The Role of Cell-Cell Contact and Intercellular Junctions in the Pathogenesis of Detrusor Overactivity

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Abstracts

Urinary Incontinence (UI) is a major cause of disability and lost autonomy in older individuals. Most incontinent elderly individuals exhibit involuntary bladder contractions, detrusor overactivity (DO). Ultrastructural studies of the overactive detrusor have revealed changes in contact between myocytes involving decreased numbers of adherens junctions, and the *de novo* appearance of junctions proposed to be aberrant gap junctions. Remarkably, former junctions has never been studied in bladder muscle cells, while, until recently, bladder muscle cells were felt not express gap junctions. We developed a primary culture system using rat bladder smooth muscle cells, and found that these formed typical N-cadherin positive junctions more typical of those formed between fibroblasts as opposed to epithelial cells. We also studied detrusor needle biopsies obtained from elderly subjects and discovered the presence of several known Connexin mRNA sequences in both individuals with DO and age-matched controls. In addition, we observed an apparent up regulation of connexin 43 mRNA in DO subjects.

L'incontinence urinaire est une source majeure de perte d'indépendance parmi les citoyens âgés. Le plus part des individus incontinents âgés soufferts des contractions de vessie involontaires, detrusor trop actif. Des enquêtes ultra structurelles ont déjà démontrée plusieurs changes dans le contact entre les cellules de muscle lisse. On y voit une perte de jonctions adhérentes, qui n'ont pas été étudie spécifiquement dans le muscle de la vessie, et l'apparence de jonctions inconnues.

Nous avons développé un système de culture de muscle lisse urinaire de rat. Nous y avons trouvé que ces cellules font des jonctions typiques expriment N-cadherin comme celles des fibroblastes, en contraste avec les cellules épithéliaux. En biopsies de vessie humaines, nous avons trouvé d'évidence que les vessies trop actives âgées expriment l'ARN messager de plusieurs connexins, et que l'ARN de connexin 43 paraisse plus abondant dans les vessies trop actives que les vessies normales.

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Introduction

Urinary Incontinence (UI) is a major cause of disability and lost autonomy in older individuals (Resnick and Yalla, 1985), (Resnick and Ouslander, 1990) yet the pathophysiology of this common and important problem is not fully understood. The most common urodynamic finding observed in older individuals who are incontinent is idiopathic detrusor overactivity (DO), a condition which refers to involuntary bladder contractions taking place in the absence of any apparent neurological deficits (Resnick et al., 1989), (Resnick, 1996). Ultrastructural studies have revealed that DO is associated with changes in the nature of contact made between bladder muscle cells involving a decreased expression of adherens junctions, as well as the appearance of novel "protrusion junctions" and "ultra-close abutments"(Elbadawi et al., 1993c), (Elbadawi et al., 1997), (Haferkamp et al., 2003). While adherens junctions have been extensively studied in some cell types (e.g. epithelial cells and fibroblasts), the composition or function of these junctions in bladder muscle cells remains unknown.

"Myogenic" Hypothesis:

The proponents of the "myogenic" hypothesis of detrusor overactivity (D.O.) have proposed that involuntary contractions during filling may be due entirely to functional changes involving bladder muscle cells, with DO developing in the absence of any apparent neurological deficits. (Elbadawi et al., 1993c). They have suggested that these involuntary contractions are the consequence of a shift from predominantly mechanical coupling of muscle cells via adherens junctions in the stable bladder to increased electrical coupling in the overactive bladder via "protrusion junctions" and "ultra-close abutments" (Elbadawi et al., 1993c).

Aim:

Our basic aim was to begin an examination of the nature of contact between bladder smooth muscle cells in the detrusor, both under normal circumstances and in the setting of idopathic detrusor overactivity.

Specific aims:

1) To develop a tissue culture model which would allow us to study the nature of contact between bladder smooth muscle cells under specific experimental conditions
2) To characterize the nature of the junctions typically formed between bladder smooth cells *in vitro*.
3) To screen for the expression of mRNA species representing both known, as well as potentially novel members of the connexin gene family, in biopsies obtained from individuals with detrusor overactivity, as well as age-matched controls.
4) To examine the hypothesis that detrusor overactivity is associated with increased connexin 43 mRNA expresssion as compared to stable bladders from age-matched controls.

Cell culture studies - rationale:

Human biopsies allow only limited possibilities for experimentation. In contrast, cell culture models facilitate experimental manipulation of the physical and chemical environment of isolated cells and also allow the subsequent observation of effects in a greatly simplified model system. Thus, using this approach, it is possible to undertake mechanistic studies and to study relevant cellular pathways in great detail. The vast majority of published studies have studied cells grown in the presence of serum, yet the use of defined medium has some distinct advantages. Serum contains many substances which facilitate tissue culture by promoting the growth and survival of cells *in vitro*. Unfortunately, it has also been recognized for some time that serum can significantly alter the phenotype of many cells. For example, in the case of vascular smooth muscle cells, even a brief addition of serum converts the cells from a "contractile" to a "secretory" phenotype more reminiscent of fibroblasts than of muscle cells (Campbell and Campbell, 1997). Moreover, both desirable and undesirable serum effects can be highly unpredictable and variable. This is not surprising given, the complexity of the normal components of mammalian serum, combined with the tremendous variability between sera obtained from different species and also different lots.

We designed a tissue culture system involving rodent bladder muscle cells with the following objectives in mind:

1) Ensure that all, or at least the great majority, of cultured cells are bladder myocytes, as opposed to uroepithelial cells.
2) Ensure that it remains possible to maintain viable and relatively stable cultures for up to 1 week.
3) Ensure that cultured bladder muscle cells maintain their typical "contractile" phenotype *in vitro*.
4) Ensure that these cells are plated at a density which is optimal for studying both the formation and maintenance of muscle-muscle contact *in vitro*.
5) Ensure that this *in vitro* system will allow for the detection of features typically seen as part of the dysjunction pattern, whether such changes occur under "basal" tissue culture conditions or following various interventions.

The value of a model system depends on the ease with which it can be studied and the extent to which it is a reflection of the human system being studied. As much as possible, we wanted our cells to reflect the contractile *in vivo* phenotype of bladder smooth muscle cells. Injured smooth muscle cells exhibit an altered phenotype (frequently used as a model of atherosclerosis) in which they lose the expression of proteins required for contraction, instead focusing their cellular activities on proliferation, as well as the synthesis and secretion of proteins, in a manner similar to that of fibroblasts (Campbell and Campbell, 1997). In the design of this model system, we initially focused our efforts on the establishment of a functional marker, the ability of our cells to contract in response to carbachol. Success had earlier been reported in the anatomically much simpler chick amnion smooth muscle (Bowers and Dahm, 1993) using very gentle dissociation and serum-free culture medium. We adapted this approach to bladder smooth muscle incorporating dissociation techniques which had produced contractile vascular smooth muscle cell cultures (Apkon et al., 1997). Our substrate choice was based on a paper demonstrating that laminin and, to a lesser extent, collagen type IV prolonged the maintenance of a contractile phenotype(Hedin et al., 1988). In contrast, fibronectin

appeared to stimulate the proliferation of arterial smooth muscle cells (Hedin et al., 1988). However, as discussed below, we had underestimated the methodological difficulties inherent in detecting a contractile response by adherent cells and ultimately we used the presence of a much more traditional marker of bladder smooth muscle cell differentiation, α-smooth muscle actin (Baskin LS et al, 1996).

Human biopsy studies - rationale:

Protrusion junctions have been described as an integral component of the ultrastructural pattern characteristic of the overactive human detrusor, yet their origin and molecular composition remains unknown. Nevertheless, it has been proposed that these structures are, in fact, aberrant gap junctions and that their appearance results in the development of increased electrical coupling between muscle cells in the unstable bladder. Although the manner in which these tissues were processed did not allow for definitive identification, some of the ultrastructural features protrusion junctions were consistent with the hypothesis that these structures are related to gap junctions. Some of these features included the lack of basal lamina, the presence of a 6-12 nm gap between inner leaflets (unfortunately, outer leaflets were not visible), and the lack of membrane specialisation.

Given the very small size of bladder biopsies and obvious limitations when conducting human research, the nature of protrusion junctions and their role in the pathogenesis of detrusor overactivity could not be studied directly. Instead, a decision was made to utilize highly sensitive molecular techniques to screen for the presence of various members of the connexin gene family, proteins whose expression defines both the presence of gap junctions, as well as their function. The connexin family of genes currently consists of 20 known members. Current proteomic techniques do not allow for the screening of very small detrusor biopsies at the protein level. However, using RT-PCR techniques it is possible to perform qualitative and, to a much lesser extent, quantitative screening for different members of the Connexin gene family even using this limited sample. Of course, once specific candidate genes are identified, future approaches could allow for the subcellular localization of their products using techniques such as immuno-electron microscopy.

Literature review

Introduction to the overactive bladder

Although urinary incontinence (UI) is a major cause of lost independence and disability in old age, it has until recently received little research attention. Nearly one-third of older individuals living in the community and more than one-half of elderly nursing home residents are incontinent (Resnick and Baumann, 1988). In addition to its considerable impact on the quality of life of individuals (Patrick et al., 1999), the annual US economic cost of dealing with UI has been estimated at over $30 (US) billion (Wagner and Hu, 1998). Nevertheless, the clinical approach to the patient suffering from UI has tended to be largely palliative. Fortunately, several studies have allowed us to begin developing a pathophysiologic understanding of bladder dysfunction in both "normal", as well as incontinent elderly individuals.

Hypothesis: A Myogenic Basis for Detrusor Overactivity

The "myogenic hypothesis" of detrusor overactivity has been proposed to explain the manner in which primary changes involving detrusor smooth muscle cells could lead to the physiologic changes involving spontaneous rises in bladder pressure which are typical of the overactive bladder (Elbadawi et al., 1993c), (Brading, 1997a).

Interestingly, the focus on bladder muscle cells as the origin of primary and principal events leading to the development of detrusor overactivity is relatively recent. In support of a "neurogenic" basis for this problem is the classical clinical observation that individuals who have suffered various cerebrovascular accidents or have developed diffuse brain lesions develop detrusor hyper-reflexia, with many symptoms resembling those found in idiopathic detrusor overactivity (de Groat, 1997). Although few clinical urodynamic studies included older individuals, for many years, the prevailing view was that detrusor instability in elderly individuals was the result of major neuronal losses thought to be associated with the development of "senility" in old age. In recent decades, the concept of "senility" has been replaced with two highly relevant observations: 1) while common, dementia is far from a universal consequence of growing old, and 2) neurodegenerative diseases which lead to dementia (e.g. Alzheimer's disease) result in a

pattern of neurodegeneration which is both limited and highly specific, involving only certain vulnerable neuronal populations (Selkoe, 2002). In the first systematic study of the relationship between urodynamic studies and cognitive function in older incontinent individuals, Resnick *et al.* (Resnick et al., 1989)failed to observe any relationship between these two parameters, with detrusor overactivity being equally common in older individuals with dementia and those who appeared to be cognitively normal. The subsequent observation of a close relationship between a specific ultrastructural pattern (dysjunction pattern) and detrusor overactivity provided further support to the concept that, at least in older individuals, changes involving detrusor muscle may represent the primary or principal element in the pathogenesis of detrusor overactivity (Elbadawi et al., 1993c).

Many aspects remain unclear and controversial, with clinical and basic research studies providing evidence to support both the "neurogenic", as well as the "myogenic" hypothesis of detrusor overactivity. Examples of the latter include studies which have demonstrated that blocking neuronal transmission in conscious rats with obstructed bladders blocks micturition, but does not block unstable pre-micturition contractions (Igawa et al., 1994). Detrusor muscle cells are spontaneously active, but in the normal bladder these contractions are uncoordinated during the filling phase, and do not lead to significant rises in pressure in the whole bladder. This activity is not blocked by the neurotoxin TTX (Bramich and Brading, 1996). Normal guinea pig detrusor cells exhibit electrical spikes at a frequency of approximately 6-30 per second. Compared to the stable bladder (Kinder and Mundy, 1987), spontaneous contractions in muscle strips from unstable detrusor were of greater amplitude, frequency and basal tension. Even when muscle strips are obtained from the same unstable bladder, they exhibit remarkably heterogeneous and variable responses (Brading, 1997a). Supersensitivity to agonists is seen in some, but not all unstable bladders (Harrison et al., 1987), (Williams et al., 1993). Finally, responses to intrinsic nerve stimulation are often significantly depressed, while increased sensitivity to direct electrical stimulation has also been observed (Coolsaet et al., 1993).

Urodymic tests: Objective measurements of bladder function

Clinical symptoms do not always provide a reliable indication of the presence of a specific type of underlying bladder condition. Previous definitions of categories of voiding dysfunctions have been useful for clinical communication, but the scientific basis of these divisions, for example between neurologically impaired versus intact cases, have been questionable, and have caused some confusion (Romanzi et al., 2001). The overactive bladder correlates most strongly with urge incontinence, but it is possible for individuals (particularly those who are older) with an overactive detrusor to be entirely asymptomatic (Elbadawi et al., 1993a). Differences in definitions have greatly complicated the ability to compare clinical studies and to develop a physiologic understanding of this problem.

Urodynamic definitions and technique

Abrams *et al.* recently proposed a list of standardised definitions for clearly describing clinical and urodynamic observations involving the lower urinary tract (Abrams et al., 2002). In addition to providing universal standards in order to improve precision and facilitate communication, the authors' goal has been to avoid terms which "imply underlying assumptions that may later prove to be incorrect or incomplete" (Abrams et al., 2002). An effort was made to keep clinical symptoms, signs and conditions separate and distinct from urodynamic observations. Urodynamic observations were purely descriptive without making any assumptions regarding the underlying cause or disease. By this definition, (Abrams *et al.*, 2002) detrusor overactivity and involuntary detrusor contractions are both urodynamic observations. Detrusor overactivity refers to any involuntary detrusor contractions during the filling phase. In the presence of any relevant concomitant neurological condition, it is termed "neurogenic detrusor overactivity" (formerly "detrusor hyperreflexia."). In the event that no concomitant condition which could contribute to detrusor overactivity is identified, it is termed "idiopathic detrusor overactivity" (formerly "detrusor instability").

The detection of detrusor overactivity requires filling cystometry (measurement of the pressure/volume relationship of the bladder during bladder filling), which requires measurement of the intravesical pressure (pressure within the bladder) and abdominal

pressure (pressure surrounding the bladder), usually estimated from rectal or vaginal pressure. Detrusor pressure is obtained by subtracting the abdominal pressure from the intravesical pressure. Detrusor function is considered normal if filling occurs without significant changes in pressure or involuntary phasic contractions despite provocation, and voiding achieves complete bladder emptying in a reasonable amount of time with a continuous detrusor contraction (Abrams et al., 2002).

Ultrastructure of the unstable bladder in older individuals: Protrusion junctions, ultra-close abutments, and the complete dysjunction pattern

A systematic urodynamic study of elderly incontinent individuals revealed that two-thirds of these subjects exhibit detrusor overactivity (Resnick et al., 1989). In the same cohort, two-thirds exhibit evidence of impaired detrusor contractility, with approximately one-third of the total showing features of both detrusor overactivity and impaired detrusor contractility (Resnick et al., 1989). This condition, termed Detrusor Hyperactivity with Impaired Contractility (DHIC) is typically seen only in older individuals, presenting unique diagnostic and management considerations. Subsequent studies conducted ultrastructural analysis of detrusor biopsies revealing the presence of several distinct morphological patterns that were predictive of a specific category of physiologic findings on urodynamic studies (Elbadawi et al., 1993a).

In these studies, the presence of urodynamically diagnosed detrusor overactivity correlated perfectly with the dysjunction pattern in older individuals (Elbadawi et al., 1993c). Ultrastructural features of the dysjunction pattern comprise of a moderate widening of intercellular spaces, a near disappearance of the normally predominant intermediate (adherens) junctions, and the *de novo* appearance of abundant previously uncharacterised junctions labelled by the authors as 'protrusion junctions' and 'ultra-close abutments'.

Protrusion junctions are long, slender, finger-like processes extending from one muscle cell toward a neighbouring cell, while ultra-close abutments have much longer contact zones. These two structures lack both sarcolemmal specialization and basal lamina, while having an extremely narrow gap of 6-12 nm separating inner leaflets of their apposed sarcolemmae (Elbadawi et al., 1993c). Although the staining techniques which have been

used to date to study these structures have lacked the ultrastructural detail needed to resolve the outer membranes, the visible features are all consistent with the possibility that these uncharacterised junctions represent aberrant gap junctions.

In the context of an overactive bladder it is frequently possible to observe the presence of multiple 'protrusion junctions' and 'ultra-close abutments' which appear to join 4-8 muscle cells one to the other in an apparent syncitium visible within the same plane of section (Elbadawi et al., 1993c). Many older individuals with stable bladders exhibited an incomplete dysjunction pattern which contains many of the qualitative elements of the full dysjunction pattern, albeit with fewer 'protrusion junctions' and 'ultra-close abutments'. A subsequent study demonstrated that the incomplete dysjunction pattern may possibly herald the development of the full dysjunction pattern and detrusor overactivity (Elbadawi et al., 1997).

A more recent study observed similar results in younger patients (Tse et al., 2000). Abnormal junctions were found in all bladders, but the average ratio of abnormal to normal junctions in patients with stable bladders was 1.1:1, while it was 9.3:1 in unstable bladders. It was proposed that detrusor biopsies with remote processing might provide a more objective and cost-effective clinical and scientific alternative to urodynamic studies in bladder dysfunction diagnosis, especially in hospitals and regions lacking urodynamic expertise.

The observation that the *de novo* appearance of 'protrusion junctions' and 'ultra-close abutments' coincides with a significant decrease in adherens junctions has prompted the suggestion that the development of the dysjunction pattern reflects a shift from predominantly mechanical coupling between detrusor myocytes in the normal bladder to mainly electrical coupling which, in turn, underlies the facilitation of the spread of involuntary excitation and muscle contraction seen in detrusor overactivity (Elbadawi et al., 1993c).

Other changes in the overactive bladder:

Other types of changes which have been observed in detrusor instability include a non-homogeneous reduction in the density of innervation (Charlton et al., 1999) with increased spontaneous contractile activity and increased sensitivity to potassium

(denervation sensitivity) (Mills et al., 2000), (Harrison et al., 1987). Evidence of a role for purinergic signalling in the unstable human bladder comes from observations of atropine-resistant, tetrodotoxin sensitive muscle-strip contractions, which are de-sensitized by the non-hydrolysable ATP analogue ABMA in bladders (Bayliss et al., 1999). These contractions are observed in obstructed bladders and in bladders exhibiting idiopathic detrusor overactivity, but not in the setting of neurogenic detrusor overactivity (Bayliss et al., 1999). ATP-initiated contractions occur by a different mechanism than acetylcholine mediated ones (Wu et al., 1999). The binding of ATP to purinergic receptors causes membrane depolarisation and opening of L-type Ca2+ channels, accounting for most of the calcium flux. In contrast to earlier results (Bayliss et al., 1999), ATP was found to elicit muscle contractions seven times more potently in detrusor overactivity as compared to the stable bladder, most likely due to decreased ATPase activity in the synaptic cleft (Harvey et al., 2002). Finally, experimental partial bladder outlet obstruction in a rabbit model leads to an increased number of ryanodine binding sites, associated with an increased surface area of sarcoplasmic reticulum in hypertrophied cells (Levin et al., 1994).

Bladder overview

The urinary bladder is a hollow muscular organ which sits on the pelvic floor (Smith, 2000). Its two major functions are to store urine produced by the kidneys during the filling phase, and then to rapidly build up sufficient pressure to completely empty the urine through the now relaxed outlet and the urethra during the voiding phase. The normal adult bladder has a capacity of roughly 400-500 ml and empties quickly and efficiently with a peak pressure of 20-40 cm H_20 and a rate of 20-30 ml/sec (Brading, 1997b). It is very compliant, able to accommodate several-fold changes in volume without building large pressures that could prevent filling and lead to renal failure. The ureters which carry urine from the kidneys enter the bladder obliquely, and are occluded by the stretching of the bladder, protecting the kidneys from backflow. Contraction of the bladder is coordinated with relaxation of the internal and external urethral sphincters, creating a difference in pressure which causes the stored urine to exit the body through the urethra. The external urethral sphincter is composed of striated muscle, while the internal sphincter is actually a thickening of smooth muscle at the bladder neck rather

than a true circular sphincter. Lining the bladder is the transitional epithelium, a unique structure which is composed of several layers of domed cells in the relaxed state, but thins out while remaining impermeable when the bladder is distended. The muscular bulk of the bladder, the detrusor, is formed by smooth muscle cells, which are remarkable for their ability to generate force over large changes in length. The disorderly wrapping of interconnected smooth muscle bundles around the bladder, except near the internal meatus, further contributes to this organ's ability to generate force over a wide range in volumes (Tanagho, 2000a).

The bladder is extensively innervated. The detrusor or body of the bladder receives sympathetic, parasympathetic and sensory innervation, while the outlet also receives somatic innervation (McAninch, 2000). Neural control of the bladder occurs at several levels. Spinal reflexes are responsible for the coordination between urethral sphincter relaxation and contraction of the detrusor, while also stimulating bladder emptying when increased urine volumes threaten to cause increase bladder pressure to an extent that could potentially increase pressure in the ureters and damage the kidneys. Higher neural centres modify these responses by contracting the sphincter and suppressing contractions of the detrusor. In view of the fact that many levels of the nervous system up to the cerebral cortex are involved in control of the bladder, voiding and bladder function can be affected by numerous diseases and injuries to the nervous system (Tanagho and Lue, 2000).

Junctions between nerve fibers and bladder smooth muscle cells consist of bead-shaped varicosities with a single basal lamina layer without any visible intercellular materials such as collagen fibrils (Gabella, 1995). These varicosities are typically packed with vesicles, located 10-100 nm from the muscle cell membrane (Gabella, 1995). The bladder has also been shown to express stretch-activated cation channels that can trigger action potentials (Wellner and Isenberg, 1993a), (Wellner and Isenberg, 1993b).

Inhibition of these channels with partially purified spider venom inhibits stretch-activated calcium signalling and spontaneous contractions, while also increasing electrical compliance with minimal effects on carbachol-induced voiding (Tertyshnikova et al., 2003). The electrical properties typical of the normal bladder include poor transverse

spread (between muscle bundles), higher impedance than has been observed for other smooth muscles, plus spontaneous impulses (10-20/minute). While such spontaneous impulses would be expected to lead to fused tetanic contraction in a tissue containing smooth muscle cells with better electrical coupling, such spontaneous impulses do not lead to fused tetanic contraction in the bladder (review: (Brading, 1997b)).

