⁹ The primary function of the RS is the initiation of voluntary micturition. Additional functions include random interruption of the urinary stream, reflex control during an increase in intra-abdominal pressure (coughing, sneezing, laughing, lifting), complete emptying of the urethra after micturition, and stabilization of the posterior urethra in the urogenital diaphragm. After prostatectomy an intact RS is important in order to support the urethra and bladder neck, which continues to be primarily responsible for continence to function as efficiently as possible.¹⁰

There are three types of muscle found in vertebrates, smooth, skeletal, and cardiac. We have shown in previous studies that the Sonic hedgehog (SHH) pathway is critical for development and maintenance of smooth muscle, in another urogenital organ, the penis.^{11–13} SHH is a morphogen- regulating crucial epithelial-mesenchymal interactions during embryonic development.¹⁴ SHH signaling is considered generally silent in post-natal life

with the exception of the urogenital organs in which they are highly expressed in the adult.¹¹ SHH is also expressed after injury and during regeneration of adult skeletal muscle¹⁴ and smooth muscle.^{13,15} The SHH signal is transduced through the interplay between PTCH1 and SMO. PTCH1 is a 12-transmembrane protein that is a receptor for SHH. PTCH1 binds to SHH but does not transduce the intracellular signal. SMO, a 7-transmembrane protein that forms a complex with PTCH1, does not bind to SHH but transduces the SHH signal through activation of GLI transcription factors. We have previously shown that SHH protein is abundant in penile smooth muscle and is decreased in the penis of prostatectomy patients and animal models.^{13,15} SHH inhibition causes a 12-fold increase in smooth muscle apoptosis that remodels penile architecture, and causes erectile dysfunction (ED),^{11–13} if SHH inhibition is removed, penile smooth muscle regenerates,¹³ and SHH treatment of the penis in a prostatectomy model, preserves and regenerates smooth muscle.¹³ Thus SHH is a critical regulator of smooth muscle. It has been suggested that the SHH pathway is also a critical regulator of skeletal muscle during embryonic limb development and that the SHH pathway is postnatally recapitulated after injury and during regeneration of adult skeletal muscle.^{14,16} SHH is upregulated 80-fold in ischemic limb skeletal muscle¹⁷ and SHH inhibition results in muscle fibrosis, increased inflammatory reaction and compromised motor functional recovery after injury.¹⁴ The RS is composed of striated skeletal muscle. Based on our previous results in penile smooth muscle and on literature findings on skeletal muscle, we hypothesize that the SHH pathway is an important regulator of human RS homeostasis and regeneration, which may directly impact urinary continence.

We have successfully adapted a novel and innovative peptide amphiphile (PA) nanofiber hydrogel delivery vehicle for SHH protein to the penis to preserve and regenerate penile smooth muscle.^{18–22} If similar SHH signaling mechanisms regulate striated RS muscle, then this innovative technology may be optimized and applied for RS regeneration at the time of prostatectomy, to prevent/treat SUI. Our preliminary data suggests that SHH signaling in RS injury/recovery has sufficient similarity to penile smooth muscle, so that our arsenal of SHH/PA delivery methods may be applicable to SUI prevention. Application of SHH PA to preserve/regenerate RS muscle is an innovative idea that may significantly impact SUI treatment strategies.

2 MATERIALS AND METHODS

2.1 RS tissue

Human RS tissue was obtained from 13 patients who underwent radical cystoprostatectomy. These patients underwent cutaneous urinary diversion, thus there was no need for preservation of the RS muscle. A small amount of the periurethral muscle was removed and saved from the specimen side during surgery. Part of the RS tissues were frozen in liquid nitrogen prior to storage at -80°C for histological examination and the remaining tissues were incubated in serum containing DMEM/F12 media for primary RS cell culture.

2.2 Primary RS cell culture

Human RS tissue was obtained from the cystoprostatectomy specimens. Primary cell culture was established using the half-dry method modified from a previously established protocol.

²³ RS cell culture is rare with only one publication in this area.²⁴ Briefly, RS tissues were cut into $1 \times 1 \text{ mm}^2$ pieces and placed into the bottom of 25 cm^2 flasks. Culture flasks containing 2 mL of medium were placed upside-down in a 5% CO_2 incubator at 37°C for 2 h to allow the tissues to attach. Flasks were gently turned over and attached tissues were slowly covered by DMEM/F12 media containing 20% heat-inactivated fetal bovine serum (FBS) and an antibiotic cocktail (Penicillin, Streptomycin, Glutamine). The culture medium was replaced twice a week for 3 weeks, with increasing volume of media (up to 7 mL). Outgrowth of RS cells (passage 1) from tissues was monitored, and at confluence cells were split into new flasks for continued passages. Cell stocks were made from passages 1–3, and cells from passage 4 were used for experiments.

