INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

ROLE OF OVARIAN HORMONES IN GERIATRIC BLADDER DYSFUNCTION

By
Qing Zhu

Supervised by
Dr. George A. Kuchel

A thesis submitted to the Faculty of Graduate studies and Research
in partial fulfillment of the requirement for the degree of Master of Science

Department of Experimental Medicine
Faculty of Medicine
McGill University
Montreal, Quebec, Canada
October, 2000

ABSTRACT

Background: Although Detrusor Hyperactivity (DH) with Impaired Contractility (IC) is a common urodynamic finding in elderly subjects, its pathogenesis remains unknown. Human detrusor biopsy studies indicate that subjects with DHIC exhibit ultrastructural evidence of both the dysjunction and degeneration patterns present in isolated DH and IC, respectively. Based on the known cellular effects of estrogen, we proposed a hypothesis that declines in ovarian hormone production could contribute to the pathogenesis of DHIC in elderly women.

Methods: In this thesis project, mature 3-14 month old female F-344 rats were studied 4 months after bilateral ovariectomy (OVx) or sham surgery. Detrusor structure was evaluated at this time point using light and electron microscopy, while classical muscle strip studies were used to measure the impact of OVx on detrusor muscle contractility. In an effort to identify estrogen-regulated proteins in the mammalian detrusor, known candidate proteins were screened using Western blotting, while identification of novel proteins was undertaken through proteomics, with two-dimensional gel protein resolution followed by microsequencing.

Findings: We observed that following OVx, detrusor smooth muscle decreased by 25%, with a 12% decrease in numbers of nucleated muscle profiles and degenerative changes observed in many axons. Muscle strips from OVx animals generated 40-50% less tension per strip or 10-20% less tension per mg wet weight in response to carbachol than did equally sized strips from sham-operated animals, with no apparent change in muscarinic receptor affinity.

We also observed that numbers of caveolae and levels of caveolin-1 protein, both markers of maturation in detrusor myocytes, are similarly down-regulated following OVx. Proteomics screening identified a string of acidic proteins (~ 110 Kd) variably charged which are down-regulated by OVx and up-regulated with 17β-estradiol replacement. Initial characterization suggests that this protein, termed DERP (Detrusor Estrogen-Regulated Protein) may be a member of the inter-alpha-trypsin inhibitor family, possibly undergoing differential post-translational modification through sialination.

Conclusions: OVx rodents exhibited many of the changes of the degenerative ultrastructural pattern, but none of the characteristic features of the dysjunction pattern. The mature rodent detrusor and its innervation both appear to be sensitive to prolonged ovarian hormonal deficiency, which could contribute to the development of IC in late life. It remains to be seen whether any of these changes or IC itself can be reversed or prevented with estrogen replacement therapy. Moreover, a full characterization of DERP may allow for the development of novel interventional strategies in geriatric bladder dysfunction.

ABRÉGÉ

Antécédents : Une hyperactivité du muscle de la parois de la vessie associée à une diminution de sa contractilité (HMDC; ou DHIC en anglais) est un phénomène urodynamique fréquement observé chez les personnes âgées incontinentes. Toutefois, la pathogénèse de l'HMDC demeure encore inconnue. L'analyse des biopsies provenant de vessies de sujets atteints d'HMDC montre, une évidence de disjonction et de dégénérescence ultrastructurale, respectivement, au niveau de l'hyperactivité et de la diminution de la contractilité du muscle de la parois de la vessie. En nous basant sur les connaissances déja acquises des effets d'une hormone ovarienne, l'oestrogène, nous proposons comme hypothèse de recherche que les périodes de déclin ou de carence de production et de sécrétion de cette hormone pourraient induire la pathogénèse de l'HMDC chez les femmes âgées.

Méthode : Nous avons étudié des rats adultes (F-344) âgées de 13 - 14 mois. Quatre mois auparavant, ces rats avaient subi une ovariectomie bilatérale (OVx) ou une chirurgie témoin. Nous avons ensuite procédé à l'évaluation de la structure de la parois de la vessie en microscope optique et électronique, ainsi que mesuré l'impact de l'OVx sur la contractilité de la parois vésicale en utilisant une technique classique d'échantillonnage de bandes musculaires. Dans le but d'identifier des protéines responsables de l'effet de l'oestrogène dans la parois de la vessie chez le rat, nous avons procédé au criblage des protéines candidates connues en utilisant la technique de transfert de type western (Western Blot). Nous avons également procédé à l'analyse et à l'identification de protéines nouvelles par recherche protéomique en utilisant l'électrophorèse bidimensionnelle (électrophorèse sur gel) suivi par le microséquençage.

Constatations : Nous avons observé qu'à la suite d'une OVx, il y a une diminution de 25% des faisseaux musculaires, une diminution de 12% du nombre de profils nucléés dans le muscle lisse, ainsi qu'une présence d'axones en dégérescence dans la parois de la vessie. En réponse au carbachol, les bandes musculaires, composées de plusieurs fibres musculaires, provenant de rats OVx ont généré (par bande) une contraction musculaire

diminuée de 40 à 50 % (ou diminuée de 10 à 20 %/mg de poids humide) comparativement aux bandes d'égale grandeur provenant des rats ayant subi une chirurgie témoin, le tout, sans changement apparent au niveau de l'affinité du récepteur muscarinique.

À la suite d'une OVx, nous avons également observé une régulation une diminution du nombre de vésicules « *caveolae* » et de protéine « *caveolin-1* » -- deux marqueurs de maturation de la fibre musculaire de la parois de la vessie. Les analyses protéomiques ont permis l'identification d'une chaîne de protéines acides (~ 110 kDa) de charge variable qui est diminuée à la suite d'une OVx, mais augmentée avec une hormonothérapie substitutive (17β-estradiol). La caractérisation initiale laisse suggérer que cette protéine, appelée protéine de régulation d'estradiol du détrusor (DERP), puisse faire partie de la famille des inhibiteurs inter-alpha-trypsine, subissant probablement une modification post-traduction différentielle par «sialination».

Conclusion : Contrairement aux sujets humains souffrants de l'HMDC, les rats OVx présentent plusieurs évidences de dégénérescence ultrastrurale, mais ne présente aucune modification des caractéristiques de disjonction. Chez le rat adult, une carence prolongée en oestrogènes qui, vraisemblablement semble affecter la parois de la vessie et son système d'innervation, conduirait éventuellement mais tardivement à une diminution de la contractilité musculaie. Toutefois, il reste à prouver si l'une de ces modifications ou si la diminution de la contractilité elle-même peut être réversible ou si l'hormonothérapie substitutive (17β-estradiol) peut la prévenir. La caractérisation complète de la DERP pourrait permettre l'élaboration de nouvelles stratégies d'intervention en gériatrie dans les cas de dysfonction vésicale.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

First, my great thanks go to my supervisor, **Dr. George A. Kuchel**, for being a wonderful mentor, for all his guidance, enthusiasm, inspiration, support, and patience.

I appreciate the valuable advice and help from **Dr. Marilyn M. Miller**.

I'm grateful to my fellow student **Jeff Ritchie** for his precious friendship, for his great companion, for helping out whenever he could, especially with his computer magic.

My friend Dr. **Dawei Huang**'s technical help made a big difference on my protein work.

My appreciations go to **Dr. Michael Ferns** for his insightful advice, to **Ms. Jacinthe Gingras** for the French translation of the abstract, and to other people in Dr. Ferns' lab for their help.

I would like to thank **Dr. Ahmad Elbadawi** for his expertise on electron microscopy; **Ms Jodell Sherman-Tamber** for processing tissue samples for electron microscopy and printing of electron photomicrographs; **Dr. Stephane Dion** and **Ms. Nadjat Marouf** for the major work on the muscle strip studies.

Further thanks go to the other members of my thesis committee: **Dr. Danuta Radzioch and Dr. Hugh Bennett** for their advice and encouragement.

I also would like to thank Dr. Peter J. Roughley and Dr. Andrés Pineiro for generously providing us the Lumican and ITIH4 antibodies.

My special thanks go to my families: my husband **Yang Cao** for his love through all these years, for his being there; my daughter **Henan Cao** for bringing sweet music to my life, and for her being understanding; my families in China for being my strong backing.

LITERATURE REVIEW

Urinary incontinence (**UI**) refers to an involuntary leakage of urine. It is a major cause of lost independence and disability in old age, particularly among elderly women. As a result, it has a considerable impact on the well being of a growing and a predominantly female aging population[1]. Nearly one-third of older individuals living in the community and more than one-half of nursing home residents are incontinent. Among the latter group, 80% are women. UI has far-reaching medical and psychological implications for the affected individual. Incontinent patients are at risk for falls, fractures, pressure ulcers, urinary infections, urosepsis, as well as embarrassment, stigmatization, isolation, depression, and even institutionalization[1,2]. In addition to affecting individuals' quality of life, UI is a major source of financial expenditure. The annual expenses of caring for all individuals suffering from UI in the US have been estimated at over $30 billion dollars, an amount greater than the combined annual expenditures for dialysis and coronary artery bypass surgery.

Nevertheless, surprisingly little is known about the pathogenesis of this problem, and the management of patients suffering from UI has been largely palliative. Fortunately, several recent seminal studies have allowed us to begin developing, for the first time, a pathophysiologic understanding of bladder dysfunction in aging.

1. Urodynamic Findings In Elderly Incontinent Subjects

The urinary bladder is composed of two anatomically and functionally distinct units: the detrusor (bladder body) and the bladder outlet. During the storage phase of micturition, the detrusor expands to store urine, while the outlet contracts to prevent urine outflow. In the voiding phase, the detrusor contracts and the outlet relaxes in order to allow for voiding to begin[3].

Resnick *et al.*[2] conducted detailed urodynamic studies in 94 elderly incontinent nursing home residents (77 women and 17 men). Among these subjects, two-thirds demonstrated evidence of detrusor hyperactivity (**DH**; the detrusor contracts involuntarily). In a similar

proportion there was evidence of impaired detrusor contractility (**IC**, the detrusor failed to contract as strongly as it should). Overall, one-third had evidence of both DH and IC, a combination of findings termed Detrusor Hyperactivity with Impaired Contractility (**DHIC**; the bladder is found to be both "unstable" and weak). Finally, some subjects (mainly men) showed evidence of bladder outlet obstruction (**BOO**, the urethra resistance is abnormally high)[2,1].

Although DHIC represents the most common constellation of urodynamic findings in this population, its pathophysiology remains unknown. Moreover, this important problem tends to be refractory to current medical therapy. In fact, antispasmodics used for DH can worsen detrusor contractility, putting the patient at risk of urinary retention resulting in possible urinary tract infection, septicemia and hydronephrosis, potentially leading to renal failure[1,2].

2. Ultrastructural Findings in Elderly Individuals with Normal and Abnormal Urodynamic Studies

Following urodynamic studies, detrusor biopsies were performed in the same group of elderly subjects. Ultrastructural features of smooth muscle cells, intrinsic nerves and the interstitium were carefully examined in large numbers of electron microscopy (**EM**) images. These systematic studies have provided the first insights into a striking and reproducible correlation between specific ultrastructural patterns and categories of urodynamic findings (Table 1) [4,5,6].

The dense band pattern was found to represent the structural norm of the aging detrusor. It is characterized by an overall normal configuration of muscle cells and cell junctions. Nevertheless, there is evidence of a slight widening of intercellular space between muscle cells. In addition, the sarcolemma becomes dominated by dense bands which appear to replace caveolae which normally present in interposed zones[5]. Since caveolae are thought to play a role in calcium signaling and muscle contraction, it was proposed that depletion of caveolae might result in or herald a decrease in myocyte contractility[5].

Table 1. E.M. Ultrastructural Patterns in Specific Urodynamic Categories

Urodynamic Category	EM Pattern	Ultrastructural Features
'Normal' Aging	Dense Band	Caveolar Depletion Dominant electron dense bands
Detrusor Hyperactivity (DH)	Dysjunction	Fewer adherens junctions Appearence of 'protrusion junctions'
Impaired Contractility (IC)	Degenerative	Axonal degeneration Myocyte degeneration
Detrusor Hyperactivity with Impaired Contractility DHIC)	Dysjunction and Degenerative	Features of both dysjunction and degenerative patterns
Bladder Outlet Obstruction (BOO)	Myohypertrophy	Enlarged myocytes Collagenosis

All subjects with detrusor hyperactivity (DH) on urodynamic studies, and none with normal detrusor, exhibited features of the **dysjunction pattern.** The EM features of this pattern include moderately widened intercellular spaces, scarce intermediate (adherens) junctions which typically dominate the normal detrusor, and the *de novo* appearance of abundant '**protrusion junctions**'[4,7]. Protrusion junctions are long, slender, finger-like processes extending from one muscle cell toward a neighboring cell. In detrusor specimens obtained from individuals with documented DH, as many as 4-8 myocytes may be joined by these junctions in a chain-like arrangement. Interestingly, these junctions have not been described previously. Nevertheless, their contact zones show some resemblance to gap junctions in that they lack sarcolemmal specialization and basal lamina, while having an extremely narrow gap separating inner leaflets of apposed sarcolemmae. As a result of these features, it has been proposed that protrusion junctions may represent aberrant gap junctions. Moreover, the fact that their appearance coincides with a decrease in adherens junctions suggests that these changes may reflect a shift from predominantly mechanical coupling between detrusor myocytes in the normal bladder to

mainly electrical coupling in the setting of DH. As a result, heightened activity of individual smooth muscle cells could lead to an involuntary contraction of the entire detrusor[1,4].

The degenerative pattern was observed only in individuals with impaired contractility (IC), while subjects with DHIC demonstrated features of both the dysjunction and the degenerative patterns. In addition to the features of the dense band pattern, the distinctive characteristics of the degenerative pattern include widespread degenerative changes involving muscle cells as well as axons, with no evidence of regeneration[5].

Finally, the **myohypertrophy pattern** was present in individuals, mainly men, suffering from bladder outlet obstruction (BOO). Its typical features include hypertrophic myocytes, a reduction in adherens junctions, as well as a widened interstitium with an abundant deposition of collagen and elastic fibers[6].

3. Bladder Innervation

Normal bladder performance is responsible for two main functions: the storage of urine, followed by bladder emptying occurring at the appropriate time and place. Although seemingly simple, these normal functions require the precise coordination of both the detrusor and the outlet, mediated predominantly through parasympathetic and sensory fibers. It is important to note that the detrusor and outlet have different, yet collaborating functions. Therefore, it is not surprising that they each receive an innervation that is somewhat distinct[8,9].

The activation of parasympathetic pathways generally promotes bladder voiding. Briefly, central facilitation of the sacral micturition reflex activates parasympathetic efferents, and the release of acetylcholine (**Ach**) contracts the detrusor and shortens the outlet, resulting in micturition[8]. There is a high concentration of sympathetic fibers innervating the bladder outlet, but only a minimal sympathetic innervation to the detrusor. Sympathetic activation promotes outlet contraction through α-adrenoceptors, promoting urine storage

and preventing leakage[10]. Sensory pathways provide information relevant for micturition, and they may also convey nociceptive information [3].

The parasympathetic preganglionic neurons residing in the brain and the sacral spinal cord (**SPN**) mediate parasympathetic outflow to the bladder. Cholinergic preganglionic neurons exit the spinal cord in the ventral spinal nerves to form the pelvic nerve, which then synapses on cholinergic postganglionic neurons in the major pelvic ganglia (**MPG**) [3] residing in close proximity to the bladder. Preganglionic fibers may also synapse on intramural ganglia within the bladder[11]. The segmental distribution of dorsal root ganglia (**DRGs**) innervating the bladder detrusor and outlet are similar, with a slight predominance of the detrusor innervation by upper lumbar DRGs and of outlet innervation by lumbar sacral DRGs. In the rat, **L6 DRGs** accounts for the majority of the afferent innervation to the bladder detrusor and outlet[3]. Table 2 summarizes autonomic and sensory innervation of bladder.

Table 2. Bladder Innervations

NEURONS	TYPE	CELL BODY	INNERVATION	
			Detrusor	Outlet
Parasympathetic	Efferent	MPG	++++	++
Sympathetic	Effer-/affer-	SC, IMG, MPG	+(β)	+++(α)
Sensory	Afferent	DRG(L6-S1)	+++	+++

Autonomic nerve fibers and nerve terminals form a dense plexus among detrusor smooth muscle cells. Most of these nerves are excitatory cholinergic type[12]. EM studies of mammalian bladders have revealed the presence of a modest number of axons in all specimens. A variety of axon terminal profiles were observed. Visible terminals included terminals which were bare and others which were either partially or completely ensheathed with Schwann's cell[5]. Cholinergic nerve terminals (with small clear synaptic vesicles) were the most frequently observed type of nerve terminal, while adrenergic

axons (with small dense core synaptic vesicles) were rarely observed, particularly in the human detrusor[5,7,13]. Neurochemical studies of rat bladders by Johnson *et al.*[14] have demonstrated the presence of a 10-fold higher norepinephrine concentration and tyrosine hydroxylase (**TH**) activity in the bladder outlet as compared to the detrusor. These findings, combined with a highly dense noradrenergic innervation at the bladder outlet, all suggest a major sympathetic role in the tonic constriction of the outlet during bladder filling. Although sympathetic innervation to the detrusor is limited, a number of studies suggest that β-adrenoceptor-mediated relaxation of the detrusor may also facilitate urine storage[8].

In other studies, a variety of cholinergic parameters, including Ach concentration and acetyltransferase (**ChAT**) activity were found to be 50% higher in the outlet as compared to the detrusor. It has been proposed that parasympathetic activity in the outlet is also important in the process of voiding[14]. Muscarinic stimulation of the outlet has been shown to facilitate voiding by inducing the contraction of longitudinal, rather than circular muscle, resulting in shortening of the outlet, and increased diameter of the lumen[15].

Although a number of investigators have examined aging-associated changes in the mammalian bladder using established animal models, these findings have not been entirely consistent. For example, a number of cholinergic and sympathetic biochemical markers remain stable with advanced age[14]. In contrast, semi-quantitative histochemical studies have revealed decreases in the sympathetic innervation to the aged rat bladder, with no significant changes in CGRP-positive sensory fibers[16]. Muscle strip studies have generally shown unchanged or increased contraction with aging by strips obtained from the detrusor and the bladder outlet, in response to muscarinic agonists and by α-adrenergic agonists respectively[17,18].

4. Contractile mechanisms in bladder smooth muscle

4.1 Transmitters and receptors

In mammals both Ach and ATP have been proposed as excitatory neurotransmitters for the activation of detrusor smooth muscle cells, through muscarinic Ach receptor (**mAchR**) and purinergic receptors, respectively. Ach, acting through smooth muscle cell mAchR, is the primary neurotransmitter controlling bladder voiding. The density of mAchR on the surface of bladder smooth muscle cells and the responsiveness of these cells to Ach stimulation are highest in the dome and lowest in the base[19]. The major subtypes of mAchRs in the bladder have been shown to be M_2 and M_3 receptors on the basis of M_2 and M_3 mRNA expression in porcine, rat and human bladders[20,20,20,21]. Wall *et al.* have utilized specific antibodies to demonstrate that M_2 receptors are the predominant mAchR (86%) in the rat bladder, while M_3 receptor expression is much lower (14%), with M_1 essentially undetectable[22]. Among adrenergic receptors, β-adrenoceptor predominates over α-adrenoceptors in the detrusor[23].

During the voiding phase, Ach release from the parasympathetic pelvic nerve endings results in a sustained contraction of the bladder. It was proposed that the M_2 receptor exerts a modulatory action on β–adrenoceptor relaxant responses by inhibiting adenylyl cyclase activation[24]. Although M_3 receptor levels are lower than those of M_2, studies indicate that M_3 is the mediator of the direct contractile effect of acetylcholine in detrusor smooth muscle, acting through IP3/Ca^{2+} signaling [24-25]. In fact, co-immunoprecipitation studies suggest that M_2 and M_3 receptors couple to members of the Gi and Gq/11 families, respectively, resulting in adenylyl cyclase inhibition and phosphoinositide hydrolysis[26]. Thus, during bladder voiding, M_3 receptor stimulation could produce direct contraction of the detrusor smooth muscle, whereas M_2 receptor may act indirectly by reversing sympathetically (β–adrenoceptor) induced relaxation of the smooth muscle. These two effects could synergise to produce a more efficient discharge of urine[27,28].

