

Bleb formation is induced by alkaline but not acidic pH in estrogen receptor silenced breast cancer cells

MAITHAM A. KHAJAH, PRINCY M. MATHEW, NADA S. ALAM-ELDIN and YUNUS A. LUQMANI

Faculty of Pharmacy, Kuwait University, Safat 13110, Kuwait

Received December 17, 2014; Accepted January 29, 2015

DOI: 10.3892/ijo.2015.2884

Abstract. *De novo* and acquired resistance to endocrine-based therapies in breast cancer occurs in parallel with epithelial to mesenchymal transition (EMT), which is associated with enhanced proliferative and metastatic potential, and poor clinical outcome. We have established several endocrine insensitive breast cancer lines by shRNA-induced depletion of estrogen receptor (ER) by transfection of MCF7 cells. All of these exhibit EMT. We have previously reported that brief exposure of specifically ER⁻ breast cancer cells, to extracellular alkaline pH, results in cell rounding and segregation, and leads to enhanced invasive potential. In this study we describe more detailed morphological changes and compare these with cell exposure to acidic pH. Morphological changes and localization of various molecules critical for cell adhesion and motility, associated with pH effects, were assessed by live cell microscopy, electron microscopy, and immunofluorescence. Exposure of either ER⁻ or ER⁺ breast cancer cells to extracellular acidic pH did not induce significant changes in morphological appearance. Conversely, brief exposure of specifically ER silenced cells, to alkaline pH, resulted in cell contractation and formation of bleb-like actin-rich structures which were evenly distributed on the outer membrane. Integrin α 2, FAK, and JAM-1 were found in the cytoplasm streaming into the newly formed blebs. These blebs appear to be related to cell polarity and movement. Pre-treatment with cytochalasin-D or inhibitors of Rho or MLCK prevented both contractation and bleb formation. Our data suggest that the effect of pH on the microenvironment of endocrine resistant breast cancer cells needs to be more extensively investigated. Alkaline, rather than acidic pH, appears to induce dramatic morphological changes, and enhances their invasive capabilities, through re-organization of cortical actin.

Introduction

About a third of breast cancer patients have tumors that either do not express estrogen receptor (ER) or very low levels and are therefore *de novo* resistant to endocrine therapy, as also are a significant proportion of patients whose tumors are ER⁺. Of the patients that do respond, the vast majority acquire resistance during therapy (1). We have previously shown that shRNA-mediated silencing of ER in MCF7 breast cancer cells leads to endocrine insensitivity (2). This is accompanied by transformation from an epithelial to a mesenchymal-like cell (commonly referred to as epithelial to mesenchymal transition; EMT) as evidenced both by their modified gene expression profile (resembling that of MDA-MB-231 cells, a line that is derived from an ER⁻ breast tumor) and their more fibroblast-like morphological appearance (3). During the EMT process described *in vivo*, epithelial cells undergo unique changes which are accompanied by diminished intracellular adhesion, resulting in enhanced motility and invasion, paralleled with poor clinical outcome (4,5). Tamoxifen resistance has been associated with changes in a large variety of molecules that include growth factors, their tyrosine kinase receptors and downstream signaling mediators such as MAPK, ERK, PI3K, AKT and SRC, cell cycle regulators and ER associated transcription factors and co-activators. Many of these are also implicated in EMT (1), providing a compelling argument for the parallelism between the two processes, as we have observed in our model system; as a result, our ER silenced cells have acquired enhanced proliferative, motile and invasive capabilities, with chemotactic attraction towards various growth factors and chemokines (6).