2.3 SHH protein treatment and SHH inhibition of RS cell growth

RS cells from passage 4 were grown in culture with Affi-Gel beads that were incubated overnight at 4°C with SHH protein (25 μL of a 1 $\mu\text{g}/\mu\text{L}$ solution, R&D Systems, Minneapolis, MN), 5E1 SHH inhibitor (388 $\mu\text{g}/\text{mL}$, Developmental Studies Hybridoma Bank, Iowa City, IA), or PBS (control) for 3–6 days. Concentration of SHH protein and inhibitor was based on previous optimization of tissue response in penis and nerve tissues.^{13,18,19,25} Matrigel (100 μL) was placed at four places along the periphery of wells in six-well plates. Affi-Gel beads (extended release vehicle) containing SHH protein, 5E1 SHH inhibitor, or PBS were placed on the Matrigel and were gelled at 37°C for 5 min. RS cells (15 000) were placed in the center of each well and the cells were allowed to adhere for 1 h prior to adding 3.5 mL of DMEM/F12 media containing 10% FBS and antibiotic. Culture plates were placed in an atmosphere-controlled incubator (5% CO_2) at 37°C for 3–6 days. Cells were quantified by hemocytometer at 3 and 6 days. Four wells for each treatment group were quantified with four hemocytometer measurements performed on cells from each well. Experiments were performed in duplicate at each time point and were replicated on cells from three patients.

2.4 Trichrome

Trichrome stain was performed as described previously²⁶ on human RS tissue from cystectomy patients ($n = 6$). Muscle tissue (smooth muscle and skeletal muscle) stains red, and collagen stains blue.

2.5 Immunohistochemical analysis (IHC)

IHC was performed on frozen RS sections which were cut to $12 \mu\text{m}$ thickness and were post fixed in acetone at 4°C for 15 min. OCT was removed with three washes of $1 \times \text{PBS}$ prior to blocking with 3% milk in PBS for 1 h at 4°C . Sections were incubated overnight at 4°C with 1/100 goat polyclonal antibody against SHH, PTCH1, and GLI-1 (sc-1194, sc-6149, sc-6152, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal against SMO (LS-A2666, Lifespan Biosciences, Seattle, WA), GLI-2, and GLI-3 (600–401695, 600–401-694, Rockland Immunochemicals, Inc., Limerick, PA). Additionally, RS cell cultures were characterized with mouse antibodies for skeletal muscle ACTIN (ab28052, Abcam, Cambridge, MA), smooth muscle ACTIN (A5691, Sigma, Burlington, MA), prolyl-4-hydroxylase (rPH, fibroblast marker, AF5110–1, Acris), nNOS (neuronal marker, 610309, Transduction Laboratories, San Jose, CA), and CD31 (epithelial cell marker, 3528S, Cell

Signaling Technology, Danvers MA). HRP secondary antibodies were 1/150 donkey anti-goat, and mouse anti-rabbit (sc-2033, sc-2491, Santa Cruz). Fluorescent secondary antibodies were 1/150 chicken anti-goat 488, and donkey anti-mouse 594 (A-21467, R37115, Thermo Fisher Scientific-Invitrogen Molecular Probes, 1/150, Carlsbad, CA). Control slides in which the primary antibody was omitted, were performed for all secondary antibodies, to ensure artifact staining was not present from the secondary antibodies. Normal penis and pelvic ganglia- cavernous nerve sections were stained as positive controls for rPH, nNOS, CD31, and α -smooth muscle ACTIN. Sections were mounted using DPX Mounting media (Electron Microscopy Sciences, Hatfield, PA) and microscopy was performed using a Leica DM2500 microscope.

2.6 Apoptosis

TUNEL assay was performed according to the ApopTag kit (Millipore, Burlington, MA) and all cells were co-stained for comparison with DAPI (0.02 μ g/mL). Slides were mounted using Fluoromount (Thermo Fisher Scientific, Waltham, MA) and photography performed using a Leica DM2500 microscope.

2.7 Statistical analysis

Statistical analysis was performed by ANOVA with a Dunnett's posthoc test using the SPSS program. Results were significantly different if $P < 0.05$.

3 RESULTS

3.1 RS tissue morphology and identification of SHH pathway in human RS

Trichrome stain was performed on human RS tissue, which showed abundant muscle (red) and collagen (blue). IHC analysis was performed on human RS tissue assaying for SHH, and its receptors PTCH1 (binding) and SMO (non-binding). SHH, PTCH1, and SMO proteins were identified in human RS muscle (Figure 1A). The downstream targets of the SHH pathway, the GLI proteins were also expressed in human RS, with GLI-1, GLI-2, and GLI-3 identified in RS skeletal muscle (Figure 1B). No primary controls (left: mouse anti-rabbit, and right: donkey anti-goat) are presented for comparison (Figure 1C).