In addition to the above classical neurotransmitters, accumulating evidence points to the importance of an atropine insensitive, nonadrenergic, non-cholinergic (**NANC**) neuronal component in bladder micturition. This NANC neurotransmission is mediated through ATP or purinergic analogs, acting mainly through P2X purinergic receptors[29-31]. These receptors belong to two major families: P2X family of ATP-gated ion channel receptors

and a P2Y family of G protein-coupled purinoceptors. In the bladder, P2X receptors were shown to localize in clusters located at the surface of detrusor muscle cells in close proximity to presynaptic varicosities[32]. These clusters represent either homo- or hetero-oligomers consisting of different subtypes of P2X receptors, including $P2X_{1-4}$[32,33]. The P2X receptors form ligand-gated cation channels through the formation of a novel trimeric subunit structure previously unobserved for any other ion channel[34]. These receptors are responsible for depolarization of the cell membrane upon stimulation by either ATP or related purine compounds[35,36]. In addition, during parasympathetic activation of the bladder, ATP may also act as cotransmitter with Ach. This occurs since released ATP acts on P2X ion channel receptors to produce excitatory junctional potentials and Ca^{2+} influx leading to rapid and transient initial contraction. Whereas activation of muscarinic receptors mediates the slow plateau phase of the response via G protein-coupled system, a mechanism which is responsible for the maintenance of contractile activity and flow[29,30,37].

Finally, the functional responsiveness of the rat bladder to these neurotransmitters appears to be also influenced by the hormonal environment. Pharmacological estrogen administration for 4 days was shown to induce significantly increased responses to an α-adrenergic agonist (methoxamine), a cholinergic agonist (bethanechol) and a purinergic agonist (ATP) in bladder strips obtained from immature female rabbits. In contrast, no change was observed in the response to the β-adrenergic agonist isoproterenol[19]. Interestingly, the density of both α-adrenergic and muscarinic receptors increased in these tissues, while β-adrenergic receptors did not change[38]. Nevertheless, in mature female rabbits or OVx rabbits 17-β-estradiol (**E2**) administration for 1-4 weeks caused marked decrease in muscarinic receptor density. The above discrepancies may be attributed to many methodological differences, including differences in the species used, in ages examined, as well as duration or dosage of E2 treatment[39,40].

4.2 Calcium flux and smooth muscle contraction

Fluxes in intracellular calcium levels represent a crucial signal for inducing bladder smooth muscle contraction. A variety of neurotransmitter stimuli lead to increased

cytosolic free Ca^{2+} concentrations $[Ca^{2+}]_i$ in detrusor myocytes. Such fluxes in intracellular Ca^{2+} levels may occur as a result of the entry of extracellular Ca^{2+} across sarcoplasma through voltage-gated or receptor-dependent Ca^{2+} channels, as well as through Ca^{2+} release from the sarcoplasmic reticulum (**SR**), its intracellular storage site. The SR releases Ca^{2+} in response to second messengers including **IP3** (inositol trisphosphate) and ryanodine[41,42]. The elevated concentration enables Ca^{2+} to bind to calmodulin (**CaM**, four Ca^{2+} bind to one CaM). This binding facilitates interaction with specific target proteins including myosin light-chain kinase (**MLCK**). The resultant $(Ca^{2+})_4$-CaM-MLCK complex (the active form of MLCK) catalyses phosphorylation on myosin light chains[43]. The reaction triggers the cycling of myosin crossbridges along the actin filaments, resulting in force or contraction of the muscle. Restoration of resting $[Ca^{2+}]_i$ is reached by removing Ca^{2+} from the cell by a sarcolemmal Ca^{2+} pump or a Na^+-Ca^{2+} exchanger, or a SR membrane Ca^{2+}pump (into the SR). This is followed by CaM dissociation and rapid MLCK inactivation, then myosin dephosphorylation and finally muscle relaxation[44].

Thus, regulation of $[Ca^{2+}]_i$ is the most effective way of controlling smooth muscle contraction, since it dictates the level of myosin phosphorylation. A variety of signals trigger an increase or reduction of $[Ca^{2+}]_i$. Among those which increase $[Ca^{2+}]_i$ are α-adrenergic agonists and membrane depolarizing neurotransmitters such as Ach, while β-adrenergic agonists and nitric oxide mediate a decrease in $[Ca^{2+}]_i$. In addition to Ca^{2+} regulation, some other mechanisms are also implicated in regulation of smooth muscle contraction, including modulations not involving phosphorylation by MLCK[44].

4.3 Intercellular communication in the bladder

Adherens junction, also known as intermediate junction or attachment plaque, consists of two dense bands matching each other in adjacent cells, with an intercellular gap of 40-60 nm occupied by a band of electron-dense material. The cell-cell adhesion properties of adherens junctions are mediated by members of the cadherin family of proteins[45]. Since cadherins are connected to the actin cytoskeleton in each muscle cell, they provide a means of mechanical coupling between individual muscle cells[46]. In view of the abundant

presence of adherens junctions and the apparent absence of gap junctions in the normal bladder, it has been proposed that the former junctions represent the predominant form of intercellular coupling among myocytes in the bladder[12]

The **gap junction** or nexus is a structure seen between smooth muscle cells in most tissues, but interestingly it has not been observed in detrusor smooth muscle cells[12]. Nevertheless, the structure of smooth muscle gap junctions in the gastrointestinal tract or vas deferens is similar to that observed in other tissues including the heart and brain[12]. A gap of 2-3 nm separates the apposed membranes. The gap junction is a hexameric hemi-channel structure that consists of transmembrane protein **connexins**. When hemi-channels of adjacent cells attach, an intercellular passage is created, which provides a hydrophilic channel directly connecting the cytoplasm of the two muscle cells. The formation of this connection allows for an exchange of ions (i.e. electrical coupling), as well as the exchange of small molecules (i.e. metabolic coupling). As a result, direct communication is established between such cells, allowing for coordinated function of the cells in a tissue.

Three major structural differences exist between the contact zones of adherens junctions (**AJ**) and gap junctions (**GJ**). These include (1) GJ sarcolemmae lack the specialization observed in adherens junctions where electron-dense bands are evident on the apposing sarcolemmae, with myofilament bundles spreading from the AJ into each of the two cells. (2) The width of the gap between the two cells is usually 40-60 nm in AJ, but only 2-3 nm in GJ. (3) The two apposing cells in AJs have a common basal lamina appearing as an electron-dense band in the separating gap. On the other hand, GJs have no lamina in the contact zone (at the periphery of the zone, the lamina of one cell becomes continuous with that of the neighboring cell and does not extend into the gap), and the sarcoplasma of the two cells are nearly continuous[12].

Protrusion junctions (**PJ**) consist of long, slender, finger-like process extending from one muscle cell toward a neighboring cell to contact either its surface or a similar process reciprocally extending from it. Protrusion junctions or similar structures have not been

described previously, but their contact zones show close resemblance to gap junctions in that they lack sarcolemmal specialization and basal lamina, while having a narrow gap separating inner leaflets of apposed sarcolemmae. In fact, the sarcoplasma of myocytes adjoined by protrusion junctions has the appearance of being continuous. As a result of these features, it has been proposed that PJs may in fact represent aberrant gap junctions. In the setting of detrusor hyperactivity and the dysjunction pattern, as many as 4-8 myocytes are joined by these junctions in a chain-like arrangement[4]. Based on the above considerations, a hypothesis has been made suggesting that the apparent replacement of AJs by PJs may mediate a conversion of cell coupling from predominantly mechanical mechanisms in the normal bladder to mainly electrical coupling in bladders exhibiting DH. Such a change in bladder structure and interface could facilitate propagation of heightened smooth muscle activity leading to involuntary detrusor contractions[1,4].

Regulation of gap junctions by estrogen. Several investigators have observed that the onset of effective labor in the myometrium of normal pregnant rats and in models of pre-term labor requires the synthesis and functional assembly of Cx43 (the major subtype of connexin proteins) into gap junctions[47]. Moreover, a variety of studies indicate that the upregulation of Cx43 and gap junction formation in this context may be, at least in part, regulated by estrogen. For example, a correlation has been noted to exist between increased Cx43 expression and elevated estrogen expression. More direct evidence indicates that treating immature rats with 17β-estradiol stimulates gap junction formation[48], a process inhibited by tamoxifen or cycloheximide[49], and that estrogens induce Cx43 mRNA levels in pregnant rats[50]. Taken together, these studies support the hypothesis that estrogens regulate gap junction formation[47]. Nevertheless, the precise mechanism of this effect remains unclear, as does the presence or absence of a functional estrogen responsive element (**ERE**) consensus site in the Cx43 promoter[51]. Several different mechanisms have been proposed for the estrogen-mediated upregulation of Cx43 expression. These include increased Cx43 gene transcription by direct mechanisms[48], as well as its induction by indirect mechanisms mediated by *c-fos* and *c-Jun* acting via putative AP-1 sites[52], or through increased synthesis of some of the prostaglandins which have been shown to induce gap junction formation[48].

5. Caveolae and Caveolin

Caveolae are 50-100 nm flask-shaped, non-clatherin-coated plasma membrane invaginations in many mammalian cell types[53]. In addition to being implicated in transcytosis (transcellular transport of macromolecules), potocytosis (the uptake of small molecules and ions via GPI-linked proteins)[54], as well as sorting of surface proteins, caveolae have been implicated as organizing centers for signaling molecules[53,54]. The number of signaling molecules apparently localized to caveolae has been increasing and includes src family kinases, Ras, phospholipase Cγ (**PLCγ**), nitric oxide sythases (**NOS**), PKC isoforms, heterotrimeric G proteins, Mek-1, Erk-2, as well as growth factor receptors such as EGFR, PDGFR[53,55].

Caveolae are abundantly expressed in most smooth muscle cells present in a variety of tissues, including the ureter, vas deferens, intestine and detrusor. Moreover, numbers of caveolae increase with maturation of these tissues[12]. The sarcolemma of mature myocytes is characterized by the presence of rows of caveolae interspersed with adjacent regions containing electron-dense material[12]. Although the rate of myocyte development and maturation varies in different tissues, caveolar appearance can be considered as one of the terminal features of a developmental-maturational process which begins when mesenchymal cells assume early myocyte characteristics such as the expression of smooth muscle-specific actin. When examining detrusor biopsies obtained from elderly individuals with normal bladder function, a "dense band" pattern was described, with caveolar depletion as a prominent feature[5]. This pattern has not been observed in younger individuals. As a result, it has been proposed that with normal aging, detrusor myocytes undergo a loss of terminal differentiation or maturation, and that this ultrastructural change represents evidence of a type of cellular "de-differentiation" in aged detrusor myocytes [5].

Caveolin-1 is a 21 Kd membrane protein, also known as VIP21, since it was originally isolated from *trans*-Gogi network-derived exocytic vesicles (**VIP21** or vesicular integral membrane protein of 21 Kd)[56] and later from caveolae (caveolin-1)[57]. Not only is caveolin-1 the major caveolar protein in many cellular populations, but its levels of

expression appear to generally correlate with numbers of caveolae present at the cell membrane. For example, both lymphocytes[53] and Fisher rat thyroid (FRT) cells[58] lack caveolae and neither cell line expresses caveolin-1 protein. However, transfection of these cells with caveolin-1 results in the formation of membrane invaginations which are morphologically indistinguishable from caveolae endogenously present in similar cells[53,58]. Both caveolar density and caveolin-1 levels increase during adipocyte differentiation and brain development[58,59]. Finally, the relationship between caveolae and caveolin-1 protein appears to be also maintained when their expression is decreased[60]. Based on these and other studies, it has been proposed that caveolin-1 protein expression is both necessary and sufficient for caveolar formation, while also contributing to the role of caveolar complex in signal transduction.

Caveolin can interact directly with many signaling molecules, and the interaction generally appears to hold the signaling proteins in an inactive conformation. For instance, caveolin interacts with epithelial NOS (**eNOS**), holding the latter inactive until activation and releasing by Ca^{++}/calmodulin[61]. In addition, caveolin also prefers the inactive conformation of Gα subunits, Ha-*Ras* and C-*Src*. Since many signaling molecules can cause cellular transformation when constitutively activated, it has been proposed that caveolin may possess transformation suppressor activity. In support of this notion, both caveolae and caveolin are most abundantly expressed in terminally differentiated cells, whereas caveolin mRNA and protein expression are reduced in NIH3T3 cell lines transformed by v-*Abl*, Bcr-*Abl* and Ras[60,62]. In studies by Thyberg *et al*[63], numbers of caveolae on the membrane surface of rat arterial smooth muscle cell decreased remarkably when the cells converted from a contractile to a synthetic phenotype, while caveolar numbers increased during the cells' conversion from a synthetic to a contractile phenotype. Furthermore, inhibition of caveolin expression with antisense oligonucleotides has the reverse effect of activation of Ras/MAPK-dependent signaling and cell transformation[64].

It has been proposed that caveolae may represent a site of calcium signal generation in endothelial cells with calcium waves emanating from caveolae-rich domains[65]. In

support of this hypothesis, IP3 receptor and calcium pump of the plasma membrane have been localized to caveolae[66,67]. These findings infer a role of caveolae in regulating cytosolic Ca^{2+} concentration, probably through a combination of channel-mediated influx and energy-dependent extrusion. Interestingly, G-protein-coupled receptors such as mAChR are clustered into caveolae upon treatment with agonists, but not with antagonists[68].

6. OVARIAN HORMONES IN THE PATHOGENESIS OF DHIC

6.1. Estrogen and Estrogen Receptors

Estrogens are produced mainly in ovaries in women and, to a lesser extent, in testes, in men. Once produced they are able to diffuse easily in and out of most cells, but undergo specific high affinity interactions through estrogen receptors (**ERs**) in target cells. At least two subtypes of nuclear estrogen receptors exist. ER-α is the "classic" estrogen receptor studied for several decades, while ER-β has only been identified quite recently[69,70]. Even more recent studies support the existence of a membrane estrogen receptor, possibly a third variety of estrogen receptor[71]. Interestingly, the ER is the only known example of the steroidal subfamily of nuclear receptors with more than one known subtype.

ER-α and ER–β can form either a homodimer or heterodimers *in vitro.* Together with the estrogen molecule, this complex can bind to the estrogen responsive element (**ERE**), the cognate target site on DNA. This type of evidence suggests the existence of estrogen target genes that are exclusively regulated by either of the homodimers or the heterodimer[72]. Moreover, in transient transfection systems these two receptors, when bound to estrogens, were each shown to exert an entirely different signal once they interact with an AP1 enhancer element. As a result, in this system, ER-α activates transcription, while ER-β inhibits transcription. In contrast to their divergent effects mediated through the AP1 enhancer element, ER-α and ER-β both demonstrate the same transactivation profile when mediating the effect of estrogen at a classical ERE[73]. The above findings have demonstrated, for the first time, that ligand-mediated responses by

ER-β at the AP1 site can act as negative regulators of transcription[73]. Moreover, these kinds of studies have provided a molecular framework that can begin to explain the often highly divergent effects of the same estrogen molecule in different cells and tissues.

6.2. Estrogen receptor structure & mechanisms of its activation.

Classical teaching views estrogen signaling as occurring through nuclear ERs, which are ligand-activated transcriptional regulators. Like other members of the superfamily, ERs have a modular structure consisting of 5 distinct functional domains named A/B, C, D, E and F. The N-terminal A/B contains the ligand-independent transcription activation function 1 (**AF-1**). The C domain or DNA binding domain (**DBD**) responsible for ERE binding has a characteristic helix-loop-helix structure stabilized by two zinc atoms. The D domain appears to be a hinge region that can modulate the DNA-binding ability of the receptor. The E and F domains are involved in the ligand-binding function (hence **LBD**) and also harbor a nuclear localization signal as well as sequences necessary for dimerization and transcriptional activation (**AF-2**). Although transcriptional activation is mediated through both AF-1 and AF–2, only the latter is ligand dependent[70,74].

The ER-α and ER–β share with each other many of structural characteristics including DNA and ligand binding (97% and 60% homology in DBD and LBD respectively). However, the molecular mechanisms that regulate the transcriptional activity profile of these two receptors are different, due to a lack of conservation among the A/B domain and F domain sequences. Such sequence divergences have profound functional consequences since the A/B and F domains are highly relevant to dimerization and transcription activation[70,75].

Lazennec *et al.*[76] analyzed the specific steps involved in the activation of ER through studies using a set of constitutively activated ER point mutants. These receptors were shown to interact with several coactivators and with members of the pre-initiation complex such as TATA box-binding protein (**TBP**) and transcription factor IIB (**TFIIB**). The consequences of the interaction were conformational changes followed by a bending of ERE-containing DNA (the bending being crucial to transcriptional ability of the

transcription factor), recruitment of RNA polymerase II to the transcription initiation site and starting of transcription. These results demonstrated that ER undergoes major conformational and functional changes when it is turned from an inactive into an active state, and that the constitutively activated ER point mutants can mimic the ligand-activated state of wild type receptors[76].

The classical concept of ER regulating genes following an obligatory step of ERE binding had been thought for a long time to be the only mechanism for ER function. However, it has increasingly been appreciated that multiple ligands for the ERs can modulate cell functions through binding to other transcription factors (such as AP1, antioxidant response element and Sp1)[77], or via non-genomic actions mediated through plasma membrane proteins[78]. However, the existence of membrane ER has only recently become accepted[79,80].

Studies have shown that 17β-estradiol (**E2**) can trigger a variety of signal transduction events, occurring over a few seconds to minutes. These events include the stimulation of cAMP[79], calcium flux [81], eNOS activation [82], PLC activation and inositol phosphate generation [83,84], as well as prolactin release[71]. Many of these rapid estrogen actions have been attributed to the ability of E2 to act at the cell membrane. Specific cell membrane effects could include E2 indirectly activating tyrosine kinase growth factor receptors such as EGFR, with subsequent signal transduction initiated through these receptors[85]. Alternatively, E2 could act directly through its own receptor(s) on cell membrane. The putative membrane receptor has been investigated by several laboratories recently and appears capable of promoting signal transduction [71,86].

Morey *et al.* showed that primary cultures of human vascular smooth muscle cells express what appear to be membrane ER[87]. Thus, estrogen could activate growth factor signaling through membrane receptors, resulting in enhanced gene transcription[88]. Recent studies from the same group showed that cells could express either ER-α and-β in the membrane. Interestingly, membrane and nuclear ERs can be derived from a single transcript and have similar affinities for E2. Nevertheless, nuclear receptors are far more

abundant than the same type of ER expressed in the cell membrane. Functionally, both membrane ER-α and ER-β activate G proteins, ERK, and induce cell proliferation, yet there is a differential regulation of c-jun kinase (JNK) activity by these two receptors, in that ER-β, but not ER-α, stimulates JNK activity[71].

6.3. ER Expression:

ERs are expressed in many cell types including neurons and smooth muscle cells.

Peripheral ganglia: Papka *et al.*[89] have demonstrated the expression of ER protein and mRNA in subpopulations of neurons in parasympathetic pelvic ganglia (**PG**), sensory dorsal root ganglia (**DRG** L6-S1), as well as vagal nodose ganglia (**NG**). Some of these ER-immunoreactive (**ER-ir**) neurons project axons to the uterus. The bladder receives a similar type of innervation originating from the same categories of ganglia (PG and DRG) and even the same DRG sensory level, as does that to the uterus. Thus, it is highly possible that some ER-ir neurons originating in these ganglia project to bladder, rather than projecting to the uterus. Moreover, recent studies have revealed that while ER-β is widely expressed in rat DRG neurons, only the smaller neurons in L6-S1 levels that are known to provide innervation to reproductive organs and the bladder express the ER-α[90]. Since neurons in autonomic and sensory ganglia also express mRNA of ER-α and ER-β[90], it is likely that estrogen could influence several functional aspects of such neurons including transmitter synthesis, receptor production, plasticity or receptivity.

