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The Role of Fibrosis and Atrophy in Age & Injury related Pelvic & Genital-Urinary
Disorders

A thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Johnny Fu

Committee in charge

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Professor Nigel Crawford, Co-Chair
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2017

The Thesis of Johnny Fu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

The Role of Fibrosis and Atrophy in Age & Injury related Pelvic & Genital-Urinary Disorders

by

Johnny Fu

Master of Science

University of California, San Diego, 2017

Professor Hemal Patel, Chair
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The most common genito-urinary and pelvic floor disorders that affect the aging population are: anal/urinary incontinence and sexual dysfunction in men. For the

analogous muscles, age-related atrophy and fibrosis have been shown to play a major role in the disorders. With increasing age comes the inability for the body to properly moderate the connective tissue and inflammatory response after an injury. Although the specific mechanisms underlying fibrosis are unclear, certain pathways such as TGF- β and the Wnt/ β -cat pathway have been known to be a key contributor in the development of fibrosis. Furthermore, in patients with erectile dysfunction, diabetes related fibrosis could be a potential source of increased collagen deposition. In order to explore these conditions, protein expression through Western blot for various atrophic and fibrotic markers of the TGF- β , Wnt/ β -cat, and Girdin pathway in skeletal muscle tissues was performed. Our findings suggest that the markers do tend to increase expression in injured/aging conditions for the particular muscles. This study may show insight into the role Wnt/ β -cat pathway may play in the spontaneous repair and regeneration in the sphincter and pelvic floor muscles that are prone to injury during childbirth for women. In addition, the Wnt/TGFB/Girdin signaling in the age related changes in the external anal sphincter and pelvic floor muscle requires more exploration with the increasing elderly population in the United States.

Introduction

The most common genito-urinary and pelvic floor disorders that affect the aging population are: anal and urinary incontinence, sexual (erectile) dysfunction in men. Age-related atrophy and fibrosis of these important muscles which regulate continence/sexual function consequent to injury are potential reasons. In the following sections the pathophysiology of these disorders are briefly discussed.

Child Birth and Anal Sphincter Injury: Childbirth related injury affects anal sphincter muscles by a variety of mechanisms, i.e., stretch, ischemia, avulsion and surgical incision (episiotomy). Vaginal delivery is associated with alterations in the anal continence function; up to 44% of women show onset of new symptoms¹⁻³. In 9% of patients, 3rd or 4th degree tears are identified during vaginal delivery and 2/3rd of these women suffer from fecal incontinence (FI) subsequently⁴⁻⁷. Of the 3 components, internal anal sphincter (IAS), external anal sphincter (EAS) and puborectalis muscle (PRM), the EAS is most commonly involved in the FI mechanism^{8, 9}. Ultrasound imaging show defects of the EAS³ and PRM muscle¹⁰ in multipara women (25-35%) and manometric data show poor voluntary squeeze pressures in these patients¹¹. Bharucha found that 73% of patients with FI have abnormal EAS muscle function and in a recent study we observed that the length-tension function of EAS and PRM is impaired in majority of women with FI¹².

Aging and FI: Aging is recognized to be a significant risk factor for late onset FI after sphincter injury¹³. A recent survey by Whitehead et al confirms a strong association

between FI and age reporting that prevalence of FI increases from 2.6% at ages 20–30 years

to 15.3% in people > 70 years¹⁴. In a recent study consisting of 999 symptomatic women, child birth related anal sphincter injury was shown to exhibit a cumulative impact on age-related sphincter dysfunction¹⁵. In addition, there has been a remarkable shift in the demographics of childbearing in the US during the last decade and the number of first births per 1000 women 40 to 44 years old leaped by 70%¹⁶. Birth related injury at an advanced age is likely to increase the risk of impaired sphincter muscle regeneration and consequent FI.

UI (involuntary urine leakage) is a major medical illness which affects a large number of men (17%)¹⁷ & women (38%)¹⁸ and the incidence increases with age^{19, 20}. Urethral sphincters (specifically striated external [EUS] or rhabdosphincter[RHB]) as well as pelvic floor muscles (PFM) play an important role in urinary continence mechanisms²¹. Age-related degenerative changes to these muscles are recognized as the most common cause for UI in the geriatric population²²⁻²⁶. In men, damage/ surgical injury to urethral sphincters is a significant cause for intrinsic sphincter deficiency (ISD). In women the major cause is childbirth related injury to the PFM. In addition, older age at time of surgery has been found to be associated with a higher prevalence of post-prostatectomy UI in men and need for longer continence recovery after prostate surgery²⁷⁻³¹. We hypothesize that impaired muscle regeneration after sphincter muscle injury and increased muscle atrophy with advancing age is mediated by a common driving molecular pathway called Wnt – β catenin signaling, a well-established phenomenon in limb muscles³².

Aging and Erectile dysfunction: Aging is an important risk factor for the onset of ED (inability to initiate or maintain adequate erection for sexual function) that affects the quality of life of men and their partners^{33, 34}. In addition, some older men respond poorly to oral pharmacotherapy. This treatment failure³⁵ often leads to invasive surgical interventions. Recent evidence suggests a significant increase in overall ED prevalence and an age-related increase in active military and VA patients^{36, 37}. Molecular mechanisms of age-related increase in ED and reasons for this refractory response to oral pharmacotherapy are unclear. Evidence-based guidelines, to prevent ED and improve pharmaco-therapeutic response in this aging population, represent an important unmet need and our studies should bridge this gap. We propose to test our hypothesis that age-related degeneration of erectile (cavernosal) tissue and supporting PFM is due to excessive atrophy/fibrosis. Fibrosis leads to derangement of muscle fiber alignment, resulting in impaired penile hemodynamic function. Evidence suggests that age-related erectile tissue fibrosis is an important cause³⁸

What is fibrosis: Fibrosis is commonly considered to be a result of deregulated tissue repair. It is a pathological condition, where processes normally occurring during tissue repair, such as formation of connective tissue and wound contracture, persist and ultimately lead to disruption of normal tissue structure and organ dysfunction. In the genital-urinary and pelvic tissues, fibrosis is known to be a primary contributor to urinary and anal incontinence. The mechanisms underlying fibrosis are still largely unclear. It is commonly thought that the excess of transforming growth factor (TGF- β 1) or/and the increased susceptibility of fibroblasts to it are possible causes of persistency of the tissue remodeling process.