3.2 Establishing a primary culture from human RS

RS tissue was obtained at the time of cystoprostatectomy and RS cells were grown from primary culture (Figure 2A). Cells had typical skeletal muscle architecture (Figure 2B) and IHC analysis of cultured cells identified staining for skeletal muscle actin (Figure 2C). Skeletal muscle actin staining was present in muscle fibers (Figure 2D). SHH protein was also identified in skeletal muscle fibers and was particularly abundant in the perinuclear region of muscle cells (Figure 2D). No primary controls are presented for comparison (Figure 2D). RS cells were additionally characterized by probing with α -smooth muscle ACTIN, prolyl-4-hydroxylase (rPH, fibroblast marker), nNOS (neuronal marker), and CD31 (endothelial marker) antibodies. The RS cells present in passage 4 stained exclusively for skeletal muscle ACTIN (Figure 2E). Penis and pelvic ganglia-cavernous nerve tissue were used as positive controls for rPH, CD31, nNOS, and α -smooth muscle ACTIN (Figure 2E), and no primary control is included in Figure 2E.

3.3 RS growth with SHH treatment and SHH inhibition

RS cells from passage 4 were grown in the wells of six well plates (Figure 3A). Affi-Gel beads containing SHH protein, SHH inhibitor (5E1), or PBS (control) were placed in Matrigel in four corners of each well and media was added (Figures 3A and 3B). Cells were grown from 3 to 6 days prior to counting cell number with a hemocytometer. The number of cells increased 1.62-fold (62%, $P = 0.0001$) after 3 days of SHH protein treatment and decreased 1.66-fold (40%, $P = 0.0001$) with SHH inhibition (5E1, Figures 3B and 3C). At 6 days, cell growth increased 1.8-fold (78%, $P = 0.0001$) with SHH treatment, and decreased 1.2-fold (18%, $P = 0.039$) with SHH inhibition (Figures 3B and 3C).

TUNEL assay was performed on cells treated with PBS, SHH protein, and 5E1 SHH inhibitor (Figure 3D). Apoptosis occurred at low abundance in all cells, irrespective of treatment (Figure 3D).

4 DISCUSSION

We sought to examine the role of SHH signaling in human RS muscle obtained from radical cystoprostatectomy specimens in patients undergoing cutaneous urinary diversion. The impact of this study is that, it may provide a novel pathway for intervention and a creative strategy to regenerate and preserve RS muscle after prostatectomy. This is the first study to identify members of the SHH pathway in skeletal muscle of human RS tissue, suggesting SHH involvement in RS homeostasis. Manipulation of the SHH pathway through inhibition of SHH binding to its receptor PTCH1, decreased RS growth of cultured patient cells, and identified the necessity of active SHH signaling for RS growth and regeneration. Exogenous SHH protein increased the number and speed of RS cell proliferation by comparison to controls, showing the responsiveness of RS muscle to SHH treatment, and suggesting that intervention with SHH may be possible to promote/enhance regeneration of RS muscle. During radical prostatectomy, RS may be excised or injured as the apex of the prostate is disconnected from the urethra and during creation of the urethrovesical anastomosis after the prostate is removed. The premise would be to apply SHH locally to the remaining RS muscle at the time of prostatectomy to enhance RS regeneration. SHH protein rather than RNA delivery is preferable since we have shown in previous studies, where there is interruption of innervation, that there is a block in SHH protein synthesis.¹² Ideally, the delivery vehicle should be capable of local, extended release of SHH protein, easily deliverable in vivo at the time of prostatectomy, and biodegradable. Our in vitro analysis of human RS cells grown in culture show a quick response in increased growth that is significantly enhanced 62% by 3 days of SHH treatment, and as much as 78% by 6 days of treatment. This suggests a short window of SHH treatment would be needed to enhance RS growth, and impact SUI.

We previously adapted peptide amphiphile nanofiber hydrogels for SHH protein delivery to the penis, to preserve and regenerate corpora cavernosal smooth muscle, which undergoes apoptosis in response to loss of cavernous innervation during prostatectomy, and causes erectile dysfunction.^{18,20} This type of hydrogel delivers SHH protein as the hydrogel breaks down and with established release kinetics of 73% cumulative protein delivery by 6 days. While protein release is dependent on ionic strength and sheer stress within the tissue, this

timing should be sufficient to promote RS regeneration and the hydrogel may be optimized for maximal RS delivery and regeneration enhancement. Our studies show that, SHH response to skeletal muscle injury is similar enough to smooth muscle recovery that our arsenal of SHH PA delivery methods may be applicable to SUI prevention, and significantly impact SUI treatment strategies.

