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Letter to the Editor

CELL-SPECIFIC ACTIVATION OF THE HB-EGF AND ErbB1 GENES BY STRETCH IN PRIMARY HUMAN BLADDER CELLS

Dear Editor:

The bladder responds to increased urethral resistance by changes in cellular composition: hyperplasia of the urothelium (Monson et al., 1992; Monson et al., 1994; Saito et al., 1994) and hyperplasia and hypertrophy of the smooth muscle cell (SMC) layer (Kojima et al., 1996). In humans, these changes occur as a progressive response to urinary outlet obstruction resulting from benign prostatic hyperplasia in adults or congenital bladder anomalies in children (e.g., posterior urethral valves). It is likely that the physical forces experienced by the bladder mediate, to some degree, the tissue growth that occurs in response to obstruction. Experiments in animal models have demonstrated that bladder outlet obstruction alters the expression of some growth-related genes, leading to an early proliferative response by the urothelial and bladder smooth muscle cells (Chen et al., 1994; Santarosa et al., 1994). However, the molecular mechanisms by which these changes occur in vivo are poorly understood.

Previously, we demonstrated that heparin-binding epidermal growth factor-like growth factor (HB-EGF) is synthesized by human bladder epithelia (urothelia) and SMC in vivo and acts as an autocrine urothelial cell growth factor (Freeman et al., 1997). HB-EGF is an activating ligand for the EGF receptor (ErbB1) and is a potent mitogen for fibroblasts, certain epithelial cells, and SMC (reviewed by Raab and Klagsbrun, 1997). Because of its biological activity and its site of synthesis, HB-EGF may be an important growth factor involved in the bladder's response to urethral obstruction. Recently, we developed an in vitro model in which we applied repetitive mechanical stretch and relaxation to bladder SMC, simulating the physical forces experienced by bladder cells as a result of bladder outlet obstruction (Park et al., 1998). These experiments demonstrated that HB-EGF is a stretch-responsive gene in rat SMC, establishing a link between mechanical forces and growth factor regulation. In the present experiments, we sought to determine whether the expression of HB-EGF and its receptor, ErbB1, in primary culture human bladder cells is stretch-inducible and if so, whether such responses are cell-type dependent.

After obtaining parental consent and institutional approval, bladder biopsies were obtained from a 2-yr-old child undergoing antireflux surgery. The patient had a negative urine culture and no other bladder pathology noted at the time of operation. Bladder urothelial and SMC were isolated and propagated in culture by methods described by Atala et al. (1993). In brief, the urothelial cells were gently scraped from the mucosal side of the bladder biopsies and cultured in keratinocyte-serum free medium (K-SFM) supplemented with human recombinant epidermal growth factor (hEGF, 5 ng/ml), bovine pituitary extract (BPE, 50 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) (all purchased from GIBCO, Gaithersburg, MD). To isolate the bladder SMC, the submucosa, serosa, and fatty tissues were carefully removed from the bladder biopsies leaving the muscularis propria behind. The remnant bladder tissues were then divided into 1-mm pieces, arranged in close proximity on a sterile culture plate, and air-dried for 10 min under sterile conditions. Dulbecco's modified Eagle medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT), penicillin (100 U/ml) and streptomycin (100 μg/ml) was then added. Both the urothelial cells and SMC were maintained in a humidified 5% CO_2-95% air atmosphere at 37° C. All experiments were performed on cells between Passages 2 and 5. Urothelial cells were characterized by morphological criteria and by their expression of cytokeratin-7, a transitional cell marker (Cilento et al., 1994), and the lack of smooth muscle α-actin. In contrast, SMC were spindle-shaped cells organized in a hill-and-valley pattern and expressed smooth muscle α-actin.

Approximately 1×10^5 urothelial cells or SMC per well were plated onto six-well silicone elastomer-bottomed culture plates coated with collagen type I (Bioflex, Flexcell, Hillsborough, NC). Cells were grown to near confluence and then were rendered quiescent by incubation for 48 h in reduced supplement medium (K-SFM with 5 μg BPE per ml but no hEGF for urothelial cells and DMEM with 1% FBS for SMC). Cells were subjected to continuous cycles of stretch-relaxation with the FX-3000 Flexercell Strain Unit (Flexcell). Each cycle consisted of 5 sec of stretch and relaxation (0.1 Hz) with a 25% maximum radial stretch at the periphery of the membrane. On selected plates, urothelial cells were subjected to greater frequency (0.5 and 1.0 Hz) and intensity of stretch (40% maximum radial stretch).