Bladder outlet obstruction

Increased outlet resistance in the human bladder is often accompanied by detrusor instability (Abrams *et al.*, 1979), and detrusor instability has been studied in this setting. Outlet obstruction leads to increased voiding pressures and prolonged voiding times. Additional short-term consequences include prolonged nerve activation, increased energy expenditure, and a greater reduction in blood flow, associated with reduced oxygen tension. Longer-term adaptive changes include detrusor hypertrophy, and increased collagen deposition, which both reverse upon removal of obstruction (review: (Brading, 1997b)). Symptoms of bladder outlet obstruction may include hesitancy, terminal dribbling, urgency, urinary retention, interruption and recurring cystitis (McAninch, 2000). Increased spontaneous activity along with supersensitivity to agonists has been observed in muscle strips obtained from obstructed human bladders (Sibley, 1984,Sibley, 1997). The mechanisms underlying these changes are unknown, but it has been proposed that the effects of hypoxia on ganglia in the bladder wall may be important since rats are different from other animal models in that they lack ganglia in bladder walls and also fail to demonstrate denervation sensitivity upon development of partial bladder outlet obstruction.

Prolonged obstruction of blood flow has been proposed as a factor in the changes observed in the obstructed bladder (Chen et al., 1996), and chronic moderate ischemia may play a role in the development of idiopathic detrusor instability (Azadzoi et al., 1999). Blood is supplied to the human bladder by the superior, middle and inferior vesicle arteries, and by branches originating from obturator and inferior gluteal arteries in both men and women plus branches of uterine and vaginal arteries in females (Tanagho, 2000a). Venous drainage takes place into the internal iliac veins (Tanagho, 2000a). Blood flow to the bladder decreases with increased detrusor pressure, and significant ischemia occurs during prolonged voiding in obstructed bladder (Greenland and Brading, 2001).

Initiation of contraction

A recent review by Fry et al. discusses the role and modulation of calcium in detrusor smooth muscle, and changes that occur in these processes in the unstable bladder (Fry et al., 2002). Synchronous contraction is achieved by dense innervation (Gabella, 1995) via parasympathetic post-ganglionic neurons. Electrical field stimulation of isolated detrusor strips leads to the release of excitatory neurotransmitters from motor neurons (which can be blocked by tetrodotoxin) resulting in contraction. In normal bladder, this can be blocked by atropine, indicating that the excitatory transmitter is likely acetylcholine (Fry and Wu, 1997).

Force generation in detrusor smooth muscle cells is initiated by changes leading to rises in sarcoplasmic calcium, and can occur in the presence or absence of action potentials (Montgomery and Fry, 1992). Excitatory signals from the nervous system are transmitted by both acetylcholine release which activates M1 and M3 receptors, as well as by ATP release which activates purinergic receptors. The importance of purinergic transmission appears to vary from species to species as well as with the state of the bladder. Activation of the M3 muscarinic G-protein coupled receptor by acetylcholine (Hegde and Eglen, 1999) leads to the activation of phospholipase Cβ at the cell membrane, releasing soluble inositol trisphosphate (IP3) and diacylglycerol (Berridge, 1993). IP3 binds ryanodine sensitive calcium channels on the sarcoplasmic reticulum, increasing intracellular calcium through release from intracellular stores (Iacovou et al., 1990). The binding of this calcium to calmodulin causes the activation of myosin light chain kinase, MLCK (Walsh, 1994) which phophorylates the contractile protein myosin that generates force through its interaction with actin filaments. The depolarisation phase of action potentials is caused by Ca^{+2} entry through voltage sensitive calcium channels (Heppner *et al.*, 1997). Action potentials occur in bursts and last 20 ms. Phasic contractions last 3-10 sec. Repolarisation depends on large conductance calcium-dependant K+ (BK) channels, and subsequent hyperpolarisation is dependant on small conductance calcium-dependant K+ (SK) channels.

Modulation of contractile force

Experiments using α-toxin show that the increase in intracellular calcium caused by M3 receptor activation is sufficient to activate contraction, but that increased calcium beyond the normal range leads to still greater contractile forces (Wu *et al.,* 1995), allowing for modulation of contractile force generation through alterations in calcium fluxes or calmodulin binding affinity. The contractile force of detrusor is also affected by intracellular and extracellular pH (Liston *et al.*, 1991).

Termination of contraction

Acetylcholine esterase keeps the extracellular stimulation short by rapidly breaking down the transmitter. Inside the cell, force generation is terminated by reduction in the intracellular concentration of calcium ion. Sarcoplasmic reticulum calcium (SERCA) pumps actively return calcium to the sarcoplasmic reticulum (Ganitkevich and Isenberg, 1992), and some calcium loss occurs across the cell membrane, possibly via a $Na^{+}/Ca2^{+}$ counter exchanger (Wu and Fry, 2001). The resting membrane potential of detrusor myocytes tends to oscillate between -40 and -55. Replenishment of intracellular calcium may occur in part through a combination of abundant L-type calcium channel (Montgomery and Fry, 1992) and T-type calcium channels (Sui et al., 2001).

Spontaneous Activity

Detrusor smooth muscle cells have been shown to develop spontaneous action potentials (Brading, 1992), (Chai and Steers, 1996), and these are thought to be important in the overactive bladder. Electrophysiological studies of guinea pig detrusor muscle found that agents that blocked voltage-dependant calcium channels abolished spontaneous contractions (Herrera et al., 2000). One proposed explanation for the development of periodic spontaneous action potentials is that Ca^{+2} oscillations may occur in Ca^{+2}-overloaded detrusor myocytes (Fry et al., 2002). $Ca+^{2}$-overloaded cardiac myocytes are known to spontaneously discharge $Ca+^{2}$from the sarcoplasmic reticulum (Eisner and Valdeolmillos, 1986). Reduced cellular ATP production, due to reduced blood flow and hypoxia, mitochondrial damage and aging (Lin et al., 1997) could initiate $Ca+^{2}$ build-up. Reduced ATP availability would cause reduced Na^{+} pump activity, and the resultant Na^{+} accumulation would cause $Ca+^{2}$ build-up via $Na^{+}/Ca+^{2}$ exchange (Fry et al., 2002).

Calcium sparks, transient calcium fluxes that affect only part of a cell, have been studied

in the bladder (Herrera et al., 2001). Localized Ca^{+2} release from intracellular stores via ryanodine receptors causes currents through BK channels in the membrane. These potasium currents would have a repolarising effect and would not cause action potentials, but changes in the dynamics of calcium may be important in the overactive bladder.

Studies of the aging bladder in rodents

The effects of aging on the bladder have been studied in a number of animal model, with most such studies conducted in rats. Unfortunately, there have often been contradictions between different studies, particularly when comparing studies conducted in different models. Moreover, since descriptions of normative bladder aging in humans have been quite recent, the appropriateness of any particular animal model for the study of bladder aging has not always been clear.

For example, bladders obtained from aged (24 month old) Fischer 344 rats have been shown to fatigue more rapidly in response to repeated electrostimulation than those from younger (3 month old) animals (Lin et al., 1997). This difference has been attributed to post-synaptic mechanisms (Lin et al., 1997). Not only are basal concentrations of phosphocreatine and ATP lower in aged bladders, but both undergo a more profound depletion following neural stimulation in the aged, as compared to the young bladder (Lin et al., 1997). Since oxygen delivery to the tissues *in vitro* was equal, it has been proposed that changes in the mitochondrial function were likely involved (Lin et al., 1997).

Longhurst *et al* described a number of structural changes in the aged rat bladder including an increased bladder mass, with evidence of increased collagen cross-linking (Longhurst et al., 1992a). At the same time, when normalizing for muscle mass, no significant changes in muscle strip contractile properties were observed (Longhurst et al., 1992a).

In other studies, rat bladder shows increased % maximum contractile force in response to acetylcholine and phenylephrine with aging, and increased density of muscarinic receptors detected by [^{3}H]quinuclidinyl benzilate binding with or without atropine to control for non-specific background (Kolta et al., 1984), but this was later shown to be limited to the base of the bladder (Ordway et al., 1986).

A number of investigators have examined the effects of estrogen on the bladder using rodent models. For example, although ovariectomy had little effect on micturition

characteristics, animals which had been ovariectomized 4 months (but not 2 months) earlier demonstrated decreased contractility in response to nerve stimulation. ATP, carbachol, and KCl (Longhurst et al., 1992b). A recent report has confirmed these findings and also demonstrated the presence of structural and ultrastructural findings in bladders obtained from animals 4 months after ovariectomy which were similar to those seen in older individuals with impaired detrusor contractility (Zhu *et al.*, 2001).

Bladder development and smooth muscle cell differentiation

A general understanding of bladder development can provide a useful context for studying the behavior of cells in culture, and for exploring the mechanisms involved in both specific pathological states, as well as normal aging (Tanagho, 2000b).

Co-culture studies using bladder epithelial cells show that diffusible growth factors released from these cells are capable of inducing differention of mesenchyme into bladder muscle cells (Liu et al., 2000). Studies conducted *in vivo* have demonstrated that differentiation of the mesenchyme to bladder smooth muscle occurs from the outside in (Baskin et al., 1996a). In the developing rat bladder, smooth muscle α–actin is first observed at embryonic day 16, followed sequentially by the smooth muscle markers myosin, vinculin, desmin, laminin and vimentin (Baskin et al., 1996a).

Smooth muscle α actin expression is apparent in the outer part of the rat bladder mesenchyme by embryonic day 18, concurrent with the appearance of robust cytokeratin staining in the urothelium, as differentiation progresses inwards. Suggested candidates for epithelium-derived signals for differentiation include TGF-α, TGF-β, FGF, and IGF (DiSandro et al., 1998). The observation that rare tumours involving intestine-augmented bladders tend to occur at the junction where urothelium is exposed to signals from both bladder and intestine stroma suggests that there may exist subtle differences in signalling between different epithelia and smooth muscle cells, which may lead inappropriate cell function(Baskin et al., 1999). Insulin-like Growth Factor IGF-1 causes hypertrophy of bladder smooth muscle (Bornfeldt et al., 1994), and transforming growth factor (TGF) alpha is thought to mediate normal bladder development, and hypertrophy due to outlet obstruction (Baskin et al., 1996b). Heparin-binding EGF-like growth factor is activated by stretch and is also thought to be involved in hypertrophy (Park et al., 1999). KGF

(keratinocyte growth factor) causes urothelial proliferation in wound healing (Baskin et al., 1997)

Bladder muscle cultures

Cell cultures provide powerful tools for studying mechanisms that occur within cells by allowing researchers to exert complete experimental control over the cells' physical and chemical environment. At the same time, an investigator must establish that cells which are being studied in vitro actually bear a reasonable resemblance to the cells which constitute a functioning organ in a living organism.

It has long been known that freshly isolated cells often behave differently than long term primary cultures or passaged cells. It is therefore important to characterise the cultured cells which are used for *in vitro* studies. Commonly used smooth muscle (SM) cell markers include SM alpha-actin, SM myosin heavy chain, h-caldesmon, and calponin (Hedin et al., 1988). Changes in smooth muscle phenotype are not only associated with changes in the above markers, but can also result in altered electrical properties (Neylon et al., 1994,Neylon et al., 1999)

In fact, the process of tissue dissociation represents a form of non-specific cellular injury, and can lead to the development of an adaptive phenotype which differs greatly from that typically observed *in vivo*. For example, cultured vascular smooth muscle typically exhibit an actively dividing and protein-secreting phenotype, which is not observed in healthy vascular smooth muscles in vivo, yet is believed to be involved in the pathogenesis of atherosclerosis (review: (Ross, 1993)).

Contractile chick amnion cultures

In a specific attempt to culture smooth muscle cells more representative of the in vivo contractile phenotype, Bowers and Dahm (Bowers and Dahm, 1993) minimized the trauma of dissociation by choosing anatomically simple chick amnion and gentle dissociation conditions in serum-free medium. These cultured cells maintained contractility in response to carbachol for 4 weeks, while the presence of added serum or the use of harsher dissociation conditions both lead to cellular flattening, proliferation and loss of contractility.

Serum response in fibroblasts

Serum is commonly used in cell cultures because of its dramatic effects on cell survival

and proliferation. Nevertheless, these effects are associated with a loss of the fully differentiated phenotype (Bowers and Dahm, 1993), demonstrating the need for the use of more specialized chemically defined media. An early gene cDNA microarray study looking at the time course of the transcriptional response of 48h serum-starved fibroblasts to 10% fetal bovine serum(Iyer et al., 1999) found evidence that serum-fed fibroblasts exhibit a wound repair response. Up regulated genes included the EDG-1 receptor, COX2, VEGF, and vimentin.

Cultured smooth muscle cell contractility

Assaying contractility in cultures of adherent cells on a rigid substrate poses a challenge. A common strategy involves the focal application, for 5-10 sec of trypsin and EDTA. While a limited digestion may leave cells attached to a rigid substrate, unable to contract, a more extensive digestion may damage the cells, impairing their ability to effect a contraction in response to an agonist One vascular smooth muscle culture system showed evidence of ability to modulate tone in response to natural VSMC stimuli despite passaging and 10 % serum. (Apkon et al., 1997), creating wrinkles in an elastic substrate. These cells contracted in response to extracellular alkalinization, and 5-HT, and relaxed with extracellular acidification.

Long term cultured smooth muscle cells have historically lost the contractile phenotype, and receptor–channel coupling in not universally conserved in culture systems(Hall and Kotlikoff, 1995). However, a number of recent studies have shown that vascular and airway smooth muscle can resume a contractile phenotype upon prolonged serum withdrawal, (Ma et al., 1998), (Li et al., 1999), (Halayko et al., 1999). The reported isolation of a contractile cell line from these studies is a milestone that has facilitated more relevant electrophysiological studies, and will contribute improved understanding of smooth muscle cells in general (Li et al., 1999). The demonstration of contractility in culture provides proof that in these cells, at least the minimal necessary apparatus for contractility is intact. The isolation of such a line makes experiments with smooth muscle more efficient and reproducible.

The ability to return to a contractile phenotype has also been documented in tracheal smooth muscle. Following passaging and prolonged serum deprivation after the attainment of confluence, a fraction (1/6) of cultured canine smooth muscle cells

elongated, aligned and expressed smooth muscle cell markers smooth muscle α actin, smooth muscle myosin heavy chain, and SM-22, resulting in maximum expression after 12 days in culture (Halayko et al., 1999). These cells expressed the M3 muscarinic acetylcholine receptor and contraction in response to carbachol when semi-adherent. Surprisingly, a detrusor cell line from hypertrophied bladder rabbit bladder has recently been isolated that maintains contractility even in the presence of 10% serum despite repeated passaging (Zheng et al., 2002). These cells uniformly express smooth muscle myosin, and exhibit other features of differentiated smooth muscle including myosin light chain kinase activity, myosin phosphatase activity, protein kinase G expression and the ability to contract in response to the muscarinic agonist bethanechol. While primary cultures were heterogeneous, the investigators were able to obtain homogeneously differentiated cells by repeated selection based on appearance under light microscopy. The contraction of these cells in response to bethanechol is sufficiently robust that it is evident despite apparent adhesion to a rigid substrate. The apparent lack of the expected injury response to serum is interesting and may merit further investigation.

Cell adhesion

Most of the studies examining the role of adhesion molecules on smooth muscle cells have been performed using vascular smooth muscles. Vascular smooth muscle has a number of different adhesion receptors, reviewed in (Moiseeva, 2001). These vary with phenotype as well as from one smooth muscle origin to another. The integrins, which bind the extracellular matrix, have been extensively studied. Those which bind laminin, a1bi and a7b1 seem to be especially important in maintaining a contractile phenotype (Yao et al., 1997), while a4b1 is expressed in development and binds fibronectin (Duplaa et al., 1997). Integrin Avb1 binds vitronectin, inhibiting contractility (Dahm and Bowers, 1998).

The dystrophin-glycoprotein complex provides mechanical stability and prevents the cell from over stretching(Petrof et al., 1993). In smooth muscle cells, dystrophin is found in the caveolar domains, and excluded from adherens junctions (North et al., 1993). However, a number of integrins, as well as CD44, a hyaluronan receptor, have been shown to be expressed in the bladder (Arafat et al., 2002), (Bagli et al., 1999).

Adherens junctions

Based on ultrastructural criteria, adherens junctions appear to provide a primary mechanism for mechanical coupling in the normal human bladder. They appear as parallel electron-dense bands on adjacent cell membranes, with variable intracellular space of about of 0.2 μm. In various cellular systems, the dense band or plasmalemmal undercoat has been found to contain many proteins including α-catenin, β-catenin, plakoglobin, vinculin, α-actinin, p120ctn, radixin, ZO-1, zyxin, spectrin, and actin (Tsukita and Tsukita, 1989,Tsukita et al., 1992,Itoh et al., 1993), (Nagafuchi, 2001). Central to these junctions, classical cadherins are transmembrane proteins that interact with identical molecules on adjacent cell membranes to mediate the calcium-dependant adhesion. The cytoplasmic portion of these molecules binds β-catenin or plakoglobin, which, in turn, binds α-catenin, with vinculin, α actinin, ZO-1 (in non-epithelial cells) and actin (Imamura et al., 1999). Nectin, an immunoglobulin-like cell-cell adhesion molecule, and the actin-binding protein afadin are also associated with the cadherin-catenin system in synapses and polarized epithelial cells, likely through a molecule whose identity has not yet been established (review: (Takai and Nakanishi, 2003). It has been proposed that mLin-7 may represent this molecule (Yamamoto et al., 2002). P120ctn is a catenin that binds classical cadherins in a highly conserved region near the membrane (review: (Anastasiadis and Reynolds, 2000)). It binds cadherins less stably than do the other catenins, but is believed to be important in increasing the half-life of the cadherins (Ireton et al., 2002).

Differences in adherens junction composition between cell types

There are differences in the composition of adherens junctions between different cell types. Knudsen *et al.* found (Knudsen et al., 1995) that in contrast to epithelial cells where both α actinin and vinculin co-immunoprecipitated with E-cadherin and catenins, α-actinin, but not vinculin co-localizes and co-immunoprecipitates extensively with the N-cadherin/catenin complex in fibroblasts.

Cadherins

Cadherins are the transmembrane glycoproteins of adherens junctions that mediate cell-cell adhesion. They are found in virtually all solid tissues (review: (Vleminckx and

Kemler, 1999)). Adhesion is calcium dependant, as the extracellular domains change conformation in response to calcium ion (Shapiro et al., 1995). The cytoplasmic domain associates with other proteins, including p120ctn, which recruits and activates small GTPases (Noren et al., 2000), and β-catenin or plakoglobin. β-Catenin links to α-catenin which links to the actin cytoskeleton. The clustering of cadherins is important for strength (Chitaev and Troyanovsky, 1998), (Adams et al., 1998), and appears to depend on posttranslational modification of p120ctn (Aono et al., 1999), which binds E-cadherin near the cytoplasmic face of the transmembrane region. E-cadherin is the major cadherin in polarized epithelial cells, while both P and E cadherins are found in squamous epithelial cells. N-cadherin plays a role in cardiac myofibrillogenesis (Soler and Knudsen, 1994) and is found in developing and mature cardiomyocytes (Volk and Geiger, 1984), as well as neurons(Matsunaga et al., 1988), developing skeletal muscle (Knudsen et al., 1990), and cultured chicken fibroblasts (Geiger et al., 1990). The linkage between N-cadherin and actin is regulated by PTP1b (Balsamo et al., 1998).

α-catenin

Alpha-catenin is a necessary component of the cadherin based cell-cell adherens junction. It appears to play a role in organising a "multi-protein complex with multiple actin-binding, bundling and polymerisation activities"(Vasioukhin and Fuchs, 2001). α-catenin migrates at 102 kDa in polyacrylamide gel electrophoresis, and is related to vinculin which is abundant in focal contacts and also found in some adherens junctions. The C-terminal binds f-actin, while the N-terminal binds β-catenin or plakoglobin and is responsible for dimerisation. The central region binds actin-bundling protein α actinin (Nieset et al., 1997), vinculin, and MAGUK (membrane associated guanylate kinase) family members ZO-1 (Itoh et al., 1997) and ZO-2(Itoh et al., 1997). VASP and mena are also recruited through α-catenin, and play a regulatory role in actin reorganisation (Vasioukhin et al., 2000). α-Catenin expression is tightly regulated through changes in translational efficiency and rates of degradation (Takahashi et al., 2000), and over expression of α-catenin disrupts wnt signalling by binding cytoplasmic β-catenin (Giannini et al., 2000).

Multiple isoforms of α-catenin exist. In addition to the widely studied α1(E)-catenin,

there are also α2(E) (Rimm et al., 1994) and α(n) I and II isoforms (Uchida et al., 1994). Deletion studies suggest that ligand binding by α-catenin is regulated (Weiss et al., 1998).

β-catenin

β–catenin is a 92-kD protein related to armadillo from drosophila and plakoglobin, another protein which associates cadherins (Peifer and Wieschaus, 1990), (McCrea et al., 1991,McCrea and Gumbiner, 1991). The binding of β–catenin to α-catenin is negatively regulated by phosphorylation, which is associated with a change in gel mobility suggestive of a conformational change (Ozawa and Kemler, 1998). Increased phosphorylation also leads to dissociation from E-cadherin and increased cytoplasmic pool (Muller et al., 1999), though in differentiating keratinocytes phosphorylation of catenins β–catenin, plakoglobin and p120ctn is associated with increased adhesive strength (Calautti et al., 1998).

Plakoglobin

Plakoglobin is a component of both adherens junctions and desmosomal plaques (Cowin et al., 1986). It has high homology to β–catenin (McCrea et al., 1991)

α-Actinin

α–Actinin is an actin cross-linking protein enriched in focal adhesion plaques that interacts with zyxin (Crawford et al., 1992). In adherens junctions, it is believed to bind classical cadherins through α–catenin (Knudsen et al., 1995).

Dense body composition

Dense bodies, the smooth muscle equivalent to cardiac and skeletal Z-disks which organise contractile units, have proteins in common with adherens junctions. These include β–catenin (Kurth et al., 1996)and α–actinin (Geiger et al., 1981), but not α–catenin (Kurth et al., 1996).

Vinculin

Vinculin has a molecular mass of 120kD, and is found both in focal contacts and in some adherens junctions. Junctions containing vinculin rather than α–catenin exhibit weaker adhesion (Herrenknecht et al., 1991), (review: (Rudiger, 1998)) Metavinculin, a larger splice variant (Rudiger et al., 1998) abundantly expressed in smooth muscle along with vinculin, stabilizes web-like structures of F-actin, compared to the parallel actin structures

stabilised by vinculin. Vinculin is phosphorylated, and its binding to F-actin is blocked by a head to tail interaction which is released by phosphatidylinositol-4,5 bisphosphate(PIP2) (Gilmore and Burridge, 1996). Immunoprecipitation evidence from an α–catenin deficient cell line suggests that α–catenin and vinculin compete to bind beta-catenin (Hazan et al., 1997).