Of the many aspects of the tumor microenvironment that play a critical role in cancer cell invasion and metastasis, one that is receiving increased attention is the pH within a growing tumor mass. Current thinking is that, in order to maintain optimum slightly alkaline cytoplasmic pH and to avoid acidosis, cancer cells undergoing excessive fermentative glycolysis (due to poor vascular perfusion and regional hypoxia) extrude lactate and protons, thereby contributing to acidification of the extracellular matrix (7-9), a phenomenon linked to increasing tumor aggressiveness (7,8,10). The associated ion movements are facilitated through various transporters and enzymes that include carbonic anhydrases, vacuolar H⁺-ATPases, the H⁺/Cl⁻ symporter, the monocarboxylate transporter, the Na⁺-dependent Cl⁻/HCO₃⁻ exchangers, and the

Correspondence to: Professor Yunus A. Luqmani, Faculty of Pharmacy, Kuwait University, Safat 13110, Kuwait
E-mail: yunus@hsc.edu.kw

Key words: breast cancer, MCF7, endocrine resistance, pH, blebs, ion channels, actin, cytoskeleton, immunofluorescence, Rho, MLCK

Na⁺/K⁺ and Na⁺/H⁺ ATPases (11-13). An acidic matrix has been suggested to promote tumor metastasis (14,15), by facilitating cancer cell clonal evolution through induction of chromosomal instability and gene mutations (16,17) and extracellular matrix degradation, in part through enhanced activity of proteases (such as cathepsins) and matrix metalloproteinases (particularly MMP2/9) (14,18). Several therapeutic interventions, aiming to reduce tumor acidity through administration of alkaline buffers (200 mM sodium bicarbonate), claim to have raised the extracellular pH (pHe) without altering the intracellular pH (pHi) (19), to have reduced lung metastasis (20), and suppressed the formation of spontaneous prostate tumors (21). In addition, several groups have found that treatment with proton pump inhibitors reduced neoplastic development of esophageal adenocarcinoma and hepatoblastoma (22,23). Other reports however, have suggested that acidosis inhibits cancer cell proliferation, induces stress response and apoptosis (24-27), and reduces activation of Akt, whose upregulation is frequently correlated with poor prognosis (28).

We have recently reported that brief exposure of specifically ER silenced (but not ER expressing) breast cancer cells, to an alkaline pH environment, can induce a marked morphological change whereby individual cells rapidly shrink and become spherical, with increased tendency to disaggregate from the cluster of cells (termed contractation). This phenomenon is paralleled by changes in the level of expression and/or activity of various signaling molecules. It is also co-incident with enhanced invasive potential, with elevated MMP2/9 activity. All of these morphological and functional changes can be inhibited by drugs targeting two major ion pumps; Na⁺/K⁺ and the Na⁺/H⁺ exchangers (29).

In the present study, we have further characterized the morphological changes mentioned above, and identify the components of the alkaline pH induced ring-like structures or blebs on the outer membrane of ER⁻ cells. These appear to be related to cell movement and polarity, and their formation is dependent upon rearrangement and integrity of the actin cytoskeleton. We show that acidic conditions fail to induce a similar response and do not modify their metastatic behaviour. These data suggest that environmental pH needs to be more extensively investigated, and the notion of a purely acidic driven mechanism for tumor dissemination may be oversimplified, at least in respect of endocrine resistant cells.

Materials and methods

Cell lines. MCF7 breast cancer cells were obtained from the American Type Culture Collection (VA, USA). YS1.2 (ER⁺) and pII (ER⁻) cell lines were established in this laboratory by transfection of MCF7 with ER directed shRNA plasmid as described previously (2,3). The YS1.2 is a transfected line that failed to downregulate the ER. For routine culture, all cell lines were maintained as monolayers in advanced Dulbecco's minimum essential medium (DMEM) containing phenol red and supplemented with 5% fetal bovine serum (FBS), 600 µg/ml L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 6 ml/500 ml 100X non-essential amino acids (all from Invitrogen, CA, USA), and grown at 37°C in an incubator gassed with an atmosphere of 5% CO₂ and maintained at 95% humidity. For YS1.2, the maintenance medium also contained

G418 (1 mg/ml) but this was omitted during experiments. The DMEM requires an atmosphere of 5% CO₂ to produce HCO₃⁻ buffering capacity to maintain pH at 7.4 for normal cell growth. Unless otherwise specified, cells were always grown under these conditions. Upon exposure to normal atmosphere, (and consequent loss of the 'CO₂-enriched' environment) this medium reaches a maximum pH value of ~8.3 within a few minutes in either flasks with a loosened cap or in tissue culture dishes/microtiter plates with free air flow. The term 'alkaline conditions' in the text refers to this pH value of 8.3. In order to maintain the medium at acidic pH (6.5), or to culture the cells at specific alkaline pH without raising it gradually through exposing the cells to the normal atmosphere, CO₂-independent medium (Invitrogen) was used to stabilize the pH value independently of the surrounding atmosphere.