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed to determine the relative levels of HB-EGF, ErbB1, and GAPDH mRNA. Total RNA was extracted from nonstretched and stretched urothelial cells and SMC with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. Reverse transcription was performed with 5 μg of total RNA, 100 U of Moloney's murine leukemia virus RT (Ambion, Austin, TX) and 1 μg of oligo$(dT)_{12-18}$ (GIBCO) as first-strand primer for 1 h at 42° C. cDNA was then precipitated with linear acrylamide, ammonium acetate, and ethanol and resuspended in Tris (10 m*M*)-EDTA (0.1 m*M*) buffer.

Primers were selected on the basis of published gene sequences for human HB-EGF (Fen et al., 1993), ErbB1 (Wainstein et al., 1994) and GAPDH (Tso et al., 1985) found in the Genbank database. A 379-nucleotide (nt) HB-EGF product was amplified with a sense 5′-GCT TTT TCA CAA CCT GTC TCT C-3′ (nt 89–113) and an antisense 5′-TGC TGA GCT GAG TTC TGT TCC G-3′ (nt 446–467) primer pair. A 724-bp ErbB1 product was amplified with a sense 5′-CAG CGC TAC CTT GTC ATT CAG-3′ and an antisense 5′-TCA TAC TAT CCT CCG TGG TCA-3′ primer pair. A 571-bp GAPDH product was amplified with a sense 5′-TCA CCA TCT TCC AGG

AGC G-3′ and an antisense 5′-CTG CTT ACC ACC TTC TTG A-3′ primer pair.

PCR reactions were performed with 22 μl of SuperMix (GIBCO), 0.5 μl of sense and antisense primer (20 pmol/μl), 0.1 μl of [^{32}P]dCTP (3000 Ci/mmol, Amersham, Arlington Heights, IL) and 2 μl of cDNA. For HB-EGF and GAPDH, PCR amplification was performed for 30 cycles at 94° C (denature), 58° C (anneal) and 72° C (extend) for 40 sec each. The conditions for ErbB1 were similar except for an annealing temperature of 50° C. The resultant PCR products were subjected to size separation by 5.1% polyacrylamide gel electrophoresis. Normalization to GAPDH expression and a limiting dilution method were used to make semiquantitative comparisons between samples. We assessed relative mRNA levels by comparing band density using the IS-100 Image Analysis System (Alpha Innotech Corporation, San Leandro, CA).

In all, 1.5×10^4 urothelial or SMC (at Passage 2) per well were seeded in 24-well tissue culture plates in fully supplemented medium and allowed to grow overnight at 37° C in a 5% CO_2 atmosphere. The plating medium was then removed and replaced with 1 ml/well of recombinant HB-EGF at 100, 25, 6.25, and 1.56 ng/ml in reduced-supplement medium. Control wells consisted of cells grown in fully supplemented or reduced-supplement medium. In selected plates, [Glu^{52}]-Diphtheria toxin (CRM197, Sigma Chemical Co., St. Louis, MO) in equimolar amount to HB-EGF was added to the culture medium at 2, 4, and 6 d following the addition of HB-EGF. To determine the rate of cellular proliferation, thiazolyl blue (MTT, Sigma) incorporation was measured every 2 d following the addition of HB-EGF. MTT (5 mg/ml) was added to each well, and the cells were allowed to metabolize the dye for 4 h at 37° C in a 5% CO_2 atmosphere, protected from light. The medium was aspirated and the converted dye was solubilized by the addition of 0.01 *N* HCl in absolute isopropanol (250 μl/well). Duplicate aliquots were transferred to a 96-well plate and absorbance at 570 nm (with background correction at 655 nm) was determined by a Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA). Values are expressed as means with standard deviations and compared by an unpaired Student's *t*-test.