Adherens junction formation

The formation of adherens junctions has been studied both in cell lines, and in primary cultures. In the well established but poorly adherent MDCK epithelial cell line, E-cadherin positive adherens junctions start as puncta with bundles of actin that progressively smooth out into a line of cortical actin (Adams et al., 1998). In contrast, Vasioukhin *et al.* found that in primary keratinocyte cultures, adherens junction formation occurs by a more active process (Vasioukhin et al., 2000). In response to increases in calcium ion concentration, filopodia extend into adjacent membranes. These processes are then clamped by desmosomes, and adjacent membranes subsequently push together by actin polymerisation. The clustering of E-cadherin at the tips of the filopodia gives these structures a distinctive zipper-like appearance when stained for E-cadherin. Vasp, Mena, vinculin and zyxin are also implicated in the formation process, and α-catenin is required (Vasioukhin et al., 2000). Similar zipper structures have been seen in vivo in drosophila (Martin-Blanco et al., 2000) and c-elegans (Raich et al., 1999). (Review; (Vasioukhin and Fuchs, 2001)). A study using N-cadherin-coated beads mimicked this effect, suggesting that the same formation mechanism may apply to N-cadherin based adherens junctions (Lambert et al., 2000).

Regulation of adherens junction formation likely involves small GTPases of the Rho family, Rho, Rac1 (Braga et al., 1999) and Cdc42. Chimeric E-cadherin fused to α–catenin is less affected by dominant negative Rac1 or Cdc42, suggesting that these small GTPases may improve the stability of the cadherin-catenin complex (Kuroda et al., 1998) by inhibiting IQGAP1 which dissociates α-catenin from E-cadherin-β–catenin complex (Fukata et al., 1999). There also exists evidence that the composition of adherens junctions changes with maturation in endothelial cells, with newly formed junctions being rich in β–catenin and p120ctn, and mature junctions of confluent cells contain less p120ctn and more plakoglobin (Lampugnani et al., 1997). Cadherin/catenin

complexes of different plakoglobin / β–catenin composition also segregate to different sites in polarized epithelial cell lateral membranes (Nathke et al., 1994). In vascular endothelial cells, sphingosine-1-phosphate acts extracellularly to induce adherens junction assembly through the membrane receptors EDG-1 and 3 (endothelial differentiation gene) and the GTPases Rac (cortical actin) and Rho (stress fibres) (Lee et al., 1999). Sphingosine 1 phosphate (SPP) is also believed to be capable of releasing calcium from both intracellular and extracellular stores (review (Young and Nahorski, 2001)). VEGF (Esser et al., 1998) and TNF-α (Wojciak-Stothard et al., 1998) have been found to disrupt adherens junctions in blood vessels.

Cell adhesion and signalling

Cellular adhesion complexes are concentrated centers for signalling (Yamada and Geiger, 1997). A vast array of signals have been implicated, including protein tyrosine phosphorylations, MAP kinase activation, Ca^{+2} influx, pH alterations and inositol lipid turn-over, with cooperation between adhesion signalling and growth-factor mediated signalling. The ability of integrins to interact with the moving cytoskeleton can be induced by ligand binding, regulated by an interaction between the α–and β–integrin tails. (Felsenfeld et al., 1996) Ligand-binding induces phosphorylations which result in recruitment of Shc and Grb2 and activation of the Ras pathway (Mainiero et al., 1995).

Clustering of β1 integrin leads to recruitment of a number of signalling molecules (Miyamoto et al., 1995), while cytoskeletal molecules α-actinin, talin, vinculin, require clustering and ligand binding.

Adherens junctions complexes are also active in signalling (Geiger et al., 1995), (Kirkpatrick and Peifer, 1995). β–Catenin interacts with EGF receptors and localises them to adherens junction regions in growth-factor activated cells (Hoschuetzky et al., 1994). It is also involved in wnt/wingless pathway (Fagotto et al., 1996), binds to transcription factor lymphoid enhancer factor-1 (LEF-1) and translocates to the nucleus where it bind to the promoter region of the E-cadherin gene (Huber et al., 1996). Nuclear translocation leads to the induction dorsal mesoderm in Xenopus (Miller and Moon, 1996). It has however been proposed that the adherens junction and wnt signalling pools of β–catenin may be distinct (Sanson et al., 1996). β–Catenin and plakoglobin also bind

actin-bundling Fascin, apparently with the same site that binds cadherin (Tao et al., 1996).

Focal adhesion kinase (FAK) has many functions in signalling and regulation of adhesion (Schlaepfer and Hunter, 1996) Inhibitors of phosphorylation inhibit focal contact formation (Burridge et al., 1992). Focal adhesions play a signalling role in the rapid stiffening and changes to adhesion and cytoskeletal arrangement in cells undergoing shear stress (Girard and Nerem, 1995). The nature of cell adhesion has important effects on smooth muscle phenotype in culture, as culturing vascular smooth muscle on fibronectin leads to a rapid modulation from a contractile to synthetic phenotype (Hedin et al., 1988), while laminin promotes the maintenance of the contractile phenotype in serum-free cultures. Cells cultured under both conditions secrete both laminin and fibronectin.

Gap junctions

Gap junctions form conduits between adjacent cells, coupling their cytoplasmic compartments electrically and metabolically to varying degrees by allowing the passage of small molecules including ions such as calcium (Toyofuku et al., 1998b), metabolites and second messengers. Six transmembrane proteins of the connexin family assemble to form a hexameric channel called a connexon, with two connexons on adjacent cell membranes assembling to form a hydrophilic channel. ((Kumar and Gilula, 1996), (Willecke et al., 2002)). Ultrastructurally, typical gap junctions appear as very close appositions, the inner membranes being separated by only 3.5 nm (Makowski et al., 1977))

Currently, 20 different connexin genes have been identified in the human genome (Willecke et al., 2002) and these have diverse properties, including different temporal and spatial expression patterns. The channels formed by various connexin proteins have different selective abilities to form functional channels with adjacent connexons of different composition (Kumar and Gilula, 1996). Additionally, studies confirm the ability of some connexin pairs to assemble into heteromeric connexons, including CX26 with CX 32 (Stauffer, 1995), connexin 43 with 37 (Brink et al., 1997), (Beyer et al., 2001), Connexin 40 with 43 in cultured vascular smooth muscle (He et al., 1999) both in vitro and in vivo (Locke et al., 2000), connexin 43 with 56 in the lens (Berthoud et al., 2001) and connexin 43 with connexin 45 (Martinez et al., 2002). Gap junctions of different

compositions differ in their permeability and gating properties, including pH sensitivity (Gu et al., 2000) and responsiveness to the gap junctional blocker halothane (He et al., 1999). Heteromeric connexin 40/connexin 43 channels have permeability properties that reflect the relative contribution of the individual connexins. Pure connexin 43 channels are ten times more permeable to lucifer yellow than pure connexin 40 channels, and heteromeric channels have intermediate values (Valiunas et al., 2002). The physiological importance of heteromeric connexons has been demonstrated in goats, where the ratio of connexin 43 to 40 has been found to be regionally modified in heart disease (van der Velden and Jongsma, 2002). In many cases examined, connexin proteins are not interchangeable (Plum et al., 2000), (Elenes et al., 1999). Most cell types express at least one connexin (Willecke et al., 2002).

Some connexins have been found to interact with different intracellular proteins (Giepmans et al., 2001b) including kinases, scaffolding proteins (Toyofuku et al., 1998a), caveolin-1 (Schubert et al., 2002) and microtubules (Giepmans et al., 2001a).

Functional and hormonal regulation of connexin

Communication between cells via gap junctions is regulated in functionally important ways. During the rat menstrual cycle, estrogens increase connexin 43 expression in endometrial smooth muscle, while progesterone inhibits connexin 43 expression and inappropriate coordinated uterine contractions in late pregnancy (Grummer et al., 1994). This inhibition is reversed with the decrease in progesterone observed immediately prior to parturition (Grummer and Winterhager, 1998). It has also been found that mechanical stimulation can dramatically induce connexin 32 expression in receptive rabbit uterine epithelium, and this may be analogous to an inflammatory reaction(Antoskiewicz et al., 1996). Changes in connexin expression in response to injury is a well known phenomenon (De Maio et al., 2002).

Connexins in the bladder: history of evidence, including electrical coupling

The extent of coupling between detrusor smooth muscle cells has been controversial, though significant advances have been made recently. Historically, it was believed that normal human and rat detrusor did not exhibit gap junctions (Gabella, 1997), (Daniel et al., 1983), (Gabella and Uvelius, 1990). This belief was based on the absence of ultrastructural evidence of typical gap junction structures in numerous electron

micrographs in studies including those of Elbadawi ((Elbadawi et al., 1993b), reviewed (Fry and Wu, 1998), (Brading, 1997a)). However, it has more recently been argued that all smooth muscle cells are electrically coupled (Hanani and Brading, 2000). In the longitudinal layer of the guinea pig ileum which also lacked ultrastructural evidence of gap junctions, dye coupling is observed, indicating the presence of intercellular channels, and this coupling was significantly reduced by low pH, further evidence that gap junctions are likely involved (Zamir and Hanani, 1990).

Until recently, ultrastructural studies (Elbadawi et al., 1993b), (Gabella and Uvelius, 1990) argued against gap junctions in the normal bladder, while electrophysiological studies were in favour of the possibility that some high resistance electrical coupling is present (Seki et al., 1992), (Fry et al., 1999).

In guinea pig, Bramich and Brading found evidence that bladder myocytes were likely coupled in bundles, but poorly between bundles (Bramich and Brading, 1996). Ultrastructural studies show that the rat bladder is densely innervated, (Gabella, 1995) and that human detrusor myocytes are each associated with several varicosities (Daniel et al., 1983), suggesting that each myocyte could be directly excited in normal voiding (Fry et al., 1999).

Intercellular electrical resistance is higher in guinea pig detrusor than in myocardium which is known to express abundant gap junctions, but current still passes and it has been calculated to be functionally electrically coupled. In human bladder it was documented that the electrophysiological properties of detrusor strips from patients with idiopathic detrusor instability differ from those of cadaveric organ-donor controls, exhibiting increased spontaneous contractile activity, in addition to evidence of denervation and increased sensitivity to potassium (denervation sensitivity) (Harrison et al., 1987).

Recent developments:

Investigators have recently found that connexin 43 is present in their cultured bladder smooth muscle, detectable at the mRNA level by northern blots, and at the protein level by western blots (Wang et al., 2001). They also detected and characterised conductance, (6.5 ± 4.6 picosiemens) and moderate, symmetrical voltage dependence, while Fry et. al

cite unpublished data showing connexin 45 protein expression by immunohistochemistry and blotting (Fry et al., 2002). More extensive evidence for connexin 45 in the bladder was provided by John et. al., who used a number of ultrastructural techniques. Though rare, they did observe the typical pentalaminar pattern between smooth muscle cells by transmission electron microscopy in the stable unobstructed bladder of patients ranging in age from 55 to 72 (John et al., 2003). Connexin 45 mRNA was identified by RT-PCR and sequencing, and localised to the smooth muscle by in situ hybridization. Connexin 45 protein was identified at the cell membrane by freeze fracture immunolabeling in cells identified as smooth muscle cells by the presence of caveolae, and was also found in spots around the perimeter of detrusor muscle cells and blood vessels by immunofluorescence.

It has been recently shown that connexin 26 and 43 are differentially regulated in the rat bladder following obstruction. Connexin 26, 32, 37, 40, 43 and 45 were found in the bladder (Haefliger et al., 2002). Connexin 26 was found to be expressed in the urothelium but not in the smooth muscle, and this expression was shown by northern blot to increase roughly 5 fold in the distended bladder compared to the empty bladder. In contrast, distended bladder smooth muscle exhibited a 4-fold increase in connexin 43 mRNA expression. The corresponding proteins were shown to increase roughly 3-fold.

In the urothelium, stress caused by increased bladder pressure due to outlet ligation leads to reduction of a scaffold protein, IB1/JIP-1 which coordinates a JNK signalling pathway, resulting in increased AP-1 binding activity and increased connexin 26 expression(Tawadros et al., 2001). Over-expression of this scaffolding molecule blocked stress induced AP-1 activation and reduced connexin 26 mRNA expression. IB1/JIP-1 expression is not seen in the smooth muscle of the bladder by *in situ* hybridization and immunofluorescence, indicating that a different mechanism must be responsible for the changes in connexin 43 expression (Tawadros et al., 2002).

Materials and methods

Cell culture and animal tissue studies

Smooth Muscle Cell Culture

Procedures involving animals were performed according to protocols approved by institutional animal care committees. P0 rat pups were anesthetised by indirect hypothermia, and sacrificed by cervical dislocation. Cell dissociation (Apkon et al., 1997) and culture (Bowers and Dahm, 1993) methods were adapted from several papers. Bladders were dissected out and collected in chilled Earle's balanced salt solution (EBSS, Gibco cat# 14015-069) and minced to roughly 1 mm cubes. Following one rinse with EBSS, tissue was dissociated for one hour at 37°C in a 0.22 μm filtered enzymatic solution consisting of 0.5 mg/ml collagenase II (Worthington LS004204), 0.5 mg/ml purified elastase (Worthington LS006365), 2 μg/ml DNAse 1 (Worthington LS002058), and 1 mg/ml soybean trypsin inhibitor (Gibco 17075-011). During the dissociation, the minced tissue was triturated 60 times through the fire-polished tip of a long Pasteur pipette at 10-15 minute intervals. Once the tissue appeared disintegrated, suggestive of an intact epithelial layer but dissociated muscle layer, it was passed through a 20μg strainer to remove the un-dissociated epithelial cells, and the filter rinsed once with EBSS. Cells were collected by centrifugation for 5 minutes at 11g , and re-suspended in plating medium consisting of Dulbeco's modified Eagle's medium with F12 supplements (DMEM/F12, Gibco 10565-018), 1% Foetal calf serum and penicillin and streptomycin (Gibco 15140-148). Cells were plated on a substrate of collagen IV (Sigma C5533) and laminin (Sigma L2020). After 18 hours to allow adhesion, cells were switched to serum-free culture medium consisting of DMEM F12 with N2 supplement (Gibco, 17502-014) and penicillin and streptomycin. Cells were fed at 2 day intervals, by replacing half of the medium with fresh serum-free medium.

Tissue sections

Tissue for sectioning was embedded in cryomolds in Tissue-Tek® O.C.T. Compound (Sakura Finetek), frozen in liquid nitrogen-chilled methylbutane, and then allowed to fully freeze at –80C overnight. 25 μm thick tissue sections were cut on a SLEE cryostat

at -20C and collected on Probe on Plus™ (Fisher Scientific) microscope slides.

Indirect Immuno fluorescence

Tissue samples were rinsed with PBS (phosphate buffered saline) and then fixed for 10 minutes with 4% para-formaldehyde in PBS. Cell membranes were made permeable by incubating for 10 minutes in 0.5% triton X-100 in PBS. Non-specific protein binding was blocked by incubating for 30 minutes with 10% FBS (Foetal bovine serum) in PBS, and primary antibody incubations were performed in 5% FBS for 2 hours at room temperature or overnight at 4 C. After 3 washes in PBS, fluorescently labelled secondary antibodies were applied in 5% FBS in PBS for 1 hour at room temperature. Following 5 more washes, including 2 with the plastic chamber removed from the chamber slides, cells were mounted with anti-fade mounting medium (ICN 622701) and cover slips sealed with colourless nail polish. Images were acquired using a Zeiss LSM 410 confocal microscope mounted on an Axiovert 135 with an argon-krypton laser with emission at 488, 568, and 647 nm and a 40x 0.95 NA air objective.

Antibodies

All antibodies were commercially available. Rabbit polyclonal primary antibodies were used against N-cadherin (R&D systems #BTA7), pan cadherin (Zymed # 18-0231), and pan cytokeratin (Zymed # 18-0059). Mouse monoclonal antibodies were used against smooth muscle α-actin (Sigma #A2547). A zonula adherens sampler kit (BD biosciences #611446) included mouse monoclonal antibodies and control protein extracts for N-cadherin, E-cadherin, β-catenin, γ-catenin, desmoglein and $p120^{ctn}$.

The secondary antibodies used in indirect immunofluorescence were an Alexa flour 488 conjugated goat anti-rabbit antibody (Molecular Probes #A-1101), and a Cy3 cong goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). Peroxidase labelled anti-mouse secondary antibodies used in western blotting were part of the ECL detection system (Amersham, #RPN2108).

Western Blots

Cultured cells were lysed and the protein extracted in buffer (5mM EDTA, 1% tritonX100, 0.1% SDS, protease inhibitor cocktail, 25 mM Tris, 25 mM glycine, 150 mM NaCl) and stored at –20°C until use. Molecular weight marker (Amersham-Pharmacia) and an equal quantity of protein samples were heat-denatured in Laemmli buffer and then resolved in 10% SDS-PAGE followed by transfer to a nitrocellulose membrane (BIO-RAD). Non-specific protein binding was blocked for at least one hour in blocking solution composed of 5% skim milk in TBST, and then blots were incubated overnight at 4°C with primary antibodies at the listed dilutions in blocking solution. After three ten-minute washes in TBST, blots were incubated with peroxidase labelled anti-mouse secondary antibodies 1:2500 in blocking solution for 1 hour. Following final washes, the blots were developed using the ECL detection system (Amersham, #RPN2108) and Kodak Biomax MR film (870 1302).

Human Studies

Urodynamic studies were performed and needle biopsies obtained by the urology labs of Dr. S. Yalla, and Neil Resnick. Samples were snap-frozen in liquid nitrogen and maintained bellow –80°C until RNA extraction. Samples were coded, with all staff and personnel being blinded to the identity of individual patients. The nature of the urodynamic diagnosis remained unknown until the completion of data analysis. Digit following the '#' in the sample code indicated the depth of the biopsy tissue, with 3 being shallowest and closest to the urothelium, and 8 being deepest into the muscle. Biopsy samples were not weighed, but it was estimated that the wet mass for a typical biopsy was approximately 3 mg.

Methods were validated using less valuable tissue animal tissue. Because of the small sample size and the low RNA yields typically obtained from detrusor, considerable effort was put into finding a method of tissue homogenisation and RNA extraction that would reliably allow maximal recovery, using rat bladder. Explosive shattering upon crushing with a standard mortar and pestle, and the need to avoid dilution to relatively large volume required for a rotor/stator such as a Polytron™ lead to the exploration of several alternative approaches. Needle biopsies were ground in liquid nitrogen in RNAse-free

ground glass micro dounce (Kimble) and then homogenised in BME-containing lysis buffer according to kit instructions (Stratagene Absolutely RNA RT-PCR miniprep kit). To maximise RNA recovery, a minimal volume of lysis buffer was used for the initial homogenisation, and then combined on the column with a larger volume wash. DNase digestion and removal were performed with Ambion "DNA-free" kit (Ambion), followed by concentration of the RNA by precipitation under ethanol with 0.5M ammonium acetate, and linear acrylamide as a co-precipitant. RNA quantification was not performed for the first set of samples, because of concerns over sample loss and degradation. However, electrophoresis of 1/20 of each sample on an agarose gel showed the RNA to be intact based on the presence of intact ribosomal RNA bands. In the second set of samples, RNA yield was estimated using absorption at 260 nm prior to precipitation.

Reverse transcription

Reverse transcription (RT) was performed using the Superscript™ system (Gibco-BRL #11904-018) according to kit instructions, using 1 µl random hexamer primers per 20 µl reaction. Sample RNA was divided equally between a reverse transcription reaction and a reverse transcriptase-negative control. Because of the low RNA yields and measurements were not considered reliable, it was not possible to adjust each sample to 1µg per reaction. Instead, ¼ of the RNA obtained from each biopsy was used for each RT+ reaction or RT- control. Assuming perfect precipitation efficiency, this corresponded to up to 0.4 µg of RNA.

Amplification of Connexin mRNA by RT-PCR:

Since the sample volume was limited, the identity of the target connexin was unknown and it was hoped that a novel connexin might be involved, PCR was performed using degenerate primers against conserved regions of the connexin family. All RT-PCR primers were synthesized by Shelton Biotechnology Center of McGill or by Gibco (Invitrogen). Degenerate connexin primer sequences were obtained from (Haefliger et al., 1992), and connexin-specific nested primers were designed using Primer3 software, available on the internet (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/primer3_www.cgi) (Steve Rozen and Helen J.Skaletsky, 2000). Primer sequences and reaction conditions are given in the table below.

Thirty to fifty cycles were performed on the samples using a MiniCycler PTC-100

Thermocycler (M.J. Research Inc., Watertown, MA, USA) as follows: 1) denaturation at 94°C for 30 s; 2) annealing at 55°C for 30 s; 3) extension at 72°C for 30 s. This was followed by a final extension cycle at 72°C for 8 min, after which samples were held at 4°C.

The PCR reaction mixture (50µl) contained 1x PCR reaction buffer, 0.2 nM dNTP mix, 0.2 nM of each primer, 1.5 nM $MgCl_2$ (except for Universal primer reaction, which had 3.0 nM), 1 unit of taq polymerase, and nuclease-free water.

PCR products were resolved by electrophoresis on agarose gels containing ethidium bromide, and visualized and photographed with UV trans-illumination using either Polaroid film (earlier studies), or an AlphaImager™ System 2200 digital imaging system.

Table 1: Predicted products of PCR with degenerate primers for human connexins

Connexin group name	Connexin molecular weight name	Amplified region	Forward primer homology (/23)	Forward primer homology (/21)	Product length	Unigene Cluster	Genbank accession / reference sequence
A1	43	385-797	23	19	433	Hs.74471	NM_000165
A3	46	178-572	23	21	415	Hs.283746	NM_021954
A4	37	242-654	22	19	433	Hs.296310	NM_002060
A5	40	299-705	23	19	427	Hs.7473	NM_005266
A7	45	175-656	22	19	502	Hs.377116	NM_005497
A8	50	178-575	23	21	418	Hs.157433	NM_005267
A9	36	181-722	22	16	562	Hs.283816	NM_020660
A10	58 or 59	178-596	21	17	439	Hs.188758	NM_030772
A12	47 = 46.6	203-783	23	19	601	Hs.100072	NM_020435
B1	32	237-595	21	21	379	Hs.333303	NM_000166
B2	26	175-536	21	20	382	Hs.323733	NM_004004
B3	31	1308-1657	22	18	370	Hs.98485	NM_024009
B4	30.3	253-599	22	19	367	Hs.351203	NM_153212
B5	31.1	342-688	20	19	367	Hs.198249	NM_005268
B6	30	175-536	21	18	382	Hs.48956	NM_006783
C1	31.9	178-533	20	19	376	Hs.135211	NM_152219
	31.3	175-536	18	16	382	Hs.367980	AF503615
	62	178-596	19	19	439	Hs.334499	NM_032602
	40.1	178-554	20	19	397	Hs.302078	XM_166122

Cloning and sequencing

PCR products for cloning were excised and purified from agarose gels using the QIAEX II gel extraction kit (Qiagen). These were then ligated into the pGEM-T Easy Vector (Promega, A1380) and transformed into JM109 cells according to kit instructions. Positive clones were identified by blue/white screening, and cultured overnight in LB broth. DNA was isolated using the Wizard Plus Mini-prep system (PROMEGA: A7100), and a portion was restriction digested with EcoR1 (Promega) in Buffer H, and colonies with bands of the appropriate size were selected for sequencing (Bio S&T).