Tissue expression: ER–β mRNA was found to be co-expressed with ER-α mRNA in ovary, prostate, brain, lung, liver, kidney, fat, bone, uterus, pituitary, and bladder[74,91,92]. The relative expression of the two receptors varies from one tissue to another. Immunohistochemical studies have revealed a marked contrast between the patterns of expression of these two receptors in the ovary and bladder, where ER-β was abundant and ER-α was almost undetectable, and in the uterus where both were present in epithelial cells lining the lumen and glands[93]. Furthermore, neonatal estrogen leads to a

significant increase in ER-α whereas ER–β levels are down-regulated[94]. This finding again speaks in favor of distinct biological roles of ER-α and ER-β in estrogen action.

6.4. Estrogen Effects:

Effects of estrogen on cell phenotype and survival

Estrogen can exert trophic and protective effects in a variety of cell types exposed to cytotoxic insults ranging from hypoglycemia, serum deprivation and β-amyloid administration to glutamate toxicity [95-99].

Bcl-2 is a potential mediator of E2 neuroprotection, as its expression has been shown to reduce oxidative stress and prevent cell death. In NT2 neurons, *Bcl-2* levels are dramatically elevated upon differentiation and are further enhanced with E2 treatment[95]. In the nervous system, estrogen can promote neurite outgrowth, neuronal sprouting, synaptogenesis, and neurotransmitter (i.e. Ach) synthesis, while influencing the expression of neurotrophic factors and their receptors (i.e. NGF)[100]. Estrogen can also influence neurons via mechanisms other than activation of a classical nuclear receptor. One example is the effect of Ach on neurotransmission, which occurs by altering the activity of membrane-bound receptor systems[100]. Furthermore, there is recent evidence that low doses of E2 may promote the survival of cultured DRG neurons deprived of NGF, possibly through anti-apoptotic mechanisms[101].

Estrogen and the bladder

Estrogen could potentially affect the bladder at multiple different levels of function. Its effects could be mediated directly via the ERs, or mediated indirectly through interaction with mediators such as growth factors, cytokines, uroepithelial cells and others[93]. Estrogens may also potentially act indirectly by having effects on the nervous system.

Levels of endogenous estrogens in postmenopausal women vary considerably. Ovarian production of estrogen essentially ceases and different estrogen levels can be attributed to

a variable estrogen synthesis in extragonadal sites, especially adipose tissues where low potency estrogens are produced by a conversion from androgens[102].

The female genital and urinary tracts develop from a common embryological precursor, the urogenital sinus. Like the vagina, the bladder outlet contains a high concentration of estrogen receptors (mainly "classical" ER-α receptors), and is therefore extremely estrogen sensitive. Estrogen deficiency can result in atrophic urethritis, a process that may result in local symptoms and contribute to the development of urinary incontinence. In individual women with urinary incontinence estrogen replacement may improve symptoms, although this benefit still remains to be proven in appropriate prospective randomized trials[103-105].

Traditionally, classical ER binding (i.e. ER-α) was detected at much lower or barely detectable levels mainly in the nuclear fractions of extracts obtained from the bladder body (detrusor) as compared to that of the bladder outlet[106]. Kuiper *et al*[74] examined both ER-α and ER-β mRNA in multiple rat tissues including ovary, uterus and bladder. Highest levels of both ER-α and ER-β mRNA were found in the ovary. In contrast, a high expression of ER-α, but only a moderate expression of ER-β mRNA were detected in the uterus. In contrast, the mRNA expression of ER-β in the bladder is much higher than that of ER-α [74]. These authors did not specify which portions of the bladder they examined. However, since the detrusor makes up, by far, the largest portion of the bladder, it would be reasonable to expect that most of the extract obtained from an entire bladder would represent detrusor tissues. Recently, investigators from the same lab[107]localized high level of ER-β mRNA and protein, but no ER-α, to the epithelium and smooth muscle in the detrusor and urethral epithelium. However, ER-α mRNA was detected, but only in the connective tissue beneath the epithelium and between the detrusor smooth muscle bundles. In another study[93], ER-β protein was localized by immunohistochemistry to the nuclei of both bladder epithelial cells, as well as smooth muscle cells.

Effects of ovariectomy on the bladder

Longhurst *et al.*[108] investigated the effects of ovariectomy (**OVx**) on bladder function in female rats. The study failed to reveal any effect of OVx on micturition characteristics, and neither OVx nor E2 treatment affected contractile function in the bladder outlet. However, muscle strips obtained from animals which had undergone OVx 4 months earlier demonstrated significant decreases in contractile responsiveness to nerve stimulation, ATP, carbachol and high KCl when compared to sham-operated rats. Treatment with E2 appears to prevent these effects of OVx. Interestingly, in contrast to the above findings, OVx appeared to have little impact at the 2 months time point. Although this study supported our overall hypothesis postulating that OVx and estrogen deficiency contribute to the pathogenesis of impaired detrusor contractility, a number of crucial issues remained unresolved. Levels of administered E2 appeared to be supraphysiologic since all such animals developed evidence of marked bladder hypertrophy. Such an effect could be mediated through a direct effect of high E2 levels on the bladder or indirectly via a diuresis induced by pharmacological E2 doses. Finally, this study did not shed any light on the cellular mechanisms mediating the effects of OVx and E2 on bladder structure, cellular function and contractility, as well as potential mediators of such effects.

In a more recent study, muscle strips obtained from rabbits only three-weeks after OVx did not differ in their contractile response to carbachol and KCl from control tissues. In the same study, a two-week treatment with E2, but not with progesterone, increased the muscle relaxation mediated by β-adrenergic receptors[109]. In another study, Diep *et al.* examined the effects of OVx and E2 treatment on micturition and detrusor strip contractility in rats of different ages[110]. The results showed that detrusor strips from OVx animals had a decreased responsiveness to cholinergic stimulation in both young and mature rats. However, there was an age-related differential response to exogenous E2, in that E2 treatment only partially restored muscle contractility in young rats, while being able to reverse the effects of OVx in the mature rats[110].

INTRODUCTION

Although urinary incontinence is an important cause of disability in late life, resulting in major financial costs, its pathophysiology, particularly at the cellular level, remains largely unknown until recently. Studies have shown that detrusor hyperactivity with impaired contractility (DHIC) was a novel, yet very common urodynamic finding in the incontinent elderly patients[2], and that patterns of ultrastructural changes in detrusor biopsies were strongly correlated with the categories of bladder dysfunction demonstrated on urodynamic studies. For instance, subjects with urodynamic evidence of detrusor hyperactivity (DH) demonstrated ultrastructural dysjunction pattern, which was characterized by evident reduction of adherens junctions, and the *de novo* appearance of "protrusion junctions"[4]. In contrast, specimens from individuals with impaired contractility (IC) exhibited features of the degeneration pattern with axonal and myocyte degeneration[5]. In subjects with DHIC features of both the dysjunction and degeneration patterns were observed [4,5].

It has been proposed that "protrusion junctions" (PJ) may be aberrant gap junctions[4]. Since their appearance coincides with the diminution of adherens junctions, It has also been suggested that such changes could convert the detrusor from a mainly mechanically coupled to a predominantly electrically coupled tissue[4]. Interestingly, mammalian parturition is preceded by a hormonally mediated *de novo* formation of gap junctions[47]. In addition, there is now also increasing evidence that estrogens can influence the survival and phenotype of many cellular populations, including vascular smooth muscle cells and specific categories of neurons[111]. The female menopause results in a loss of ovarian estrogen production[112]. However, since many non-ovarian tissues continue to produce lower potency estrogens, both the levels, and the relative proportions of endogenous residual estrogens in elderly women not receiving hormonal replacement therapy are variable[112]. We have therefore proposed a hypothesis suggesting that estrogen deficiency in elderly women could contribute to the development of DHIC.

In this thesis project, a systematic evaluation was conducted examining the effects of prolonged ovariectomy upon the physiologic, ultrastructural and biochemical properties of the mammalian detrusor. In the following pages, evidence is presented that detrusors of chronically ovariectomized mature rats develop structural and physiologic changes that support a role of ovarian hormones, most likely estrogen, in the pathogenesis of IC. Efforts have also been made to screen for molecules that could be potential mediators for the effects of ovarian hormones, including 17β-estradiol, on the structural integrity and function of the aged bladder. As a result, we also present preliminary data identifying one such candidate protein, which we termed Detrusor Estrogen-Regulated Protein (**DERP**).

MATERIALS AND METHODS

1. Animal Procedures

Female Fischer 344 rats (retired breeders) were used in order to replicate the findings published by Longhurst *et al* [108]. Moreover, F-344 rats represent a well-established and characterized rodent model for the study of aging[113]. In addition to a wealth of available background information, in contrast to other strains such as the Sprague Dawley rat, F-344 rats are inbred resulting in lesser variability and do not develop major obesity.

The animals were obtained at the age of 9-10 months from a single colony (Harlan Sprague Dawley Inc., Indianapolis, IN), they were housed 2 per cage in an approved animal care facility with water and food provided *ad lib*. Vaginal examinations and cytological studies were conducted prior to the surgery in order to standardize timing within the menstrual cycle. Under anesthesia with pentobarbital, either sham or bilateral ovariectomy (**OVx**) surgery was performed using a dorsal surgical approach. Vaginal observation and vaginal smears were performed post-surgery and one week prior to sacrificing (4 months after surgery). The presence of mature cells on vaginal cytology in OVx animals was indicative of estrogen stimulation from residual ovarian tissue and such animals were excluded from further studies. Most sham animals were in a state of

irregular cycling at this age, while a few demonstrated a constant estrus pattern[114]. Efforts were made to sacrifice the animals at the stage of proestrus whenever possible.

Upon sacrificing of the rat under deep anesthesia, the bladder detrusor was immediately removed at the level of the ureter, and then cut sagitally while maintained cold. One-half was transferred to pre-chilled fixative for light and electron microscopy processing, the remaining half was then cut into two quarters and transferred into the Eppendorf tubes and snap frozen in liquid N_2, stored at –80°C until processing for protein studies. For muscle strip contractility studies, the entire bladder was used. After bladder removal, the uterus was removed and weighed.

Some animals underwent 17β-estradiol (E2) treatment before sacrificing. Silastic capsules containing E2 (for OVx rats) or placebo (for sham or OVx rats) were implanted subcutaneously on the back of the neck. The protocol used for silastic capsule preparation and placement followed a well-characterized and established method for the physiological and long-lasting administration of E2[115].

In our study of maturation, the detrusor portions of the bladders were removed from newborn (2 days old) and young adult (1 month old) female rats immediately after sacrifice. When examining estrogen receptor expressions, bladder detrusor, bladder outlet and ovary were dissected and removed from 5 adult female rats. The samples were snap frozen in N2 and stored at –80°C until processing.

2. Light and Electronic Microscopic Studies

2.1 Tissue processing

Bladder samples were processed for electronic microscopic (EM) as previously described in human biopsy studies by Elbadawi *et al*[116]. Hemi-bladders were immersed overnight in freshly made pre-chilled 2.5% glutaraldehyde at 4°C, and subsequently transferred into pre-chilled 0.2 M cacodylate buffer. Samples were stored refrigerated with buffer changed weekly until trimmed. Two tissue blocks (~ 1 mm^3), trimmed to the size

appropriate for electron microscopy, were obtained from the dome regions of detrusor specimens. The blocks underwent standard processing by osmication, dehydration and embedment in Araldite. Semi-thin (1 μm thick) sections stained with Toluidine blue were examined by light microscopy for orientation of smooth muscle profiles, and also for quantitative light microscopic studies. Ultrathin sections (silver to silver-gold) were obtained from the trimmed block that best represented the specimen and had optimal orientation (dominant obliquely and longitudinally sectioned) of muscle cell profiles. The sections were then mounted on uncoated 150-mesh grids (5 to 10 sections per grid) and stained by the standard uranyl nitrate/lead citrate sequence. At least 2 grids from each of the 2 blocks were studied in each case.

2.2. **Quantitative light microscopic studies**

A computer controlled image analysis system (*Star I*, Photometrics Inc.) was used by a "blinded" trained individual to capture a total of 716 low magnification images from "coded" semi-thin (1 μm thick) toluidine blue stained sections obtained from 7 sham and 7 OVx animals. Resultant images were stored on floptical disks. *ImagePro* Software (Media Cybernetics Inc.) was used to manually delineate the boundaries of the visible muscle tissue in each image and to determine the proportion of pixels in each field represented by muscle tissue. Similarly the numbers of myocyte nuclei in each image were independently counted by two different "blinded" observers. The code was only broken upon completion of all data analysis.

2.3. **Qualitative Electronic microscopic studies**

Detrusor tissues from 7 pairs of sham/OVx animals were fully processed for electron microscopic analysis. Under a JEOL JEM 100SX or a Philips EM300 electron microscope, structure of the 3 compartments: muscle cells, interstitium and intrinsic nerves of the detrusor were examined in detail and amply documented photographically (average of 90 photographs per specimen). The specific features evaluated qualitatively in each of the 3 compartments have been described previously[116]. All EM photographs used in qualitative studies were scanned by a scanner (SnapScan 310, Agfa). Using low (4,000 x) magnification images a "blinded" individual manually delineated the

boundaries of individual nucleated muscle cells using image analysis software (Media Cybernetics Inc.). Only those muscle cell profiles with a whole perimeter and nucleus were traced and analyzed. The length of the muscle cell perimeter and area were measured in pixels and these values were subsequently converted to μm and μm^2 using internal standards. All samples and images were coded until the completion of analysis.

2.4. Caveolar analysis

Sarcolemmal caveolae were manually counted in a "blinded" way in 72 coded high magnification photographs (10,000 x) obtained from the same 7 pairs of OVx/sham rats. Structures counted as caveolae included all non-coated vesicles present within 100nm of the myocyte membrane. Counts were only performed in the single myocyte in each high magnification photograph displaying an entire membrane perimeter cut in cross-section. These photographs were then scanned (SnapScan310; Agfa) and analyzed using image analysis software (ImagePro I, Media Cybernetics; Silver Spring, MD). Myocyte profiles were traced manually with a mouse and their length in pixels was determined using this software. Each 1000 pixels of image represented 7.1 μm of sarcolemmal length.

3. Muscle Strip Studies

The method has been adapted from Longhurst *et al*[108]. Upon removal, the entire detrusor was placed in oxygenated (95% O_2, 5% CO_2) 4°C Tyrode solution (NaCl 135 mM; KCl 2.8 mM; $CaCl_2$ 1.9 mM; $MgCl_2$ 0.4 mM; Na_2HPO_4 0.4 mM; NaH_2PO_4 0.4 mM; $NaHCO_3$ 1.3 mM and Dextrose 6 mM). Muscle strips measuring 50 mm x 2 mm (8-15 mg) were prepared. A total of 2-4 strips were cut longitudinally from each detrusor in a standardized fashion. The carbachol-stimulated contractility was compared using muscle strips obtained from sham vs. OVx rats, each group having 12 muscle strips from 4 animals. Each strip was suspended between paired hooks and placed in a 5 ml organ baths containing warm (32°C) oxygenated Tyrode solution. The strips were connected to a Grass force displacement transducer (FTO3C) and an initial tension of 1 gm was applied during an equilibration period of 60-90 minutes. Responses were recorded on a Grass Polygraph (Model 7). Concentration-response curves were measured by applying

increasing concentrations of Carbachol (# C4382; Sigma; St. Louis, MO) and waiting for the response to plateau. The interval between applications increased from 15-30 minutes as higher doses were reached. The addition of each concentration of carbachol was followed by several washes with drug-free buffer. Muscle strips were then blotted dry and weighed.

4. Western Blot Studies

Frozen sample tissue quarters were homogenized in liquid nitrogen. A lysis buffer (recipe from Dr. M. Ferns's lab) was used to dissolve the homogenate (25mM Tris, 150mM NaCl, 5mM EDTA, 1%TritonX-100, 0.1%SDS, plus a protease inhibitor cocktail from Roche, Inc.). After sonication and extracting at 4°C for 20 min, the lysates were centrifuged (10,000g at 4°C for 15 min) and supernatants were transferred. Following protein quantification using the Bradford method (BIO-RAD #500-0006), equal amounts of 2x Laemmli buffer (Sigma; #S-3401) was added to each sample, mixed, and the samples were snap frozen in small aliquots in liquid nitrogen, and stored at –80°C.

Molecular weight marker (Amersham-Pharmacia) and an equal quantity of protein samples were heat-denatured and then resolved in 10% SDS-PAGE followed by transfer to a nitrocellulose membrane (BIO-RAD). For detection of caveolin and actin, the blot was blocked with 5% skim milk in TBST, then cut into two portions at approximately 30 Kd molecular weight. The upper portion was incubated with monoclonal antibody to α–smooth muscle actin (SIGMA; #A2547), and the lower one with a polyclonal antibody to caveolin-1 (Transduction Laboratories; #C13630) at a concentration of 1:1000. Incubation was carried out for 1 h at RT or 4°C overnight. The blots were washed with TBST 10 min x3 and then incubated with peroxidase labeled anti-mouse (upper part) or anti-rabbit (lower part) secondary antibodies for 1h. Following final washes, the blots were developed using the ECL detection system (Amersham, #RPN2108). Quantitation of Western blot bands of interest was performed using a phosphoimager/densitometry image analysis system (Molecular Dynamics). Quantitative comparisons were made only between bands resolved on the same membrane.

In identifying the protein of interest, anti-lumican primary antibody was kindly sent by Dr. Peter J. Roughley[117]; and the anti-serum against pig ITI-H4 was generously provided by Dr. Andres Pineiro[118]. When performing Western blotting with the latter antibody, 5% Ovalbumin in TBST was used as blocking solution.

5. RT-PCR

Frozen tissue obtained from rat detrusor, bladder outlet or ovary was ground in liquid N2 into a fine powder. RNA extraction was then performed using the "RNAwiz" kit (Ambion; #9736). RNA quantification was performed using spectrometer optical density at 260/280. Aliquots were resolved using agarose gels in order to check the integrity of the RNA samples. A total of 2μg of total RNA was used for reverse transcription. This was accomplished using the Superscript system (Gibco-BRL #11904-018) with oligo (dT) primer followed by PCR amplification. The primers specific for ER-α, ER-β [74], and ITI-H4 cDNA (designed on Gene Runner) were synthesized by Shelton Biotechnology Center of McGill. (Table 3).