Antibodies. The following antibodies were obtained from Cell Signaling, USA: Akt (cat no. 9272), FAK (cat no. 9330), vimentin (cat no. 3932), and actin (cat no. 4967). The following antibodies were obtained from Abcam: integrin-α2 (cat no. ab55340), and JAM-1 (cat no. ab52647).

Microscopic analysis of morphological changes in response to pH. For each cell line, 10⁵ cells were seeded into individual wells of a 12-well plate and allowed to settle at 37°C for 24 h. To induce a gradual increase in pH, culture plates were removed from the gassed incubator (i.e., from the 5% CO₂ atmosphere needed to maintain the buffering capacity of the DMEM) and placed in an un-gassed incubator at 37°C. In another experimental set-up, cells were cultured at specific acidic or alkaline pH by using the CO₂-independent medium adjusted to pH 6.5 or 8.3 and placed in an un-gassed incubator at 37°C. Several fields containing colonies were marked and photographed immediately and after several time-points (as indicated in the text) using an Olympus inverted microscope fitted with a camera. Resultant changes in cell size and shape in each photographed field were quantified in terms of the field area occupied by cells, using Adobe Photoshop CS4 Measuring Tool.

Live cell imaging. The effect of pH change on pII cells was monitored by time-lapse photography using a live cell imager (Cell Observer HS, Zeiss, Germany). Cell monolayers grown in 5% CO₂ in a 25-cm² tissue culture flask were placed inside the imaging chamber maintained at 37°C and then exposed to normal atmosphere by loosening the cap (to induce a gradual increase in the pH to 8.3). In separate experiments, images were recorded every 5 min over a 72-h period, at either x20, x40 or x60 magnification. AxioVision software (Zeiss) was used to combine all the individual snap shots to generate a video using Windows Movie Maker software (Microsoft). The effect of amiloride or zonisiporide (10 µM) was tested by pre-treatment of cells with either agent for 1 h before placing in the imager.

Electron microscopy. Coverslips (22x22 mm) were coated with 50 µg/ml of poly-D lysine for 4 h, air-dried at room temperature and sterilized by UV light for 15 min. After that, pII cells (1x10⁴) were seeded in 6-well plates containing the coated coverslip and allowed to grow for 2 days. Cells were

washed with PBS and fixed with 3% glutaraldehyde for 3 h at room temperature. After that, cells were washed with Millong's phosphate buffer three times and then treated with 1% osmium tetroxide for 2 h. Then the cells were washed with Millong's phosphate buffer three times. The coverslips were carefully removed and adhered to double-sided carbon tabs on aluminum stubs. The coverslips were then gold coated with Autofine coater JFC-1600 (Jeol, Japan) for 40 sec to have a uniform fine conducting layer of electrons. The coated coverslips was observed under scanning electron microscope (SEM) Carryscope JCM-5700 (Jeol).

Apoptosis assay. pII cells were cultured in 6-well plates at various pH (7.4, 8.3 and 6.5) using the CO₂-independent medium. In addition, another batch of cells were cultured in normal DMEM and then transferred to an ungasged incubator at 37°C for 1 h to induce a gradual increase in the pH to 8.3. All of the cells were then trypsinized, pelleted by centrifugation at 1000 g for 3 min and washed twice by re-suspension and centrifugation in ice-cold PBS and once in Annexin V binding buffer [10 mM HEPES/NaOH (pH 7.4), 0.14 M NaCl, 2.5 mM CaCl₂]. The final cell pellet was re-suspended in 100 µl of Annexin V binding buffer at 5x10⁶ cells/ml and processed for FACS analysis using the PE Annexin V apoptosis detection kit I from BD Pharmingen (USA). Cells were stained in the following manner: a) cells only (negative control); b) with 10 µl of Annexin V-PE; c) with 20 µl of 7AAD; and d) with 10 µl of Annexin V-PE plus 20 µl 7AAD. All incubations were performed in the dark at room temperature (RT) for 15 min.