Band-identification strategies

Amplification of known connexins using the degenerate primers is known to produce bands of different sizes depending on the identity. A simple computer program was written with Visual Basic™ in Microsoft Access (see appendix B) to calculate the predicted amplicon sequences (see appendix A) and their sizes, and to view the degree of homology between the degenerate primers and the mRNA (See Table 1). Specific nested primers were designed for the known connexins (See Table 2) within the sequences amplified by the universal primers, and these were used to re-amplify the product of PCR with the universal primers.

For resolution of potentially closely spaced bands of less than 500 bp on the agarose gel, 3% low melt agarose was used. When this produced a single band, other methods for increasing sensitivity and resolution were used, including running the samples on 6% denaturing polyacrylamide gels, staining with the more sensitive nucleic acid dye, SybrGold (Molecular Probes), and incorporating radioactive dATP in the PCR reaction.

For increased sensitivity over ethidium bromide labelling of PCR products, gels were incubated for 10 minutes with rocking in 0.1% SybrGold (molecular probes) in TBE.

Table 2: Connexin-specific primers internal to degenerate primers

GROUP NAME	MW-NAME	Internal Sense	Internal Antisense	Predicted size
A1	43	ACCTGGCTCATGTGTTCTATG	AAGAAGGCCACCTCAAAGATAG	235
A3	46	CACGCCCACCCTCATCTAC	CTTCAGCTCGAAGCCGTACA	271
A4	37	CATCTCCCACATCCGCTACT	CCATGGTCCAGCCGTACA	332
A5	40	CATCTCCCACATTCGCTACT	TGGTCAGGAAGATTCCGTAGAT	329
A7	45	GAGGAGGACAACGAAGAGGA	ATAAAACGGGTGGACTTGGA	231
A8	50	CAGCAGGCGGGGACTAAC	CGGAACCCGTACAGGAAGTAG	182
A9	36	CCCCTGAGTCCATAGGAGGT	TGCCTTCTGAGCTTGGATTT	217
A10	58 (59)	ACGACCAGGCCTTTCCTATC	TGGCCATGGCACTTAAATAG	379
A12	47 46.6	ATCTCCACTCCCTCGGTCA	GTCTCCTCCTCCTCCTCCTC	218
B1	32	TCCCTGCAGCTCATCCTAGT	CCCTGAGATGTGGACCTTGT	156
B2	26	AGCCCAGCGCTCCTAGTG	GAAGCCGTCGTACATGACATAG	228
B3	31	ACTTCCCCATCTCCAACATC	AGGCTGAACAGGTAGGTCCA	202
B4	30.3	TTGTCTGCAACACCAAGCA	CTCAGCAAGTACGTCCACCA	250
B5	31.1	GGGAGGTTCAGGAGAAGAGG	TTTGGGGTAGAATGAGTGGAA	173
B6	30	CCCTCCAGCTGATCTTCGT	ACCCCTCTATCCGAACCTTG	159
C1	31.9	GCTTCTGGCTCTTCCACATC	CACGCTCAGCAGGTAGCA	194
	31.3	AAGGGGAAGGAGGAGGAGA	GGCATCTGGAACCCATACAG	170
	62	ACTTGGAGGAGCAGCAAAGA	GGTGCATTTGAAACCCATAGA	183
	40.1	GCCGTCTTCAGCGTCTATGT	GAGGAGGAGGTGGATGATGT	180
	25	-	-	-

(Primers were designed to have melting temperatures of 60 +/- 1 to facilitate parallel reactions.)

S^{35} labelling

For maximum sensitivity, PCR products were radioactively labelled, run on a 6% denaturing polyacrylamide sequencing gel, dried and exposed to film (Kodak X-Omat AR). PCR reaction component were the same as for cold reactions with universal connexin primers, except that 1 µl of s35 labelled dATP was added per 20 µl reaction, cold dATP was reduced 100-fold to 2uM, and 1:100 DTT was added. Following exposure, the bands were aligned and cut out. DNA was recovered by incubating with 100 µl of water at room temperature for 10 minutes and then boiling for 15 minutes to rehydrate, and centrifuging at 12,000 g for 2 minutes to elute. The supernatant was concentrated by precipitation overnight at –20C with 0.5 M Ammonium acetate and 2%

linear acrylamide under 2.5 volumes of ethanol. The concentrated DNA was re-amplified by PCR with the universal connexin primers as described above.

Real time Quantitative PCR

Real time PCR experiments were carried out on an Applied Biosystems ABI 7000 sequence detection system. Custom primers and Fam-MGB labelled probe were designed using Applied Biosystem's Primer Express™ software. Sequences are given in Table 3. Pre-optimised FAM labelled eukaryotic 18S ribosomal control reagents (4333760T) were used to normalize for differences in amounts of total RNA. Reverse transcription was performed as described above. Real-time PCR reactions contained commercially prepared 1x TaqMan® Universal PCR Master Mix (4304437) (AmpliTaq Gold® DNA Polymerase, AmpErase® UNG, dNTPs with dUTP, Passive Reference 1, and optimized buffer components) and the indicated dilutions of cDNA. Control reagents were used according to supplier directions at (dilution), and connexin 43 forward primer, reverse primer and probe concentrations were (900 nM, 900 nM and 250 nM) respectively. Each condition was prepared in triplicate. Statistical strength for the null hypothesis was assessed using a two-tailed unpaired *t*-test assuming unequal variances.

Table 3: Real-time PCR primers and probe

Probe: Humcx43-158T	FAM-ACTTGGCGTGACTTCA-MGB
Forward primer: Humcx43-125F	TTCATTTTACTTCATCCTCCAAGGA
Reverse primer Humcx43-223R	CGCTCCAGTCACCCATGTT

Briefly, real-time PCR monitors the rate of accumulation of PCR product after each thermal cycle through the detection of the release of a fluorescent reporter from the probe upon incorporation into a product. Rather than comparing the amount of accumulated product after a predetermined number of cycles as in traditional PCR, a threshold is chosen, and the number of cycles (Ct) for each sample to accumulate sufficient product to reach this threshold is compared. Since each cycle permits a doubling of the DNA, each unit difference in Ct between comparable samples represents a relative difference of 2, with lower Ct values corresponding to greater concentration. Problems of saturation which make standard RT-PCR quantitatively less reliable are avoided.

Results

Establishment and Characterization the Bladder Muscle Tissue Culture System:

One of our primary objectives in establishing a physiologically relevant tissue culture model was to ensure that bladder muscle cells do not lose their typical functional and phenotypic features while maintained *in vitro*. Although few studies have been conducted using bladder muscle cells, when exposed to serum vascular smooth muscle rapidly lose their typical "contractile" appearance, assuming a morphological appearance more typical of "secretory" cells such as fibroblasts. With these considerations in mind, we established a tissue culture system which minimized the exposure of our cells to serum, while providing good yields of bladder muscle cells. As described below, we have succeeded at culturing cells which express the expected typical smooth muscle cell markers. Not only do these cultures yield reliable numbers of cells, but the vast majority of cells present in out cultures are bladder muscle cells, with very few contaminating urothelial cells. We had been hopeful that it could be possible to demonstrate the ability of these cells to contract in response to an exogenous stimulus such as carbachol. Nevertheless, in spite of several attempts, we were unable to observe contractility by these cells, possibly due to the fact that they had rapidly become highly adherent through the formation of focal adhesions (Figure 2).

Initial attempts at culturing cells without any exposure to serum resulted in extremely low yields. In subsequent studies, 1% serum was added to cells while being plated, and the serum was then gradually replaced with defined medium. Cells were then generally studied after being maintained in defined medium for 3-5 days. Under these conditions, we have been able to reliably obtain high yields of bladder muscle cells expressing smooth muscle actin (Figure 1) and forming intercellular connections typical of bladder myocytes (Figures 2-5). As expected, the addition of 5% fetal bovine serum to these cells leads to massive cellular proliferation and a drastic reduction in smooth muscle α actin expression (Figure 1B) when compared to control cells which been grown in defined serum-free medium for 5 days (Figure 1A).

Figure 1: Serum induces loss of smooth muscle actin expression. (A) Control primary detrusor cultures exhibit smooth muscle actin staining typical of differentiated smooth muscle cells. (B) Following 48 hour treatment with 5% FBS, cells are confluent with markedly diminished smooth muscle actin expression in individual cells.

Formation of Inter-cellular Junctions:

Bladder muscle cells are typically coupled mechanically through abundant adherens junctions. In contrast to smooth muscle cells in most other tissues, gap junctions are typically rare and electrical coupling difficult to document in bladder muscle cells. Once again, bladder muscle cells maintained many of these typical features *in vitro*. Numerous attempts at staining cells plated at various densities with antibodies against connexins 26, 37 and 43 failed to produce evidence of connexin protein expression at cell-cell junctions between bladder muscle cells. However, as expected we did observe the presence of intense connexin immunoreactivity at areas of contact between rare urothelial cells which were much smaller, densely packed, and polygonal in shape.

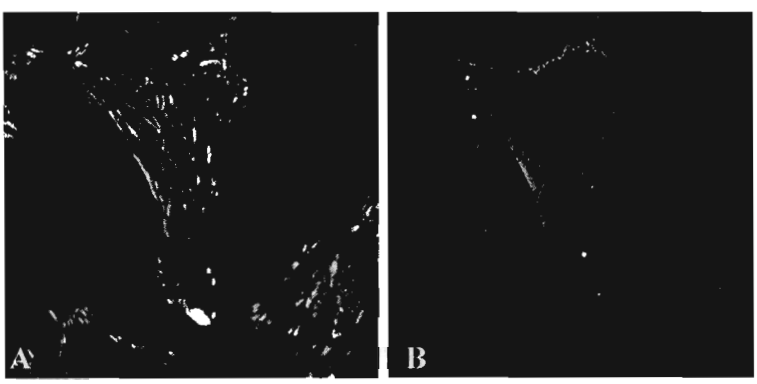


Figure 2: Bladder muscle cells form vinculin positive contacts to extra-cellular matrix. (A) Vinculin expression is limited to areas of focal contact of muscle cells with extra-cellular matrix, representing likely focal adhesions. (B) Pan-cadherin staining is limited to areas of focal contact between muscle cells. No evidence of significant co-localization of vinculin and pan-cadherin.

Vinculin staining demonstrated the presence of extensive focal contacts attaching the cells to the laminin and collagen substrate. Vinculin has been shown to co-localize with E-cadherin into the adherens junction complex of epithelial cells, while it does not appear to localize into N-cadherin-positive adherens junctions found in fibroblasts(Knudsen et al., 1995). Although investigators have assumed that vinculin immunoreactivity is indicative of the presence of adherens junctions in the bladder(Carey et al., 2000), to date, the nature and composition of adherens junction complexes in bladder muscle cells has remained unknown. The lack of co-localization of vinculin with pan-cadherin (Figure 2) or N-cadherin immunoreactivity (not shown) in our bladder muscle cultures suggests that the composition of N-cadherin-positive adherens junctions in bladder muscle cells and fibroblasts likely differs from that of E-cadherin-positive adherens junctions in keratinocytes or urothelial cells.

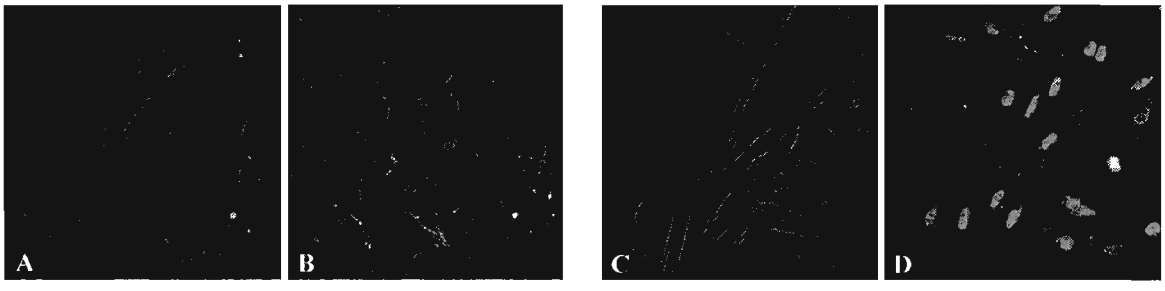


Figure 3: Formation of N-cadherin positive junctions between bladder muscle cells. (A,C) Smooth muscle actin-positive bladder muscle cells. (B) Pan-cadherin immunoreactivity is limited to areas of bright, punctate staining representing areas of pan-cadherin positive focal contact between bladder muscle cells (likely adherens junctions). (D) Similar immunoreactivity pattern, combined with non-specific nuclear staining, is observed with antibody to N-cadherin.

Double labelling with antibodies against smooth muscle α actin and N-cadherin revealed the presence of multiple areas of focal N-cadherin-positive cell-cell contact, most likely representing the formation of adherens junctions between cells. Bright, punctate staining was visible along the muscle perimeter at regions of intercellular contact (Figure 3), though the polyclonal N-cadherin antibody also produced a considerable background of nuclear staining that is also visible in other reports using this antibody (Vasioukhin et al.,

2000). An interesting aspect of our studies was the occasional observation of N-cadherin-positive "zipper-like" structures between muscle cells (Figure 4). The appearance of these structures resembled that of "adhesion zippers" which had been described as areas of new cell-cell contact and active adherens junction formation between keratinocytes (Vasioukhin et al., 2000). To the best of our knowledge, our observation of "adhesion zippers" in bladder muscle cells is the first demonstration of the ability of N-cadherin expressing cells to form this type of junctional contact.

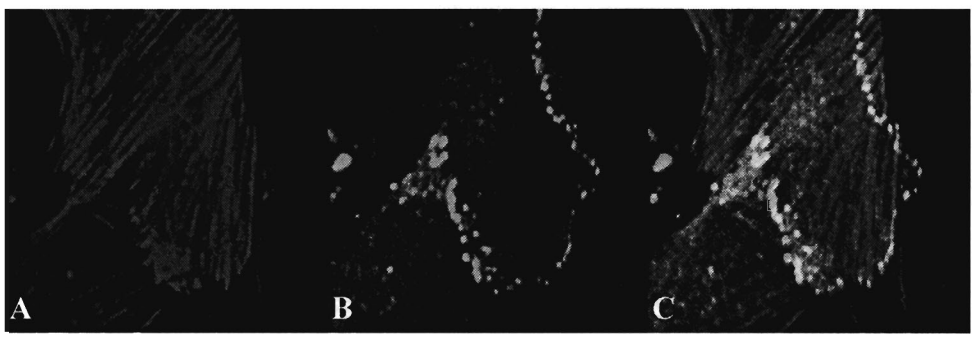


Figure 4: Formation of N-cadherin positive zipper-like contacts: (A) High magnification image of smooth muscle actin positive bladder muscle cells observed in early stages of cell-cell contact. (B) N-cadherin immunoreactivity is limited to areas of "zipper-like" contact between muscle cells. (C) Smooth muscle actin-positive fibres terminate in areas of N-cadherin-positive contact.

A great deal of knowledge has been gained in recent years regarding the biochemical composition of adherens junctions, as well as the specific signals which lead to the assembly of these components during adherens junction formation. Unfortunately, most of this information has been obtained in studies of epithelial cells. While some studies have addressed these issues in N-cadherin-expressing cells including fibroblasts and skeletal muscle, very little is known about the composition of these junctions in smooth muscle cells. Adherens junctions appear to represent a very important form of muscle-muscle contact in the bladder and to be involved in the pathogenesis of some categories of detrusor dysfunction. Nevertheless, the composition of adherens junctions expressed by bladder muscle cells remains unknown. With these considerations in mind, we undertook an initial examination of the composition of adherens junctions in cultured bladder smooth muscle.

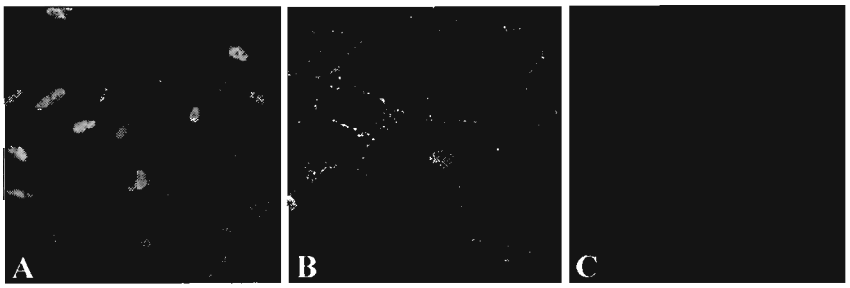


Figure 5: Cultured rat detrusor smooth muscle cells express N-cadherin, but not E-cadherin. N-cadherin positive intercellular contact is evident in cultured bladder muscle cells using both a polyclonal (A) or monoclonal (B) antibody. No E-cadherin immunoreactivity was observed (C).

We observed that detrusor smooth muscle cells express N-cadherin, but not E-cadherin protein. This was evident both in tissue culture where the vast majority of cells are muscle cells (Figure 5), and also in tissue sections where both urothelial and muscle cells are present (Figure 6). Urothelial cells, which were quite rare in tissue culture (not shown) and common in tissue sections (Figure 6), stained intensely for both an epithelial marker (pan-cytokeratin) as well as for E-cadherin. In contrast, smooth muscle cells expressed both smooth muscle α actin and N-cadherin (Figures 3, 4).

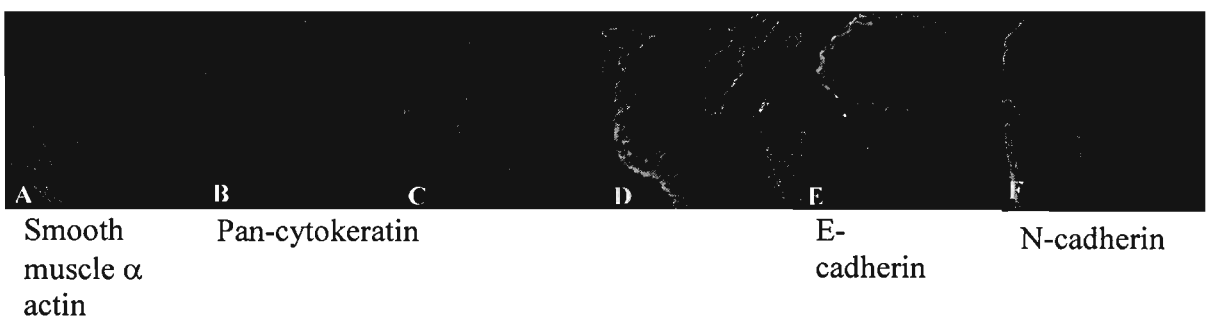
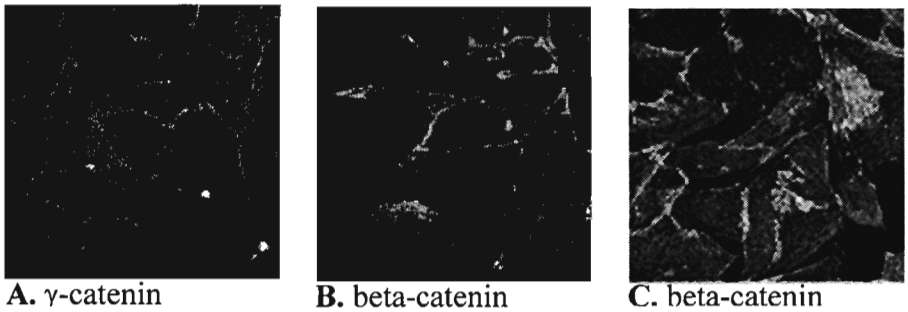


Figure 6: Cadherin Expression in Bladder Tissue Sections. (A) Smooth muscle actin staining seen throughout bladder muscle cells. (B, C, D) Pan-cytokeratin immunoreactivity is limited to urothelial layer. E-cadherin staining is entirely limited to pan-cytokeratin-positive urothelial layer (E), while N-cadherin immunoreactivity is present throughout the muscle layer.

The expression of a number of known adherens junction components was evaluated using both western blots and immunohistochemical staining (Summarised in Table 4, Table 5).

Although immunoprecipitation studies were not performed, a number of conclusions could be drawn. Alpha catenin staining was attempted. Staining was very weak and was likely non specific, but may be consistent with the staining of cytoplasmic dense bodies. It showed no preferential localisation to the junctions (not shown). Western blots with this antibody produced a single strong band of inappropriate size in both cultured detrusor cell extracts and in the protein extract from human endothelial cells provided with the antibody as a positive control, casting further doubt over the specificity of the staining (Figure 8D). β–catenin immunoreactivity labelled the cytoplasm (Figure 7B), but was especially strong at the membrane in regions of intercellular contact. Western blots show a strong band of appropriate size (Figure 8C). γ–catenin (also called plakoglobin) exhibited a similar staining pattern (Figure 7A), and an appropriately sized band (~82 kDa) on western blots (Figure 8G).



A. γ-catenin **B.** beta-catenin **C.** beta-catenin

Figure 7: Localization of known adherens junction components in bladder muscle cultures. (A) γ-Catenin (plakoglobin) immunoreactivity exhibits a diffuse membrane expression. (B, C) β-catenin staining was largely localized to areas of junctional contact between muscle cells.

Desmoglein produced a strong band of appropriate size on western blots of the expected size (~165kDa) on western blots, but immunostaining appeared noisy and non-specific.

A western blot for p120ctn showed strong bands of the appropriate size (~120-130 kDa).