Damage to skeletal muscle leads to the activation of multiple pathways that work to repair the injury. However, when the pathways are disrupted, the buildup of permanent connection tissue and inflammatory responses can lead to disruption of muscle function. Although fibrosis commonly occurs in the normal healing process after injury, aging has been known to complicate the cellular mechanisms. While the formation of scars eventually dissipates in young animals, the build-up of additional connective tissue tends to persist with age. This age-related fibrosis can lead to dysfunction in major organ systems and result in multiple diseases. Complicated diseases such as diabetes are widespread among the elderly and is a known contributor to fibrosis. The inability to produce significant insulin in diabetic subjects can eventually develop injuries to tissues due to the abundance of glucose. If the injuries become persistent, tissue fibrosis can occur and may compound the initial complications.

Environmental stress may also factor into progression of irregular tissue repair. In particular, oxidative stress has been implicated to play a role in the production of fibrosis in several different organs particularly the skin and lungs. However, whether oxidation produces fibrotic disorders in skeletal muscles, specifically the muscles that regulate GU function is still unclear.

The primary markers known to be associated with the fibrotic phenomenon are Collagen-I, TGF-beta, Beta-Catenin, and the Wnt Pathway. These constituents will be assessed among the EAS, bladder neck, and mid-urethra tissues of different aged rabbit samples and the penile cavernosal tissue of humans (obtained from men undergoing penile prosthesis). Additionally, we examined a marker previously shown to be significantly involved in atrophy: Atrogin-1. Atrophy, similarly to fibrosis, is the wasting,

loss, or degeneration of muscle tissue. This is most commonly caused by lack of muscle use due to inactiveness or lack of physical activity, injuries, diseases causing immobilization, or aging, which is what we are aiming to focus on with our study. In muscle atrophy, the Ubiquitin-proteasome pathway (UPP) is a major determinant of muscle wasting, or atrophy, as its up regulation is associated with muscle breakdown. Major components of this pathway and other closely associated pathways are all the markers that we are aiming to test for an existing correlation.

TGF- β is found in all tissues, but is particularly abundant in bone, lung, kidney and placental tissue. TGF- β is produced by many but not all parenchymal cell types, and is also produced or released by infiltrating cells such as lymphocytes, monocytes/macrophages, and platelets. Following wounding or inflammation, all these cells are potential sources of TGF- β . In general, the release and activation of TGF- β stimulates the production of various extracellular matrix proteins and inhibits the degradation of these matrix proteins, although exceptions to these principles abound. These actions of TGF- β contribute to tissue repair, which under ideal circumstances leads to the restoration of normal tissue architecture and may involve a component of tissue fibrosis.

The role of Collagen-1 in the muscle is to provide strength and structure for moving and functioning as a structural protein that helps form body structures, gives strength to muscle tissue and maintains elasticity. As aging occurs, Collagen-I works as a necessity for retaining muscle strength and functioning.

β -catenin is a molecule required for muscle differentiation. Satellite muscle cells express Beta-catenin and this expression is maintained as proliferation occurs. Beta-

catenin interacts significantly with MyoD, a transcription factor involved in muscle differentiation, by enhancing MyoD binding to E box elements in transcription. As studies have shown, the interaction is so critical that when Beta-catenin is not present or the Beta-catenin and MyoD interaction is disrupted, MyoD is inhibited in cells.³⁹ In the absence of Wnt signaling, β -catenin is constantly being suppressed by the Axin protein which prevents β -catenin from entering a cell nucleus affecting gene expression.⁴⁰

The Wnt/ β -catenin dependent pathway controls key developmental gene expression programs by modulating the amount of β -catenin through regulating its degradation or accumulation and its translocation from the adherens junction and cytoplasm to the nucleus. In the absence of a Wnt signal, the cytoplasmic β -catenin is tightly maintained at a low level by a multiprotein destruction complex consisting of Axin, the adenomatous polyposis coli protein (APC), casein kinase 1 α (CK1 α), and Glycogen Synthase Kinase 3 β (GSK3 β).³⁹ The complex phosphorylates cytoplasmic β -catenin leading to its degradation by the ubiquitin-proteasomal system. The continuous elimination of β -catenin prevents its accumulation in the cytoplasm and the consequent translocation into the nucleus. In the presence of a Wnt signal, the destruction complex is disassembled leading to an increment of β -catenin levels and allowing its translocation into the nucleus where it activates Wnt target gene expression.⁴¹ The regulation of the Wnt/ β -catenin pathway plays a role in the pathogenesis of several diseases including cancer, birth defect disorder, skeletal diseases, and fibrotic diseases. For this reason, Wnt/ β -catenin signaling is tightly regulated and kept under strict control at different levels of the Wnt cascade. Wnt/ β -catenin signaling can up-regulate the expression of an important fibrogenic growth factor (TGF- β) and TGF- β 1 can promote β -catenin signaling^{42, 43}. Thus, targeting the Wnt/ β -catenin pathway may be a novel approach to treat fibrosis.