Actin staining. YS1.2 and pII cells grown overnight at 37°C, 5% CO₂ in 8-well glass chambered slides (Lab-Tek, USA) were treated in the following manner: a) continued to be cultured at 37°C, 5% CO₂ (pH 7.4); b) medium changed to CO₂ independent media pH 6.5 for 1 h at 37°C; or c) medium changed to CO₂ independent media pH 8.3 for 1 h at 37°C. All cells were then fixed with 3.7% paraformaldehyde and stained with either phalloidin (red stain) or phallotoxin (green stain) to visualize F-actin, and examined by confocal microscopy.

In a separate experimental set-up, cells were pre-treated with various concentrations of cytochalasin-D (StressMarq, USA), a rho kinase inhibitor (Millipore, USA), or myosin light chain kinase (MLCK) inhibitor peptide 18 (Millipore) for 1 h and either fixed directly with 3.7% paraformaldehyde, or after exposure to alkaline pH (as above) for 1 h. To visualize the nuclei, cells were finally incubated with DAPI (blue stain).

Immunostaining. YS1.2 and pII cells were cultured and fixed as described above, washed with 1% BSA and incubated overnight at 4°C with primary antibody diluted in 1% BSA: anti-integrin α-2 (1:50 dilution), anti-vimentin (1:150 dilution), anti-AKT (1:500 dilution), and anti-JAM-1 (1:100 dilution). After several washes in PBS, followed by incubation with secondary antibody (1:500 dilution in 1% BSA) for 1 h in the dark at RT, cells were mounted and examined by fluorescence confocal microscopy.

Membrane ruffling and polarity assays. For assessment of membrane ruffling, pII cells were stimulated with EGF

(Sigma, USA) (10 ng/ml) for 10-30 min at 37°C, 5% CO₂ followed by paraformaldehyde fixation and phalloidin staining. For polarity experiments, pII cells were cultured on a polylysine coated glass coverslip and allowed to adhere overnight. The coverslip was then embedded in a 10-cm² petri dish in contact with previously added solidified 0.5% ultra-pure agarose (Invitrogen) containing either PBS (control) or 0.1 mg/ml EGF. DMEM was carefully pipetted into the dish and cells incubated overnight at 37°C, 5% CO₂ followed by paraformaldehyde fixation and phalloidin staining. In another experimental setup, after the overnight incubation with EGF, cells were incubated for 1 h in a 37°C ungasged incubator to induce a gradual increase in pH to 8.3, then fixed and stained with phalloidin.

Statistical analysis. Differences between means of individual groups were assessed by the Student's two tailed unpaired t-test: p≤0.05 was considered statistically significant.

Results

Effect of acidic pH on cell morphology. No specific morphological changes were observed by exposure of either the ER⁺ YS1.2 or the ER⁻ pII cells to extracellular acidic pH. This is illustrated for pII cells in Fig. 1A. The left panel shows pII cells photographed immediately after removal from the incubator when the culture medium was at pH 7.4, while the right panel shows pII cells after transfer to acidic pH medium for 30 min at 37°C.