Table 4: Summary of Immunohistochemical Studies Using Antibodies to Potential Adherens Junction Components

Protein	Observed Pattern
N-cadherin (P) (monoclonal)	Punctate membrane staining, (nuclear background-as expected) Monoclonal has less nuclear staining
E-cadherin	None in cultured smooth muscle. Labels urothelial cells in rat bladder sections
α-catenin	Weak, primarily cellular staining. Possibly of cytoplasmic dense bodies, but likely non-specific
Beta-catenin	Strong junctional staining (some cytoplasmic)
γ-catenin (plakoglobin)	Membrane staining. weaker background
Vinculin	Focal contacts
SM α actin	Strong labelling of filaments in cultured cells
Keratin	Strong labelling of urothelial cell layer
Pan-cadherin	Punctate membrane labelling in regions of cell-cell contact

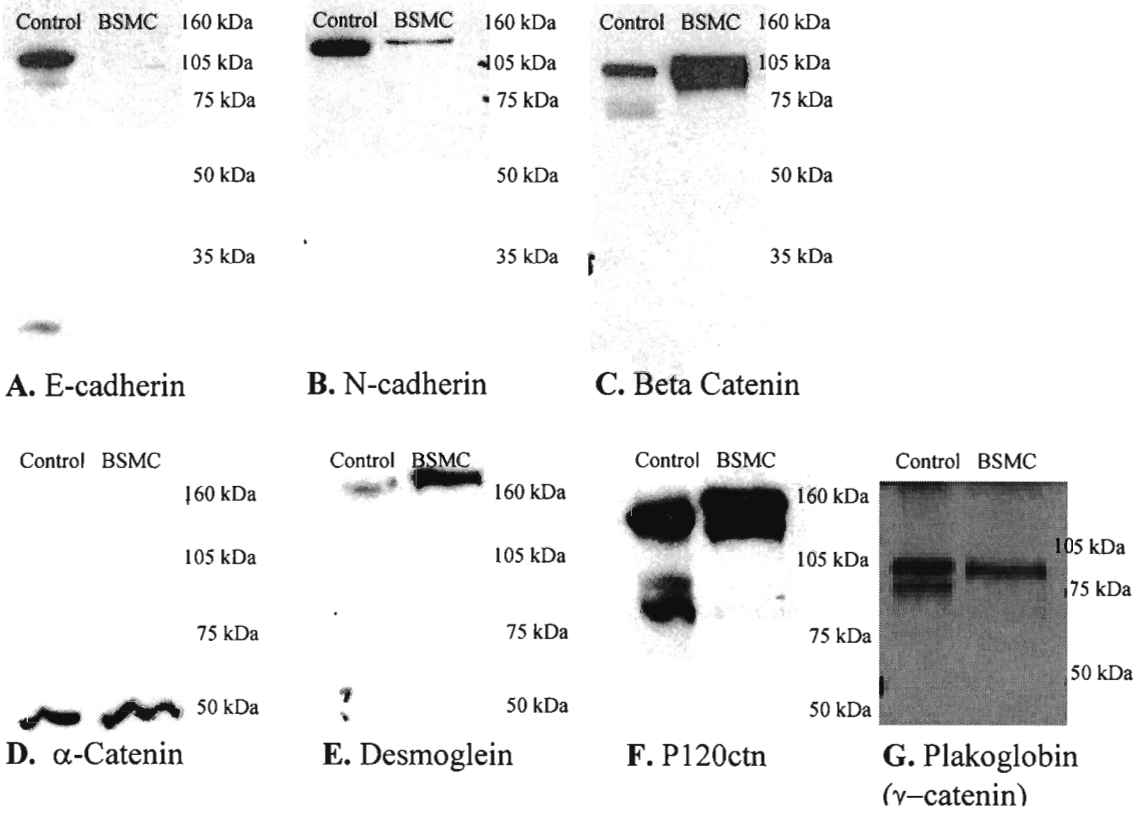


Figure 8: Western blots performed with antibodies for known adherens junction components using control protein extracts as positive controls (left) and total protein extracts prepared from cultured rat bladder smooth muscles (right- BSMC)

Table 5: Summary of Western blots

Protein	Observations (Expected size (kDa))
N-cadherin	130 kDa (130) weaker in cultured smooth muscle cells than in control extract, but very distinct
E-cadherin	120 kDa (120) strong in control, but almost undetectable in cultured smooth muscle
α-catenin	50 kDa (102) a single identical band of inappropriate size is observed in both cultured smooth muscle and control cell extract, indicating that the antibody is not specifically detecting the desired product.
Beta-catenin	92 kDa (92) very strong in cultured smooth muscle
γ-catenin (plakoglobin)	82 kDa (82). single band (vs. double in control)
Desmoglein	165 kDa (165) Strong in cultured smooth muscle
P120ctn	120 kDa+ (120) Strong in cultured smooth muscle, appears as a doublet

Human studies

Clinical data: Urodynamic profiles of subjects

Since no protrusion junctions or other features of the disjunction pattern was observed during the course of our tissue culture studies, techniques were developed to evaluate the connexin gene expression of human biopsies containing these junctions. In the course of this project, two groups of human biopsies were obtained for connexin gene profiling. The first group of 6 biopsies (group 1; Table 6) included only biopsies from individuals who had overactive bladder. In contrast, of the 13 biopsies in the second group (group 2; Table 7), 8 were from individuals with stable and 5 from individuals with overactive bladders. Surprisingly, biopsies in the first group tended to vary in appearance and size (Table 6). Biopsies in the second group were much smaller, yet more uniform in size and texture (Table 7).

Table 6: Urodynamic profiles of subjects and biopsy description- group 1

Sample code	bladder capacity (ml)	Volume (ml) at 1st UC	Estimated biopsy size (mg)	Notes regarding biopsy appearance	Urodynamic outcome
A: GC 0046	727	727	4		UC provoked, with leakage
B: LL 0263	770	703	2		UC provoked, with leakage
C: CD 0261	819	548		Dark red	UC spontaneous, inhibitable, no leak
D: MC 0258	562	546	4	lighter in colour	UC provoked, with leakage
E: CK 0264	439	–	2		no UC (stable)
F: KO 0148	1099	128	8	Very large	UC spontaneous, non-inhibitable, with leakage

UC= unstable contraction

Table 7: Urodynamic profiles of subjects and biopsy description- group 2

Blind codes	Sample code	Contractility	Obstruction	Overactive	Pre-precipitation Calculated RNA yields (µg)
4	2008#3	Normal	-	+	0.76
7	2008#4				0.78
6	2008#7				1.20
13	2008#8				1.06
5	2009#6	Normal	-	+	0.34
14	2012#5	Slightly impaired	-	+	0.88
15	2012#7				0.63
2	2015#6	Normal	-	-	0.34
11	2028#6	Normal	-	-	0.19
10	2034#6	Normal	-	-	0.77
3	2037#6	Normal	-	+	0.31
1	2040#6	Normal	-	-	0.25
12	2045#6	Normal	-	-	0.40

Initial Qualitative PCR Analysis of Biopsy Samples

RNA yields, especially those from samples included in the second group, were low, but detectable. The code was broken and clinical data such as specific urodynamic diagnosis was revealed only after the completion of S^{35} radiolabelling studies for the first group, and following the completion of real-time PCR analysis in the case of the samples obtained in the second group.

Following RNA extraction, samples from the first group all revealed the presence of intact ribosomal RNA bands, with no apparent genomic DNA contamination when resolved in agarose gels. PCR amplification using the universal connexin primers produced a single strong band for all biopsy samples. No visible amplicons were produced in paired samples (RT- controls) in which the RT enzyme was excluded, further confirming the absence of any significant contamination with genomic DNA (Figure 9). Agarose gels used to resolve PCR products obtained from second group biopsies revealed a similar pattern (Figure 10). Samples were still coded at this point, yet amplicon size was similar in the case of all biopsy samples (Figure 10). The size of all amplicons obtained at this point was ~430-440 bp was consistent with the possible presence of connexin 43 (433 bp), connexin 37 (433 bp), connexin 40 (427 bp), connexin 59 (439 bp) or connexin 62 (439 bp). Amplicons representing connexin 26 (382 bp) sequence, which were present in urothelium (not shown), were not observed in bladder biopsy samples.

A custom designed program (Appendix B) was used to identify regions of homology between the degenerate primers used in this study and members of the human connexin gene family, and predicted amplicon sizes (Table 1) and sequences were calculated. These calculated amplicon sizes agreed with experimental results (Urban et al., 1999) for sequences that had been previously examines using these primes. While many molecules exhibited high homology with the primers, only connexin 46 and 50 exhibited perfect homology to both. Connexin 36, which was reported to not amplify with these primers and conditions, has especially low homology with the 3' end of the antisense primer, which is consistent with this result.

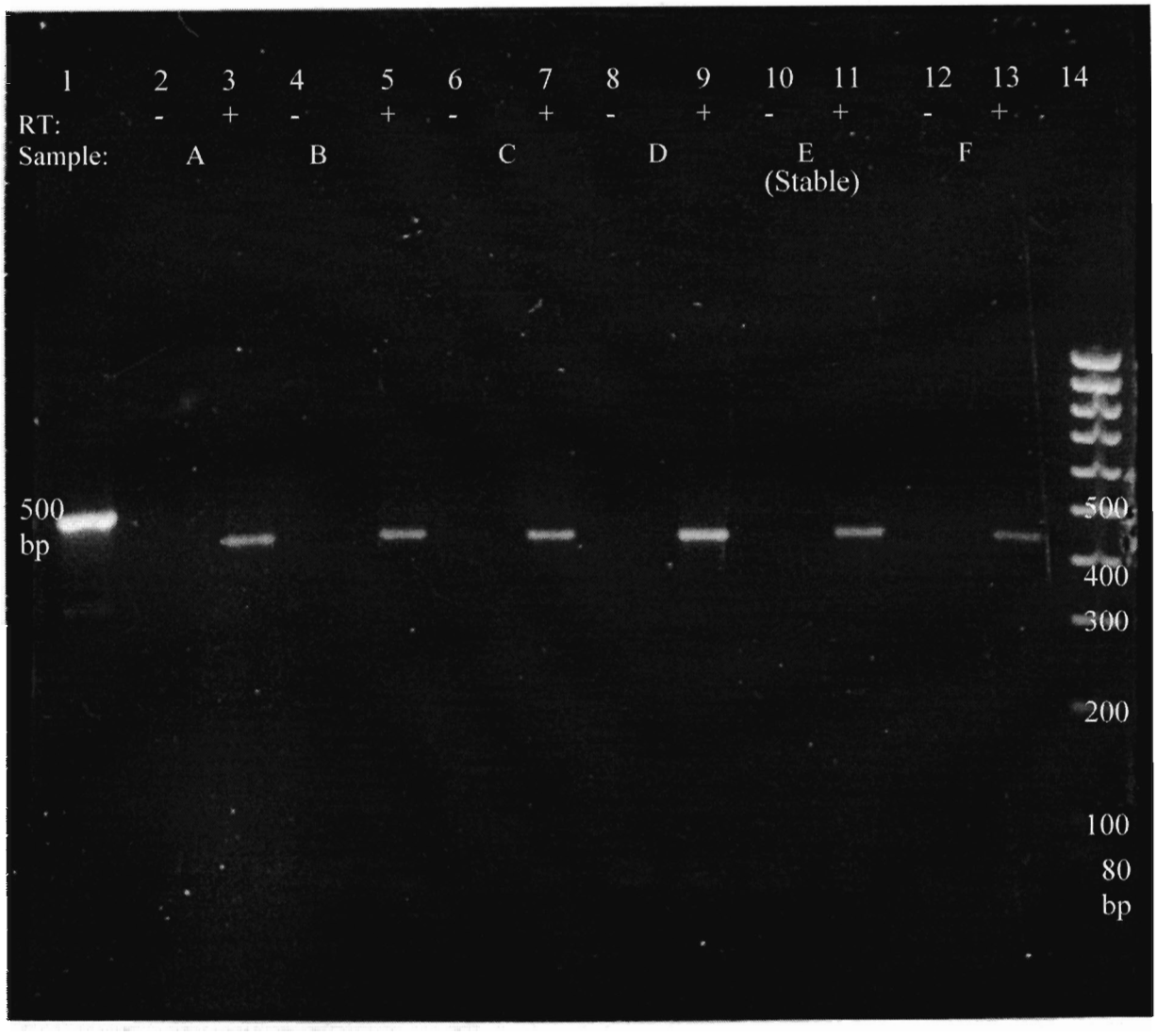


Figure 9: Degenerate PCR primers against conserved regions of the connexin family produced strong bands from cDNA from all samples (batch 1), but not from their RT-negative controls. The size of roughly 433 is consistent with connexins 43, 37 (433 bp), 40 (427 bp), 59 or 62 (439 bp). Bands for connexin 26 (382 bp), present in the urothelium, are not seen.

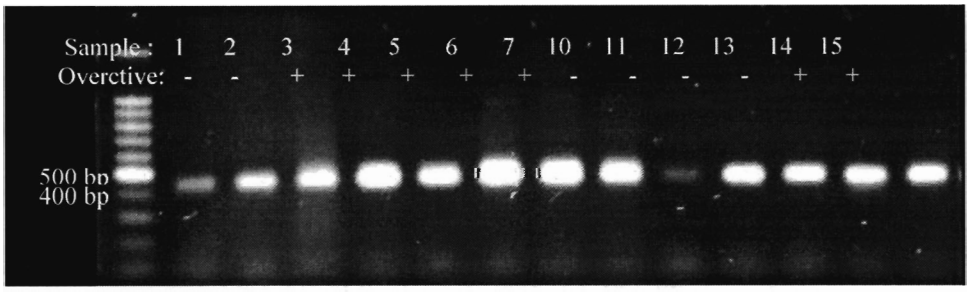


Figure 10: RT-PCR with degenerate connexin primers on bladder biopsies, batch 2

In order to obtain better amplicon resolution, electrophoresis was also performed using 6% poly-acrylamide mini-gels, followed by staining with SybrGold. While clearly being much more sensitive than traditional ethidium bromide staining, neither this approach nor one using larger sequencing gels, resulted in the identification of any additional bands using the universal connexin PCR primers (not shown).

PCR reactions were also performed in the presence of α-S^{35} dATP in an attempt to further increase the sensitivity of this detection system and to begin obtaining sequence information. This strategy did produce additional faint bands on a sequencing gel (Figure 11) Unfortunately, PCR results were not consistently reproducible. Additional bands (e.g. lane E in Fig. 11) could not be obtained on a consistent basis and no single amplicon was able to reliably and consistently differentiate overactive and stable bladders.

Establishing Sequence Identity of Individual Amplicons

Radioactive bands were excised from the sequencing gel, and re-amplified using the degenerate connexin primers. Using this strategy, a single band of an unexpected size was excised and sequenced from an S^{35}-labelled reaction (Figure 11). Analysis of sequence data revealed the presence of an artefact which resulted from the insertion of an additional forward primer 5' of the human connexin 43 sequence (98% homology to the published sequence). Errors, including the amplification of a primer-dimer in the antisense direction, were likely due to the combination of high magnesium concentration, the use of degenerate primers and an enzyme lacking proofreading activity, as well as the high number of cycles used.

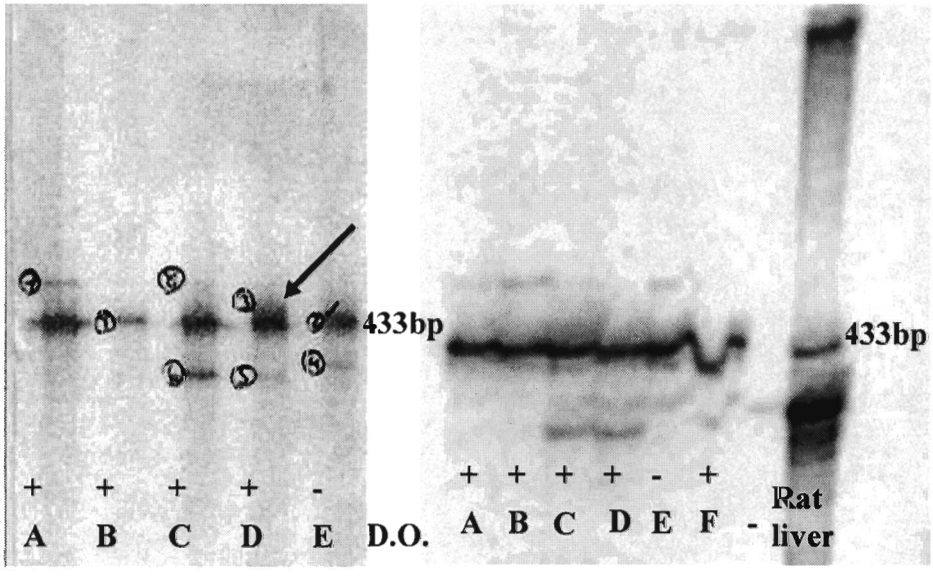


Figure 11: S^{35}-dATP labelled RT-PCR on bladder biopsy samples with degenerate connexin primers did not reveal consistent novel bands associated with detrusor overactivity. Arrow indicates sequenced band.

Pooled PCR products obtained using degenerate connexin from all six samples was further amplified with specific nested primers to various human connexins. Nested PCR using primers specific for connexins 40 and 43 produced strong bands consistent with the expected sizes 329 and 235 bp respectively. Primers specific for connexins 26 and 37 resulted in the presence of expected bands which were distinct but faint. In contrast, primers specific for connexins 46, 50 and 59 did not produce any visible bands in ethidium bromide stained agarose gels.

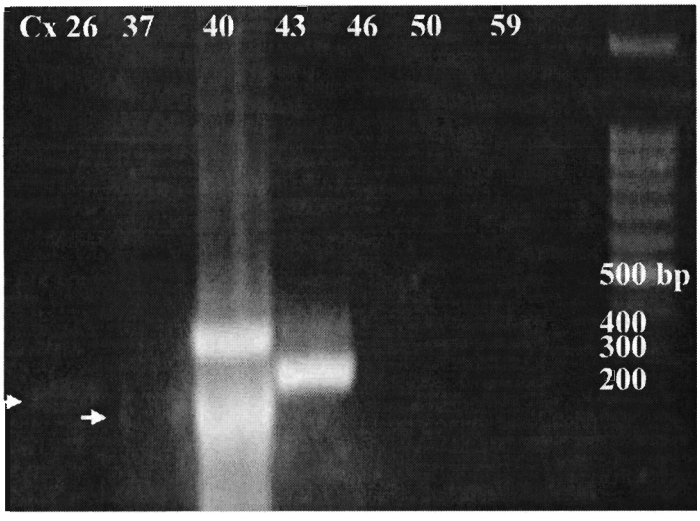


Figure 12: Nested PCR for specific connexins: Pooled degenerate connexin PCR product from all six samples was further amplified with specific nested primers to various human connexins. Connexins 40 and 43 produced strong bands, while connexins 26 and 37 produced faint but detectable band (white arrows), and connexins 46, 50 and 59 did not produce bands visible on ethidium bromide stained agarose gel.

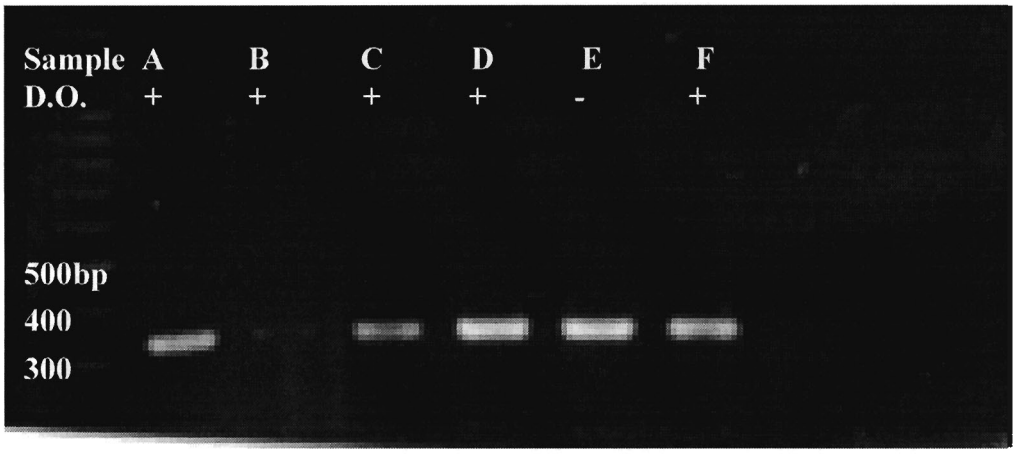


Figure 13: Connexin 40 is detected in all samples by RT-PCR. The weakest band, in the lane for sample b, does not correspond to the stable bladder. Expected size is 329 bp

Nested PCR produced strong bands for both connexin 43 (Figure 12) and connexin 40 (Figure 12 & 13). The identity of connexin 43 was further confirmed using PCR cloning and direct sequencing.

Connexin 37 and 26 produced very weak bands, while evidence of 46, 50 and 59 was absent. (Figure 12). Connexin 40 is detected in all samples by RT-PCR (Figure 13). The weakest band, (sample B) does not correspond to the stable bladder (sample E).

Sequencing of multiple colonies cloned from gel-purified degenerate connexin RT-PCR product revealed primarily sequences highly homologous with connexins 43, with several identifying connexin 37 (Figure 14).

A.Human connexin 43 (reverse orientation) 100% homology

TGGGGCGGGAGATGAAGCAGTCCACCTGATGTGGGCAGGGNATCTCTTTTGCAAGTGTAAACAGCACTC
AAGCTGAATCCATAGATGTACCACTGGATCAGCAAGAAGGCCACCTCAAAGATAGACTTGAAGAGGATA
CTGATGATGTAGGTTCGCAGCAACCCCCCTCGCATTTTCACCTTACCATGCTCTTCAATACCGTACTTG
AACTTCTTTATCTCAATCTGCTTCAAGTGCATGTCCACATTGACACCATCAGTTTGGGCAACCTTGAGT
TCTTCCTCTTTCTTGTTCAGTTTCTCTTCCTTTCGCATCACATAGAACACATGAGCCAGGTACAAGAGT
GTGGGTACAGACACAAATATGATCTGCAGGACCCAGAAGCGCACATGAGAGATTGGGAAAGACTT**GTCA**
TAGCAGACGTTTTTACAGCCAG

B.Human connexin 37, variant 1, 99.7% homology

GGCTGTGACAACGTCTGCTACGACCAGGCCTTCCCCATCTCCCACATCCGCTACTGGGTGCTGCAGTTC
CTCTTCGTCAGCACACCCACCCTGGTCTACCTGGGCCATGTCATTTACCTGTCTCGGCGAGAAGAGCGG
CTGCGGCAGAAGGAGGGGGAGCTGCGGGCACTGCCGGCCAAGGACCCACAGGTGGAGCGG**C**CGCTGGCG
GCCGTAGAGCGTCAGATGGCCAAGATCTCGGTGGCAGAAGATGGTCGCCTGCGCATCCGCGGAGCACTG
ATGGGCACCTATGTCGCCAGTGTGCTCTGCAAGAGTGTGCTAGAGGCAGGCTTCCTCTATGGCCAGTGG
CGCCTGTACGGCTGGACCATGGAGCCCGTGTTTGTGTGCCAGCGAGCACCCTGCCCCTACCTCGTGG**AC**
TGCTTCCTCTCCAGCCCCA

Figure 14: Examples of sequences identifying connexin 43 and 37 in PCR product of pooled degenerate RT-PCR product from the second batch of samples. A) Human connexin 43 sequence, inserted in reverse orientation, 100% homology with the reference sequence NM_000165 (excluding primer sequence). B) Human connexin 37, variant 1, 99.7% homology with the published sequence AF139100 (excluding primer sequence).