Mechanical stretch induces HB-EGF expression in human SMC but not urothelial cells. Bladder SMC were subjected to cyclical stretch-relaxation at 0.1 Hz and 25% maximal radial stretch. Relative HB-EGF mRNA levels in these cells were determined by semiquantitative PCR at 0, 2, 4, 6, and 12 h following initiation of the stretch stimulus (n = 4 wells for each time point) with normalization to their respective GAPDH mRNA levels. Compared to nonstretched controls, bladder SMC exposed to stretch demonstrated a time-dependent increase in levels of HB-EGF mRNA (Fig. 1). By 6 h, stretched bladder SMC exhibited a 3.5 (± 0.41)-fold increase in HB-EGF mRNA levels ($P < 0.001$ compared to time-matched, nonstretched controls; Fig. 2 *a*). However, this increase in HB-EGF expression was not sustained and by 12 h, HB-EGF mRNA levels returned to baseline. In contrast, urothelial cells subjected to cyclical stretch-relaxation under the same conditions (0.1 Hz and 25% maximal radial stretch) did not demonstrate any increase in HB-EGF mRNA levels compared to nonstretched controls (Fig. 1, 2 *b*). Neither increasing the frequency of stretch to 0.5 and 1.0 Hz nor increasing the intensity of stretch to 40% resulted in changes in HB-EGF mRNA expression in urothelial cells (Fig. 2 *c*).

Mechanical stretch induces ErbB1 expression in both human SMC and urothelial cells. In contrast to HB-EGF mRNA levels, mechanical stretch resulted in a time-dependent increase in ErbB1 mRNA levels in both human bladder SMC and urothelial cells (Fig. 1). ErbB1 is the primary cognate receptor for HB-EGF. By 4 h following stretch stimulation, there was a 7.5 (± 3.5) and 10.1 (± 1.1)-fold increase in ErbB1 mRNA levels in the bladder SMC and urothelial cells, respectively ($P = 0.015$ and $P < 0.001$ compared to time-matched nonstretched controls) (Fig. 2 *d,e*). Furthermore, in contrast to the pattern of HB-EGF mRNA expression observed following mechanical stimulation of bladder SMC, increases in ErbB1 mRNA levels were maintained up to 12 h following initiation of the stretch stimulus.

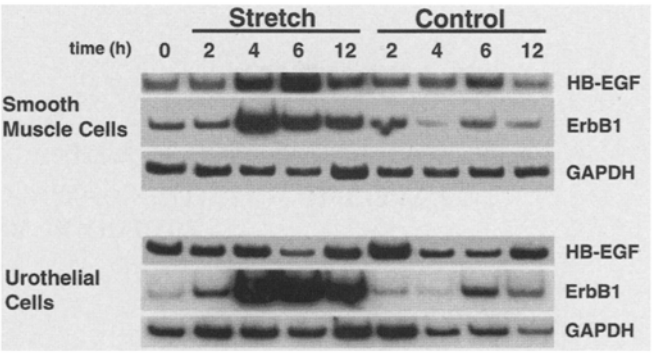

FIG. 1. RT-PCR analysis of HB-EGF and ErbB1 mRNA levels in human SMC and urothelial cells subjected either to continuous cycles of stretch-relaxation for various durations or no stretch (control); representative result from four independent experiments. In SMC, HB-EGF mRNA was increased 4 and 6 h following stretch. No change in HB-EGF expression was observed in stretched urothelial cells. In contrast, ErbB1 mRNA expression was increased in both stretched SMC and urothelial cells.

HB-EGF stimulates cellular proliferation in both human SMC and urothelial cells. To evaluate the potential physiologic relevance of HB-EGF in this system, human bladder SMC and urothelial cells were grown in reduced-supplement medium in the presence of HB-EGF. Cellular proliferation was measured by determining the amount of thiazolyl blue dye (MTT) incorporation by these cells at 2-d intervals (n = 4 wells for each time point). In SMC, MTT incorporation was maximal at 8 d following the addition of 25 ng HB-EGF per ml ($P < 0.001$ compared to cells grown in reduced-supplement medium alone; Fig. 3 *a*). Interestingly, higher concentrations of HB-EGF (100 ng/ml) resulted in lower rates of cellular proliferation. In contrast, urothelial cell proliferation was more modest in response to HB-EGF stimulation (Fig. 3 *b*). A significant increase in MTT incorporation was not evident until 6 d following HB-EGF stimulation and was maximal after 12 d. We postulated that this delay in cellular proliferation may result from HB-EGF-mediated induction of a secondary factor that in turn stimulates cell growth. To determine whether HB-EGF was directly responsible for the observed increase in urothelial cell proliferation, CRM197, a specific inhibitor of human HB-EGF (Mitamura et al., 1995), was added 2, 4, or 6 d following HB-EGF stimulation. MTT incorporation was markedly reduced (comparable to urothelial cells grown in reduced-supplement medium) independently of the time of CRM197 addition (Fig. 3 *c*). This finding suggests that HB-EGF can directly stimulate urothelial cell proliferation.