Wnt-TGF- β pathway interactions in fibrosis: Recent reports confirm co-operation and cross-regulation between TGF- β and canonical Wnt pathways^{44, 45}. Expression of Wnt1, β -catenin and their target genes were upregulated following TGF- β -induced podocyte injury and this process was blocked by a Wnt antagonist⁴⁵. Inhibition

of TGF- β signaling by a selective TBRI inhibitor strongly reduced the activation of the canonical Wnt pathway in experimental fibrosis⁴⁴. Though Wnt and TGF- β pathways cooperatively mediate fibrosis, recent studies implicate multi-receptor involvement in organ fibrosis^{46,47}.

Girdin or also known as GIV has a central role within the fibrogenic-signaling network. Once expressed, the protein enhances profibrotic pathways such as TGF β -SMAD while inhibiting antifibrotic pathways. Recently Ghosh et al identified GIV/Girdin protein as a platform for receptor cross-talk that integrates many signals and modulates several key pathways within downstream signaling network, all via activation of trimeric G proteins⁴⁶. Her studies show that GIV/Girdin is a central hub of fibrogenesis in the liver, kidney and skin, thus raising the possibility that it is a potential target for the reversal of skeletal muscle fibrosis in the EAS^{47,48} as well. Furthermore, by virtue of its ability to activate trimeric G protein Gi, GIV simultaneously modulates pro and anti-fibrotic pathways that function antagonistically to balance collagen synthesis and its removal in a delicate equilibrium.

What is atrophy?: Atrogin-I is a protein involved in the Ubiquitin-Proteasome Pathway where, when signaled, is activated and leads to protein degradation alongside MuRF-I (another UPP component). These two proteins are stimulated by FOXO1/FOXO3, which when inhibited by a protein synthesis pathway, is activated to perform this phenomenon. Myostatin is a member of the transforming growth factor-beta superfamily with a crucial role in the negative regulation of muscle mass. Overexpression has been shown in literature to cause muscle atrophy. Myostatin signaling activates Smad 3, which has shown to be necessary for myostatin-induced atrogin-1 expression and

atrophy. Smad3 is a signaling mediator protein for TGF β and myostatin which are responsible for regulating skeletal muscle growth. It has a signaling role in skeletal muscle regeneration. In previous literature on Smad3, mice deficient in Smad3 had incomplete recovery after muscle injury and had reduced fibrotic tissue formation suggesting that Smad3 deficiency leads to impaired muscle regeneration.

Connection between atrophy and fibrosis pathways: Wnt- β -catenin pathway is implicated in both age related fibrosis as well as atrophy^{32, 49-51}. In addition, GIV fibrosis pathway involves some of the key mediators⁴⁶ (TGF- β , SMADs etc). The patterns that have shown to be observed for each marker will be tested on various aged and injured rabbits as well as human and mice penile tissue and should help to reach an overall understanding on how each of these factors contribute to fibrosis and atrophy in regulatory muscle functioning. In addition, the use of an siRNA agent to silence the Girdin gene will be used to determine whether a therapeutic treatment can be produced for manipulating the fibrotic pathways.

Connection between Fibrosis and Diabetic pathways: The presence of long term hyperglycemia has been shown to alter the extracellular matrix function (ECM) in various tissues.⁵² A chief factor of fibrosis in the kidneys is TGF- β . As a growth factor, TGF- β , is implicated to be involved in both the early and later stages of diabetic neuropathy and ECM accumulation.^{53, 54} Although the effects of diabetes in the renal, cardio, and liver pathways have been studied, how the urinary pathway and its collagen deposits are affected are still unclear.

The goals of our study were to evaluate: (i) role of TGF β WNT signaling pathway for fibrosis in the genital-urinal and pelvic disorders, (ii) role of the atrogen pathway for

aging in similar tissues, (iii) role TGF β /WNT pathway plays in diabetes in relation to fibrosis, and (iv) role girdin plays in controlling the fibrotic pathways and whether it's reduction or elimination could potentially provide therapy for fibrotic diseases.

Materials and Methods

Aging Study: Adult New Zealand white female rabbits (n=12; 6 young-6M, 6 old-36M) were anesthetized with an intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg). Animals were sacrificed at the end of the study and the anal canal was harvested to perform Western blots to quantify relevant proteins.

Injury Study: Rabbit - Adult New Zealand white female rabbits (n=15; 4 - 5 kg) were anesthetized with an intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg). Of these, twelve rabbits were subjected to EAS myotomy and three were subjected to sham surgery (uninjured controls). The control animals were maintained and sacrificed at the end of the study (15 weeks) and EAS tissue was used for molecular analysis. Mice - We used adult wild-type male C57BL/6J (WT) (n=6) for our study. Of these, two were old (3 years), two were subject to penile myotomy, and two were subjected to sham surgery (young controls). The control animals were maintained and sacrificed at the end of the study (15 weeks) and penile tissue was used for molecular analysis.

Diabetic Study: We used adult wild-type male C57BL/6J (WT) and age-matched T2DM, CAV-1 KO, CAV-3 KO mice in this study. To create the T2DM mouse model, 3 month old WT male mice were injected with a single dose of streptozotocin (STZ; 75mg/kg in 0.1 M citrate buffer, pH=4.5, i.p., non-fasted) and then switched to a high fat diet (HFD; 60% kcal of total calories). Control mice received injections of 0.1 M citrate buffer and were maintained on standard chow (4% of calories from fat). Two months after streptozotocin injection, serum glucose and glucose tolerance tests (GTT) were performed to confirm metabolic disorder. For serum analysis in non-fasted mice, blood

was collected via the retro-orbital route for glucose and insulin measurements. Glucose tolerance tests (GTT) were performed in a subset of fasted mice (12-14 weeks after high fat/normal diet). For GTT, mice were fasted for 11-12 hrs, fasting glucose levels were assessed via tail snips followed by i.p injection of 1 g/kg glucose in 0.9% NaCl and monitoring of blood glucose [every 30 min via tail vein for 3 hrs]. Insulin ELISA kit was used to measure plasma insulin levels. Animals were maintained on HFD for 4 months and euthanized to harvest penile tissues.