Nested PCR:

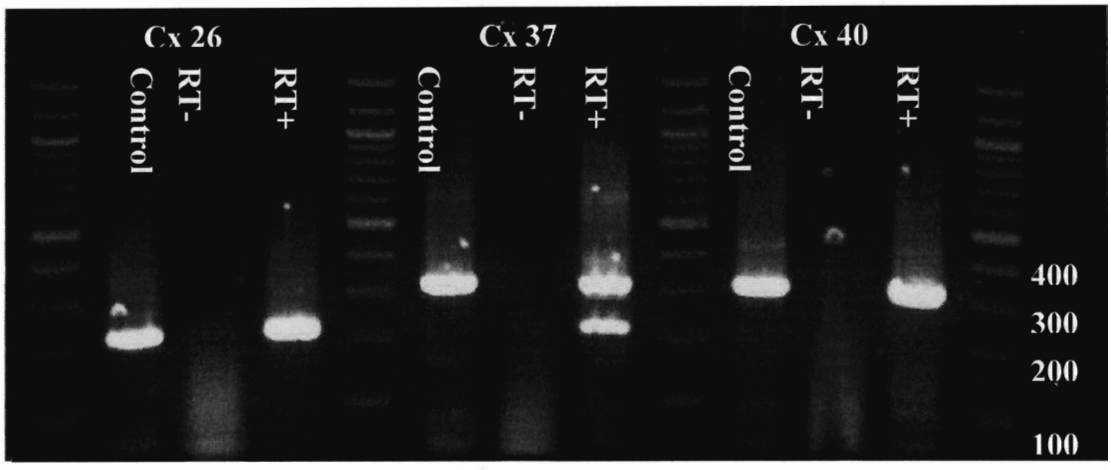


Figure 15: Nested PCR for connexin 26, 37 and 40. Expected sizes: 228, 332, 329 bp

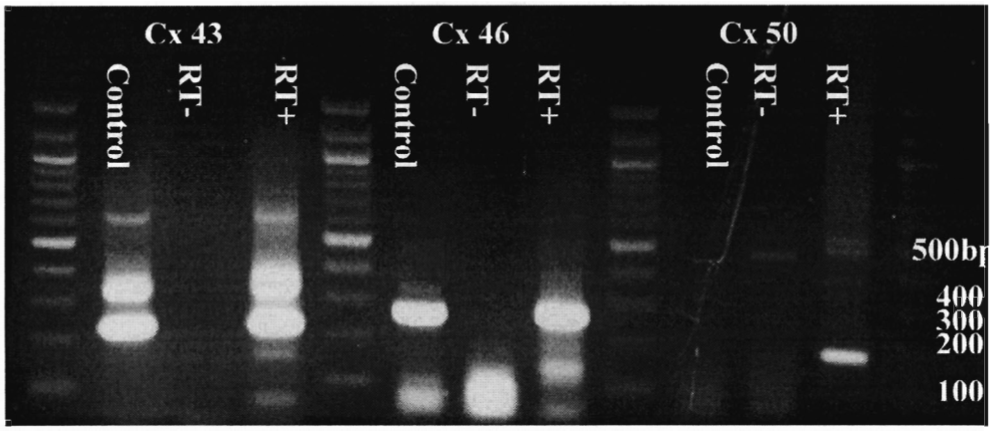


Figure 16: Nested PCR for connexin 43, 46 and 50. Expected sizes: 235, 271, 182

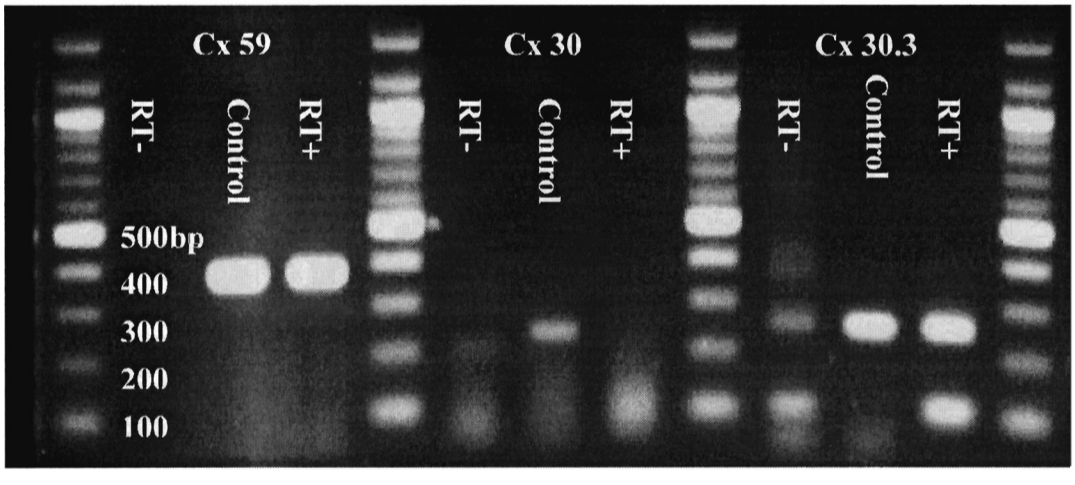


Figure 17 Nested PCR for connexin 59, 30, 30.3. Expected sizes: 379, 159, 250

A fraction of the degenerate connexin PCR product from either the RT+ or RT- reaction was pooled, ensuring that each pooled sample contained product from an unstable bladder. This pooled DNA was then screened by nested PCR for presence of specific connexins. Oligos specific for connexins 26, 37, 40, 46, 59 and 30.3 revealed the presence of appropriately sized bands in the human bladder biopsy RT+ nested reaction and the human reference RNA control RT+ nested reaction (Figures 15-17), but not in the biopsy RT- control nested reaction. These assays were performed using a high cycle number. Although this approach improved PCR sensitivity it did also reduce the specificity of this assay producing a number of weaker inappropriately-sized bands. Connexin 30 primers produced a single weak band of inappropriate size in the positive control reaction, but not the bladder biopsy reaction, while connexin 50 primers produced a distinct but relatively weak band in the bladder biopsy reaction but not the reference RNA control reaction.

In summary, qualitative PCR screening of these bladder biopsies revealed the presence of several known members of the connexin gene family. However, using this approach we did not observe the presence of any potential novel connexins, nor were any consistent connexin gene mutations apparent.

Quantitative Analysis of Connexin 43 mRNA Using Real-time PCR

In view of the fact that major qualitative difference in connexin gene expression could not be demonstrated in our initial analyses, subsequent efforts were devoted to examining the hypothesis that the development of an unstable bladder was associated with increased connexin 43 mRNA expression.

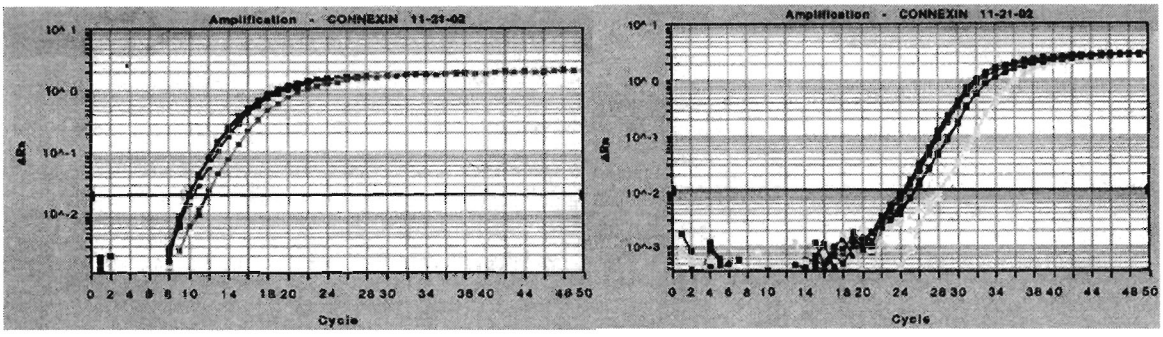


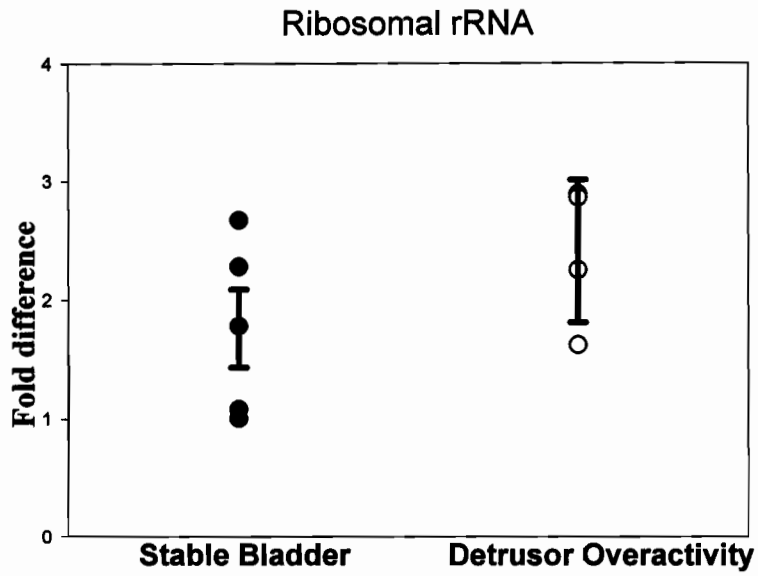
Figure 18: Example of raw output obtained from a Real-time quantitative PCR reaction for 18S ribosomal RNA (left) and Connexin 43 mRNA (right) using cDNA obtained from a human bladder biopsy.

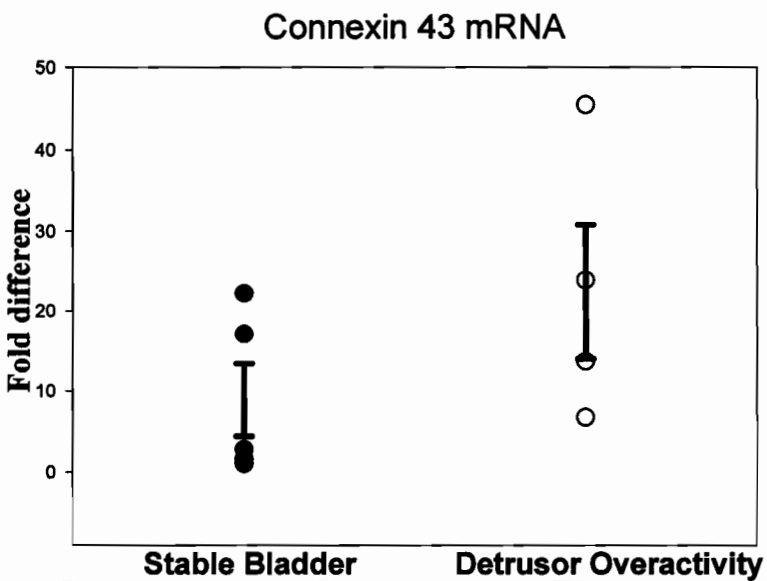
Real-time quantitative PCR for connexin 43 mRNA was performed using primers and TaqMan® probe. This approach resulted in the amplification and detection of an expected 99 bp fragment, representing nucleotides 125 to 223bp of the connexin 43 mRNA sequence. Resultant amplicons produced clean amplification with superimposible curves for replicate wells from the same biopsy samples (Figure 18). A threshold of 0.02 was chosen for ΔRn (relative change in fluorescent signal intensity per cycle) was chosen. A threshold cycle (Ct) number was identified by ABI 7000 Sequence detector software as the extrapolated cycle number at which the signal would have intersected the threshold. Relative differences in mRNA between samples were calculated according to Equation 1.

$$\text{Relative concentration} = 2^{\Delta Ct}, \text{ Where}$$

$$\Delta Ct = (\text{(maximum sample Ct) - (given sample Ct)})$$

Equation 1





	Stable					Overactive			
Sample:	2040	2015	2034	2028	2045	2037	2009	2008	2012
Cx43 mRNA	1.60	2.75	22.21	1.00	17.11	6.73	13.67	45.46	23.86
S18 rRNA	1.78	1.08	2.67	1.00	2.28	1.62	2.25	2.89	2.88

Averages	Stable Bladder	standard error of the mean	Detrusor Overactivity	standard error of the mean	fold increase
Cx43 mRNA	8.93	4.99	22.43	9.75	2.51
S18 rRNA	1.76	0.37	2.41	0.35	1.37

Figure 19: relative amounts of ribosomal and connexin 43 mRNA detected in biopsy samples by Real-time PCR, including only the deepest biopsies available from each subject.

Relative amounts of both connexin 43 mRNA and 18S ribosomal mRNA varied between biopsy samples (Figure 19). In part, this was due to the fact that the highly limited amount of mRNA present in each sample did not allow for the determination of total mRNA levels prior to the performance of real time quantitative PCR.

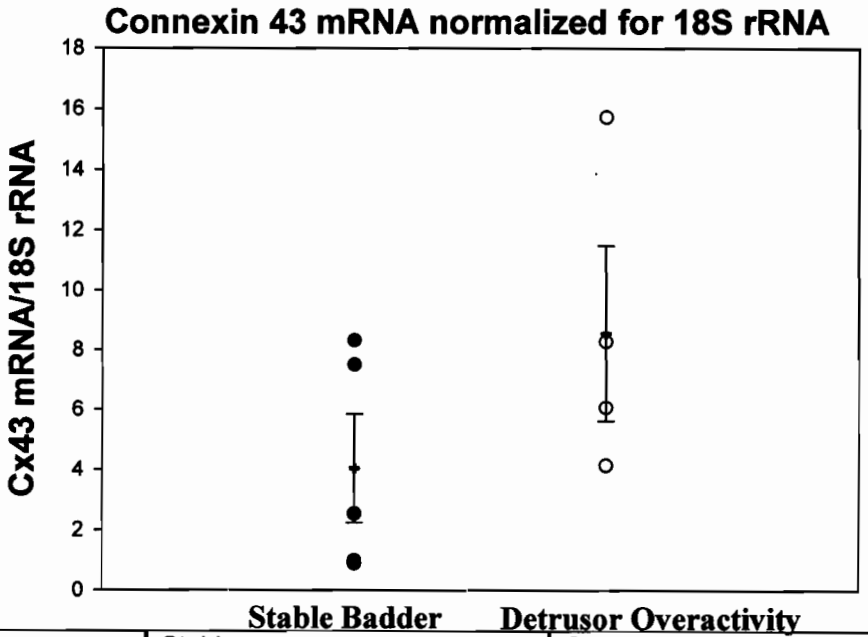
As a result, the amount of connexin 43 mRNA measured in each sample was normalised to the amount of 18S ribosomal mRNA present in the same sample (Equation 2).

$$\text{Relative connexin mRNA expression} = 2^{\Delta(\Delta Ct)}$$

Where

$\Delta(\Delta Ct)$ = -(given sample (Cx43 Ct – Ribosomal Ct) – minimum sample (Cx43 Ct – Ribosomal Ct))

Equation 2



	Stable					Overactive			
Sample:	2040	2015	2034	2028	2045	2037	2009	2008	2012
Cx43/rRNA	0.90	2.55	8.32	1.00	7.50	4.15	6.08	15.73	8.28

Averages	Stable Bladder	standard error of the mean	Detrusor Overactivity	standard error of the mean	fold increase
Cx43/rRNA	4.05	1.80	8.56	2.93	2.11

Figure 20: Scatter plot of connexin 43 mRNA expression normalised to 18S rRNA examining the deepest biopsy samples available for each subject.

Comparing the average relative values for the two groups, samples from overactive elderly bladders contained an average of 2.1 times more connexin 43 mRNA than those from stable bladders (Figure 20), but the variability within each of these groups was high. The two means were less than one standard deviation apart, and $p = 0.19$ using a two-tailed unpaired *t*-test assuming unequal variances. Interestingly, there appeared to be a bi-modal distribution of Cx43 mRNA in the stable bladder group. While samples from 2 stable bladders (mean = 8.81) demonstrated Cx 43 mRNA expression nearly comparable to that obtained from unstable bladders (mean= 10.25), in 3 biopsies from stable bladders Cx 43 mRNA expression was much lower (mean = 1.67).

Discussion

Cell culture

Overview of results

A tissue culture model system was established using rat bladder muscle cells. The primary goal of this effort was to provide us with opportunities to begin addressing questions related to the pathogenesis of detrusor overactivity and to do so in a mechanistic fashion simply not possible when using human needle biopsies. Since no single published tissue culture protocol fully met our study objectives, our approach included potentially desirable features of several published studies. A period of development and validation of our tissue culture protocol was also necessitated by the fact that it is uncommon for investigators to culture smooth muscle cells in serum-free defined media and that studies focussing on bladder muscle cells are very rare.

Our initial conditions were based on a report by (Bowers and Dahm, 1993) who had established an *in vitro* tissue culture system which allowed chick amnion smooth muscle cells to retain their ability to contract for extended periods of time. Our choice of gentle dissociation conditions and defined N2 serum-free medium (DMEM 1:1 with Ham's nutrient mixture F-12) was based on their observation that harsher dissociation conditions and exposure to serum lead to loss of contractility, while HEPES and TES buffers induce the formation of vacuoles in these cells(Bowers and Dahm, 1993).

The initial low cell density of $8x10^3$ cells/cm^2, the feeding schedule of replacing half of the medium every second day, and some of the dissociation strategies including the use of specific purified elastase, triturating through the tip of a fire-polished pipette and the removal of undissociated epithelial cells with a 70 μm nylon mesh strainer also came from this paper(Bowers and Dahm, 1993). Adaptation of tissue dissociation methods to the bladder, an anatomically more complex tissue than chick amnion, was influenced by methods used to obtain cultured rat cerebral vascular smooth muscle that develops tension in response to pH changes (Apkon et al., 1997). In this report, tissues were dissociated into cells by incubating for 1 hour in collagenase type II, elastase, DNase I and soybean

trypsin inhibitor dissolved in a saline solution of similar composition to the Earle's balance saline solution (EBSS). Laminin and collagen type IV were chosen as our substrate in response to the demonstration by Hedin *et al* (Hedin et al., 1988) that collagen type IV and laminin prolonged the maintenance of a contractile phenotype of arterial smooth muscle cells in contrast to a substrate of fibronectin which promoted proliferation and protein synthesis.

Interpretation of results

Data presented here clearly demonstrates that our protocol allows us to maintain rat detrusor smooth muscle cells *in vitro* for several days. Moreover, in contrast to detrusor muscle cells grown in the presence of serum, these cells appear to retain the morphologic and phenotypic features (e.g. strong smooth muscle α-actin expression) typical of contractile smooth muscle cells. We had hypothesized that cultured detrusor muscle might be induced to express connexins and form connexin-positive cell-cell contact as a result of the injury response associated with cell dissociation and culture. Under our culture conditions rat bladder muscle cells did not express connexin 26, 37 or 43 protein, nor did we observe the presence of any connexin-positive structures which could represent cell-cell contact through protrusion junctions. Rare instances of connexin 26 staining were exclusively associated with apparent epithelial cell contamination (based on cell shape, size, and absence of smooth muscle α–actin expression - data not shown), which decreased significantly as technique improved. Interestingly, other groups have recently reported connexin-43 protein expression by cultured guinea pig and human bladder muscle cells(Neuhaus et al., 2002a),(Neuhaus et al., 2002b), (Wang et al., 2001). These differences may be attributed to harsher dissociation conditions, the presence of 10% serum in culture medium, a much higher plating density with cells being confluent or to species differences.

We were able to observe ample cadherin-positive cell-cell contact in our cultured bladder smooth muscle cells. Although we did not perform ultrastructural examination of these junctions, the appearance of cadherin-positive immunoreactivity suggested both the presence of established cadherin-positive intercellular junctions (adherens junctions), while also being able to observe the establishment of inter-cellular contact through the

assembly of such junctions ("adhesion zippers"). Our results indicate that following careful dissociation, bladder muscle cells cultured using our protocol are able to re-establish precisely the type of cell-cell contact which is observed in the normal (stable) bladder. Having established tissue culture conditions which allow bladder muscle cells to establish and maintain all relevant aspects of their normal phenotype *in vitro*, has allowed us to begin characterizing, for the first time, the nature of adherens junctions in bladder muscle cells, while also exploring ways of potential inducing the formation of "protrusion" junctions and other aspects of the dysjunction pattern in our model system. As discussed below, in contrast to E-cadherin-positive adherens junctions formed by urothelial cells, bladder muscle cells formed N-cadherin-positive contacts *in vitro*. Moreover, these bladder muscle junctions more closely resembled adherens junctions described in fibroblast than those which had been extensively studied in epithelial cells.

The need for animal models of Detrusor Overactivity

The most popular existing model of detrusor overactivity is the partial surgical obstruction of rat or guinea pig bladder outlet(Williams et al., 1993). However, this operation leads to a large degree of bladder hypertrophy in addition to the unstable contractions. Although men with bladder outlet obstruction can develop an over-active detrusor together with associated symptoms, in most men and women detrusor overactivity develops without any evidence of associated bladder obstruction, muscle hypertrophy or neurological lesion (Elbadawi et al., 1993c),(Elbadawi et al., 1993d). Given the fact that the animal model of partial bladder outlet obstruction results in major degrees of muscle hypertrophy, as well as dramatic changes in gene expression (Chaqour et al., 2002), on first examination, partial bladder outlet obstruction does not seem to represent a good model to study the pathogenesis of the type of detrusor instability seen in most older individuals who are incontinent.

Ovariectomized Rat Develops Changes Typical of Impaired Detrusor Contractility, but not of Detrusor Overactivity

Detrusor Hyperactivity with Impaired Detrusor Contractility (DHIC) is a commonly observed urodynamic pattern in older incontinent individuals. Most such individuals are older post-menopausal women whose estrogen levels or often low. Since estrogens have been shown to influence neuronal survival (Brinton, 2001), we proposed that estrogen

depletion could promote the development of the degeneration pattern described with impaired detrusor contractility. Moreover, since estrogens have also been shown to influence the expression of gap junctions and connexins in the uterus (Grummer et al., 1994), we also proposed that estrogen depletion could also contribute to the development of the dysjunction pattern and associated detrusor overactivity. In this earlier study, female Fischer 344 rats underwent sham or bilateral ovariectomy surgery at 9 months of age and were sacrificed 4 months later (Zhu et al., 2001). Bladder muscle strips from ovariectomized animals demonstrated decreased contraction in response to carbachol, while morphologic evaluation revealed evidence of diminished muscle bulk and muscle quality, as well as axonal degeneration in ovariectomized, but not sham-operated animals (Zhu et al., 2001). Thus, we believe that the ovariectomized bladder provides an appropriate model system for the study of impaired detrusor contractility, while not representing a useful model for the study detrusor overactivity.