Table 3. RT-PCR for Detecting mRNA Expression

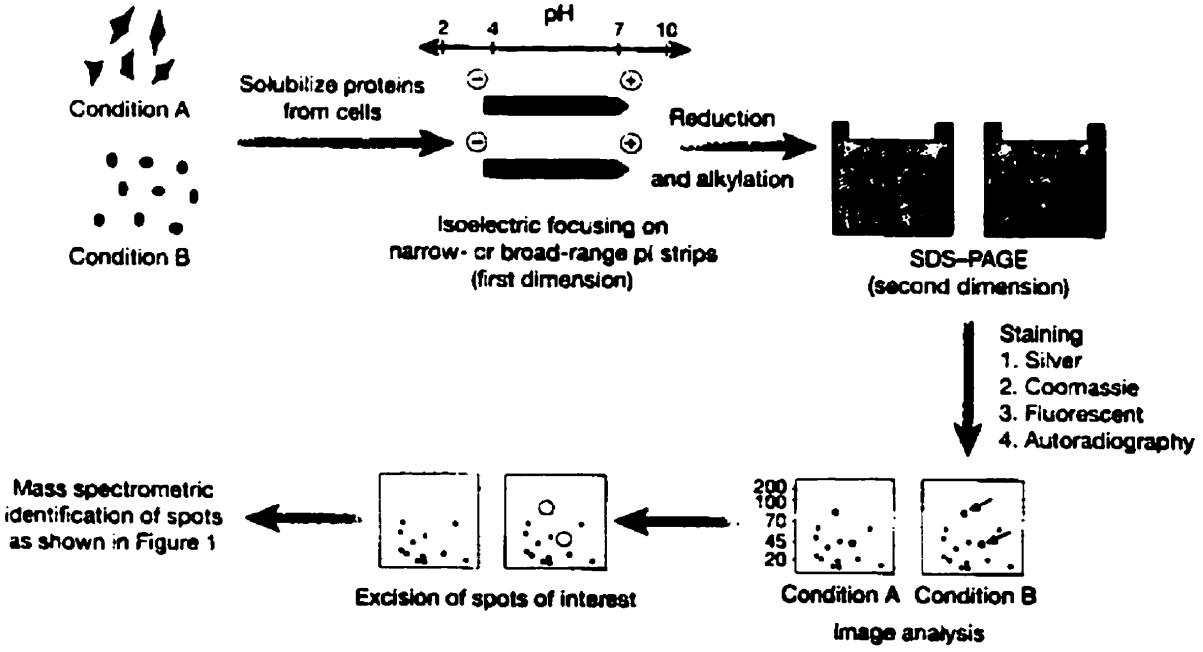
mRNA	Primers	PCR Program
ER–α	5'-AATTCTGACAATCGACGCCAG (nt 472-492) 5'-GTGCTTCAACATTCTCCCTCCTC(nt 794-116)	Denature: 95°C, 1 min Annealing: 57°C,1 min Extension: 72°C,1 min
ER–β	5'-TTCCCGGCAGCACCAGTAACC (nt 38-58) 5'-TCCCTCTTTGCGTTTGGACTA (nt 279-300)	Cycles: 30
ITI-H4	5'-TAGACAATACATGCCTCCTC-3'(nt 1986-2005) 5'-GTCACCACCAGTCGTTCAG-3' (nt 2432-2450)	Denature: 94°C, 1 min Annealing: 55°C, 1 min Extension: 72°C, 1 min Cycles: 35

The PCR reaction mixture (50μl) contained 25mM $MgCl_2$, 10μM each of the sense and antisense primers and 1 unit of *Taq* DNA polymerase. The PCR program was carried out in the DNA thermal cycler following a denaturation step at 94°C /95°C for 4 min, and

was concluded by a single extension step at 72°C for 10 min (see Table 3 for details of individual PCR reactions). Finally, PCR products were resolved and analyzed using ethidium bromide/ 1% Agarose gel electrophoresis.

6. 2-D Gel Analysis And Protein Microsequencing

Schema For The Proteomic Approach (Pandey *et al.* [112])



6.1. Study design

The schematic figure adapted from Pandey *et al.* [119] summarizes the experimental strategy which allowed us to identify one candidate protein in our search for Detrusor Estrogen-Regulated Protein (DERP), a protein down-regulated by OVx and up-regulated by E2 treatment.

6.1. **2-D gel electrophoresis**

Approximately one quarter of a rat detrusor was homogenized in liquid N2 and then resolved in IEF buffer, followed by disruption by sonication and extraction for 20min at RT before centrifugation (10,000g at RT for 15 min). The protein in the supernatant was quantified at a wavelength of A280 on the spectrometer and then stored at –80°C in aliquots.

The Mini-PROTEIN II 2-D system (BIO-RAD) was used to resolve protein extracts in two dimensions. Equal amounts (120μg) of protein samples from detrusors of sham, OVx, OVx+E2 treated animals were resolved first through isoelectric focussing (**IEF**), followed by SDS-PAGE electrophoresis[120]. After electrophoresis the gels were fixed in 50% methanol for 1 hr with 3 changes and then subjected to silver staining (protocol provided by Harvard Microchemistry Facility).

6.2. **Preparation of protein sample for amino acid sequencing**

Visual analysis of Ag-stained 2-D gels allowed us to identify several distinct protein spots, which were consistently down-regulated by OVx and then up-regulated or re-expressed following E2 treatment. The spots of interest were then excised and pooled, following the protocol on microsample handling for peptide internal sequence analysis provided by Harvard Microchemistry Facility. Standard 2-D gels that optimize for the density (i.e. maximizing the protein to gel volume ratio) were run, fixed, Ag stained. Only the gels with sharp protein spots and clear background were chosen and used for sample pooling.

Working in the fume hood with surgery cap, mask and surgery gown (to avoid contamination including that of the ubiquitous keratin), hearts of the chosen spots were excised with a "neuro punch" (0.65 mm, Fine Science tools inc.). Excised spots were aspirated by connecting the neuro punch with a Butterfly-19 needle (Venisystems, #4590), and the latter with a 1ml syringe. The gel spots were then transferred to a labeled 1.5ml Eppendorf tube. An equivalent number of spots were excised from the background to serve as controls, and transferred to a separate labeled tube). Gel slices were then

washed in the tube twice using 50% HPLC grade acetonitrile in water for 2-3 min. The supernatant was aspirated after each wash, leaving the gel slices moist. The cap was closed, snap frozen in N2 and then stored at –80°C. Gel spots were accumulated in a single tube using this approach until a total concentration of 0.5-1 *p*Mol was achieved for each sample.

6.3. Internal peptide sequencing

The pooled protein sample was subjected to protein microsequencing analysis, performed at the Harvard Microchemistry Facility by microcapillary reverse-phase HPLC nano-electrospry tandem mass spectrometry (μLC/MS/MS) on a Finnigan LCQ quadrupole ion trap mass spectrometer. The basic steps include:

7. Characterization of the protein of interest

Upon receipt of sequencing data, data base (such as Entrez PubMed of NCBI) and literature researches were performed. The protein of interest was further confirmed and characterized using immunoblotting and other biochemical approaches.

8. Statistical analysis

Comparisons between groups were performed using Student *t*-test, chi-square, 1-way analysis of variance (1-way ANOVA) or 2-way analysis of variance (2-way ANOVA), as appropriate.

RESULTS

1. Ensuring the completeness of the castration

All of the OVx animals studied demonstrated both evidence of mucosal atrophy on visual inspection, as well as a complete absence of mature epithelial cells in vaginal cytological smears. In addition, during a post-mortem examination no residual ovarian tissue was

observed in any of the OVx animals, while obvious uterine atrophy was seen in all. In contrast, cytological smears from most sham-operated animals exhibited constant diestrus, a pattern typical for animals of this age. As expected, following OVx animal body weight increased, while the uterine weights decreased ($p<0.05$, Table 4).

Table 4. Body Weight and Uterus Weight of the Rats

	Sham	OVx	OVX+E2
Body Weight (g)	274.7 +/- 29.9*	305.0 +/- 24.7*	288.0 +/- 30.3
Wet Weight Uterus(g)	0.8 +/- 0.1 *	0.2+/- 0.1*	0.5+/- 0.163*

* $p < 0.05$

2. Long-term Ovariectomy Results in Sarcopenia of Detrusor

Low magnification EM images did not reveal any striking changes in the detrusor 4 months after ovariectomy. Muscle fascicles and cells appeared to be largely intact in both sham-operated (Fig.1A) and ovariectomized (Fig.1B) animals. In the latter, the smooth muscle compartment appeared to be decreased, with possible widening of spaces between muscle fascicles. Adherens junctions joining detrusor muscle cells were visible at high magnification in both sham and ovariectomized tissues, but seemed fewer in the latter. No ultraclose abutments were observed in either group, but a single structure resembling a "protrusion junction" was seen in a single field in one ovariectomized animal.

Smooth muscle represented 130,816 $\pm$ 1957 pixels in images obtained from sham-operated animals (59.1% of the field; Fig. 2A), but only 103,888 $\pm$ 1897 (47.0 %, $p<0.001$) in equal numbers of images from OVx animals. Counts of myocyte profiles were somewhat lower (149 $\pm$ 6) in specimens from OVx tissues than those from sham-operated animals (169 $\pm$ 8, Fig. 2B; $p<0.05$). In low magnification EM images from OVx animals (Fig. 2C), there appeared to be fewer small-sized myocyte profiles (< 50 μm^2)

than in images from sham-operated animals, with an apparent shift towards medium-sized (50-100 μm^2) and large-sized (>100μm^2) myocyte profiles.

3. Ovariectomy Also Results in Degeneration of Detrusor Axon Profiles

Axonal profiles were generally intact with no evidence of degeneration in detrusors from sham-operated rats (Fig.3A). Degeneration was observed in some intrinsic axons in OVx detrusors (Fig.3B). The degeneration was characterized by depleted synaptic vesicles, disrupted mitochondria, axoplasmic multivesicular electron-dense bodies, and disrupted axolemma with fragmentation or lysis of some profiles. The identity of axonal profiles undergoing degenerative changes could not be determined since no secretory vesicles or myelination was observed in any. Neither axon sprouts nor regenerated axon terminals were observed in any specimen. Among intact axons observed in OVx bladders were both myelinated and nonmyelinated profiles.

4. Detrusor Muscle Strips From OVx Rats Generate Less Tension In Response To Carbachol

The tension generated at different carbachol concentrations by detrusor strips from OVx rats was 30-50% less than that by strips of the same length from sham-operated animals (Fig. 4A; $p < 0.001$). Although cut to the same length, strips obtained from OVx animals tended to weigh less (9.3 ± 0.8 mg vs. 11.4 ± 0.7mg, NS). When tension generated was corrected for strip weight, the differences in tension became considerably smaller, yet still statistically significant (Fig. 4B; $p < 0.05$). Expression as a percentage of the maximum tension generated by each muscle strip, per entire strip (Fig.4C) or per weight of the strip (Fig.4D) revealed no significant differences between sham and OVx groups.

5. Caveolar / Caveolin Regulation

5.1. Caveolar Depletion Following OVX

Ultrastructural examination of myocytes in detrusors from sham-operated controls revealed that the sarcolemma displayed the characteristic features of normal mature smooth muscle cells (*i.e.* alternating thick electron dense bands and much thinner less

dense zones, the latter containing rows of uniform sized, flask-shaped surface vesicles-caveolae; Fig. 5A). In contrast, in tissues from OVx animals, the sarcolemmae appeared to be dominated by electron dense band patterns (sometimes in long stretches that covered almost the whole sarcolemma) which were interposed by unevenly spaced thin zones with fewer caveolae (Fig. 5B). Image analysis revealed a 28% decrease in the numbers of caveolae per 1000 pixels of sarcolemmal length ($p<0.005$; Fig.5C).

5.2. Caveolin-1 protein expression was down-regulated after OVx

Western blots for caveolin-1 and α–smooth muscle actin were performed using equal amounts of detrusor protein extracts obtained from paired OVx/sham-operated rats. Coomassie blue stain was used to ensure both protein integrity and equal loading. A comparison of α–smooth muscle actin signal intensity showed no evident difference between such paired samples. In contrast, there was a modest, but consistent decrease in caveolin-1 expression with OVx in the same blots (Fig.6A). Quantitative analysis using samples from 7 pairs of OVx/sham-operated rats revealed a 30% decrease in caveolin-1 expression after OVx ($p<0.005$), whereas α–smooth muscle actin expression showed no change (Fig.6B).

5.3. Down regulation of Caveolin-1 is reversed by administration of 17β-estradiol

When equal quantities of total protein were examined, the down regulation of caveolin-1 protein 4 months after OVx was effectively reversed by a 1-month administration of E2. In contrast, α–smooth muscle actin signal intensity showed no evident difference between such paired samples (Fig.7).

5.4. The relationship between caveolin-1 and myocyte maturation

Levels of α–smooth muscle actin were similar in equal quantities of protein extracts obtained from newborn and young adult female rats. In contrast, caveolin-1 protein levels were significantly higher in young adult as compared to newborn animals (Fig.8A). Quantitative analysis using samples from a total of 8 animals revealed a greater than 3-fold increase in caveolin-1 protein in adult, as compared to newborn detrusors ($p<0.001$), while α–smooth muscle actin expression showed no change (Fig.8 B).

6. Expression of Estrogen Receptors In Bladder

As shown in Fig.9, ER-α and ER-β mRNA were both abundantly expressed in the ovary. The expression of ER-α mRNA is evident in the bladder outlet, yet is barely detectable in the detrusor (**A**). In contrast, ER-β mRNA is significantly expressed in both the detrusor and the outlet (**B**).

7. Identification of DERP

7.1. Patterns of protein expression following OVx

2-D gel resolution of protein extracts from OVx and sham-operated rats revealed relatively subtle changes in protein expression patterns. Four months after the surgery, the expression of most proteins did not change. No proteins appeared to be consistently expressed *de novo*, while a few proteins were upregulated, and some proteins were shown to be downregulated. Of particular note, a string of protein spots was observed at ~110 Kd with a p*I* varying between 4-5. Interestingly, this string of proteins nearly disappeared 4 months after OVx, but was restored to approximately normal expression following the subcutaneous implantation of E2 for only 1 week (Fig.10). As a result of these consistent findings, this protein string was chosen as candidate for peptide sequencing.

7.2. Sample pooling of the protein of interest for sequencing

Fresh protein samples from sham rats and the highest quality and fresh reagents/solvents were used throughout the procedures. Standardized 2-D gels were stained with Ag, and the ones with optimized conditions were chosen for gel pooling. The string of spots at ~110 Kd with p*I* of 4-5 were carefully excised and processed. A total of nearly 100 spots (~0.5 *p*Mol) were pooled from 30 gels, which were, in turn chosen from over 100 gels.

7.3. Microsequencing of the protein of interest

The following sequences resulted from the microsequencing of the pooled protein extract.

A. (S52284) Rat **lumican** precursor[121]:

1). ITNIPDEYFNR

2). NNQIDHIDEK

3). EEAVSASLK

B. (y11238). Rat Inter-alpha-inhibitor H4 heavy chain (or **ITI-H4**)[122]:

1). SQNEQDTVLDGDFIVR

2). IPAQGGTNINK

C. Single sequences (No Groups)

1). (p04264) HUMAN CYTOKERATIN1: SKAEAESLYQSK

2). (z29074) HUMAN CYTOKERATIN9: TLNDMRQEYEQLIAK

7.4. **Identification and characterization of DERP**

First, we set out to see if Lumican may be our protein of interest. We ran SDS-PAGE and performed Western blotting using a polyclonal antibody generated against rat lumican. Visible bands were observed at ~60-80 Kd. 2D gels combined with Western blotting for lumican using 5 pairs of samples from sham and OVx resulted in a string of "smeared" signals in each of the blots and no evident difference between the two groups (Fig.11). Careful observation of the Ag stained 2D gels revealed a "smeared" string of signals at ~80 Kd, immediately below the string of clear spots which were of primary interest to us. Based on lumican studies conducted in Dr. Peter Roughley's lab, we concluded that the string of signals appeared to be "smeared" vertically due to differential glycosylation. As a result of this "smearing" and the great sensitivity of the microsequencing method lumican was sequenced at a greater than expected size (Fig.10).

Subsequent efforts focussed on the 2nd candidate protein: Inter-alpha-inhibitor H4 heavy chain (**ITI-H4**). Using antisera generated against pig ITI-H4, 2D-Western blots were performed. Our results demonstrated a clear string of signal at ~110 Kd with p*I* of 4-5. Moreover, signals from the sham sample appeared to be of a stronger intensity than those obtained from the OVx group (Fig.12).

DISCUSSION

1. Long-term OVx results in sarcopenia involving detrusor muscle

Our results indicate that the rodent detrusor loses smooth muscle mass (sarcopenia) following ovariectomy. Until now, the term "sarcopenia" has only been used to describe age-related loss of skeletal muscle mass[123,124]. However, our data indicate that the hormonal changes associated with the female menopause and aging may also result in sarcopenia involving detrusor smooth muscle. As in the case of skeletal muscle sarcopenia, detrusor sarcopenia can result in decreased muscle strength that may then contribute to impaired contractility. Although we did not observe any ultrastructural features of apoptosis[125] or necrosis, fewer nucleated muscle profiles were observed in detrusors from OVx than sham-operated animals, suggesting that the observed sarcopenia is, at least in part, the result of a loss of myocytes. Nonetheless, the observed shift towards medium and large muscle profiles after OVx suggests a potentially heterogeneous response of individual detrusor myocytes to ovarian hormone depletion. This finding could reflect selective loss of certain muscle cells with compensatory hypertrophy of some of the remaining ones. In a recent study, Hashimoto *et al.* demonstrated the presence of decreased smooth muscle cell density in bladders from rabbits which had undergone OVx 4 weeks earlier[126]. As expected, estrogen replacement increased smooth muscle cell density within the bladder wall[126]. Although performed in a different species, this study provides support for our observation of the presence of detrusor muscle sarcopenia following long-term OVx surgery.

2. Degeneration of some axonal profiles in the detrusor follow OVx

Unlike the changes seem in the neuropathic humandetrusor[5], the axonal degeneration observed in our OVx rats was neither widespread nor accompanied by axonal regeneration after OVx. The degenerated axons in detrusors from OVx animals are more likely parasympathetic, afferent sensory, or both than sympathetic in nature, in view of scarcity of sympathetic fibers in the dome of the normal rat bladder[127]. In the present study, the identity of the degenerated axons could not be determined in the absence of their histochemical characterization or ultrastructural definition of their synaptic vesicles.

It has been shown that androgens influence the morphology of parasympathetic neurons controlling bladder function[128] and that estrogen induces the expression of detrusor muscarinic receptors[39]. Moreover, 17β-estradiol can clearly promote the survival of dorsal root ganglia neurons *in vitro*, possibly through an anti-apoptotic mechanism[101]. The presence of intact axonal projections in the mammalian bladder is necessary for both the initiation of normal detrusor contractility following neurotransmitter release, as well as for the trophic maintenance of its target (i.e. smooth muscle cells). Thus, axonal degeneration could contribute to diminished detrusor contractility both through direct effects (diminished neurotransmitter release) and potential indirect effects (diminished trophic support for detrusor myocytes, with their possible degeneration).

3. **Muscle strips from OVx animals generate less tension in response to carbachol**

Tension generated by detrusor muscle strips in response to nerve stimulation, ATP, carbachol, and high KCl was lower at 4 months, but not 2 months after OVx, when compared to strips from sham-operated animals[108]. Our studies performed in mature rodents confirm that 4 months of ovarian hormonal deprivation results in major (nearly 50%) declines in muscle strip contractility. In neither our, nor the cited study[108], did ovariectomy result in a statistically significant decrease in bladder muscle weight. Nevertheless, expressing contractility per strip weight diminished the ovariectomy-induced overall decline in contractility. These findings, supported by our quantitative morphological data suggest that, in addition to an axonal degeneration, ovariectomy-related decline in detrusor contractility also results from sarcopenia, as well as potential contractile deficits in individual muscle cells. The latter do not appear to be related to altered receptor affinity to carbachol, but could be the result of muscarinic receptor loss, specific deficits in post-receptor signal transduction, or both.

4. Regulation of caveolae and caveolin-1 following OVx

As discussed in the literature review, caveolar depletion was a prominent feature observed in the "dense band" pattern identified with normal aging of the detrusor. Furthermore, caveolae play a role in Ca^{2+} signaling and the entry of extra-cellular Ca^{2+}[55].

Based on the above considerations, we postulated that caveolar depletion could contribute to diminished contractility of individual myocytes and perhaps the entire detrusor. Thus, we undertook an examination of caveolae and their major component protein, caveolin-1, on our animal model.

Our findings demonstrate a down-regulation in caveolae and caveolin-1 expression in detrusor myocytes 4 months after OVx. Although the role of estrogen and other hormones in the development and maturation of responsive tissues is well established, this is, to our knowledge, the first demonstration of either caveolar expression or caveolin protein being regulated hormonally. The similar magnitude of the decrease in caveolar expression at the sarcolemmae (28%) and the lowering of caveolin-1 protein levels (30%) supports the concept of a coupling between caveolin-1 levels and the numbers of caveolae[53,58].

