

## ISOLATION, IMMUNOHISTOCHEMICAL CHARACTERIZATION AND ATP RELEASE FROM MYOFIBROBLAST CELL CULTURES FROM PIG BLADDER MUCOSA

### Hypothesis / aims of study

ATP release from the bladder urothelium in response to stretch has been suggested to be responsible for initiating afferent signalling. Two key cell populations of the mucosa, urothelial cells and suburothelial myofibroblasts, are closely associated with afferent nerves. We hypothesised that myofibroblasts were a key cell type which contributes to afferent signalling. The aim of this study was to prepare and characterise myofibroblast cell cultures, and examine their ability to release ATP in response to stretch and other stimuli.

### Study design, materials and methods

**Cell culture:** Three separate cell cultures were established. For myofibroblast cell cultures, the urothelial cell layer was scraped away from the luminal surface of the bladder mucosa. The remaining thin layer of the mucosa then minced and treated with 0.25% trypsin-EDTA supplemented with 0.15% collagenase. The resulting cells were filtered and centrifuged then resuspended in RPMI 1640 with 10% FBS, antibiotic/antimycotic.

To obtain urothelial cells, the mucosa was digested from the luminal surface with 0.25% trypsin-EDTA. The released cells were cultured in the media described above, supplemented with EGF (25ng/ml) [1].

Detrusor muscle was minced and digested (0.25% trypsin-EDTA with 0.15% collagenase) to yield muscle cell cultures [1]. All cells were plated at approximately  $10^6$  cells per well in 48-well plates and incubated at 37°C in 5% CO<sub>2</sub> until confluent (approximately 10 to 14 days).

**Immunohistochemistry:** Cells were washed in PBS, fixed with absolute ethanol, rewashed with PBS and then incubated with primary antibody (AE1/AE3,  $\alpha$ -smooth muscle actin, and vimentin, Dako) for 2 h at room temperature. After washing (x 3) with PBS, cells were incubated with FITC-conjugated anti-mouse IgG antibody for 1 h, and then counterstained with DAPI (nuclear stain) for 5 min. After washing (x 3) with PBS, staining was visualized using fluorescence microscopy.

**ATP release from myofibroblast cell cultures:** Cells were equilibrated with sterile carbogenated Krebs solution for 1 h. Cells were then treated with normal Krebs, hypotonic Krebs (1:2 dilution of Krebs), acid (Krebs with the pH adjusted to 6.4 or 5.5) or agonists (all at 1 $\mu$ M). Where appropriate, control cells were treated with vehicle: DMSO for capsaicin, in 0.01M acetic acid for neurokinin A (NKA) and substance P (SP). After 10 min stimulation, the supernatant was collected for ATP determination using a bioluminescence assay kit (Sigma) [2]. The median ATP concentration (in nM) per treatment was determined. Data were compared using a Wilcoxon matched pairs test or Kruskal-Wallis test, as appropriate.

### Results

**Immunohistochemistry:** Urothelial cell cultures stained with the cytokeratin marker AE1/AE3 (Figure 1A) but not with the contractile protein  $\alpha$ -smooth muscle actin or the cytoskeletal filament vimentin. Myofibroblast and detrusor muscle cell cultures did not stain for AE1/AE3 but did stain positively for vimentin (Figure 1B and C) and  $\alpha$ -smooth muscle actin (Figure 1E and F). A feature to differentiate myofibroblasts from muscle was seen - staining of individual cytoskeletal filaments could be clearly seen in vimentin positive myofibroblast cells (Figure 1B and D), but not in detrusor muscle cells (Figure 1C).

**ATP release from myofibroblast cell cultures:** ATP release was significantly stimulated by stretch (hypotonic media, Fig 2A,  $p = 0.002$ ,  $n=14$ ), but not by acid (Fig 2B,  $p = 0.56$ ,  $n=9$ ), capsaicin (Fig 2C,  $p = 0.31$ ,  $n=18$ ), NKA or SP (Fig 2D,  $p = 0.59$ ,  $n=8$ ) (ANOVA).