Current state of the art treatments to improve RS function involve isolation of muscle satellite cells to try and re-grow the muscle.²⁷ The methodology we are proposing would be a significant improvement over these strategies since the hydrogel delivery of SHH is a non-invasive, and biodegradable method of preserving/regenerating RS muscle. During embryogenesis, the SHH pathway is essential for muscle development, and SHH improves age associated impairment of skeletal muscle in mice.¹⁶ A potential mechanism for SHH function in RS may involve the GLI proteins. Myf5, a skeletal muscle regulatory gene, is a direct target of long-range SHH signaling during muscle development through positive regulation by GLI transcription factors.²⁸ Delayed ischemic muscle repair observed in old mice was associated with an impaired upregulation of GLI-1.²⁹ GLI signaling is complex, with several isoforms that are activated by the SMO part of the SHH receptor. Upon SHH binding to PTCH1, this releases the repression of PTCH1 on SMO, allowing SMO to preferentially upregulate the formation of GLI-2 and GLI-3 to their active forms. Active GLI-2 and GLI-3 regulate activation of SHH target genes, including GLI-1,^{30–31} which is indicative of high level SHH signaling. In our study GLI-1, GLI-2, and GLI-3 were identified in human RS muscle, indicating active SHH signaling through GLI-1, which may be an exploitable avenue for intervention to further enhance RS regeneration.

5 CONCLUSIONS

This is the first study to identify the SHH pathway in skeletal muscle of human RS. Inhibition of the SHH pathway decreased RS growth, and identified the necessity for active SHH signaling in RS tissue. RS growth increased with SHH treatment, indicating intervention may be possible to enhance regeneration and improve SUI. SHH signaling in RS has sufficient similarity to penile smooth muscle, so that our arsenal of SHH PA delivery methods may be applicable to regenerate RS muscle. The impact of this study is that it may provide a novel pathway for intervention and a creative strategy to regenerate and preserve RS muscle after prostatectomy, thus minimizing and potentially preventing SUI development.

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SUMMARY SENTENCE

The sonic hedgehog pathway is active and regulates growth of human rhabdosphincter muscle, thus providing a novel avenue for intervention to regenerate and preserve rhabdosphincter muscle after prostatectomy, and a creative strategy to minimize/prevent stress urinary incontinence.

SIGNIFICANCE STATEMENT

This is the first study to identify the sonic hedgehog pathway in human RS. Inhibition of the SHH pathway, decreases RS growth, and identifies the necessity for active SHH signaling. RS growth increases with SHH treatment, indicating intervention may be possible to enhance RS regeneration, and impact SUI.

FIGURE 1.

Trichrome stain and IHC analysis for SHH pathway in human RS muscle. A, Trichrome stain of human RS indicates abundant muscle and collagen. Arrows indicate muscle. IHC analysis of human RS shows SHH, PTCH1, and SMO protein localization in RS muscle. B, GLI-1, GLI-2, and GLI-3 are abundant in RS muscle. C, No primary controls (left: mouse anti-rabbit, and right: donkey anti-goat) are presented. Arrows indicate protein. 100–200× magnification

FIGURE 2.

Primary culture was set up from human RS tissue. A, Photo of cells outgrowing in the primary culture. Muscle cells have characteristic skeletal muscle shape (B) and stain intensely with skeletal muscle ACTIN (C). Nuclei were stained with DAPI (blue). Arrows indicate muscle cells. 100–400× magnification. D, Skeletal muscle fibers stained intensely for skeletal muscle ACTIN (red) whereas SHH protein (green) was abundant both in the cytoplasm and perinuclear region of the RS muscle cells. Nuclei were stained with DAPI (blue). No primary controls were included for comparison. Arrows indicate protein. 400×

magnification. E, RS cells were additionally characterized with skeletal muscle ACTIN, α -smooth muscle ACTIN, rPH (fibroblast marker), CD31 (epithelial marker), and nNOS (neuronal marker). RS cell culture only stained for skeletal muscle ACTIN. No primary control for donkey anti-mouse is included for comparison and penis and pelvic ganglia-cavernous nerve were used as positive controls for rPH, CD31, nNOS, and α -smooth muscle ACTIN. Arrows indicate staining. 100–200 $\times$ magnification

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FIGURE 3.

RS cells were grown in 6 well plates in the presence of Affi-Gel beads containing, SHH protein, SHH inhibitor or PBS (control). Affi-Gel beads were placed in matrigel to hold them in place and RS cells were grown in the center of the wells (A). SHH treatment significantly increased the number of RS cells at 3 and 6 days of treatment, while SHH inhibition significantly decreased the number of RS cells (B and C). TUNEL assay was performed on cells treated with PBS, SHH protein and 5E1 SHH inhibitor (D). Apoptosis

occurred at low abundance in all cells, irrespective of treatment. Arrows indicate apoptotic nuclei. 200× magnification

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