We have also shown that caveolin-1 levels increase significantly with post-natal maturation of the female rat detrusor, while levels of α-smooth muscle actin remain unchanged. The timing and sequence of events during rat detrusor myocyte differentiation have been described previously[129]. Rat bladder mesenchymal cells begin to acquire smooth muscle features at day 16 of a 22 day gestation and α-smooth muscle actin protein is one of the earliest biochemical markers of this differentiation[129]. In contrast, our data support the concept that caveolin-1 protein is a marker of late differentiation (maturation) rather than earlier differentiation in smooth muscle cells [12,130]. Meanwhile, expression of α-smooth muscle actin protein appears to be more reflective of the presence of a general myocyte phenotype. Taken together, our observations indicate the presence of a loss of terminal differentiation or maturation (early "de-differentiation") in cells which still retain the general features of the smooth muscle cell phenotype [5].

Our observation that 17-β-estradiol administration increases caveolin-1 protein levels after OVx further implicates estrogen in the regulation of this protein and caveolae. However, at this time, our findings do not allow us to draw conclusions about the level at

which this regulation takes place. Future studies need to utilize quantitative methods such as Northern blots, quantitative RT-PCR techniques or the RNase protection assay to clarify this issue. It also remains to be established whether this regulation is a direct effect of estrogen or is mediated indirectly through the expression of another intermediate molecule by myocytes or by other cellular populations. The promoter of the human caveolin-1 gene has been shown to contain three regions which are capable of binding SREBP-1 (sterol regulatory element binding protein-1), in addition to CAAT and SP1 consensus sequences[131]. These findings do not rule out an interaction of the estrogen-estrogen receptor complex with this promoter, particularly in the 5' region of the published sequence.

Our results so far are the first to demonstrate that ovarian hormonal production is an important factor in maintaining the integrity and phenotype of the adult detrusor, as well as its innervation in the female mammal. Since ovariectomy (after 4 months) leads to sarcopenia of detrusor with impaired contractility, degeneration of its intrinsic axons, as well as possible phenotype change like "de-differentiation" reflected by caveolar change and cavelin-1 regulation, deficiency of ovarian hormones may influence the development of impaired detrusor contractility in women late in life. This notion is supported by the clinical studies on elderly women which revealed the presence of highly variable endogenous estrogens in elderly women, and which also suggested that these hormones may favor the maintenance of normal detrusor structure and contractility in late life[132]. In contrast to the effects of ovarian hormones on impaired contractility, neither our animal studies, nor our preliminary human results have provided any data to support a role for these hormones in the pathogenesis of detrusor hyperactivity. For example, there was no evidence for the development of the dysjunction pattern in the OVx animals. Nevertheless, our findings do not completely rule out the possibility that estrogen deficiency may play a role in DH through mechanisms other than the dysjunction pattern (such as neurogenic) or its development at time points after OVx other than the one studied.

5. Estrogen receptors expression and estrogen effects

The effects of estrogen on the integrity of detrusor smooth muscles and intrinsic axons, as well as myocyte growth and contractility may or may not be mediated directly. It is well known that both the female bladder outlet and urethra require estrogen to achieve and maintain normal function[103]. In contrast, the detrusor has not been traditionally considered to be an estrogen-dependent target[103]. Nevertheless, studies by Longhurst *et al.*[108] demonstrated decreased *in vitro* contractility of detrusor muscle strips obtained from OVx rat as compared to sham-control. Radioligand binding studies have demonstrated high levels of ER-α in human vagina, cervix and uterus, as well as in the bladder outlet regions, but specific ER-α binding is extremely low in the detrusor[103].

The newly identified β-estrogen receptor, however, is more abundant in the rat bladder[74], with significant expression in detrusor myocytes[93], as well as uroepithelium[93]. Our RT-PCR results demonstrated with clarity that the mRNAs of the two subtypes of estrogen receptors (ER-α and ER-β) are differentially expressed in the bladder, in that ER-α is abundantly expressed in the outlet but barely detectable in the detrusor, while ER-β is expressed in both regions. These differences in expression and distribution could partly explain the diversity and complexity of estrogen functions.

6. Identification of DERP (Detrusor Estrogen-Regulated Protein)

6.1. Strategies for scanning molecules of interest

In searching for the mediator(s) for the estrogen effects, we had originally started with Western blotting using antibodies to examine molecules that could potentially play a role in such effects. These molecules included various connexins, cadherins, growth factors including NGF, PDGF and CNTF, as well as cytokines such as IL-6. To date, we have not obtained any positive results using this strategy. This may be the result of a low protein expression of these substances, the poor sensitivity of some of the antibodies utilized or the fact that only a single and late time point after OVx was studied.

The 2D gel-microsequencing strategy proved to be much more advantageous. It was chosen over differential display[133], although both approaches are powerful methods to detect differentially expressed molecules (protein in the former example and gene in the latter). 2D gel-sequencing furnishes additional information such as identification of potentially relevant expressed proteins[134]. Moreover, differential display has three most noticeable drawbacks: high frequency of false positives, the short size of the differentially displayed fragments and the fact that cloned sequences tend to be at the 3' end of sequences, regions which are often poorly characterized and sometimes even unpublished. As a result, it is often necessary to use cloned short fragments to screen a full-length cDNA library in order to obtain larger clones and more useful sequence information[135]. Finally, controversy also exists regarding the reproducibility of differential display, whereas 2D gel approaches at the protein level tend to yield much more consistent results.

Our studies using 2D gels have proved to be successful. The string of protein spots described above was consistently expressed in the sham control samples, but was barely detectable in the OVx group, with a return to sham levels in the OVx+E2 group. Microsequencing of this string of gel spots indicated two relevant molecules: lumican and ITIH4.

6.2. Studies on lumican

Lumican is a member of the small interstitial proteoglycan proteins with leucine-rich repeat (LRR), and is widely expressed in the extracellular matrix (ECM) of many tissues including connective tissues, arteries, lung and others[136]. The presence of LRR suggests that lumican may be important for the ECM formation and maintenance due to its role in regulating collagen fibrillogenesis and mediating interactions with other ECM components[137,138]. Growth factors/cytokines have been shown to modulate the molecular form of lumican probably via a direct effect on synthesis within cells[139]. Furthermore, age-related variation in growth factors and hormones present in the ECM or their receptors present on the cell surface could contribute to the changes in lumican structure observed with age[117,139]. Based on the above information, it is reasonable to speculate that

lumican may have a role in mediating some of the effects of estrogen on the mammalian bladder.

Western blotting combined with protein resolution in 1D or 2D gels revealed no difference between OVx and sham controls, but a "smeared" signal was observed in 2-D gels at ~80 Kd with vertically long streaks. Careful observations matched this string of spots with the "smeared" string on Ag-stained 2D gels localized right beneath the clear spot string of our interest. Personal communication with Dr. Roughley (who provided us with the lumican antibody) also confirmed the size and the "smeared" character of the lumican protein. Thus, lumican was excluded as our protein of interest.

6.3. Studies on ITIH4

As a result of more recent studies, we believe that "DERP" (Detrusor Estrogen-Regulated Protein) is closely related to ITIH4. Combining 2D gel protein resolution with Western blot detection using antiserum raised against pig ITIH4 we observed a clear string of signal at ~110 Kd and a p*I* of 4-5. In addition, signals from the sham samples appeared to be stronger than those from the OVx group.

ITIH4 belongs to the ITI (Inter-alpha trypsin inhibitor) protein family of plasma serine protease inhibitors, with a highly conserved N-terminal 2/3 and a variable C-terminal 3rd [140-142]. Proteins of this family harbor a von Willebrand type A (VWA) domain suggesting a heterophilic binding capacity[140], a calcium binding site and the reactive site as α-2-thiol-protease inhibitors[141]. Analysis of amino acid sequences revealed that ITIH4 possesses several potential N-glycosylation sites, although there are no Cys-rich domain as are often found in many plasma proteins. Interestingly, some unique properties have been found in its C-terminal 3rd. These include a proline-rich, bradykinin-like domain (suggestive of vulnerability to cleavage)[143], and a region homologous to the ATP-dependent proteases, with the significance of the latter remaining to be confirmed[140,143].

Being a recently cloned protein [140,141], ITIH4 is not well characterized, and little is known about its regulation and function[144]. Our data suggest that it may be regulated by OVx and estrogen, and that it may play a potential role in bladder muscle integrity. For instance, the presence of a site with potential protease inhibitory activity suggests that down-regulation of DERP following OVx could result in diminished inhibition of endogenous proteases with increased proteolysis, as well as muscle compartment loss (sarcopenia) and impaired contractility. We have not yet obtained full sequence on our proteins. We have assigned the name "DERP" (Detrusor Estrogen-Regulated Protein) to this protein in order to emphasize its relationship with estrogen and its origin from detrusor tissue, while also acknowledging the possibility that this protein may be closely related to ITI-H4 rather than being ITIH4 itself.

Recently, endometrial gene expression of ITI-H4 was detected during the estrous cycle and early pregnancy in pig, suggesting an ITI-H4 protective role of the uterus from the inflammatory response induced by conceptus attachment to the uterine epithelium[145]. This also suggests that ITIH4 may be regulated by estrogen (supporting our data), and that uterine tissues can synthesize ITIH4. Combined with studies that showed ITI-H4 mRNA expression in such smooth muscle tissues as stomach and intestine in addition to liver, its major expression site[146], it is reasonable to speculate that bladder can synthesize ITIH4. However, as ITIH4 was first identified as a serum protein [118], and was shown to act as an acute phase protein[144,146,147], it could also function as a serum protein binding nonspecifically to the bladder. Our initial RT-PCR studies using primers for ITIH4 have yielded signals that demonstrate ITIH4 mRNA expression in the rodent bladder. Efforts are underway to examine whether OVx affect ITIH4 mRNA expression.

The mechanism whereby DERP appears as a string of differentially charged protein spots and subsequently disappears after OVx currently remains unexplained. Nevertheless, several potential explanations can be considered. It is possible that the string of spots represents several entirely distinct protein molecules all with the same molecular weight, yet with 5-7 different p*I*s. This is unlikely due to the fact that the 5-7 distinct spots are all regulated in the same pattern by OVx and E2 treatment, and that the sequencing of the

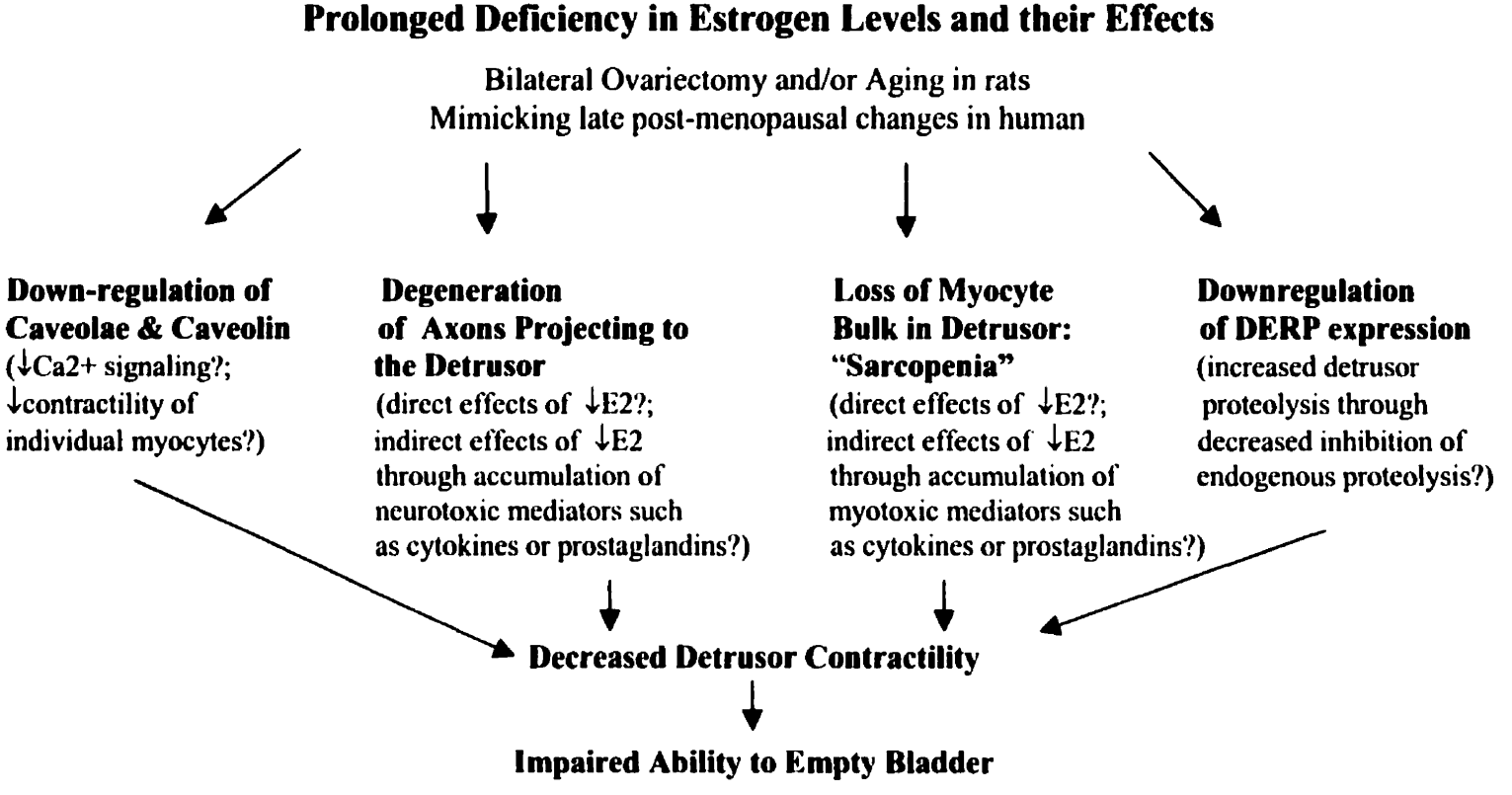
pooled protein homogenate representing all of the spots did not point to several different proteins. Thus, this string of protein spots more likely represents isoforms of the same protein resulting from a variety of possible post-translational modifications. One possible mechanism is through variable glycosylation. Not only can this in some cases (i.e. addition of sialic acid residues) modify the overall charge on a protein, but a variety of studies point to differences in glycosylation of specific molecules (i.e. NCAM) which occur in the setting of development and pathological events and which can have important functional consequences[148,149]. Phosphorylation can also cause alterations in charge, but this is unlikely in our case because phosphorylation usually takes place in intracellular proteins (signaling molecules in particular), but not in serum proteins.

We have consistently shown by 2D and silver staining that DERP was nearly undetectable 4 months after OVx, but returned to sham levels with E2 treatment as a string of protein spots at ~110 Kd with p*I* range at 4-5. This regulation could take place at different levels varying from transcription, RNA processing, translation, to post-translational modifications. Our experiment combining 2D gels with Western blots revealed a string of signals at the apparently correct position in the OVx sample, although of much lower intensity when compared to sham controls. Unfortunately, this particular experiment was not entirely convincing since the background surrounding the signals was very strong in the sham control. Due to technical difficulties, we have not been able to reproduce this result with more samples. Nevertheless, this single experiment suggests downregulation of DERP at either the level of mRNA or protein production, or through more rapid protein degradation, or through changes in its ability to enter and bind bladder tissue. Other potential experiments to explain our findings include alteration of the degree of a type of glycosylation (sialination), since the addition of small numbers of sialic acid residues to a large protein can alter its p*I* without changing its kD detectable on routine gel electrophoresis[149]. Future studies need to aim at clarifying the following questions by *in vivo* and *in vitro* studies: What is the identity of DERP exactly? Why does it normally appear as a string of spots? How is it regulated by OVx and estrogen? What else regulates it and how? Which cells in the detrusor produce it and how is it released into the serum? What is its role in ensuring normal detrusor structure and function?

SUMMARY

In the course of our studies we have observed the development of a number of changes involving both the detrusor and its innervation following chronic bilateral ovariectomy. As illustrated in the schematic figure presented in the next page, a number of these changes, including decreases in numbers of myocyte caveolae, axonal degeneration, as well as sarcopenia of the detrusor could all contribute to decreases in detrusor contractility. We have also searched for molecules that could mediate some of the effects of E2 in the bladder. As a result of these efforts, we have identified DERP (possibly ITI-H4) as a potential candidate. Our results provide a pathophysiologic basis whereby post-menopausal declines in ovarian hormones could contribute to the pathogenesis of IC in women late in life. Future studies will be required to clarify the relationship between hormonal status and IC in elderly women, while characterising the potential roles played by DERP and other possible mediators such as prostaglandins and cytokines.

Model: Schematic summary highlighting those cellular effects of prolonged estrogen deficiency which may contribute to the pathogenesis of impaired detrusor contractility in late life. Unproven or speculative effects and mechanisms are presented in brackets.



FIGURES AND LEGENDS

FIG. 1. Structure of detrusor muscle fascicles and extracellular spaces in sections obtained from sham and bilaterally ovariectomized female rats. A. Sham-operated animal. Fascicles (F) of several muscle cells, many nucleated; narrow largely empty spaces between fascicles. X 5,050, No. J-52719.

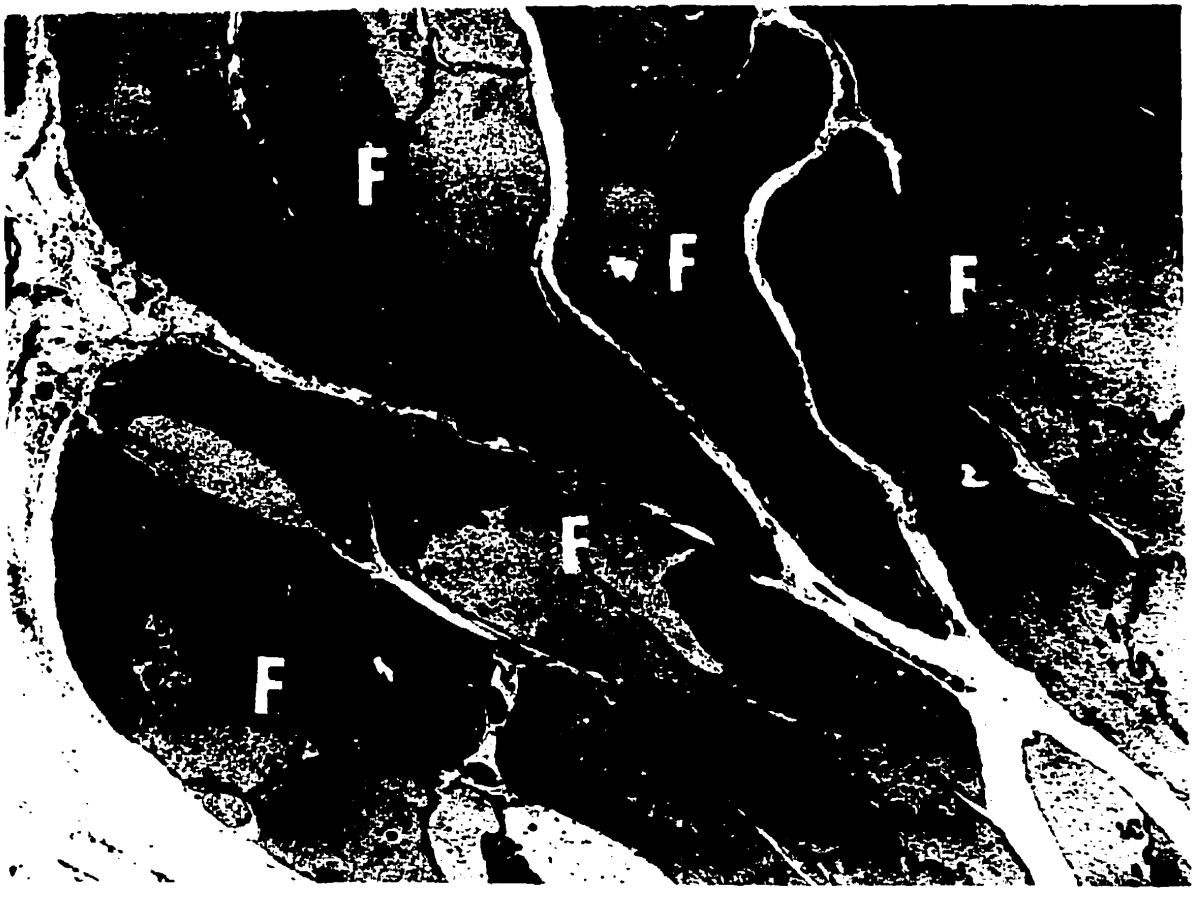


Fig. 1B. 4 m OVx animal. Spaces between fascicles wider and contain collagen. X 5,050, No. J-52689.