Effect of short exposure to alkaline pH on morphology and viability of pII cells. The left panel in Fig. 1B shows pII cells photographed immediately after removal from the incubator when the culture medium was at pH 7.4, while the right panel shows the same cells cultured in an ungasged incubator at 37°C for 30 min, at which time the pH of the medium had become noticeably alkaline as evidenced by the purple color of the phenol red indicator and confirmed by pH meter. A dramatic morphological change was observed, where cells separated from each other and became spherical (contractation). These alkaline pH-induced morphological changes also occurred when cells were cultured in serum-free media (data not shown). Fig. 1C shows higher magnification views; multiple ring or vesicle-like bleb structures were observed on the outer cell membrane, which all disappeared within 2-3 h of returning the cells to pH 7.4. Prior exposure of cells to either amiloride or zoniporide inhibited both contractation and bleb formation as shown in Fig. 1D and E, respectively, which suggests that these two processes occur simultaneously. Fig. 1F shows that there was a significant reduction in the culture area occupied by pII cells (30-40%) after exposure to alkaline but not acidic pH. Since membrane blebbing has been observed as an early event preceding cell death, we analysed the cells for any indication that the short exposure to alkaline or acidic pH had a damaging effect. Fig. 1G shows the percentage of viable, apoptotic and necrotic pII cells after exposure to neutral, acidic or alkaline pH for 1 h. In the experiment performed, most of the cells (90-95%) were viable when cultured at pH 7.4 and no significant difference was observed after exposure to either acidic or alkaline pH, suggesting that the formation of blebs