Western Blot: To quantify levels of fibrogenic markers, samples were first prepared on ice with non-reducing tris-glycine SDS sample buffer (Novex) and heated on a heat block at 95 degrees C for 10 minutes. When reducing conditions were required, -mercaptoethanol (5% v/v) was added to the sample buffer. Denatured samples (10 g) were loaded onto 4–20% NuPAGE tris-glycine SDS polyacrylamide gels and subjected to electrophoresis in tris-glycine running buffer in the Xcell II Minicell, according to the manufacturer's instructions (Novex Australia Pty Ltd, French's Forest, NSW, Australia). Protein standards were included in each gel: 5 L Precision Plus Dual Color standards. Proteins were transferred to PVDF (Biorad Immunoblot PVDF membrane) overnight at 25 V.

Western blots were probed for various protein markers as follows, all steps being carried out at room temperature, with gentle shaking. After 60 min in blocking buffer (5% Diploma skim milk in PBS), filters were incubated overnight with the following optimal dilutions of primary antibodies in blocking buffer: 1 in 1500 for rabbit anti-human TNF- α , 1 in 150 for rabbit anti-human IL-1 β , and 1 in 1000 for rabbit anti-human IL-1 α (all Genzyme). After thorough washing in PBS containing 1% Tween 20 (wash buffer),

filters were incubated for 2 h in a 1 in 3000 dilution of the appropriate second antibody, horseradish peroxidase (HRP)-conjugated donkey anti-rabbit, rabbit anti-mouse IgG (H + L; Jackson Immunosearch, West Grove, PA, USA) in wash buffer. Blots were developed by the enzyme chemiluminescence (ECL) method according to the manufacturer's instructions.(Amersham)

Results

Aging Related Studies: To determine the effects of aging in the pelvic and genital-urinary tissues, we examined the protein expression of the various fibrotic and atrogen pathways for young and old subjects in the rabbit and human model.

External Anal Sphincter: We quantified the age-related change in fibrogenic proteins by Western blot for the external anal tissue for young and old rabbits (Figure 3A-B). The changes between young and old for each marker were all significant with Collagen-I ($p=0.004$), TGF-Beta ($p=0.0104$), and β -catenin ($p<0.0001$).

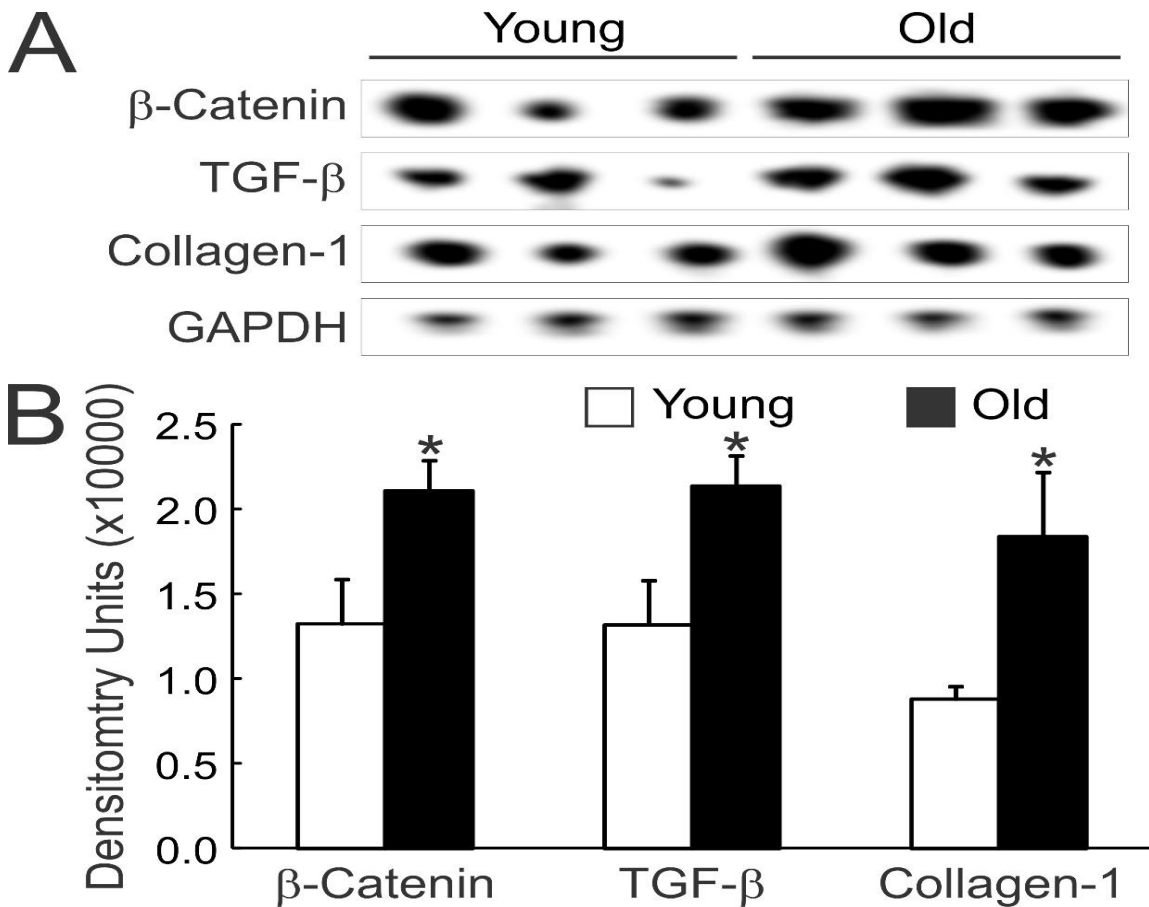


Figure 1: A. Western Blot of rabbit external anal tissue for young and old animals probed for B-catenin, TGF-B, and Collagen. B. Densitometry levels for the probed fibrogenic markers. All three markers depict a significance between young and old animals.