Cultured Rat Bladder Muscle Cells From Cadherin-Positive, but not Connexin-positive Contact.

In the course of our studies bladder muscle cells expressed cellular markers typical of fully-differentiated smooth muscle cells (e.g. smooth muscle actin, caveolin-1) and were able to form cadherin-positive junctions *de novo*. In contrast to urothelial cells, none of these bladder muscle cells appeared to express connexin 26, 37 or 43 protein. Bladder biopsies obtained from older individuals with normal bladder function have been shown to express abundant adherens junctions, with no apparent gap junctions (Elbadawi et al., 1993b), (Elbadawi et al., 1993c). John *et al.* (John et al., 2003) have recently demonstrated the presence of small gap junctional plaques expressing connexin 45 in muscle cells in the tumour-free resection margins of individuals with bladder cancer. A recent report also reported that presence of low levels of Connexin 43 expression in adult male rat bladder muscle cells and a significant up-regulation of this signal following partial bladder outlet obstruction (Haefliger et al., 2002). Thus, our tissue cultures provide a good model system for the study of the mechanisms involved in the development and maintenance of normal bladder structure and function. At the same time, given the apparent potential of both human and rodent bladder muscle cells to express connexin proteins and to form gap junctions, future efforts will be undertaken to

identify and characterize specific signals capable of inducing connexin expression and gap junction formation in this model system.

Our tissue culture protocol was developed using a variety of elements which had been previously shown by other investigators to be important in allowing different types of muscle cells to maintain their contractile properties. Factors which were felt to be important in our opinion included a gentle digestion-dissociation protocol using specific high purity enzymes, relevant extracellular matrix substrates laminin and collagen, and chemically defined serum-free culture medium. Adjustment of different mechanical, chemical and biological parameters of cellular dissociation, plating and maintenance lead to a protocol for reliably producing viable primary detrusor smooth muscle cultures from rat pup bladders. Morphology and appropriate α–smooth muscle actin expression identified these cells as smooth muscle cells in nature, and cultures were generally free of SM-actin negative polygonal epithelial cell contamination, which was important for experimental techniques involving the bulk properties of the cultures, such as western blots and RT-PCR. It was shown here that bladder smooth muscle cells could be maintained under the desired serum-free conditions, while the addition of serum lead to a phenotypic modulation involving rapid proliferation and reduced intensity of smooth muscle α–actin filament staining (Figure 1). Smooth muscle α actin expression decreased with serum exposure, as expected, and its presence was taken as a marker of cell type, while its intensity and organisation were taken as indicators of cell phenotype. In response to prolonged exposure to 5% fetal bovine serum, cells exhibited a decrease in smooth muscle-α-actin staining intensity, combined with a robust proliferation response.

We were unable to observe a clear-cut contractile response by these muscle cells in response to carbachol. At this point, it is unclear whether this lack of a visible contraction was due to the inability of carbachol to induce a contractile response in these cells or whether the ability of these cells to affect a visible shortening was due to their high degree of attachment to a fixed matrix. As shown in Figure 2, these cells exhibited a high degree of attachment to a fixed matrix (glass slides), through vinculin-positive focal adhesions. In the future, this issue could be resolved by either again attempting bulk

partial digestion of cell-substrate adhesion with trypsin solution or by using flexible matrices for tissue culture. It should be noted that care will need to be taken in interpreting any negative results since a significant change in tissue culture conditions such as the addition of a trypsin digestions step or any change in the adherence properties of the matrix could induce phenotypic changes in the bladder muscle cells and the loss of a contractile response.

Components of the cadherin cell-cell adhesion complex in bladder muscle cells.

Positive staining with a pan-cadherin antibody showed that these cells were forming adherens junctions in culture, and that they were re-establishing specific contact de *novo* between adjacent bladder muscle cells. Not only are adherens junctions abundant in the normal (stable) adult bladder, but these mechanically-coupled junctions have been thought to be responsible for the majority of cell-cell coupling between bladder muscle cells. Moreover, one of the typical features of the dysjunction pattern present in detrusor overactivity is a dramatic decrease in the numbers of adherens junctions between bladder muscle cells (Elbadawi et al., 1993c). Our interest in examining the formation of adherens junctions in cultured bladder muscle cells arose from the fact that these junctions define both the development and the maintenance of normal bladder structure.

In addition to these considerations, our studies have provided us with a remarkable opportunity to begin an examination of a type of adherens junctions whose biology and composition had not been previously studied. In recent years, a great deal of information has been obtained regarding both the biology and composition of adherens junctions with most studies performed in epithelial cells (Vasioukhin et al., 2000). While most such studies were done in keratinocytes, there has been considerable interest in the role of E-cadherin-positive adherens junctions in the pathogenesis of bladder cancer arising from bladder urothelial cells (Sun and Herrera, 2002).

Interestingly, in spite of a dearth of information regarding bladder muscle adherens junctions some have incorrectly assumed that these junctions are in all aspects identical to those which have been extensively characterized in keratinocytes. For example, Carey *et*

al. used the fact that vinculin has been shown to be a component of E-cadherin-positive adherens junctions in keratinocyte to justify the use of vinculin as a marker for adherens junctions in the human bladder (Carey *et al.*, 2000). While urothelial cells express E-cadherin, our studies conclusively demonstrate that bladder muscle cells express N-cadherin and that cell-cell contact between these cells is formed through N-cadherin-positive contact points. In addition, while vinculin immunoreactivity in bladder muscle cells localizes to areas of focal contact between muscle cells and the matrix (focal adhesions), vinculin immunoreactivity does not co-localize with cadherin immunoreactivity with no localization to areas of cell-contact (Figure 2).

Our results also suggest that the composition of N-cadherin-positive adherens junctions in the bladder differs from that of E-cadherin-positive junctions typically studied in keratinocytes. Additionally, they are fully consistent with the earlier observation made by Knudsen *et al.* (Knudsen et al., 1995) that the composition of fibroblast adherens junctions differs from that of adherens junctions in epithelial cells.

Both E- and N-cadherin had been shown to co-localize extensively and/or co-precipitate with α-, β– and γ–catenin (plakoglobin) (Knudsen et al., 1995). In contrast, while E-cadherin co-localizes with vinculin, N-cadherin does not (Knudsen et al., 1995). Our studies with bladder myocyte adherens junctions are consistent with these findings. In addition to establishing that vinculin does not co-localize with N-cadherin in these cells, we were able to demonstrate co-localization of both β–catenin and γ–catenin (plakoglobin) with N-cadherin at areas of cell-cell contact. Unfortunately, the results of α–catenin and P120ctn western blotting and immunostaining were inconclusive. While our findings (see Table 8 summary) will need to be pursued further using immunoprecipitation approaches, they do indicate that the adherens junctions found in detrusor muscle cells are more like those found in fibroblasts than those found in epithelial cells.

Table 8: Adherens junction composition in epithelial cells, fibroblasts and smooth muscle

Epithelial cells (Knudsen et al., 1995)	Human Fibroblasts	Bladder Smooth Muscle
E-cadherin	N-cadherin	N-cadherin
alpha-actinin	α–actinin	?
Vinculin	Not vinculin	Not vinculin
α-catenin	α-catenin	?
β-catenin	β-catenin	β-catenin
Plakoglobin	plakoglobin	plakoglobin

Connexin gene expression in human bladder biopsies

We were able to extract mRNA and perform both qualitative and quantitative RT-PCR on human bladder biopsies containing a very small amount of tissue. In the course of our studies, we had clearly demonstrated that connexin 37, 40 and 43 are expressed in human bladder biopsies. These three connexins appeared to be expressed both in bladder biopsies from individuals with evidence of detrusor instability, as well as those obtained from normal controls with stable bladders. The degenerate "universal" primers did not detect the presence of any novel connexin sequence, nor did we identify any significant mutation in a connexin gene using this approach.

The degenerate "universal" connexin primers used in our studies were designed against a consensus connexin sequence and do not match any of the human connexin sequences perfectly. The addition of a relatively high $MgCl_2$ concentration (e.g. 3.0 mM) increases their sensitivity in terms of amplifying related sequences, but decreases their specificity, as well as quantitative representativeness (as mis-priming is necessary in order to obtain even the desired product). In earlier studies these same primers had been used to identify other previously unknown connexins (Haefliger et al., 1992), rat connexins 31.1, 33, 37 and 40.Thus, if any previously unidentified connexin were associated with the development of detrusor overactivity, these degenerate primers might have provided an opportunity of detecting such novel sequences. Computer analysis (appendix B) revealed the regions of homology between the widely used degenerate connexin primers and all known human connexin mRNA sequences (Willecke et al., 2002). Predicted sizes were calculated for the amplification product of each (Table 1). Of course, this strategy would

not have identified connexins whose DNA sequence differed from known sequences only outside of the region spanned by these degenerate "universal" connexin primers, nor would this approach detect any novel post-translational alterations of known connexin proteins.

Our RT-PCR studies using these degenerate "universal" connexin primers produced a single major band in all samples (Figures 9-10). Subsequent sequence analysis of these amplicons revealed the presence of connexin 43 sequence. The use of a more sensitive radioactive detection system in higher resolution polyacrylamide gels revealed the presence of additional, yet weaker bands. These additional bands were present in some, but not all of the biopsy samples. We had the opportunity to analyze only a limited number of bladder biopsy samples and, to date, in no case was the expression of one of these bands specific for either normal or unstable bladders (Figure 11). Sequence analysis of one "larger than expected" RT-PCR amplicon (see arrow Figure 11) revealed the presence of connexin 43 DNA sequence combined with an artefact involving a primer-dimer product. This and the several point mutations in the sequences obtained were probably the product of high magnesium concentrations and cycle number, as well as the use of Taq DNA polymerase lacking proof-reading activity. Other possible sources of weak bands could also include potential contaminating cell types. Such potential contaminating populations other than bladder muscle cells include occasional urothelial cells, blood vessel cells (endothelial or smooth muscle cells), as well rare fibroblasts. All of these potentially contaminating cell populations express connexins and may be variably present in different biopsies due to differences in biopsy technique, biopsy depth or may simply represent sampling differences.

Nested PCR

While cloning and sequencing of bands from degenerate RT-PCR reactions favoured the detection of the more abundantly expressed and efficiently amplified connexin sequences, it also permitted the possibility of the detection of less abundantly represented connexins using nested PCR. Internal sequences were predicted (Table 1- methods section, appendix), and primers were designed against all known connexins (Table 2-methods).

Preliminary results suggest that connexins 26, 30.3, 46, 50, and 59 might also be present. However, our interpretation of these results is complicated by the low specificity of RT-PCR reactions adjusted for optimum sensitivity. These results were obtained using nested PCR on the degenerate product. While a high number of PCR cycles greatly increased assay sensitivity, it did so at the expense of specificity and a loss of any possible quantitativity. Many of the bands appeared saturated, and while RT- controls prepared under identical conditions were negative indicating the absence of genomic DNA contamination, it is possible that rare mRNA from contaminating cell types could have lead to strong bands. The use of larger amounts of bladder mRNA in future studies could allow the amplification of less abundant connexin mRNA levels with fewer amplification cycles. Moreover, in the future additional biopsies could be sectioned, allowing for the localization of given connexins to specific cellular elements using immunohistochemistry, *in situ* hybridization or *in situ* PCR.

Real-time PCR

Because consistent qualitative differences between connexin expression in the stable and overactive bladder were not observed, quantitative studies were pursued next. Connexin 43 was selected as the best candidate based on sequence data, and TaqMan™ probe and primers were designed for real-time PCR.

Interpretation of the real-time PCR results

Our results demonstrated that connexin 43 mRNA is detectable in transurethral bladder biopsies from elderly human detrusor. Connexin 43 mRNA was detectable both in superficial bladder biopsies (containing both urothelial and muscle cells), as well as deep biopsies (containing predominantly bladder smooth muscle cells). In the course of our preliminary studies we were able to first demonstrate our ability to quantitatively measure both connexin 43 and 18S rRNA using real time PCR.

Once the quantitative nature of these reactions was established, a first study using biopsy samples was performed. A total of 4 biopsies from individuals with detrusor instability were chosen, as were 5 biopsies from normal controls with stable bladders. In each case, whenever several biopsies were available from any individual subject, only the deepest

was chosen for real time PCR analysis. Measurement of 18S rRNA levels in these biopsy samples revealed a small, statistically insignificant increase in rRNA levels in biopsies from individuals with detrusor instability (mean 1.76 $\pm$ 0.37 s.e.m. vs. mean 2.41 $\pm$ s.e.m.; NS; Figure 19). The amount of total mRNA obtained from these bladder biopsies (~ 1-2 μg) did not permit measurement of total mRNA levels using standard techniques. Therefore, since ribosomal RNA constitutes the majority of RNA expressed in each cell, we used 18S rRNA to adjust for the total amount of total RNA obtained from each biopsy.

Although our findings did not reach statistical significance, they demonstrated a roughly two-fold increased expression in connexin 43 mRNA levels in biopsies from individuals with detrusor instability, as compared to normal controls (22.43$\pm$9.75 s.e.m. vs. 8.93 $\pm$ 4.99 mean $\pm$ s.e.m.; NS; Figure 19). Our studies revealed that correcting connexin 43 mRNA levels for 18S rRNA levels did not significantly alter the data obtained (8.56$\pm$2.93 vs. 4.05$\pm$1.80 mean $\pm$ s.e.m.; NS; Figure 20). This observation is consistent with the hypothesis that the overactive bladder becomes less stable due to increased electrical coupling through connexin 43–positive protrusion junctions.

Biological variability and sample size

Interestingly, both the unstable and stable groups demonstrated considerable variability in connexin 43 mRNA levels, even after adjustments for made for 18S rRNA levels. It remains to be seen whether this variability is the result of variability in the performance of the real time PCR assay or whether, in fact, this represents true biological variability in either or both of these groups.

Ultrastructural studies conducted within both the stable and unstable groups point to considerable degrees of potential variability in connexin 43 mRNA expression which may have a biological explanation. For example, while all individuals with detrusor instability exhibit some features of the dysjunction pattern, the extent of these changes does appear to vary as do the numbers of protrusion junctions (Elbadawi et al., 1993c), (Elbadawi et al., 1997). At the same time, it has been reported that many individuals with urodynamically stable bladders have evidence of what has been termed an incomplete dysjunction pattern (Elbadawi et al., 1993c), (Elbadawi et al., 1997). Although many of

the features of the full dysjunction pattern are seen in these subjects, protrusion junctions are much less abundant and they do not appear to join muscle cells in chains as is observed with the complete dysjunction pattern (Elbadawi et al., 1993c), (Elbadawi et al., 1997). Although this preliminary study included only a small number of subjects are findings could potentially be attributed to this type of biological variability.

Interestingly, the five biopsies obtained from individuals with stable bladders demonstrated a potentially bimodal distribution, with three low normalised values (1.02, 2.83 and 1.17; Cx mRNA/18S rRNA) and two high values (9.24 and 8.38; Cx mRNA/18S rRNA). Ultrastructural analyses of biopsies from these subjects are currently underway, but we believe that while the former will likely represent biopsies with no significant features of the dysjunction pattern, the latter group may represent individuals with the incomplete dysjunction pattern. Although the results obtained from the four subjects with urodynamically demonstrated detrusor instability varied greatly, all four values (17.49, 9.35, 4.78 and 6.75; Cx mRNA/18S rRNA) were higher than any of the three low values obtained from stable subjects (1.02, 2.83 and 1.17; Cx mRNA/18S rRNA). Thus, future studies will be required to: 1) correlate connexin 43 mRNA expression with ultrastructural changes; 2) to increase the sample size studied; 3) to localize the expression of connexin genes to individual cellular populations and 4) to begin a prospective study of these findings.

Such studies are absolutely essential since the mere usefulness of bladder biopsies as a diagnostic tool for detrusor dysfunction remains unclear and controversial (Lu et al., 2002), (Prieto et al., 1999), (Mastropietro et al., 2001), (Carey et al., 2000), (FitzGerald et al., 2000) and (Haferkamp et al., 2003). It has been proposed that many of these controversies could be attributed to major methodological differences between groups of investigators (Haferkamp et al., 2003).

Methodological variables to be considered in future studies

Given the observed variability, several changes to the methodology might be useful. First, RNA levels used in the RT reaction would ideally be higher, and more strictly controlled. Though the amounts of ribosomal RNA detected between samples were similar, they were not identical. By necessity, less than 1 microgram of was used per RT

reaction, and this was not adjusted per RT reaction at the time due to limits in the sensitivity of the UV spectrophotometer. Attempts could be made in future studies to use higher amounts of RNA. However, given the small size of biopsies obtained from human subjects and the nature of bladder tissue, this objective could be difficult to accomplish. Biopsy samples could be pooled to obtain greater amounts of RNA, however, unless samples were pooled according to earlier ultrastructural assessment, combining tissues from individuals with normal pattern and an incomplete dysjunction pattern could merely increase variability. Surgical specimens could be obtained from the tumour-free margins of individuals who underwent cystectomy for bladder cancer. Although these samples can be proven to be tumour-free, it is not clear how representative these samples are of bladder tissue in older individuals with idiopathic detrusor instability.

Secondly, the concentration of samples by ethanol precipitation using linear acrylamide as a co-precipitant would be omitted. In retrospect, this manipulation appears to have been unnecessary, as the achieve 4-fold concentration of the RNA was probably not worth the increased risks that come with additional sample manipulation. Despite the care taken, this step increased the risk of differential sample loss, degradation and contamination with salt or ethanol. Increased noise is of greater concern in instances of even weaker signals.

Follow-up studies:

Though the expression of mRNA is an absolute prerequisite for the protein to be expressed, changes in mRNA expression alone are insufficient to prove the hypothesised increase in electrical coupling. Screening for connexin mRNA can therefore provides a good indicator of which connexins proteins to look for using antibodies. Antibodies to several, but not all human connexins are available. Connexin 45 protein expression was recently examined in tissue obtained from stable bladders undergoing radical cystectomies (John et al., 2003), and it would be of interest to repeat these experiments , including immunoelectron microscopy, for other connexins including connexin 43.

If larger amounts of tissue became available from unstable bladders as well, westerns or ELISA assays would provide means of acquiring quantitative assessment of protein expression, though contamination with other connexin-containing cell types such as

endothelial cells and fibroblasts could still interfere with interpretation if expression levels are low.

The translation of mRNA to protein and the translocation of this protein to the cell membrane are both known to be regulated for connexins. At least two connexin mRNAs, connexin 43 and 32, contain internal ribosomal entry sites (IRES) suggesting complex post-translational regulation (Schiavi et al., 1999,Hudder and Werner, 2000). Additionally, many connexin proteins including connexin 43 have half-lives in the range of 1-5 hours (review: (Saffitz et al., 2000)). It could be interesting to examine if and how these are regulated in the bladder.

Future prospects

The historical controversy over connexin expression in the bladder appears to be resolving with the application of modern, more sensitive techniques, though many fundamental questions still remain. While earlier ultrastructural studies (Elbadawi, 1982,Daniel et al., 1983,Elbadawi et al., 1993c) generally failed to reveal any typical gap junctions, ultrastructural evidence supporting the presence of gap junctions in the bladder has recently grown stronger (Elbadawi et al., 1993c), (Tse et al., 2000), (John et al., 2003).

Electrophysiological studies have suggested that the bladder is electrically coupled, though far less extensively than other tissues (Bramich and Brading, 1996,Fry et al., 1999), and that changes occur in the overactive bladder which are consistent with increased coupling. The finding that connexin levels increase in the obstructed bladder supports both the significance of this model to studies of non-obstructed idiopathic overactive bladder, and the involvement of connexins, protrusion junctions and increased electrical coupling in the changes occurring in the overactive bladder. Though the statistical power of our results is weak, they are supported by published electrophysiological results and recent results from surgically obstructed rodent bladder.

<u>Follow-up questions:</u>

Having found evidence of connexin 43 mRNA expression and possible up-regulation in the overactive human bladder (a finding which must be confirmed using additional biopsies), studies at the protein level will need to follow. Tissue availability from cancer-free characterised bladders is insufficient to support western blotting. Immunoelectron

microscopy to localize the connexin expression to the protrusion junctions and ultra-close abutments is feasible and more meaningful because it would avoid the problem of interference by contaminating cells from blood vessels. In rat small intestine smooth muscle immunoelectron microscopy has successfully been performed, and it was found that connexin 43 but not connexin 45 immunoreactivity labelled junctions with remarkably similar appearance to protrusion junctions (Seki and Komuro, 2001). Very recently, the expression of connexin 45 was localised to junctions in the stable non-obstructed human bladder by immunoelectron microscopy (John et al., 2003). Since the methodology has been worked out, it would be of great interest to expand this study to include unstable bladders, and also to examine the expression of connexin 43.

Why are protrusion junctions so hard to find?

In the first report published in 1992, protrusion junctions were felt to be unique to the overactive bladder (Elbadawi et al., 1993c), while subsequent studies by this and other groups indicate the presence of protrusion junctions in bladders other than those with documented detrusor instability. Nevertheless, the observation that protrusion junctions are more prominent in the unstable bladder has remained.

It has been proposed that methodological factors could play a role in the relative scarcity of ultrastructurally observed gap junctions in most studies of the human or rodent bladder. In their study, John *et. al.* have proposed the intriguing suggestion that the empty state of the bladder at the time that the tissue was obtained may be a factor, and that in a filled state, the number and visibility of gap junctions could increase (John et al., 2003). This question could be addressed in animal models, or in artificially stretched tissue strips or cultured cells.

Pictures of structures resembling protrusion junctions in chronically obstructed guinea pig bladder were published in a review article, but methods were not discussed (Brading, 1997a).With respect to protrusion junctions in the human bladder, it could be speculated that the inconsistencies seen between studies may relate to the history of the bladder prior to the biopsy, including the length of time between biopsy and the most recent distension of the bladder, as well as the duration and magnitude of this pressure rise and distension.

Is N-cadherin down regulated in the overactive detrusor?

While ultrastructure has demonstrated decreased adherens junctions in the overactive bladder, quantitative evidence might strengthen the case. Electron microscopy is a powerful technique, but it is demanding, and can be very sensitive to differences in processing (Mastropietro et al., 2001), (Holm et al., 1997).