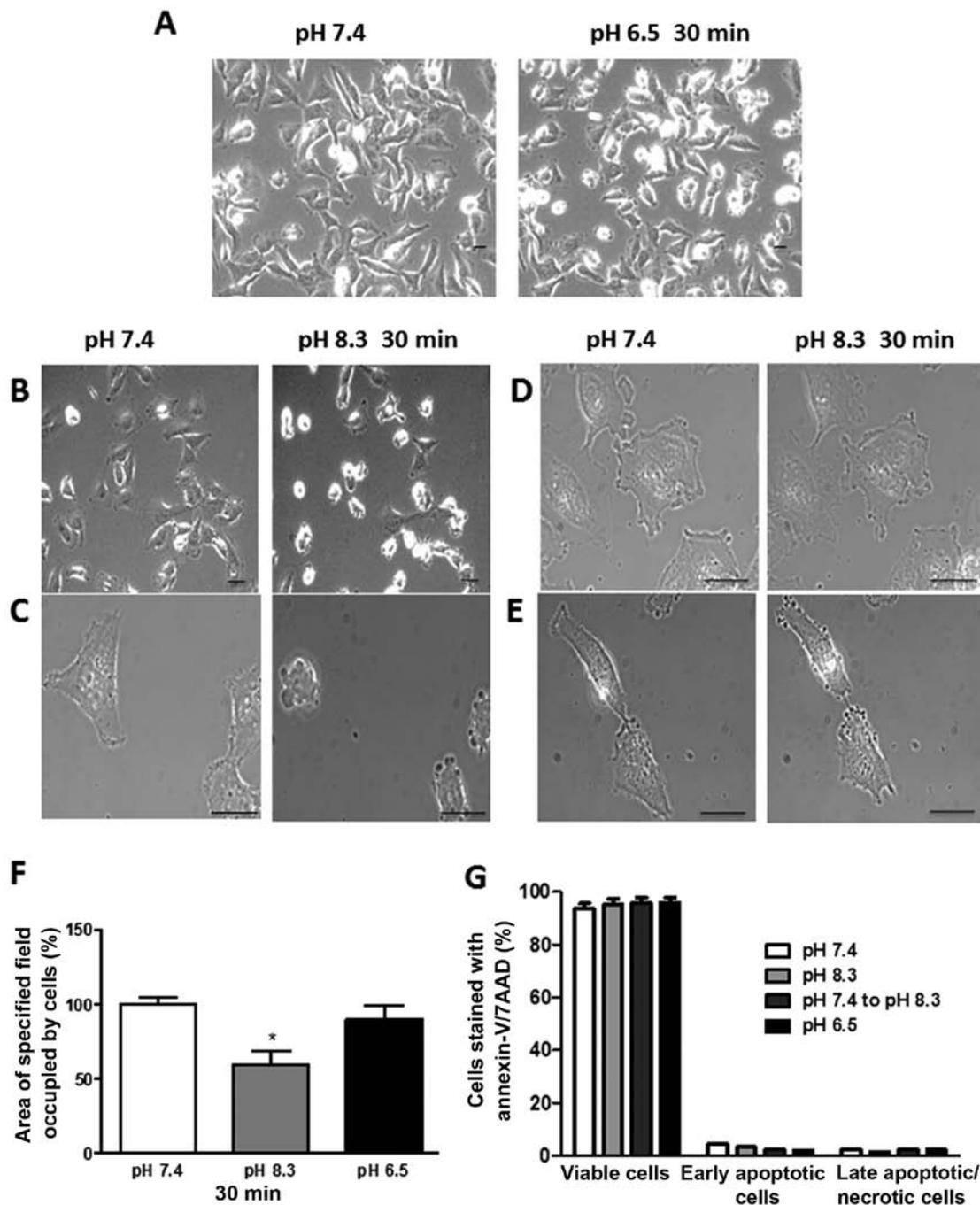


Figure 1. Effect of acidic and alkaline pH on contractotolation and apoptosis of pII cells. (A) pII cells grown in complete DMEM photographed immediately after removal from the CO₂ gassed incubator (pH 7.4) and after exposure to acidic pH at 37°C for 30 min by exchanging with CO₂-independent medium. (B and C) pII cells (at x20 and x40 magnifications) grown in complete DMEM photographed immediately after removal from the CO₂ gassed incubator (pH 7.4) and after exposure to atmospheric conditions for 30 min (which increased the pH to 8.3). (D and E) The effect of pre-treatment for 1 h with 10 μ M amiloride (D) or 10 μ M zoniporide (E). Scale bars represent 40 μ m (A and B) and 20 μ m (C-E). (F) Histograms show the quantified area in selected fields (mean \pm SEM of at least 3 separate fields of at least 3 independent experiments) occupied by pII cells cultured at pH 7.4 and then exposed to pH 8.3 and 6.5 for 30 min; values expressed relative to control (pH 7.4). Asterisk denotes significant difference from the pH 7.4 condition with $p \leq 0.05$. (G) Cell viability determined by flow cytometry using Annexin V/7AAD staining of cells cultured at pH 7.4 harvested either: i) immediately after removal from the CO₂ gassed incubator (pH 7.4, open bar); ii) after exposure to alkaline condition for 1 h by exchange with CO₂-independent medium (light gray bar); iii) after exposure to atmospheric conditions for 1 h (as the pH gradually increased to 8.3; dark gray bar); or iv) exposure to acidic condition by exchange with CO₂-independent medium (black bar).

seen rapidly after exposure to alkaline pH is not associated with cell apoptosis.

Effect of prolonged (overnight) exposure to alkaline pH. Fig. 2A shows pII cells photographed at various times after

exposure to alkaline pH. Cells started to detach from the plate after 4 h and beyond and they were unable to re-adhere or grow when they were subsequently placed back at pH 7.4. Fig. 2B shows phalloidin staining of the actin cytoskeleton for pII cells at neutral pH (left panel), after brief exposure to alkaline pH