Mid Urethra & Bladder Neck: We quantified the age-related change in fibrogenic proteins by Western blot for the mid urethra and bladder neck tissues for young, middle-aged, and old rabbits (Figure 4 A-B). The changes between young and old for each marker were all significant with TGF-Beta ($p=0.0104$), and β -catenin ($p<0.0001$).

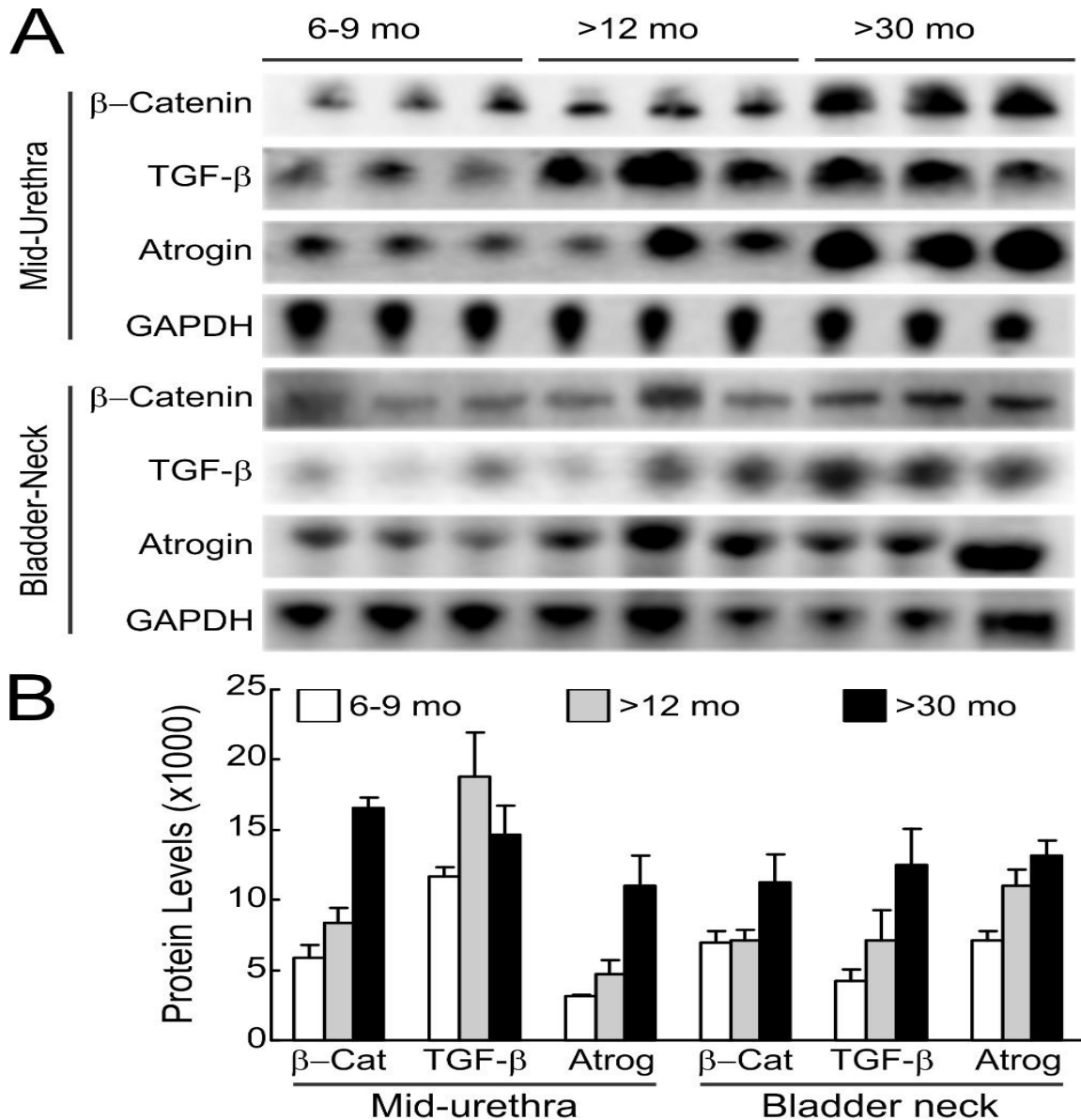


Figure 2: A. Western Blot of Mid-Urethra and Bladder-Neck for young, middle-aged, and old animals probed for B-catenin, TGF-B, and Atrogin. B. Densitometry levels for the probed fibrogenic markers.

Human Penile: We quantified the age-related change in fibrogenic proteins by Western blot for the human penile cavernosal tissues for subjects younger and older than 65 years. The changes between young and old for each marker all showed an increase for GIV, Stat3, Smad1,2,3, TGFB, and CTGF as shown in Figure 5.