In this study, we observed that expression of HB-EGF mRNA is inducible by mechanical stretch in human bladder SMC. This is consistent with our previously reported results in rat bladder SMC,

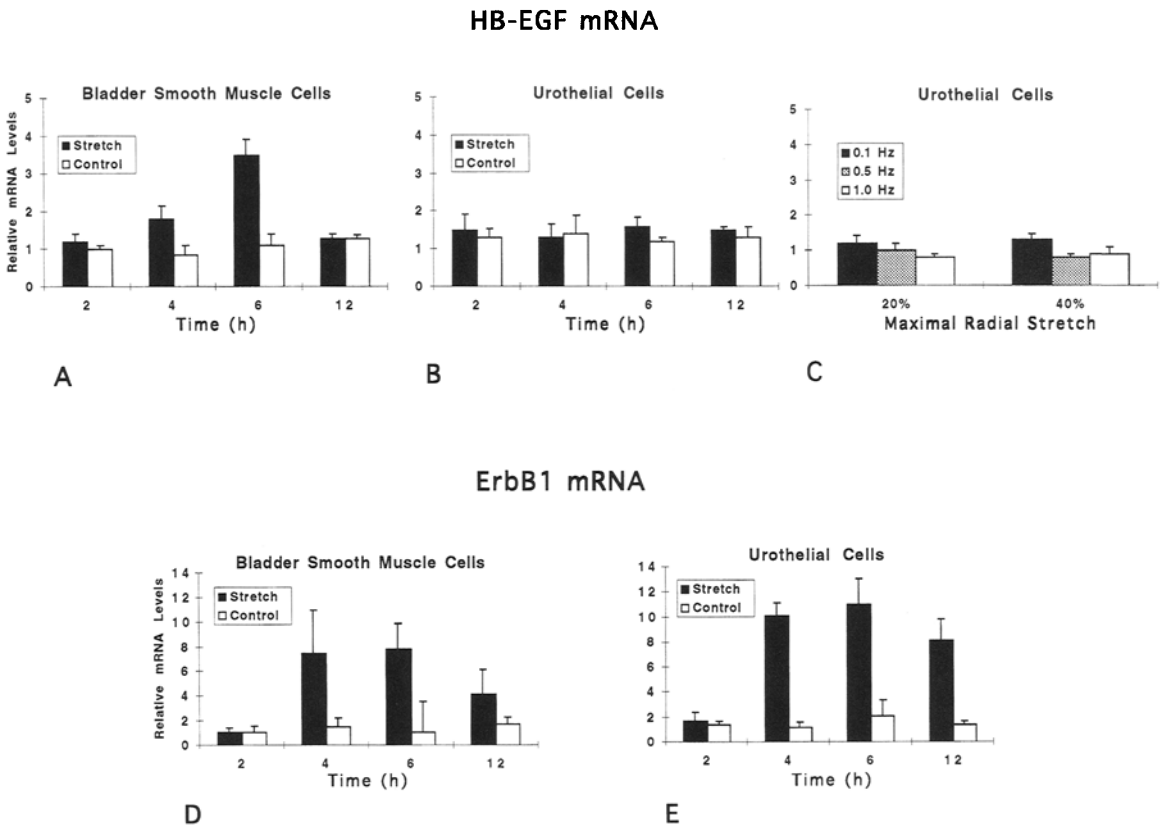

FIG. 2. Densitometric analysis of the HB-EGF and ErbB1 mRNA expression as determined by RT-PCR (n = 4 for each time point). All samples were normalized to GAPDH mRNA levels and expressed as a dimensionless ratio. *A*, HB-EGF mRNA expression in response to stretch in human bladder SMC and *B*, in human urothelial cells. *C*, HB-EGF mRNA expression in response to increasing frequency and maximal radial stretch in human urothelial cells. *D*, ErbB1 mRNA expression in response to stretch in human bladder SMC and *E*, in human urothelial cells.