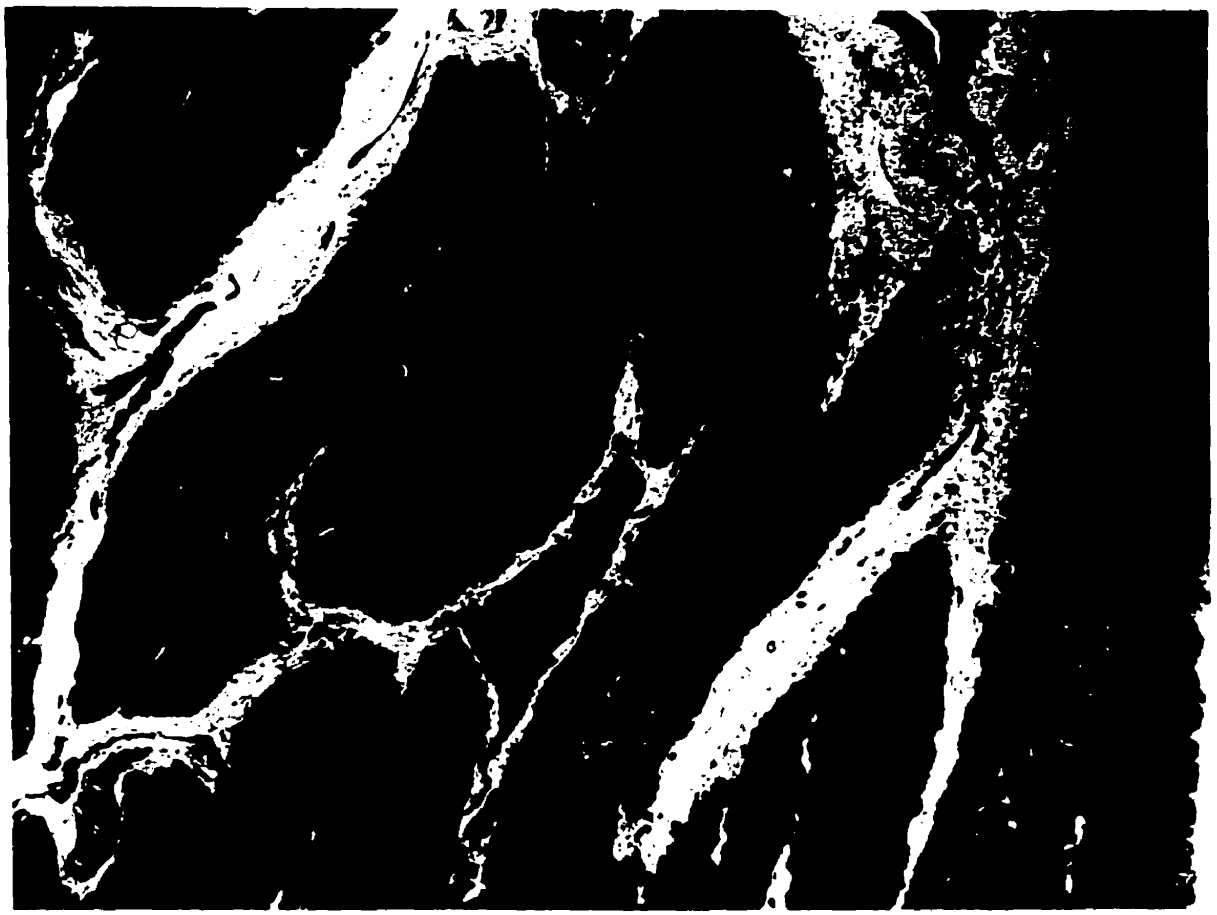


FIG. 2A. Loss of detrusor muscle bulks (sarcopenia) 4 m after OVx. Computer assisted analysis of light microscopic images from sham-operated (n=7) vs. OVx (n=7) animals; statistically significant ($p<0.001$; double asterisk).

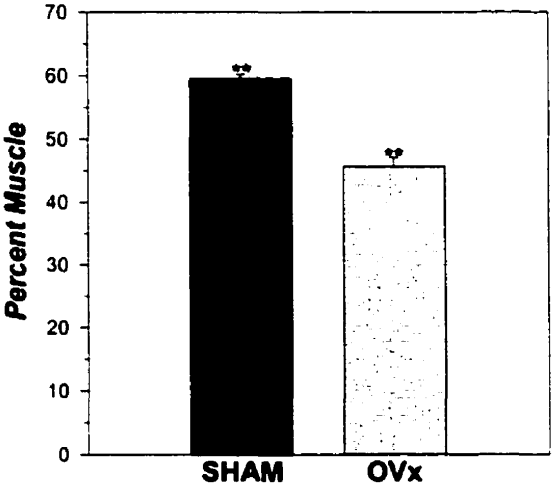


FIG. 2B. Reduced nucleated detrusor muscle cells (nucleated) after OVx. Number of cells significantly lower (asterisk) after OVx (n=7), as compared to sham-operated (n=7) animals ($p < 0.05$).

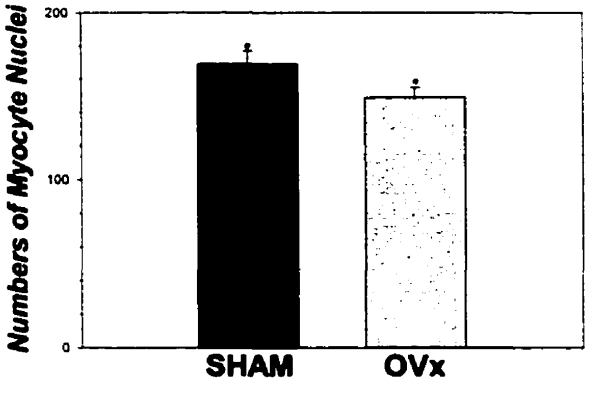


FIG. 2C. Fewer smaller muscle profiles (< 50 μm^2) images from OVx (n=6) than images from sham-operated animals (n=6); apparent shift towards medium-sized (50-100 μm^2) and larger (>100μm^2) profiles in the former.

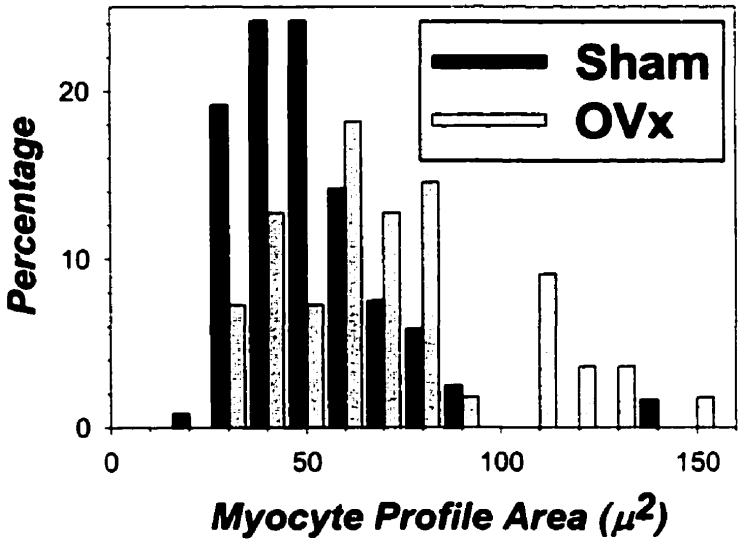


FIG. 3. Detrusor after OVx. Profiles of degenerated (arrows) and intact (thick arrow) axons (X 20,050 , No. J-47569).

FIG. 4. Comparison of carbachol-stimulated contractile force in 12 muscle strips from 4 sham-operated and 12 from 4 OVx animals.

A. Generated tension greater in muscle strips from sham-operated than strips from OVx animals (2-way ANOVA; $p < 0.001$). **B.** Tensions expressed per muscle strip weight reduced but differences still statistically significant (2-way ANOVA; $p < 0.05$). **C. & D.** Generated tension as a percentage of maximal contraction for that strip expressed per strip (C) or strip weight (D); no significant differences.

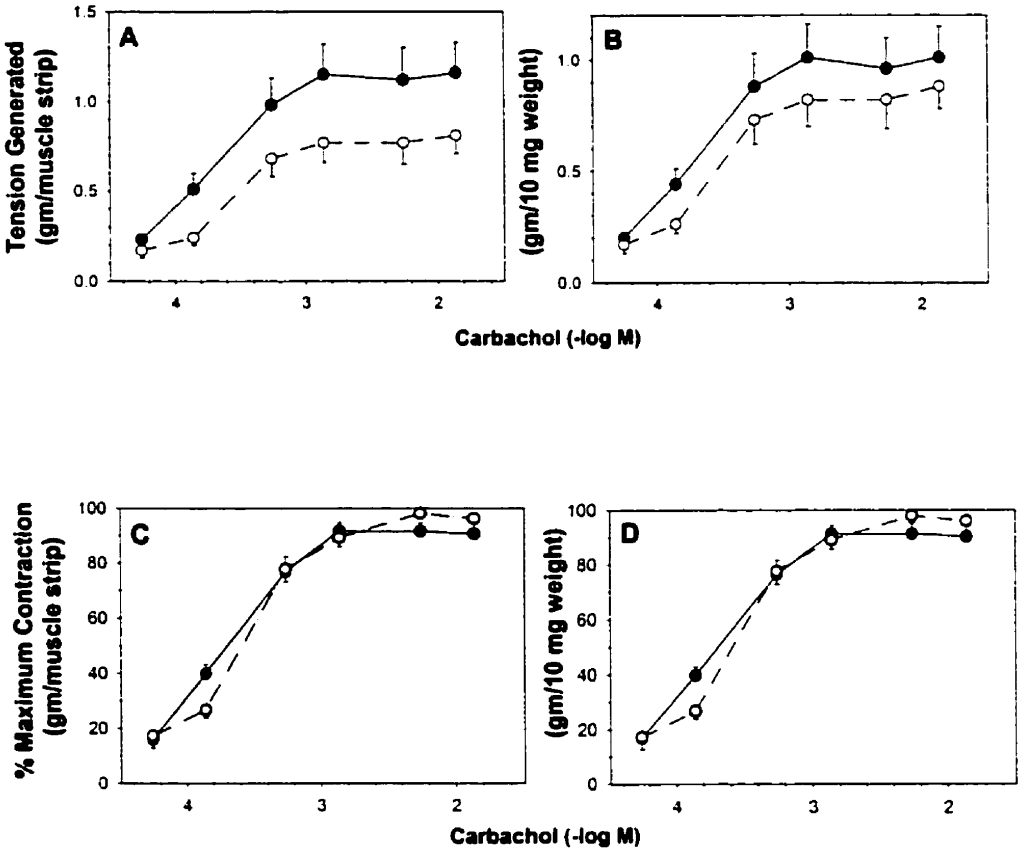


Fig. 5. Caveolar downregulation following OVx.

A. Detrusor tissue from a sham-operated rat displaying the characteristic features of a normal sarcolemma. These include alternating electron dense and thinner zones, with the latter containing many caveolae characterized as flask-shaped, relatively uniform, ordered arrangement (arrows). Cisternae of endoplasmic reticulum and mitochondria are often observed beneath caveolae. Bar=0.21μm

B. In OVx specimen, the myocyte sarcolemma was dominated by long electron dense bands with fewer interposed caveolae (arrows).

C. Numbers of caveolae per 1000 pixels of membrane perimeter in high magnification photomicrographs of detrusor tissues from 5 pairs of sham and OVx rats ($p<0.005$).

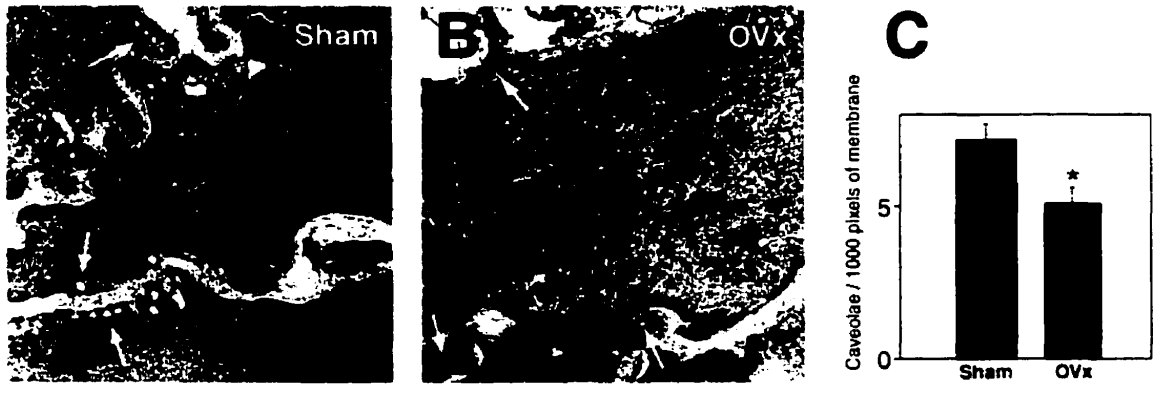


Fig. 6. Expression of Caveolin-1 protein is downregulated by OVx

A. Western blot for caveolin-1 and α-smooth muscle actin protein using equal amount of protein extract from detrusors of paired sham-operated and OVx animals. **B.** Graph of densitometry results from a total of 14 animals. The caveolin-1 or α-smooth muscle actin band intensity in samples from 7 OVx animals was expressed as a percentage of the band intensity in their paired sham-operated controls ($p<0.05$).

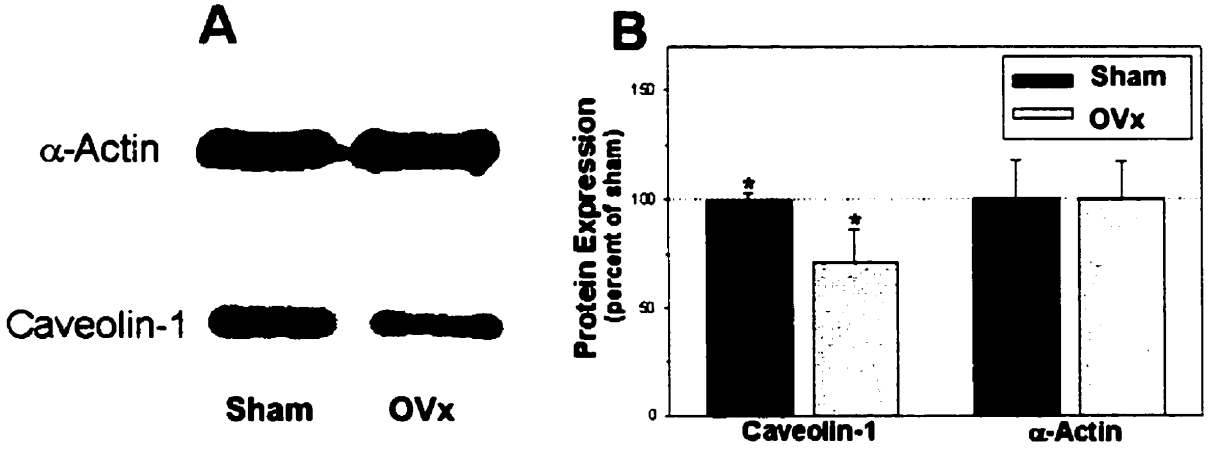


Fig. 7. Caveolin-1 downregulation is reversed by E2 treatment. Western blot of caveolin-1 and α-smooth muscle actin in animals treated for 1 month with subcutaneous silastic implants containing placebo (OVx) or E2 (OVx+E2) four month after OVx surgery.

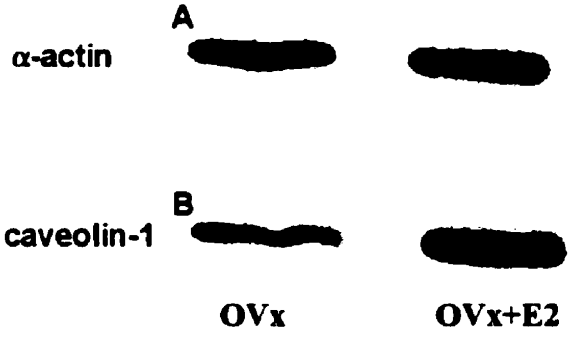


Fig. 8. Caveolin-1: a marker of myocyte maturation? A. Western blot for caveolin-1 and α-smooth muscle actin in detrusors obtained from newborn (day 2) and young adult (one month old) female rats. Results are representative of studies involving a total of 8 rats. **B.** The caveolin-1 or α-smooth muscle actin band intensities in samples from a total of 8 animals are expressed as a percentage of the band intensity in young adult as a percentage of that in paired newborn animals ($p<0.001$).

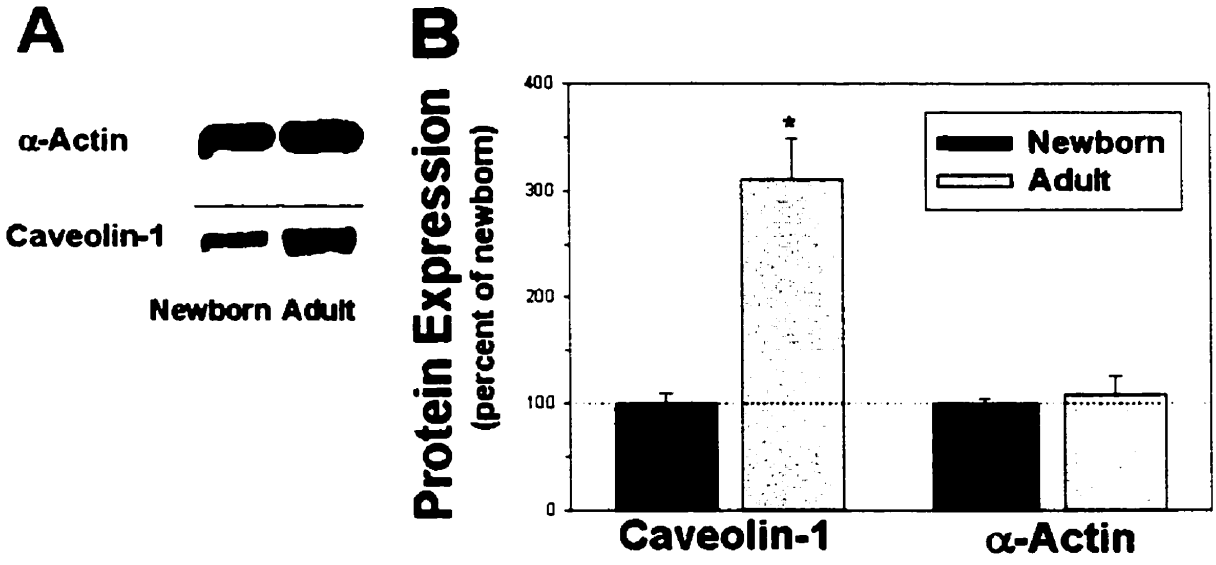


Fig. 9. Estrogen receptor mRNA expression in detrusor, bladder outlet and ovary. Total RNA was extracted from bladder detrusor, outlet and ovary of adult female rat, the latter serving as positive control and negative control ("ovary RT-"). RT-PCR was carried out with ER-α (**A**) and ER-β (**B**) primers. The final products were resolved in 1% agarose/ethidium bromide gel.