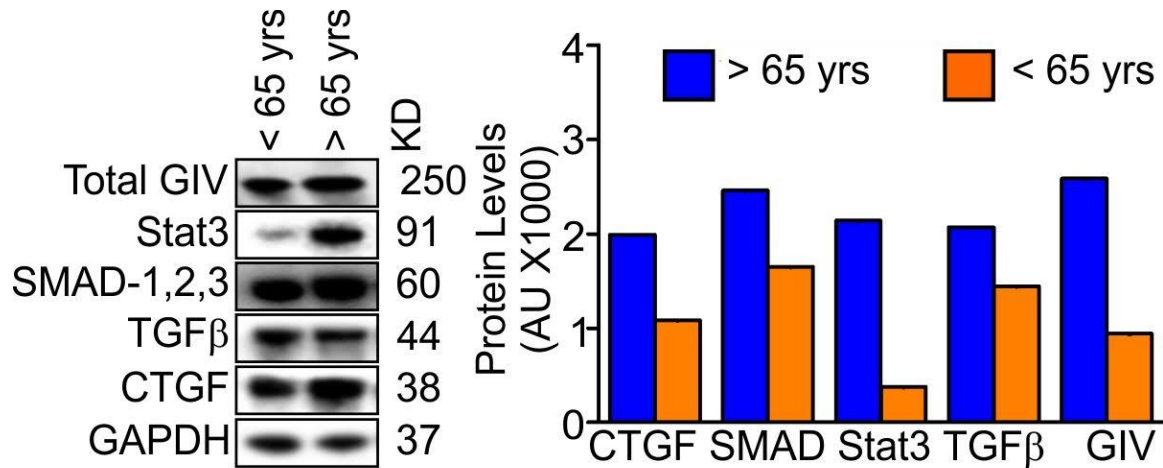


Figure 3: Left: Western blot analysis for young and old subjects probed for Total Giv, Stat3, Smad 1,2,3, TGFB, and CTGF. Right: Densitometry levels for the probed fibrogenic markers.

Injury Study: To determine the effects of injury on the fibrotic pathways, we examined the protein expression in the external anal sphincter in control and injured subjects of the rabbit model.

Rabbit EAS: Figure 6 A-B show representative images depicting injury related changes in protein levels of important fibrogenic proteins, β -catenin, collagen-I and TGF- β . After EAS myotomy there was significant increase in all three fibrogenic proteins (Figure 6B).

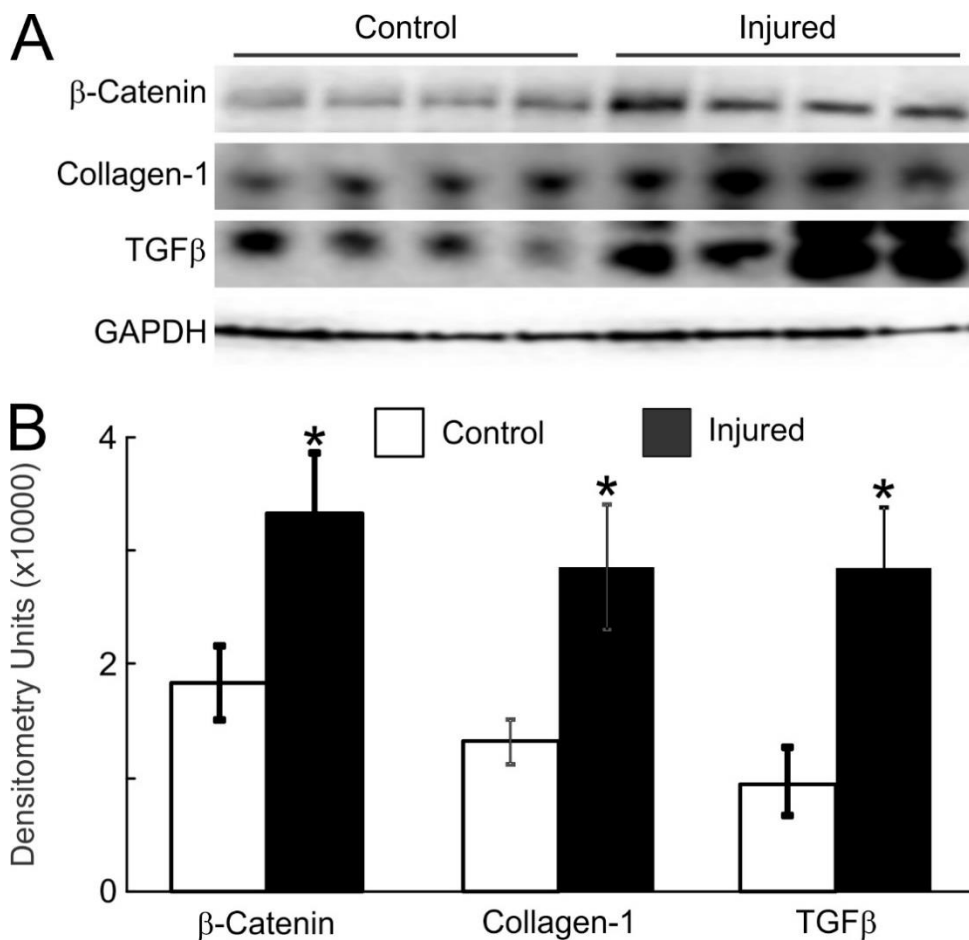


Figure 4: Rabbit EAS tissue protein levels estimated by Western blot (a) followed by (b) densitometry and of β -catenin collagen-I, and TGF- β from control and animals subjected to EAS myotomy estimated at 15 weeks post-myotomy (3-4 from each group)

Diabetic Study: To determine the role for caveolins in diabetes and how diabetes may lead to increasing collagen deposits, western blot analysis in diabetic and control mice penile tissues were probed for specific markers.

Mice Penile: Western blot analysis performed in penile mice tissue showed a significant decrease in both Cav-1 and Cav-3 levels in the T2DM mice (Figure 7 A,B). In addition, there was a significant increase in β -CAT, GSK3, SMAD3, and TGF- β levels in the T2DM mice (Figure 7 C,D).