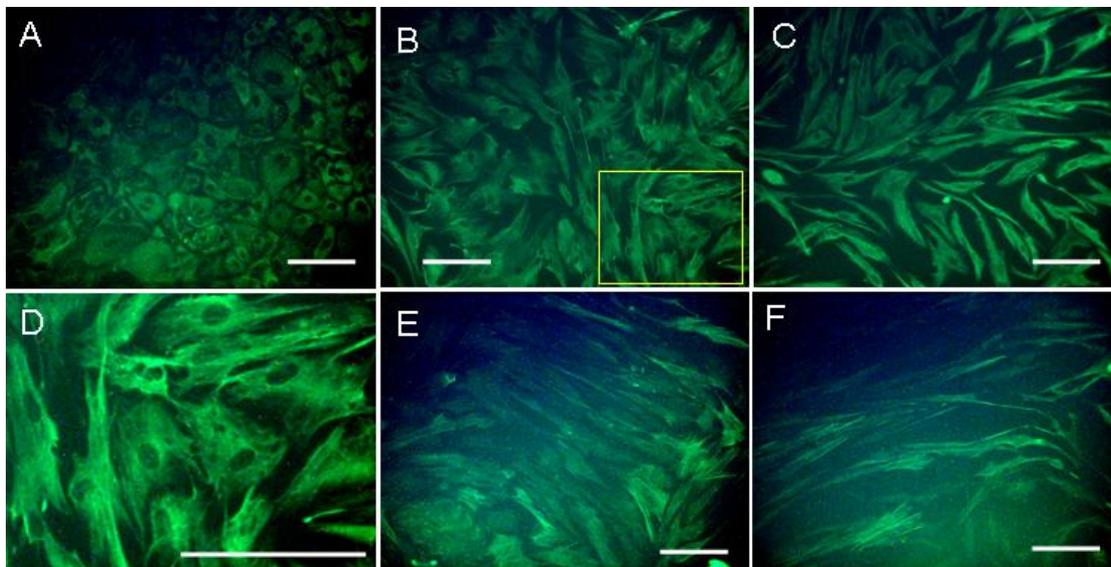


Figure 1. Fluorescence immunohistochemistry. A: urothelial cells stained with AE1/AE3; B and D (cropped): myofibroblast cultures stained with vimentin; C: detrusor muscle cultures stained with vimentin; E: myofibroblast cultures stained with  $\alpha$ -smooth muscle actin; F: detrusor muscle cultures stained with  $\alpha$ -smooth muscle actin. Bar, 200  $\mu$ m.

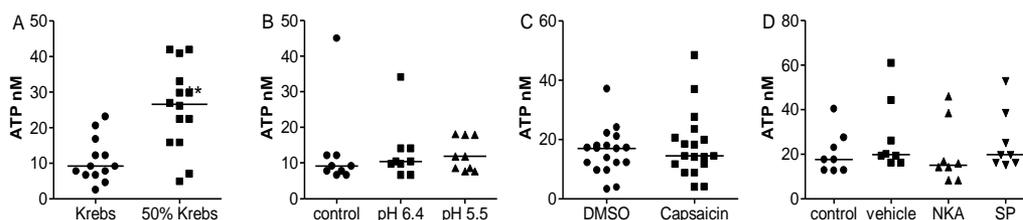


Figure 2. ATP release (nM) from myofibroblast cell cultures elicited by (A) hypotonic media but not by (B) acid, (C) 1  $\mu$ M capsaicin or (D) 1  $\mu$ M NKA or SP.

#### Interpretation of results

Myofibroblast cell cultures were distinct from urothelial cells in that they did not stain with cytokeratin markers AE1/AE3. Instead, myofibroblasts stained with  $\alpha$ -smooth muscle actin, previously used to demonstrate sub-urothelial myofibroblasts [2], and with vimentin, which has been used previously to identify these cells [3]. This staining pattern was similar to detrusor muscle cell cultures, but morphologically the myofibroblast cells had more distinct cytoskeletal filaments revealed by vimentin staining.

Our functional results show that stretch leads to release of ATP from myofibroblast cell cultures, similar to results from urothelial cell cultures [1]. In contrast, acid was ineffective as a stimulant for ATP release in the myofibroblast cultures whereas it was effective in porcine urothelial [1]. Myofibroblasts, similar to porcine urothelial cells, did not release ATP in response to capsaicin or neuropeptides [1]. However, it has been reported that the myofibroblast cell layer contracts in response to NKA [2].

#### Concluding message

Three cell populations have been cultured from porcine bladder and characterised immunohistochemically. As for urothelial cells, myofibroblast cell cultures also appear to be capable of releasing ATP in response to stretch. Therefore, myofibroblasts may contribute to generation of afferent impulses from the bladder in response to stretch during filling.

#### References

1. Y Cheng, KJ Mansfield, E Burcher and KH Moore. ICS abstract 2009 (#310)
2. Sadananda P, Chess-Williams R, Burcher E. Br J Pharmacol. 2008. 153(7):1465-73.
3. Sui GP, Wu C, Fry CH. 2004. J Urol. 171:938-43.

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<b>Name of ethics committee</b>	<b>University of New South Wales</b>