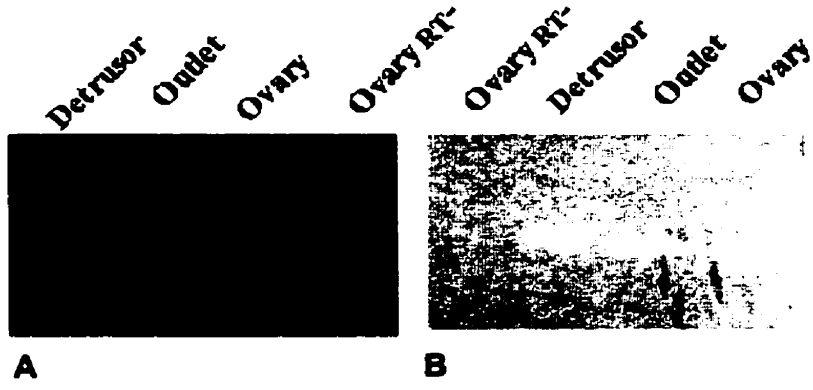


Fig. 10. 2D gel protein patterns. Protein extracts from detrusors of sham (**A**), OVx (**B**) and OVx +E2 (**C**) animal were resolved in first dimension by IEF followed by SDS-PAGE for the second dimension. The protein signals were visualized by Ag staining. The arrows indicate the position of the protein string of interest (~110 kD, p*I* 4-5), and the white arrows the string of fuzzy signals (lumican, 84kDa).

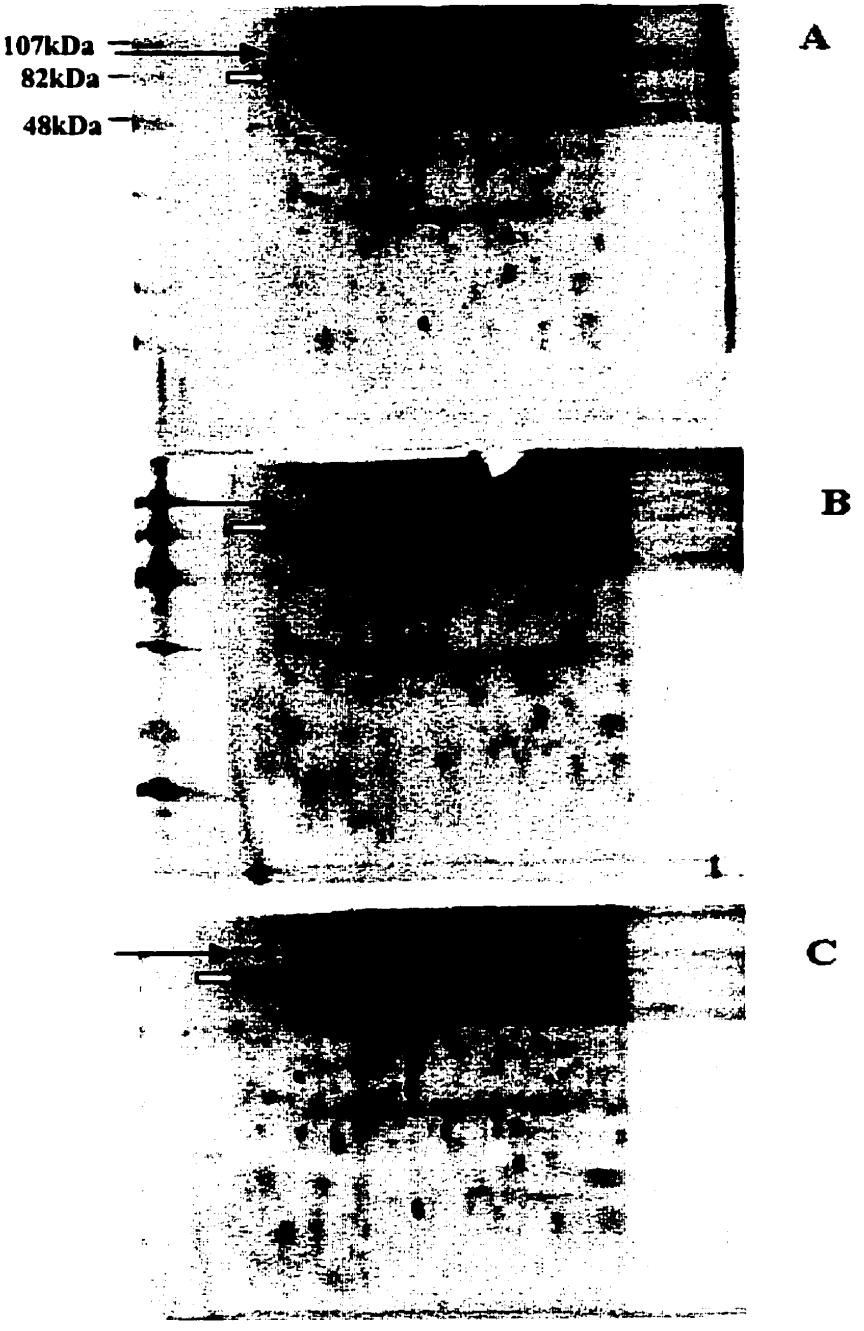


Fig. 11. Lumican excluded. Western blotting was carried out following SDS-PAGE (**A**) and 2D-gel electrophoresis (**B**) using detrusor protein extracts from sham or OVx animals, probing with polyclonal antibody against lumican.

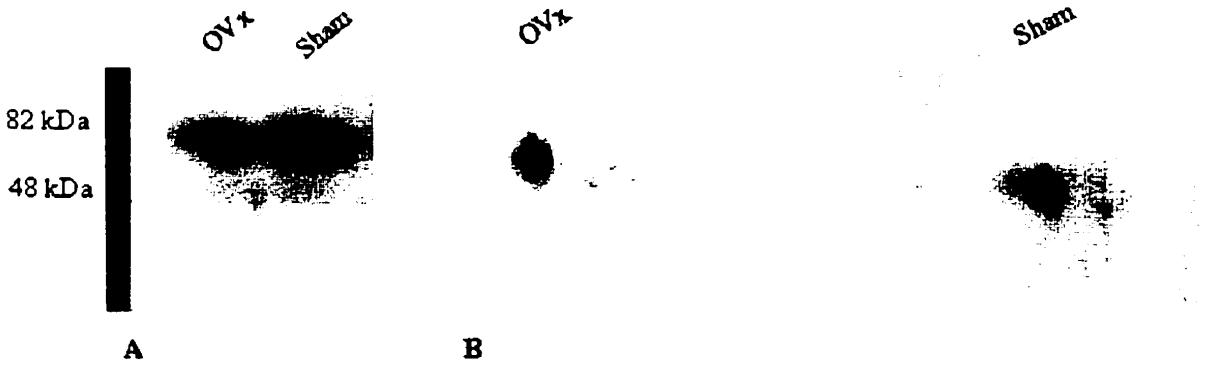
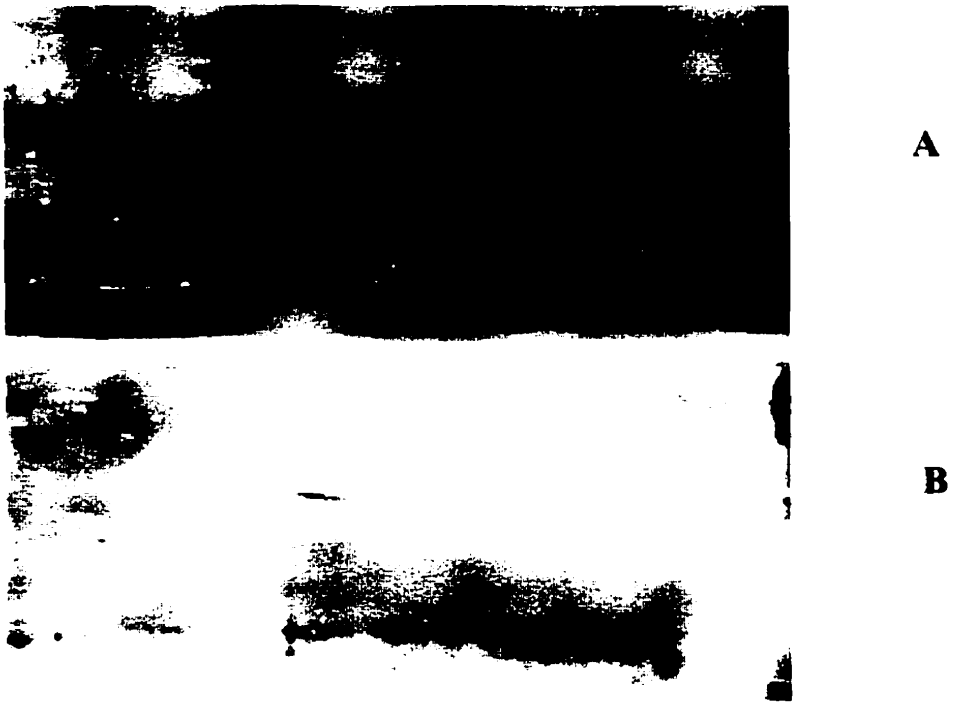


Fig. 12. Studies on ITIH4. 2D gel electrophoresis of protein samples from sham (**A**) or OVx (**B**) rat, followed by Western blotting using antiserum against pig ITIH4.



REFERENCES

1. Resnick,N.M. Urinary incontinence. *Lancet* **346**, 94-99 (1995).

2. Resnick,N.M., Yalla,S.V. & Laurino,E. The pathophysiology of urinary incontinence among institutionalized elderly persons [see comments]. *N. Engl. J. Med.* **320**, 1-7 (1989).

3. Vera,P.L. & Nadelhaft,I. Afferent and sympathetic innervation of the dome and the base of the urinary bladder of the female rat. *Brain Res. Bull.* **29**, 651-658 (1992).

4. Elbadawi,A., Yalla,S.V. & Resnick,N.M. Structural basis of geriatric voiding dysfunction. III. Detrusor overactivity. *J. Urol.* **150**, 1668-1680 (1993).

5. Elbadawi,A., Yalla,S.V. & Resnick,N.M. Structural basis of geriatric voiding dysfunction. II. Aging detrusor: normal versus impaired contractility. *J. Urol.* **150**, 1657-1667 (1993).

6. Elbadawi,A., Yalla,S.V. & Resnick,N.M. Structural basis of geriatric voiding dysfunction. IV. Bladder outlet obstruction. *J. Urol.* **150**, 1681-1695 (1993).

7. Resnick,N.M. & Yalla,S.V. Management of urinary incontinence in the elderly. *N. Engl. J. Med.* **313**, 800-805 (1985).

8. de Groat,W.C. Nervous control of the urinary bladder of the cat. *Brain Res.* **87**, 201-211 (1975).

9. Vera,P.L. & Nadelhaft,I. Conduction velocity distribution of afferent fibers innervating the rat urinary bladder. *Brain Res.* **520**, 83-89 (1990).

10. Levin,R.M. & Wein,A.J. Distribution and function of adrenergic receptors in the urinary bladder. *Mol. Pharmacol.* **16**, 441-448 (1979).

11. Chai,T.C. & Steers,W.D. Neurophysiology of micturition and continence. *Urol. Clin. North Am.* **23**, 221-236 (1996).

12. Dixon,J.S. & Gosling,J.A. Ultrastructure of Smooth Muscle. Motta,P.M. (ed.), pp. 153-169 (Kluwer Academic Publishers,1990).

13. McConnell,J., Benson,G.S. & Wood,J.G. Autonomic innervation of the urogenital system: adrenergic and cholinergic elements. *Brain Res. Bull.* **9**, 679-694 (1982).

14. Johnson,J.M., Skau,K.A., Gerald,M.C. & Wallace,L.J. Regional noradrenergic and cholinergic neurochemistry in the rat urinary bladder: effects of age. *J. Urol.* **139**, 611-615 (1988).

15. Hassouna,M., Abdel-Hakim,A., Abdel-Rahman,M., Galeano,C. & Elhilali,M.M. Response of the urethral smooth muscles to pharmacological agents. I. Cholinergic and adrenergic agonists and antagonists. *J. Urol.* **129**, 1262-1264 (1983).

16. Warburton,A.L. & Santer,R.M. Sympathetic and sensory innervation of the urinary tract in young adult and aged rats: a semi-quantitative histochemical and immunohistochemical study. *Histochem. J.* **26**, 127-133 (1994).

17. Ordway,G.A., Esbenshade,T.A., Kolta,M.G., Gerald,M.C. & Wallace,L.J. Effect of age on cholinergic muscarinic responsiveness and receptors in the rat urinary bladder. *J. Urol.* **136**, 492-496 (1986).

18. Ordway,G.A., Kolta,M.G., Gerald,M.C. & Wallace,L.J. Age-related change in alpha-adrenergic responsiveness of the urinary bladder of the rat is regionally specific. *Neuropharmacology* **25**, 1335-1340 (1986).

19. Levin,R.M., Shofer,F.S. & Wein,A.J. Estrogen-induced alterations in the autonomic responses of the rabbit urinary bladder. *J. Pharmacol. Exp. Ther.* **215**, 614-618 (1980).

20. Maeda,A., Kubo,T., Mishina,M. & Numa,S. Tissue distribution of mRNAs encoding muscarinic acetylcholine receptor subtypes. *FEBS Lett.* **239**, 339-342 (1988).

21. Yamaguchi,O. *et al.* Evaluation of mRNAs encoding muscarinic receptor subtypes in human detrusor muscle. *J. Urol.* **156**, 1208-1213 (1996).

22. Wall,S.J., Yasuda,R.P., Li,M. & Wolfe,B.B. Development of an antiserum against m3 muscarinic receptors: distribution of m3 receptors in rat tissues and clonal cell lines. *Mol. Pharmacol.* **40**, 783-789 (1991).

23. Wein,A.J. Campbell's Urology. (1992).

24. Longhurst,P.A., Leggett,R.E. & Briscoe,J.A. Characterization of the functional muscarinic receptors in the rat urinary bladder. *Br. J. Pharmacol.* **116**, 2279-2285 (1995).

25. Mutoh,S., Latifpour,J., Saito,M. & Weiss,R.M. Evidence for the presence of regional differences in the subtype specificity of muscarinic receptors in rabbit lower urinary tract. *J. Urol.* **157**, 717-721 (1997).

26. Wang,P., Luthin,G.R. & Ruggieri,M.R. Muscarinic acetylcholine receptor subtypes mediating urinary bladder contractility and coupling to GTP binding proteins. *J. Pharmacol. Exp. Ther.* **273**, 959-966 (1995).

27. Hegde,S.S. *et al.* Functional role of M2 and M3 muscarinic receptors in the urinary bladder of rats in vitro and in vivo. *Br. J. Pharmacol.* **120**, 1409-1418 (1997).

28. Mimata,H. *et al.* Muscarinic receptor subtypes and receptor-coupled phosphatidylinositol hydrolysis in rat bladder smooth muscle. *Int. J. Urol.* **4**, 591-596 (1997).

29. Chancellor,M.B., Kaplan,S.A. & Blaivas,J.G. The cholinergic and purinergic components of detrusor contractility in a whole rabbit bladder model. *J. Urol.* **148**, 906-909 (1992).

30. Theobald,R.J., Jr. Purinergic and cholinergic components of bladder contractility and flow. *Life Sci.* **56**, 445-454 (1995).

31. Tong,Y.C., Hung,Y.C., Shinozuka,K., Kunitomo,M. & Cheng,J.T. Evidence of adenosine 5'-triphosphate release from nerve and P2x- purinoceptor mediated contraction during electrical stimulation of rat urinary bladder smooth muscle. *J. Urol.* **158**, 1973-1977 (1997).

32. Dutton,J.L., Hansen,M.A., Balcar,V.J., Barden,J.A. & Bennett,M.R. Development of P2X receptor clusters on smooth muscle cells in relation to nerve varicosities in the rat urinary bladder. *J. Neurocytol.* **28**, 4-16 (1999).

33. Maggi,C.A., Santicioli,P. & Meli,A. Postnatal development of myogenic contractile activity and excitatory innervation of rat urinary bladder. *Am. J. Physiol* **247**, R972-R978 (1984).

34. Nicke,A. *et al.* P2X1 and P2X3 receptors form stable trimers: a novel structural motif of ligand-gated ion channels. *EMBO J.* **17**, 3016-3028 (1998).

35. Fujii,K. Evidence for adenosine triphosphate as an excitatory transmitter in guinea-pig, rabbit and pig urinary bladder. *J. Physiol (Lond)* **404**, 39-52 (1988).

36. North,R.A. & Barnard,E.A. Nucleotide receptors. *Curr. Opin. Neurobiol.* **7**, 346-357 (1997).

37. Hoyle,C.H. & Burnstock,G. Atropine-resistant excitatory junction potentials in rabbit bladder are blocked by alpha,beta-methylene ATP. *Eur. J. Pharmacol.* **114**, 239-240 (1985).

38. Levin,R.M., Jacobowitz,D. & Wein,A.J. Autonomic innervation of rabbit urinary bladder following estrogen administration. *Urology* **17**, 449-453 (1981).

39. Shapiro,E. Effect of estrogens on the weight and muscarinic cholinergic receptor density of the rabbit bladder and urethra. *J. Urol.* **135**, 1084-1087 (1986).

40. Batra,S. & Andersson,K.E. Oestrogen-induced changes in muscarinic receptor density and contractile responses in the female rabbit urinary bladder. *Acta Physiol Scand.* **137**, 135-141 (1989).

41. Furuichi,T., Kohda,K., Miyawaki,A. & Mikoshiba,K. Intracellular channels [published erratum appears in Curr Opin Neurobiol 1994 Oct;4(5):758]. *Curr. Opin. Neurobiol.* **4**, 294-303 (1994).

42. Gong,C., Zderic,S.A. & Levin,R.M. Ontogeny of the ryanodine receptor in rabbit urinary bladder smooth muscle. *Mol. Cell Biochem.* **137**, 169-172 (1994).

43. Sobieszek,A. & Small,J.V. Myosin-linked calcium regulation in vertebrate smooth muscle. *J. Mol. Biol.* **102**, 75-92 (1976).

44. Murphy,R.A. What is special about smooth muscle? The significance of covalent crossbridge regulation. *FASEB J.* **8**, 311-318 (1994).

45. Steinberg,M.S. & McNutt,P.M. Cadherins and their connections: adhesion junctions have broader functions. *Curr. Opin. Cell Biol.* **11**, 554-560 (1999).

46. Troyanovsky,S.M. Mechanism of cell-cell adhesion complex assembly. *Curr. Opin. Cell Biol.* **11**, 561-566 (1999).

47. Hendrix,E.M., Mao,S.J., Everson,W. & Larsen,W.J. Myometrial connexin 43 trafficking and gap junction assembly at term and in preterm labor. *Mol. Reprod. Dev.* **33**, 27-38 (1992).

48. Puri,C.P. & Garfield,R.E. Changes in hormone levels and gap junctions in the rat uterus during pregnancy and parturition. *Biol. Reprod.* **27**, 967-975 (1982).

49. Mackenzie,L.W. & Garfield,R.E. Effects of tamoxifen citrate and cycloheximide on estradiol induction of rat myometrial gap junctions. *Can. J. Physiol Pharmacol.* **64**, 703-706 (1986).

50. Lefebvre,D.L., Piersanti,M., Bai,X.H., Chen,Z.Q. & Lye,S.J. Myometrial transcriptional regulation of the gap junction gene, connexin-43. *Reprod. Fertil. Dev.* **7**, 603-611 (1995).

51. Piersanti,M. & Lye,S.J. Increase in messenger ribonucleic acid encoding the myometrial gap junction protein, connexin-43, requires protein synthesis and is associated with increased expression of the activator protein-1, c-fos. *Endocrinology* **136**, 3571-3578 (1995).