Are other connexins expressed and up regulated?

Many tissues express multiple connexins, and it would be worth investigating whether any other connexins are up regulated in the overactive bladder. Connexin 45 is found in the stable bladder (John et al., 2003), and we have designed Real-time PCR primers and probe to investigate the potential regulation of connexin 45 mRNA.

What is the mechanism underlying this up-regulation?

It is difficult to identify upstream changes in a signalling pathway by rational design when triggering events are not known. Questions of this nature are likely to benefit from the rapid accumulation of genetic information and gene expression data. A solid understanding of the elements of the connexin 43 promoter region would narrow the search, and a cross-sectional examination of gene expression array studies for patterns associated with the up regulation could also prove enlightening.

Is the up regulation of connexin 43 necessary and sufficient?

Two transgenic mice exist which could permit the creation an inducible connexin 43 knock-out targeted to smooth muscle. A connexin 43 "floxed" mouse (Theis et al., 2001) could be bred with the SM-CreER^{t2}(tg) mouse which expresses Cre recombinase fused with an engineered ligand binding domain specific for tamoxifen, in smooth muscle cells under the influence of smooth muscle specific SM22 promoter (Kuhbandner et al., 2000). Failure of these mice to develop unstable contractions in response to outlet obstruction would suggest that connexin 43 expression is necessary for the development of detrusor overactivity. However, the demonstration of other connexins in the rodent and human(John et al., 2003) bladder makes this scenario unlikely. Moreover, the SM22 promoter will target this gene to all smooth muscle cells, including vascular, gut, bronchial and bladder. Currently, no promoters specific for bladder smooth muscle cells are available. Thus, gene over-expression or deletion which would be bladder muscle specific is not specific at this time.

Final conclusion and summary;

We were able to grow primary cultures of rat bladder smooth muscle under serum-free conditions. These cells expressed relevant smooth muscle markers, including smooth muscle α–actin. These cultures were largely free from urothelial cell contamination.

Smooth muscle cells formed N-cadherin positive adherens junctions, and also expressed vinculin in a pattern suggestive of focal adhesion contacts to the matrix.

Cultured smooth muscle cell adherens junctions are similar to those of epithelial cell line MDCK in that they express β catenin and plakoglobin, but they appear to differ in their expression of N-cadherin instead of E-cadherin. Moreover, unlike in E-cadherin positive cells, vinculin did not appear to co-localize with cadherin to areas of cell-cell contact in bladder muscle cells.

The characterization of these junctions specifically in bladder smooth muscle will help to build a solid foundation for understanding changes in the intercellular junctions of bladder smooth muscle cells observed in the bladders of elderly individuals with detrusor hyperactivity versus stable age-matched controls.

Junction components in human detrusor biopsies and rat detrusor primary cultures have been examined. Connexin mRNA was found to be present in the human bladder, and connexin 43 mRNA appears to be up-regulated, though variability between samples was high, possibly reflecting biological differences between stable bladders with normal ultrastructure and those with an incomplete dysjunction pattern. Further studies will be required in order to determine that the protein is expressed and localised to protrusion junctions.

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Appendix A:

Sequence of potential connexin amplicons using universal primers (used for creating specific nested primers)

A1 cx43 433bp
GGTTGTGAAA ATGTCTGCTA TGACAAGTCT TTCCCAATCT CTCATGTGCG
CTTCTGGGTC CTGCAGATCA TATTTGTGTC TGTACCCACA CTCTTGTACC
TGGCTCATGT GTTCTATGTG ATGCGAAAGG AAGAGAAACT GAACAAGAAA
GAGGAAGAAC TCAAGGTTGC CCAAACTGAT GGTGTCAATG TGGACATGCA
CTTGAAGCAG ATTGAGATAA AGAAGTTCAA GTACGGTATT GAAGAGCATG
GTAAGGTGAA AATGCGAGGG GGGTTGCTGC GAACCTACAT CATCAGTATC
CTCTTCAAGT CTATCTTTGA GGTGGCCTTC TTGCTGATCC AGTGGTACAT
CTATGGATTC AGCTTGAGTG CTGTTTACAC TTGCAAAAGA GATCCCTGCC
CACATCAGGT GGACTGTTTC CTCTCTCGCC CCA

A3 cx46 415bp
GGCTGCGAGA ACGTCTGCTA CGACAGGGCC TTCCCCATCT CCCACATCCG
CTTCTGGGCG CTGCAGATCA TCTTCGTGTC CACGCCCACC CTCATCTACC
TGGGCCACGT GCTGCACATC GTGCGCATGG AGGAGAAGAA GAAAGAGAGG
GAGGAGGAGG AGCAGCTGAA GAGAGAGAGC CCCAGCCCCA AGGAGCCACC
GCAGGACAAT CCCTCGTCGC GGGACGACCG CGGCAGGGTG CGCATGGCCG
GCGCGCTGCT GCGGACCTAC GTCTTCAACA TCATCTTCAA GACGCTGTTC
GAGGTGGGCT TCATCGCCGG CCAGTACTTT CTGTACGGCT TCGAGCTGAA
GCCGCTCTAC CGCTGCGACC GCTGGCCCTG CCCCAACACG GTGGACTGCT
TCATCTCCAG GCCCA

A4 cx37 433bp
GGCTGCACCA ACGTCTGCTA TGACCAGGCC TTCCCCATCT CCCACATCCG
CTACTGGGTG CTGCAGTTCC TCTTCGTCAG CACACCCACC CTGGTCTACC
TGGGCCATGT CATTTACCTG TCTCGGCGAG AAGAGCGGCT GGCGCAGAAG
GAGGGGGAGC TGCGGGCACT GCCGGCCAAG GACCCACAGG TGGAGCGGGC
GCTGGCCGGC ATAGAGCTTC AGATGGCCAA GATCTCGGTG GCAGAAGATG
GTCGCCTGCG CATTCCGCGA GCACTGATGG GCACCTATGT CGCCAGTGTG
CTCTGCAAGA GTGTGCTAGA GGCAGGCTTC CTCTATGGCC AGTGGCGCCT
GTACGGCTGG ACCATGGAGC CCGTGTTTGT GTGCCAGCGA GCACCCTGCC
CCTACCTCGT GGACTGCTTT GTCTCTCGCC CCA

A5 cx40 427bp
GGCTGCCAGA ATGTCTGCTA CGACCAGGCT TTTCCCATCT TCCACATTCG
CTACTGGGTG CTGCAGATCA TCTTCGTCTT CACGCCCTCT CTGGTGTACA
TGGGCCACGC CATGCACACT GTGCGCATGC AGGAGAAGCG CAAGCTACGG
GAGGCCGAGA GGGCCAAAGA GGTTCGGGGC TCTGGCTCTT ACGAGTACCC

GGTGGCAGAG AAGGCAGAAC TGTTCTGCTG GGAGGAAGGG AATGGAAGGA
TTGCCCTTCA GGGCACTCTG CTCAACACCT ATGTGTGCAG CATTCTGATT
CGCACCACCA TGGAGGTGGG CTTCATTGTG GGCCAGTACT TCATCTACGG
AATCTTTCTG ACCACCCTGC ATGTCTGCCG CAGGAGTTCC TGTTCCCACC
CGGTCAACTG TTACGTATTC CGGCCCA

A5 cx59 439bp
GGCTGCAGAA ATGTATGCTA CGACCAGGCC TTTCCTATCT CCCTCATTAG
ATACTGGGTT CTGCAGGTGA TATTTGTGTC TTCACCATCC CTGGTCTACA
TGGGCCATGC ATTGTACCGA CTGAGAGTTC TTGAGGAAGA GAGGCAAAGG
ATGAAAGCTC AGTTAAGAGT AGAACTGGAG GAGGTAGAGT TTGAAATGCC
TAGGGATCGG AGGAGATTGG AGCAAGAGCT TTGTCAGCTG GAGAAAAGGA
AACTAAATAA AGCTCCACTC AGAGGAACCT TGCTTTGCAC TTATGTGATA
CACATTTTCA CTCGCTCTGT GGTTGAAGTT GGATTCATGA TTGGACAGTA
CCTTTTATAT GGATTTCACT TAGAGCCGCT ATTTAAGTGC CATGGCCACC
CGTGTCCAAA TATAATCGAC TGTTTTGTCT CAAGACCAA

A7 cx45 502bp
GGCTGTGAGA ATGTCTGTTA TGATGCGTTT GCACCTCTCT CCCATGTACG
CTTCTGGGTG TTCCAGATCA TCCTGGTGGC AACTCCCTCT GTGATGTACC
TGGGCTATGC TATCCACAAG ATTGCCAAAA TGGAGCACGG TGAAGCAGAC
AAGAAGGCAG CTCGGAGCAA GCCCTATGCA ATGCGCTGGA AACAACACCG
GGCTCTGGAA GAAACGGAGG AGGACAACGA AGAGGATCCT ATGATGTATC
CAGAGATGGA GTTAGAAAGT GATAAGGAAA ATAAAGAGCA GAGCCAACCC
AAACCTAAGC ATGATGGCCG ACGACGGATT CGGGAAGATG GGCTCATGAA
AATCTATGTG CTGCAGTTGC TGGCAAGGAC CGTGTTTGAG GTGGGTTTTC
TGATAGGGCA GTATTTTCTG TATGGCTTCC AAGTCCACCC GTTTTATGTG
TGCAGCAGAC TTCCTTGTCC TCATAAGATA GACTGCTTTA TTTCTAGACC CA

A8 cx50 418bp
GGCTGCGAGA ACGTCTGCTA CGACGAGGCC TTTCCCATCT CCCACATTCG
CCTCTGGGTG CTGCAGATCA TCTTCGTCTC CACCCCGTCC CTGATGTACG
TGGGGCACGC GGTGCACTAC GTCCGCATGG AGGAGAAGCG CAAAAGCCGC
GACGAGGAGC TGGGCCAGCA GGCGGGGACT AACGGCGGCC CGGACCAGGG
CAGCGTCAAG AAGAGCAGCG GCAGCAAAGG CACTAAGAAG TTCCGGCTGG
AGGGGACCCT GCTGAGGACC TACATCTGCC ACATCATCTT CAAGACCCTC
TTTGAAGTGG GCTTCATCGT GGGCCACTAC TTCCTGTACG GGTTCCGGAT
CCTGCCTCTG TACCGCTGCA GCCGGTGGCC CTGCCCCAAT GTGGTGGACT
GCTTCGTGTC CCGGCCCA

B1 cx32 379bp
GGCTGCAACA GCGTTTGCTA TGACCAATTC TTCCCCATCT CCCATGTGCG
GCTGTGGTCC CTGCAGCTCA TCCTAGTTTC CACCCCAGCT CTCCTCGTGG
CCATGCACGT GGCTCACCAG CAACACATAG AGAAGAAAAT GCTACGGCTT
GAGGGCCATG GGGACCCCCT ACACCTGGAG GAGGTGAAGA GGCACAAGGT
CCACATCTCA GGGACACTGT GGTGGACCTA TGTCATCAGC GTGGTGTTCC

GGCTGTTGTT TGAGGCCGTC TTCATGTATG TCTTTTATCT GCTCTACCCT
GGCTATGCCA TGGTGCGGCT GGTCAAGTGC GACGTCTACC CCTGCCCCAA
CACAGTGGAC TGCTTCGTGT CCCGCCCCA

B2 cx26 382bp
GGCTGCAAGA ACGTGTGCTA CGATCACTAC TTCCCCATCT CCCACATCCG
GCTATGGGCC CTGCAGCTGA TCTTCGTGTC CAGCCCAGCG CTCCTAGTGG
CCATGCACGT GGCCTACCGG AGACATGAGA AGAAGAGGAA GTTCATCAAG
GGGGAGATAA AGAGTGAATT TAAGGACATC GAGGAGATCA AAACCCAGAA
GGTCCGCATC GAAGGCTCCC TGTGGTGGAC CTACACAAGC AGCATCTTCT
TCCGGGTCAT CTTCGAAGCC GCCTTCATGT ACGTCTTCTA TGTCATGTAC
GACGGCTTCT CCATGCAGCG GCTGGTGAAG TGCAACGCCT GGCCTTGTCC
CAACACTGTG GACTGCTTTG TGTCCCGGCC CA

B3 cx31 370bp
GGCTGCACCA ACGTCTGCTA CGACAACTAC TTTCCCATCT TCAACATTCG
CCTCTGGGCC CTGCAGCTCA TCTTCGTCAC ATGCCCCTCG CTGCTGGTCA
TTCTGCACGT GGCCTACCGT GAGGAGCGGG AGCGCCGGCA CCGCCAGAAA
CACGGGGACC AGTGCGCCAA GCTGTACGAC AACGCAGGCA AGAAGCACGG
AGGCCTGTGG TGGACCTACC TGTTCAGCCT CATCTTCAAG CTCATCATTG
AGTTTCTCTT TCTCTACCTG CTGCACACTC TCTGGCATGG CTTCAATATG
CCGCGCCTGG TGCAGTGTGC CAACGTGGCC CCCTGCCCCA ACATCGTGGA
CTGCTACATT GCCCGACCTA

B4 cx30.3 367bp
GGCTGCCCCA ACGTCTGCTA TGACGAGTTC TTCCCCGTGT CCCACGTGCG
CCTCTGGGCC CTACAGCTCA TCCTGGTCAC GTGCCCCTCA CTGCTCGTGG
TCATGCACGT GGCCTACCGC GAGGAACGCG AGCGCAAGCA CCACCTGAAA
CACGGGCCCA ATGCCCCGTC CCTGTACGAC AACCTGAGCA AGAAGCGGGG
CGGACTGTGG TGGACGTACT TGCTGAGCCT CATCTTCAAG GCCGCCGTGG
ATGCTGGCTT CCTCTATATC TTCCACCGCC TCTACAAGGA TTATGACATG
CCCCGCGTGG TGGCCTGCTC CGTGGAGCCT TGCCCCCACA CTGTGGACTG
TTACATCTCC CGGCCCA

B5 cx31.1 367bp
GGCTGCTCCA ACGTCTGCTT TGATGAGTTC TTCCCTGTGT CCCATGTGCG
CCTCTGGGCC CTGCAGCTTA TCCTGGTGAC ATGCCCCTCA CTGCTCGTGG
TCATGCACGT GGCCTACCGG GAGGTTCAGG AGAAGAGGCA CCGAGAAGCC
CATGGGGAGA ACAGTGGGCG CCTCTACCTG AACCCCGGCA AGAAGCGGGG
TGGGCTCTGG TGGACATATG TCTGCAGCCT AGTGTTCAAG GCGAGCGTGG
ACATCGCCTT TCTCTATGTG TTCCACTCAT TCTACCCCAA ATATATCCTC
CCTCCTGTGG TCAAGTGCCA CGCAGATCCA TGTCCCAATA TAGTGGACTG
CTTCATCTCC AAGCCCT

B6 cx30 382bp
GGATGCAAAA ATGTGTGCTA TGACCACTTT TTCCCGGTGT CCCACATCCG

GCTGTGGGCC CTCCAGCTGA TCTTCGTCTC CACCCCAGCG CTGCTGGTGG
CCATGCATGT GGCCTACTAC AGGCACGAAA CCACTCGCAA GTTCAGGCGA
GGAGAGAAGA GGAATGATTT CAAAGACATA GAGGACATTA AAAAGCACAA
GGTTCGGATA GAGGGGTCGC TGTGGTGGAC GTACACCAGC AGCATCTTTT
TCCGAATCAT CTTTGAAGCA GCCTTTATGT ATGTGTTTTA CTTCCTTTAC
AATGGGTACC ACCTGCCCTG GGTGTTGAAA TGTGGGATTG ACCCCTGCCC
CAACCTTGTT GACTGCTTTA TTTCTAGGCC AA

C1 cx36 562bp
GGCTGTAACC AGGCCTGCTA TGACCGGGCC TTCCCCATCT CCCACATACG
TTACTGGGTC TTCCAGATCA TAATGGTGTG TACCCCCAGT CTTTGCTTCA
TCACCTACTC TGTGCACCAG TCCGCCAAGC AGCGAGAACG CCGCTACTCT
ACAGTCTTCC TAGCCCTGGA CAGAGACCCC CCTGAGTCCA TAGGAGGTCC
TGGAGGAACT GGGGGTGGGG GCAGTGGTGG GGGCAAACGA GAAGATAAGA
AGTTGCAAAA TGCTATTGTG AATGGGGTGC TGCAGAACAC AGAGAACACC
AGTAAGGAGA CAGAGCCAGA TTGTTTAGAG GTTAAGGAGC TGACTCCACA
CCCATCAGGT CTACGCACTG CATCAAAATC CAAGCTCAGA AGGCAGGAAG
GCATCTCCCG CTTCTACATT ATCCAAGTGG TGTTCCGAAA TGCCCTGGAA
ATTGGGTTCC TGGTTGGCCA ATATTTTCTC TATGGCTTTA GTGTCCCAGG
GTTGTATGAG TGTAACCGCT ACCCCTGCAT CAAGGAGGTG GAATGTTATG
TGTCCCGGCC AA

Appendix B:

Visual basic code for finding the best match for universal primers:

These two simple pieces of code were custom made to identify sequences of DNA that most closely match a short given sequence, allowing for specific degenerate nucleic acid codes. The first function, "Homology" takes two sequences, compares them position by position, and returns an integer that increases with each match and decreases with each mismatch.

The second function scans a longer sequence, and using the "homology" function at each position, it identifies the starting position producing the highest homology between the long and short sequences

```
'gene sequence analysis tools
Function Homology(strWant As String, strHave As String) As Integer
  Homology = 0
  Dim i As Integer
  i = 1

  While i <= Len(strWant)
      If Mid(strWant, i, 1) = "A" Or Mid(strWant, i, 1) = "C" _
      Or Mid(strWant, i, 1) = "G" Or Mid(strWant, i, 1) = "T" _
      Then
        If Mid(strWant, i, 1) = Mid(strHave, i, 1) Then
          Homology = Homology + 6
        Else
          Homology = Homology - 1
        End If
      End If
      If Mid(strWant, i, 1) = "?" Or Mid(strWant, i, 1) = "Y" _
      Or Mid(strWant, i, 1) = "R" Or Mid(strWant, i, 1) = "V" _
      Then
        Homology = Homology + 1
      Else
        Homology = Homology - 1
      End If
      i = i + 1
  Wend
End Function

Function SCAN(strPrimer As String, strSeq As String) As Integer
  Dim intScore As Integer
```

```
    Dim intHomology As Integer
    Dim intMatch As Integer
    Dim strMatch As String
    Dim intLength As Integer
    Dim i As Integer
    i = 1
    intMatch = 1
    intLength = Len(strPrimer)
    intScore = Homology(strPrimer, Mid(strSeq, 1, intLength))

      While i < Len(strSeq) - 5
        i = i + 1
        intHomology = Homology(strPrimer, Mid(strSeq, i, intLength))
        If intHomology > intScore Then
          intMatch = i
          intScore = intHomology
        End If
      Wend

     SCAN = intMatch

    'MsgBox "Highest score: " & intScore & Chr$(13) & "position: " & SCAN & Chr$(13)
& _
    'strPrimer & Chr$(13) & strMatch
End Function
```

Appendix C: Ethics Certificates

ACTION	✓	DATE
P.I.	✓	01
FACC	✓	11
RGO	✓	4
VET	✓	4
INOST		
PROTO		

FOR OFFICE USE ONLY

Project #	3702
Investigator #	729
Approval End Date	NOV. 30 2001
Facility Committee	MGH

McGill University Animal Use Protocol

☐ New Application

☐ Renewal of Project # 3702

1. Investigator Information

Principal Investigator: GEORGE KUCHEL, M.D. Telephone: 934-8015

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Animal Use: Research [x] Teaching [ ] Specify Course number:

Project Title: ***PATHOGENESIS OF DETRUSOR OVERACTIVITY AND IMPAIRED CONTRACTILITY***

2. Funding Source

External [x] Internal [ ]

Source(s): National institute on Aging; National Institutes of Health (USA)

Peer Reviewed source: Yes [x] No [ ] *If no, see instructions - section 2

Awarded [x] Pending [ ]

Funding Period: From: July 1999 To: April 2003

Proposed Start Date of Research: (Day/Month/Year) 1 December, 1999

Expected Date of Completion: (Day/Month/Year) April 2003

3. Emergency: Person(s) designated to handle emergencies (2 emergency telephone numbers must be indicated)

Name:	Qing Zhu	Phone #: Work:	937-6011 ext. 4621	Alternative #:	935-4914 (home)
Name:	Jeff Ritchie	Phone #: Work:	937-6011 ext. 4621	Alternative #:	483-8997 (home)

Certification:

The information in this application is exact and complete. I agree to follow the policies and procedures set forth by the Facility Anima Care Committees and McGill University, as well as those described in the "Guide to the Care and Use of Experimental Animals" prepared by the Canadian Council on Animal Care. I shall request the Animal Care Committee's approval prior to any deviations fro the procedures described within.

Principal Investigator/Course Director		Date	00/11/29

Approval:

Chairperson, Facility Animal Care Committee			Date	Nov. 30/00	
University Animal Care Officer			Date	1/12/01	
Approved period for animal use	Beginning	Dec. 1 2000	Ending	NOV. 30 2001	

☐ NOTE REVIEWER'S MODIFICATION(S) ON PAGE 2

Revised 02/97

University of Connecticut Health Center
School of Medicine

Office of Research Compliance

Office of Research Compliance
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Principal Investigator: Dr. George Kuchel
Center on Aging 5215

Co-Investigators: Neil Resnick, Derek Griffiths, Ahmad Elbadawi, Charles Pound, Halina Syczysnki, Elizabeth Logue

Reference Number: 02-253

Progress Report Due: 1/31/04

Type of Review: Continuation Expedited

IRB Meeting Date: 3/17/03

Proposal: The Pathogenesis of Detrusor Overactivity
(Sponsor: United States Public Health Service)

- Approved for continuation on 3/17/2003. Study determined to qualify for expedited review under the revised Expedited Federal Regulation (rev. 11/98) Title 45 Code of Federal Regulation Part 46, section 46.110(8). The specimens collected for this study are for a part of research approved at the University of Pittsburgh.
- IRB approval is valid for one additional year through 3/31/04.
- With approval of a proposal, the committee requests that should any untoward effects occur, the committee be informed immediately. In addition, should there be any modifications or changes contemplated, it is necessary that the committee be informed in writing, for review and approval prior to their inception.
- It is the committees' understanding that this proposal will be terminated as of 3/31/04 unless the investigator notifies the committee that the study will be continued.

Health And Human Services Multiple Project Assurance Number 1345-02.

Richard Simon, MD, Chair, Institutional Review Board

cc: Department Chair, Center on Aging