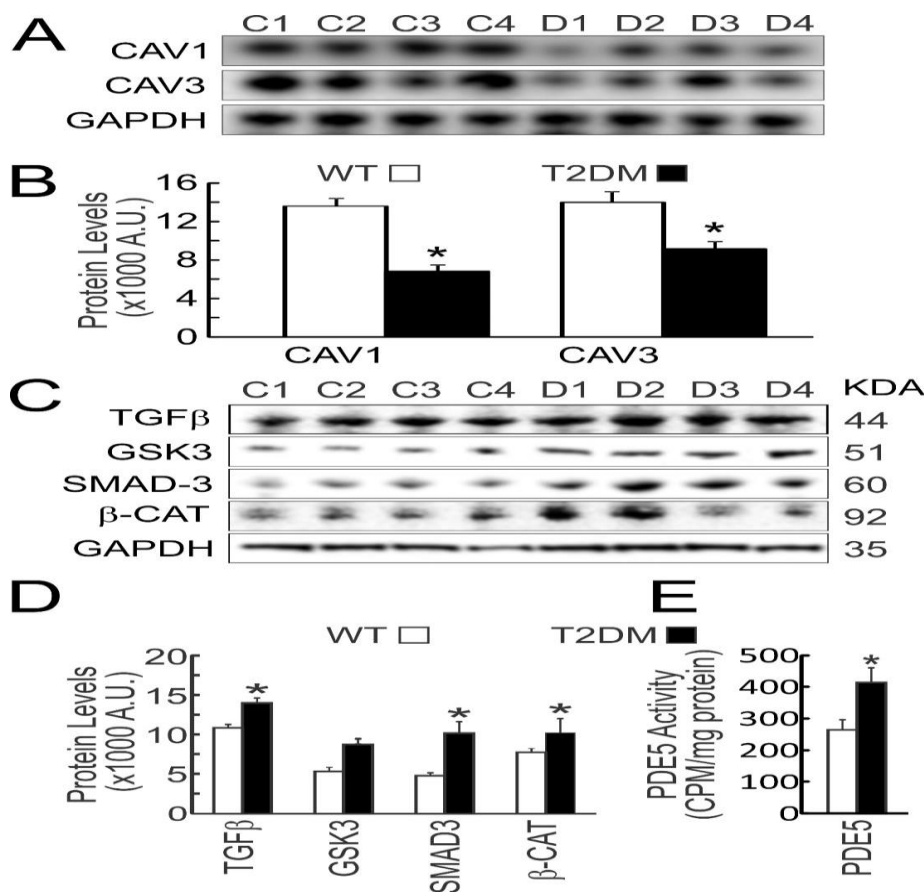


Figure 5: (A-B): Western blot analysis of Cav 1,3 shows a significant decrease in protein levels for both Cav-1 and Cav-3 in T2DM (D1-D4) mice compared to WT-control mice (C1-C4). (C-D): Western blot analysis also shows a significant increase in β -Catenin, GSK3, Snad 3, and TGF- β in control mice compared to diabetic mice.

Girdin Study

Rabbit External Anal Sphincter: Western blot was performed for old, young (injured), and young rabbit EAS tissues and probed for Total Girdin, Collagen, Stat-3, β -cat, Smad 1,2,3, TGF- β , and CTGF. Our results show increased protein levels of all the fibrosis markers in EAS muscles of old animals as well as in young animals after injury. (Figure 8) Furthermore, increase in expression for Girdin is accompanied by increase in expression of the Wnt/ β -Cat pathway markers. This supports our hypothesis that EAS muscle fibrosis after injury and during aging likely involve common profibrogenic cascade that include Giv/Girdin-STAT3- Wnt signaling pathways.

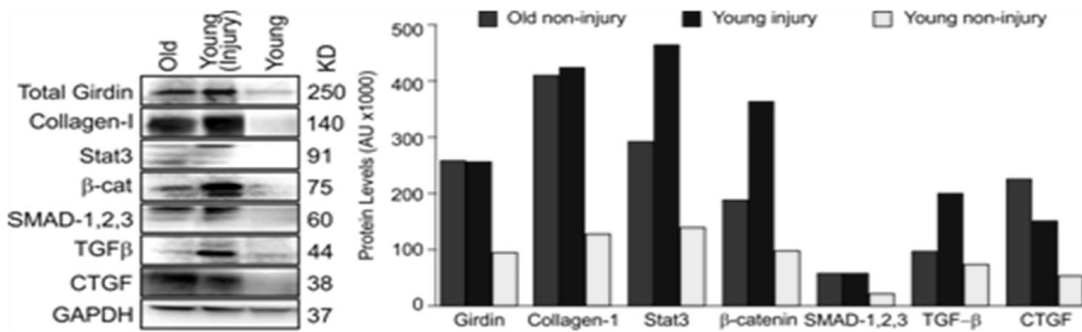


Figure 6: Western blot analysis of Total Girdin, Collagen-1, Stat3, B-catenin, Smad1,2,3, TGF β , and CTGF shows an increase in both old and young (injured) animals compared to young noninjured.

Discussion

Aging Study

The results of this study confirm previous reports that with advanced age, there is upregulation of major fibrogenic signaling, i.e. Wnt- β catenin and TGF- β pathways in the EAS muscle. Wnt/ β -catenin signaling is activated as a consequence of injury as well as during normal aging and is reported to play a major role in several types of injuries induced fibrosis in several organ system, i.e., ischemia induced myocardial fibrosis, idiopathic and bleomycin induced pulmonary fibrosis, fibrosis seen in chronic kidney failure, liver fibrosis and abnormal skin wound healing^{42, 55}. In all of the above examples Wnts and positive regulators of β -catenin signaling are up-regulated and inhibitors of Wnt/ β -catenin signaling are down-regulated. Antagonists of Wnt have been found to reduce fibrosis in the above organ systems. Akhmetshina et al (2012) show that canonical Wnt signaling is critical for TGF- β -mediated fibrosis and implicate a key role for the interaction of both pathways in the pathogenesis of fibrotic diseases. Findings from our study that age- related EAS muscle fibrosis involves both Wnt and TGF- β -mediated signaling pathways support these observations. Targeted anti-fibrotic therapy has been shown to reduce fibrosis and improve organ function^{56, 57}.