although the magnitude of HB-EGF mRNA induction was observed to be less in the human SMC (3.5 vs. 10.2-fold induction in the rat) (Park et al., 1998). This may represent an inherent difference between species, or alternatively, a difference in the chronological age of the bladder specimens from which the cells were obtained. Furthermore, we found that HB-EGF mRNA expression was not altered by stretch in human urothelial cells despite attempts to induce its expression by increasing the strength and/or frequency of the mechanical stimulus. This suggests that either mechanoregulation of HB-EGF gene expression is cell type-dependent or that gene expression in urothelial cells is not *inducible* by mechanical stretch. However, we also observed that mRNA expression for ErbB1, the cognate receptor tyrosine kinase for HB-EGF, was increased following stretch stimulation in both bladder SMC and urothelial cells. This indicates that mechanical stretch can, in fact, stimulate gene expression in urothelial cells and that the stretch signal acts to regulate genes in a cell-specific manner.

The finding that stretch-relaxation can regulate gene expression in human SMC and urothelial cells further supports the concept that mechanical forces can mediate local regulation of tissue growth. This concept is not unique to bladder SMC. Using similar in vitro stretch systems, investigators have demonstrated that mechanical signals can alter the expression of certain growth factors (e.g., angiotensin II and platelet-derived growth factor) in cardiac myocytes and fibroblasts, vascular SMC and endothelial cells, and glomerular mesangial cells (reviewed by Sadoshima et al., 1993; Booz and Baker, 1995; Osol, 1995; Cortes et al., 1996; Chien et al., 1998). These cells respond to mechanical stimuli by increasing rates of gene expression, protein synthesis and/or cell proliferation (Sadoshima et al., 1993; Wilson et al., 1993).

In this study, we also demonstrated that HB-EGF was directly mitogenic to bladder SMC and urothelial cells, as measured by MTT incorporation. The HB-EGF concentration (1 ng/ml) that resulted in the greatest degree of growth stimulation in urothelial cells was similar to that of a previous report from our laboratory (Freeman et al., 1997). The addition of CRM197, a specific inhibitor of HB-EGF, negated the mitogenic effects of HB-EGF. Previously, it has been shown that HB-EGF is synthesized by the bladder detrusor muscle based on immunohistochemical analysis of human bladder tissue (Freeman et al., 1997). Furthermore, HB-EGF synthesis also occurs in the urothelium and HB-EGF has been identified as an autocrine urothelial cell growth factor (Freeman et al., 1997). Moreover, the predominant receptor for HB-EGF, ErbB1, has been localized to both the smooth muscle and urothelial layers of the bladder (Borer et al., submitted; Baskin et al., 1996). These findings implicate HB-EGF as a potentially important mediator of bladder SMC and urothelial cell proliferation.