52. Geimonen,E., Boylston,E., Royek,A. & Andersen,J. Elevated connexin-43 expression in term human myometrium correlates with elevated c-Jun expression and is independent of myometrial estrogen receptors. *J. Clin. Endocrinol. Metab* **83**, 1177-1185 (1998).

53. Parton,R.G. Caveolae and caveolins. *Curr. Opin. Cell Biol.* **8**, 542-548 (1996).

54. Lisanti,M.P. *et al.* Caveolae, transmembrane signalling and cellular transformation. *Mol. Membr. Biol.* **12**, 121-124 (1995).

55. Razani,B., Schlegel,A. & Lisanti,M.P. Caveolin proteins in signaling, oncogenic transformation and muscular dystrophy. *J. Cell Sci.* **113**, 2103-2109 (2000).

56. Kurzchalia,T.V., Dupree,P. & Monier,S. VIP21-Caveolin, a protein of the trans-Golgi network and caveolae. *FEBS Lett.* **346**, 88-91 (1994).

57. Glenney,J.R., Jr. & Soppet,D. Sequence and expression of caveolin, a protein component of caveolae plasma membrane domains phosphorylated on tyrosine in Rous sarcoma virus-transformed fibroblasts. *Proc. Natl. Acad. Sci. U. S. A* **89**, 10517-10521 (1992).

58. Lipardi,C. *et al.* Caveolin transfection results in caveolae formation but not apical sorting of glycosylphosphatidylinositol (GPI)-anchored proteins in epithelial cells. *J. Cell Biol.* **140**, 617-626 (1998).

59. Scherer,P.E. *et al.* Induction of caveolin during adipogenesis and association of GLUT4 with caveolin-rich vesicles. *J. Cell Biol.* **127**, 1233-1243 (1994).

60. Koleske,A.J., Baltimore,D. & Lisanti,M.P. Reduction of caveolin and caveolae in oncogenically transformed cells. *Proc. Natl. Acad. Sci. U. S. A* **92**, 1381-1385 (1995).

61. Feron,O. *et al.* Modulation of the endothelial nitric-oxide synthase-caveolin interaction in cardiac myocytes. Implications for the autonomic regulation of heart rate. *J. Biol. Chem.* **273**, 30249-30254 (1998).

62. Engelman,J.A. *et al.* Recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage-independent growth. *J. Biol. Chem.* **272**, 16374-16381 (1997).

63. Thyberg,J. *et al.* Expression of caveolae on the surface of rat arterial smooth muscle cells is dependent on the phenotypic state of the cells. *Lab Invest* **77**, 93-101 (1997).

64. Galbiati,F. *et al.* Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade. *EMBO J.* **17**, 6633-6648 (1998).

65. Isshiki,M. *et al.* Endothelial Ca2+ waves preferentially originate at specific loci in caveolin-rich cell edges. *Proc. Natl. Acad. Sci. U. S. A* **95**, 5009-5014 (1998).

66. Fujimoto,T., Nakade,S., Miyawaki,A., Mikoshiba,K. & Ogawa,K. Localization of inositol 1,4,5-trisphosphate receptor-like protein in plasmalemmal caveolae. *J. Cell Biol.* **119**, 1507-1513 (1992).

67. Fujimoto,T. Calcium pump of the plasma membrane is localized in caveolae. *J. Cell Biol.* **120**, 1147-1157 (1993).

68. Strosberg,A.D. Structure/function relationship of proteins belonging to the family of receptors coupled to GTP-binding proteins. *Eur. J. Biochem.* **196**, 1-10 (1991).

69. Kuiper,G.G., Enmark,E., Pelto-Huikko,M., Nilsson,S. & Gustafsson,J.A. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. U. S. A* **93**, 5925-5930 (1996).

70. Tremblay,G.B. *et al.* Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol. Endocrinol.* **11**, 353-365 (1997).

71. Razandi,M., Pedram,A., Greene,G.L. & Levin,E.R. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. *Mol. Endocrinol.* **13**, 307-319 (1999).

72. Pettersson,K., Grandien,K., Kuiper,G.G. & Gustafsson,J.A. Mouse estrogen receptor beta forms estrogen response element-binding heterodimers with estrogen receptor alpha. *Mol. Endocrinol.* **11**, 1486-1496 (1997).

73. Paech,K. *et al.* Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites [see comments]. *Science* **277**, 1508-1510 (1997).

74. Kuiper,G.G. *et al.* Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **138**, 863-870 (1997).

75. Mosselman,S., Polman,J. & Dijkema,R. ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett.* **392**, 49-53 (1996).

76. Lazennec,G., Ediger,T.R., Petz,L.N., Nardulli,A.M. & Katzenellenbogen,B.S. Mechanistic aspects of estrogen receptor activation probed with constitutively active estrogen receptors: correlations with DNA and coregulator interactions and receptor conformational changes. *Mol. Endocrinol.* **11**, 1375-1386 (1997).

77. Gustafsson,J.A. An update on estrogen receptors. *Semin. Perinatol.* **24**, 66-69 (2000).

78. Wehling,M. Specific, nongenomic actions of steroid hormones. [Review] [151 refs]. *Annual Review of Physiology* **59**, 365-393 (1997).

79. Aronica,S.M., Kraus,W.L. & Katzenellenbogen,B.S. Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. *Proc. Natl. Acad. Sci. U. S. A* **91**, 8517-8521 (1994).

80. Pappas,T.C., Gametchu,B. & Watson,C.S. Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. *FASEB J.* **9**, 404-410 (1995).

81. Tesarik,J. & Mendoza,C. Nongenomic effects of 17 beta-estradiol on maturing human oocytes: relationship to oocyte developmental potential. *J. Clin. Endocrinol. Metab* **80**, 1438-1443 (1995).

82. Chen,Z. *et al.* Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen [published erratum appears in J Clin Invest 1999 May;103(9):1363]. *J. Clin. Invest* **103**, 401-406 (1999).

83. Lieberherr,M., Grosse,B., Kachkache,M. & Balsan,S. Cell signaling and estrogens in female rat osteoblasts: a possible involvement of unconventional nonnuclear receptors. *J. Bone Miner. Res.* **8**, 1365-1376 (1993).

84. Le,M., V, Grosse,B. & Lieberherr,M. Phospholipase C beta and membrane action of calcitriol and estradiol. *J. Biol. Chem.* **272**, 11902-11907 (1997).

85. Nelson,K.G., Takahashi,T., Bossert,N.L., Walmer,D.K. & McLachlan,J.A. Epidermal growth factor replaces estrogen in the stimulation of female genital-tract growth and differentiation. *Proc. Natl. Acad. Sci. U. S. A* **88**, 21-25 (1991).

86. Collins,P. & Webb,C. Estrogen hits the surface [news] [see comments]. *Nat. Med.* **5**, 1130-1131 (1999).

87. Morey,A.K. *et al.* Estrogen and progesterone inhibit vascular smooth muscle proliferation. *Endocrinology* **138**, 3330-3339 (1997).

88. Morey,A.K. *et al.* Oestrogen and progesterone inhibit the stimulated production of endothelin-1. *Biochem. J.* **330 (Pt 3)**, 1097-1105 (1998).

89. Papka,R.E., Srinivasan,B., Miller,K.E. & Hayashi,S. Localization of estrogen receptor protein and estrogen receptor messenger RNA in peripheral autonomic and sensory neurons. *Neuroscience* **79**, 1153-1163 (1997).

90. Taleghany,N., Sarajari,S., DonCarlos,L.L., Gollapudi,L. & Oblinger,M.M. Differential expression of estrogen receptor alpha and beta in rat dorsal root ganglion neurons. *J. Neurosci. Res.* **57**, 603-615 (1999).

91. Petersen,D.N., Tkalcevic,G.T., Koza-Taylor,P.H., Turi,T.G. & Brown,T.A. Identification of estrogen receptor beta2, a functional variant of estrogen receptor beta expressed in normal rat tissues. *Endocrinology* **139**, 1082-1092 (1998).

92. Maruyama,K. *et al.* A novel isoform of rat estrogen receptor beta with 18 amino acid insertion in the ligand binding domain as a putative dominant negative regular of estrogen action. *Biochem. Biophys. Res. Commun.* **246**, 142-147 (1998).

93. Saunders,P.T., Maguire,S.M., Gaughan,J. & Millar,M.R. Expression of oestrogen receptor beta (ER beta) in multiple rat tissues visualised by immunohistochemistry. *J. Endocrinol.* **154**, R13-R16 (1997).

94. Prins,G.S. *et al.* Estrogen receptor-beta messenger ribonucleic acid ontogeny in the prostate of normal and neonatally estrogenized rats. *Endocrinology* **139**, 874-883 (1998).

95. Singer,C.A., Rogers,K.L. & Dorsa,D.M. Modulation of Bcl-2 expression: a potential component of estrogen protection in NT2 neurons. *Neuroreport* **9**, 2565-2568 (1998).

96. Bishop,J. & Simpkins,J.W. Estradiol treatment increases viability of glioma and neuroblastoma cells in vitro. *Mol. Cell Neurosci.* **5**, 303-308 (1994).

97. Green,P.S., Bishop,J. & Simpkins,J.W. 17 alpha-estradiol exerts neuroprotective effects on SK-N-SH cells. *J. Neurosci.* **17**, 511-515 (1997).

98. Persky,A.M. *et al.* Protective effect of estrogens against oxidative damage to heart and skeletal muscle in vivo and in vitro. *Proc. Soc. Exp. Biol. Med.* **223**, 59-66 (2000).

99. Spady,T.J., McComb,R.D. & Shull,J.D. Estrogen action in the regulation of cell proliferation, cell survival, and tumorigenesis in the rat anterior pituitary gland. *Endocrine.* **11**, 217-233 (1999).

100. Behl,C. & Holsboer,F. The female sex hormone oestrogen as a neuroprotectant. *Trends Pharmacol. Sci.* **20**, 441-444 (1999).

101. Patrone,C., Andersson,S., Korhonen,L. & Lindholm,D. Estrogen receptor-dependent regulation of sensory neuron survival in developing dorsal root ganglion. *Proc. Natl. Acad. Sci. U. S. A* **96**, 10905-10910 (1999).

102. Judd,H.L. Hormonal dynamics associated with the menopause. *Clin. Obstet. Gynecol.* **19**, 775-788 (1976).

103. Klutke,J.J. & Bergman,A. Hormonal influence on the urinary tract. *Urol. Clin. North Am.* **22**, 629-639 (1995).

104. Batra,S.C. & Iosif,C.S. Female urethra: a target for estrogen action. *J. Urol.* **129**, 418-420 (1983).

105. Elia,G. & Bergman,A. Estrogen effects on the urethra: beneficial effects in women with genuine stress incontinence. *Obstet. Gynecol. Surv.* **48**, 509-517 (1993).

106. Iosif,C.S., Batra,S., Ek,A. & Astedt,B. Estrogen receptors in the human female lower uninary tract. *Am. J. Obstet. Gynecol.* **141**, 817-820 (1981).

107. Makela,S. *et al.* Differential expression of estrogen receptors alpha and beta in adult rat accessory sex glands and lower urinary tract [In Process Citation]. *Mol. Cell Endocrinol.* **164**, 109-116 (2000).

108. Longhurst,P.A., Kauer,J., Leggett,R.E. & Levin,R.M. The influence of ovariectomy and estradiol replacement on urinary bladder function in rats. *J. Urol.* **148**, 915-919 (1992).

109. Yono,M. *et al.* Effects of ovarian hormones on beta-adrenergic receptor-mediated relaxation in the female rabbit bladder. *Urol. Res.* **28**, 38-45 (2000).

110. Diep,N. & Constantinou,C.E. Age dependent response to exogenous estrogen on micturition, contractility and cholinergic receptors of the rat bladder. *Life Sci.* **64**, L-89 (1999).

111. Miller,M.M. & Franklin,K.B. Theoretical basis for the benefit of postmenopausal estrogen substitution. *Exp. Gerontol.* **34**, 587-604 (1999).

112. Judd,H.L. & Fournet,N. Changes of ovarian hormonal function with aging. *Exp. Gerontol.* **29**, 285-298 (1994).

113. Hazzard,D.G., Bronson,R.T., McClearn,G.E. & Strong,R. Selection of an appropriate animal model to study aging processes with special emphasis on the use of rat strains [editorial]. *J. Gerontol.* **47**, B63-B64 (1992).

114. Lu,K.H., Hopper,B.R., Vargo,T.M. & Yen,S.S. Chronological changes in sex steroid, gonadotropin and prolactin secretions in aging female rats displaying different reproductive states. *Biol. Reprod.* **21**, 193-203 (1979).

115. Legan,S.J., Coon,G.A. & Karsch,F.J. Role of estrogen as initiator of daily LH surges in the ovariectomized rat. *Endocrinology* **96**, 50-56 (1975).

116. Elbadawi,A., Yalla,S.V. & Resnick,N.M. Structural basis of geriatric voiding dysfunction. I. Methods of a prospective ultrastructural/urodynamic study and an overview of the findings. *J. Urol.* **150**, 1650-1656 (1993).

117. Sztrolovics,R., Alini,M., Mort,J.S. & Roughley,P.J. Age-related changes in fibromodulin and lumican in human intervertebral discs. *Spine* **24**, 1765-1771 (1999).

118. Gonzalez-Ramon,N. *et al.* The major acute phase serum protein in pigs is homologous to human plasma kallikrein sensitive PK-120. *FEBS Lett.* **371**, 227-230 (1995).

119. Pandey,A. & Mann,M. Proteomics to study genes and genomes. *Nature* **405**, 837-846 (2000).

120. Lorenzo,H.K. 2-D Proteome Analysis Protocols. Link,A.J. (ed.), pp. 111-127 (HUMANA PRESS,1999).

121. Krull,N.B. & Gressner,A.M. Differential expression of keratan sulphate proteoglycans fibromodulin, lumican and aggrecan in normal and fibrotic rat liver. *FEBS Lett.* **312**, 47-52 (1992).

122. Soury,E. *et al.* The H4P heavy chain of inter-alpha-inhibitor family largely differs in the structure and synthesis of its prolin-rich region from rat to human. *Biochem. Biophys. Res. Commun.* **243**, 522-530 (1998).

123. Holloszy J.O. Workshop on sarcopenia: Muscle atrophy in old age. *Journal of Gerontology, Series A: Biological Sciences and Medical Sciences* **50A Supple.**, (1995).

124. Roubenoff,R. & Hughes,V.A. Sarcopenia: current concepts.[In Process Citation]. *J. Gerontol. A Biol. Sci. Med. Sci.* **55**, M716-M724 (2000).

125. When Cells die. Wiley-Liss,Inc., (1998).

126. Hashimoto,T., Ishigooka,M., Zermann,D.H., Sasagawa,I. & Nakada,T. Effects of estrogen and progesterone on urinary bladder in female rabbit: evaluation by quantitative morphometric analysis. *Urology* **53**, 642-646 (1999).

127. Elbadawi A. & Schenk E.A. Dual innervation of the mammalian bladder. A histochemical study of the distribution of cholinergic and adrenergic nerves. *American Journal of Anatomy* **119**, 405 (1966).

128. Keast,J.R. & Saunders,R.J. Testosterone has potent, selective effects on the morphology of pelvic autonomic neurons which control the bladder, lower bowel and internal reproductive organs of the male rat. *Neuroscience* **85**, 543-556 (1998).

129. Baskin,L.S., Hayward,S.W., Young,P.F. & Cunha,G.R. Ontogeny of the rat bladder: smooth muscle and epithelial differentiation. *Acta Anat. (Basel)* **155**, 163-171 (1996).

130. Munoz,P. *et al.* Expression and insulin-regulated distribution of caveolin in skeletal muscle. Caveolin does not colocalize with GLUT4 in intracellular membranes. *J. Biol. Chem.* **271**, 8133-8139 (1996).

131. Bist,A., Fielding,P.E. & Fielding,C.J. Two sterol regulatory element-like sequences mediate up-regulation of caveolin gene transcription in response to low density lipoprotein free cholesterol. *Proc. Natl. Acad. Sci. U. S. A* **94**, 10693-10698 (1997).

132. Kuchel, G.A., Tannenbaum, C., Zhu, Q. et al. Role of Ovarian Hormones in the Pathogenesis of Impaired Detrusor Contractility.
II. Studies in Elderly Women. *Journal of Urology* (2000).

133. Liang,P. & Pardee,A.B. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction [see comments]. *Science* **257**, 967-971 (1992).

134. Cremona,O. *et al.* Differential protein expression in aortic smooth muscle cells cultured from newborn and aged rats. *Exp. Cell Res.* **217**, 280-287 (1995).

135. Christine Debouck. Differential display or differential dismay? *Current opinion in biotechnology* **6**, 597-599 (1995).

136. Grover,J., Chen,X.N., Korenberg,J.R. & Roughley,P.J. The human lumican gene. Organization, chromosomal location, and expression in articular cartilage. *J. Biol. Chem.* **270**, 21942-21949 (1995).

137. Rada,J.A., Cornuet,P.K. & Hassell,J.R. Regulation of corneal collagen fibrillogenesis in vitro by corneal proteoglycan (lumican and decorin) core proteins. *Exp. Eye Res.* **56**, 635-648 (1993).

138. Funderburgh,J.L. *et al.* Macrophage receptors for lumican. A corneal keratan sulfate proteoglycan. *Invest Ophthalmol. Vis. Sci.* **38**, 1159-1167 (1997).

139. Melching,L.I. & Roughley,P.J. Modulation of keratan sulfate synthesis on lumican by the action of cytokines on human articular chondrocytes. *Matrix Biol.* **18**, 381-390 (1999).

140. Salier,J.P., Rouet,P., Raguenez,G. & Daveau,M. The inter-alpha-inhibitor family: from structure to regulation. *Biochem. J.* **315 (Pt 1)**, 1-9 (1996).

141. Saguchi,K. *et al.* Cloning and characterization of cDNA for inter-alpha-trypsin inhibitor family heavy chain-related protein (IHRP), a novel human plasma glycoprotein. *J. Biochem. (Tokyo)* **117**, 14-18 (1995).

142. Luck,M. *et al.* Identification of plasma proteins facilitated by enrichment on particulate surfaces: analysis by two-dimensional electrophoresis and N- terminal microsequencing. *Electrophoresis* **18**, 2961-2967 (1997).

143. Hashimoto,K. *et al.* Primary structure of the pig homologue of human IHRP: inter-alpha- trypsin inhibitor family heavy chain-related protein. *J. Biochem. (Tokyo)* **119**, 577-584 (1996).

144. Pineiro,M. *et al.* ITIH4 serum concentration increases during acute-phase processes in human patients and is up-regulated by interleukin-6 in hepatocarcinoma HepG2 cells. *Biochem. Biophys. Res. Commun.* **263**, 224-229 (1999).

145. Geisert,R.D., Yelich,J.V., Pratt,T. & Pomp,D. Expression of an inter-alpha-trypsin inhibitor heavy chain-like protein in the pig endometrium during the oestrous cycle and early pregnancy. *J. Reprod. Fertil.* **114**, 35-43 (1998).

146. Daveau,M. *et al.* Hepatic and extra-hepatic transcription of inter-alpha-inhibitor family genes under normal or acute inflammatory conditions in rat. *Arch. Biochem. Biophys.* **350**, 315-323 (1998).

147. Gonzalez-Ramon,N. *et al.* Pig MAP/ITIH4 and haptoglobin are interleukin-6-dependent acute-phase plasma proteins in porcine primary cultured hepatocytes. *Eur. J. Biochem.* **267**, 1878-1885 (2000).

148. Paech,K., Balsan,S. & Devine P.L. 2-D Proteome Analysis Protocols. Link,A.J. (ed.), pp. 341-352 (Humana Press,1999).

149. Rutishauser,U. Polysialic acid and the regulation of cell interactions. *Curr. Opin. Cell Biol.* **8**, 679-684 (1996).