However, our study is the first one to show the role of Wnt signaling in the aging EAS muscle, and should stimulate other studies whether similar mechanisms are operative in injuries to the anal sphincter and pelvic floor muscle. If proven to be correct, our findings have tremendous clinical implications, i.e., Wnt antagonist may be useful in preventing EAS and other pelvic floor muscle dysfunction following surgical and obstetrical injuries, which are extremely common and have devastating consequences. A

number of Wnt antagonists are available for systemic, oral and topical use⁵⁸, and merit clinical trials to prevent trauma (surgical, sports and obstetrical) induced anal sphincter muscle dysfunction. We propose that Wnt antagonists are novel potential therapeutic agents for the prevention of anal sphincter dysfunction resulting from various types of anal sphincter injuries. In summary, our results suggest noteworthy details between aging and various muscle parameters including muscle thickness, anal canal pressure/tension, muscle to connective tissue ratio, and expression of fibrotic markers which have all implied muscle weakness related to old age compared to young age.

Injury Study

Our studies show that following EAS myotomy, there is upregulation of Wnt- β catenin and TGF- β signaling pathways. We evaluated EAS muscle function by determining changes in fibrogenic markers. Overall, our results reveal involvement of Wnt- β catenin signaling pathways in mediating surgical myotomy induced fibrosis and EAS muscle dysfunction. After injury, skeletal muscles such as EAS regenerate through a series of well-coordinated events⁵⁹. Regeneration starts with activation of quiescent satellite cells that reside in the muscle. The later leads to formation of new muscle fibers, which go through proliferation and differentiation⁶⁰. Major events of the healing processes are: **1)** an initial inflammatory reaction & invasion of macrophages, **2)** activation, differentiation & fusion of satellite cells & **3)** maturation of newly formed myofibers and remodeling of regenerated muscle. There are several important signaling pathways that regulate muscle repair and among them Notch and canonical Wnt signaling⁶¹⁻⁶³ are thought to be critical. Notch pathway is believed to be involved in the myoblast proliferation and Wnt signaling modulates differentiation process. Canonical Wnt

signaling cascade involves soluble Wnt ligands interaction with Frizzled receptors and low-density lipoprotein receptor-related protein co-receptors (LRP). The later stimulates phosphorylation of disheveled (a cytosolic phosphoprotein) protein that inactivates glycogen synthase kinase 3 β (GS3K β) phosphorylation of β catenin. With the assistance of axin, the de-phosphorylated and stable β - catenin does not undergo ubiquitination and degradation; instead it is translocated to the nucleus where it binds to TCF/LEF1 transcription factors^{64-66, 65}.

Injury to the EAS muscle in humans may result from several possible etiologies, 1) surgical trauma, i.e., following operations in this area for anal fissure and hemorrhoids, 2) accidental and sports injury (e.g. goring during bull fight) and 3) most commonly from obstetrical trauma. The latter is extremely common and can be related to excessive stretch during spontaneous passage of fetus through the birth canal, use of forceps and surgical episiotomy. The disruption of anal sphincter muscles at the time of vaginal delivery may be obvious or occult (OASIS). One can argue that our model of muscle injury, i.e., surgical EAS myotomy may not be completely representative of what happens during various types of injuries. However, our study is the first one to show the role of Wnt signaling in the EAS muscle injury and repair, and should stimulate other studies whether similar mechanisms are operative in other types of injuries to the anal sphincter and pelvic floor muscle. If proven to be correct, our findings have tremendous clinical implications, i.e., Wnt antagonist may be useful in preventing EAS and other pelvic floor muscle dysfunction following surgical and obstetrical injuries, which are extremely common and have devastating consequences. A number of Wnt antagonists are available for systemic, oral and topical use⁵⁸, and merit clinical trials to prevent trauma

(surgical, sports and obstetrical) induced anal sphincter muscle dysfunction. We propose that Wnt antagonists are novel potential therapeutic agents for the prevention of anal sphincter dysfunction resulting from various types of anal sphincter injuries.

Diabetic Study

The goals of our study were to understand the molecular mechanisms of T2DM related changes to male erectile tissue and to evaluate the role of fibrosis. We evaluated caveolae, Cav-1, Cav-3, and fibrosis levels in the T2DM mouse penile tissue. Overall, we found: 1) significantly decreased caveolae, Cav-1 and Cav-3 levels in T2DM mice and 2) increased fibrosis in T2DM mice. In conclusion, our results suggest a regulatory role for caveolins in penile hemodynamics of endothelial dysfunction and the possibility of diabetes compounding the issues already faced by those with aged-related fibrotic disorders.

Girdin Study

Our preliminary studies into the Girdin and Wnt- β -cat pathway indicate a strong likelihood that the two pathways interact in a profibrogenic fashion. To further expand on this study, we are currently performing interventional studies with GIV/Girdin siRNA through an atelocollagen transfection reagent injected immediately after injury to the EAS. By silencing GIV through siRNA oligos, we hope to intervene in the fibrotic cascade through the attenuation of collagen deposition and improving the EAS muscle function. We believe that excessive muscle fibrosis causes muscle dysfunction, which can be the basis for future pharmacological interventional studies. We also believe that our studies have important therapeutic implications for the prevention (injection of a Wnt

antagonist as a possible prophylactic measure) and treatment strategies for patients with symptoms of anal incontinence. It is very possible that the Wnt/STAT3/Girdin inhibition after EAS injury may become an important therapeutic strategy to gain anal continence function.

Sphincter and pelvic floor muscles are prone to injury during childbirth and thus strongly believe that a study on the role of Wnt signaling in the spontaneous repair and regeneration of the sphincter muscle following surgical injury is urgently warranted because it has strong clinical implications for women health. Furthermore, with increase in the aging population in the USA the role of Wnt/STAT3/Girdin signaling in the age related changes in the external anal sphincter and pelvic floor muscle requires immediate attention.

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