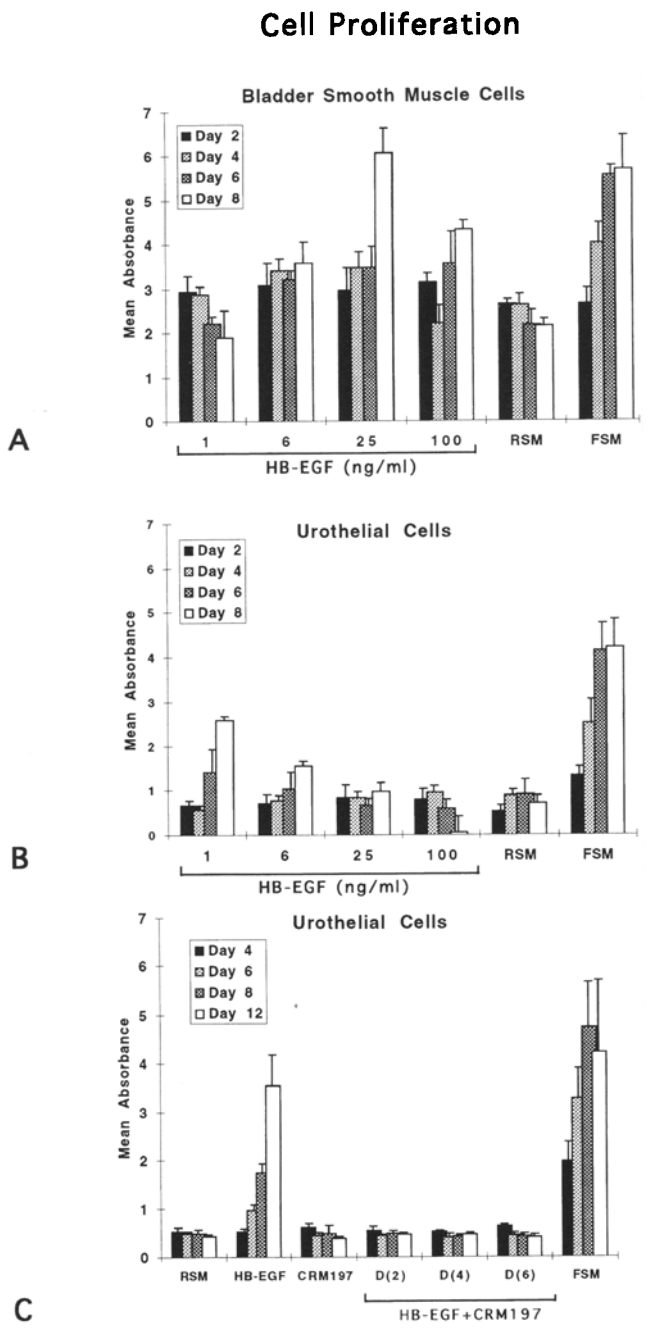

FIG. 3. Proliferation of human bladder cells in response to HB-EGF. Proliferation was evaluated by examining the uptake of MTT (noted as Absorbance) during incubation in reduced-supplement medium with various concentrations of HB-EGF (1, 6, 25, and 100 ng/ml) at respective time points (2, 4, 6, and 8 d following the addition of HB-EGF) (n = 4 for each time point). All values were expressed as a ratio to the absorbance of the corresponding sample at time 0. Cells grown in full supplement (FSM) or reduced supplement (RSM) medium served as positive and negative controls, respectively. *A*, MTT incorporation in response to HB-EGF in human bladder SMC and *B*, in human urothelial cells. *C*, The effects of CRM197 (C) on proliferation when added at 2 (H + C 2), 4 (H + C 4) and 6 (H + C 6) d following stimulation with 1 ng HB-EGF (H) per ml. Cells grown in HB-EGF (1 ng/ml) or CRM197 (44 ng/ml) alone served as additional controls.

Taken together, the results of the present study suggest a potential mechanism by which the bladder responds to outlet obstruction. Mechanical stretch, evoked by bladder outlet resistance and distention, may induce the expression of HB-EGF (and possibly other mitogens) in SMC and the expression of its receptor in both the muscle and urothelial tissue compartments. HB-EGF, in turn, would stimulate the growth of SMC and urothelial cells in an autocrine and paracrine fashion, respectively. Furthermore, HB-EGF, which is also a potent mitogen for fibroblastic cells, may also stimulate the growth of undifferentiated fibroblasts, which have been shown to proliferate in the lamina propria following bladder outlet obstruction (Monson et al., 1992). As a result, hyperplastic and/or hypertrophic growth would be induced in both the urothelial and muscle layers in response to obstruction. In support of the physiologic relevance of the present findings, we recently observed that following acute bladder outlet obstruction in mice, the expression of HB-EGF mRNA and its protein are increased, primarily in the bladder smooth muscle compartment (Borer et al., submitted).

In conclusion, we have found that: 1) both HB-EGF and ErbB1 are mechanically regulated genes in normal human bladder cells; 2) urothelial cells as well as SMC regulate gene expression in response to mechanical signals; 3) mechanoinduction of gene expression is regulated in a cell-specific manner; and 4) HB-EGF has a direct mitogenic effect on both SMC and urothelial cells. These findings suggest a role for HB-EGF in the response of the bladder to injury and as one potential mediator of bladder wall thickening.

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