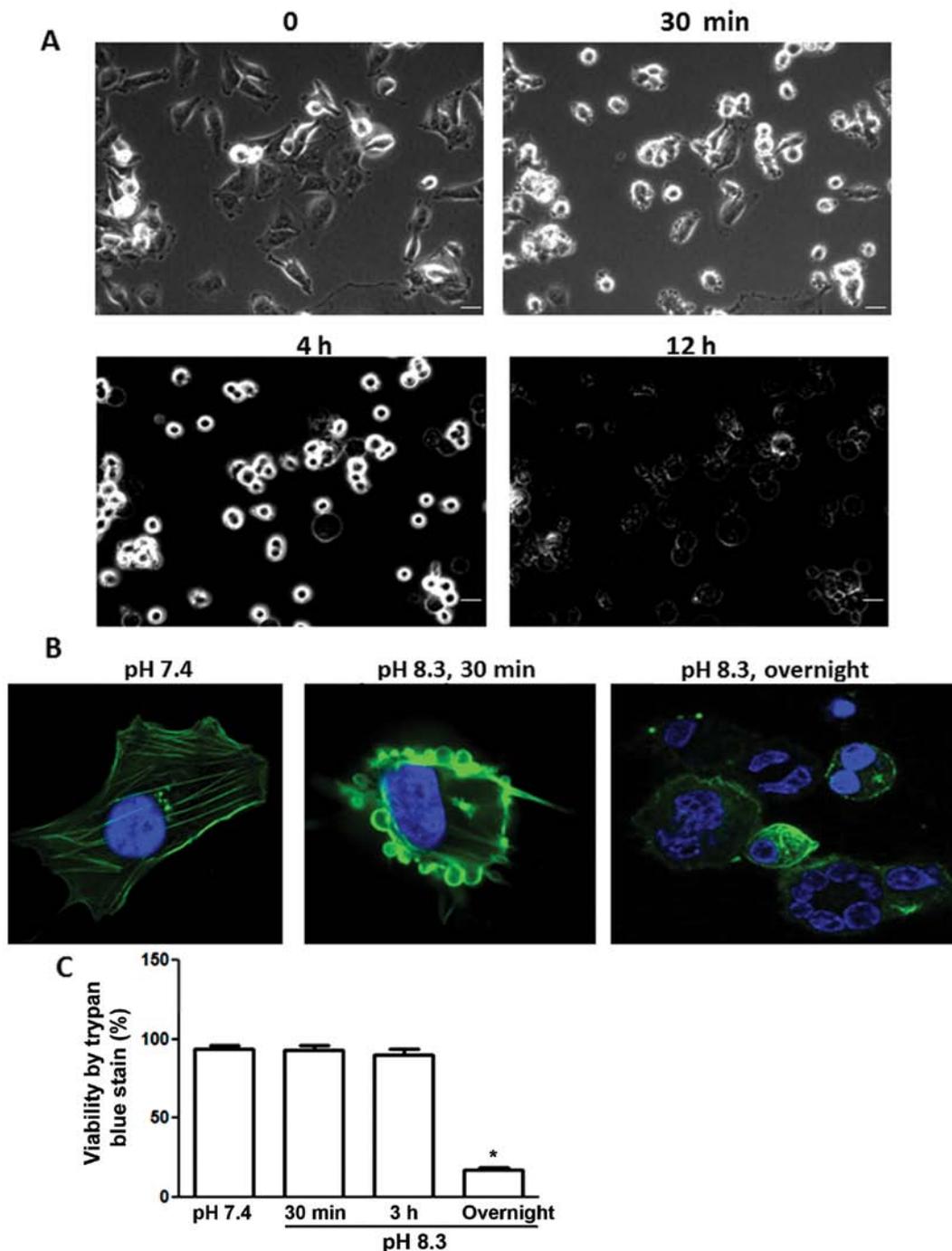


Figure 2. Effect of prolonged incubation of pII cells at alkaline pH. pII cells were cultured overnight in DMEM medium in a 12-well plate and then photographed immediately before transfer to an ungasged incubator and after 30 min, 3 h and overnight incubation (i.e., under alkaline conditions) (A). (B) Cells stained with phalloidin (green stain) at pH 7.4 or after exposure to alkaline pH for 30 min or overnight. Nuclei are highlighted by the blue DAPI staining. (C) Cell viability determined by trypan blue exclusion. Asterisk indicates significant difference from pH 7.4 condition with $p < 0.001$. Scale bars represent 20 μm (A) and 40 μm (B).

(contractation, middle panel) or, after prolonged (overnight) exposure to alkaline pH (right panel) when cells lost their membrane integrity and demonstrated nuclear condensation, suggesting cell death. Fig. 2C shows the percentage of viable pII cells after various exposure times to alkaline pH as determined by trypan blue exclusion. Approximately 80% of cells were stained with trypan blue after an overnight exposure, confirming the immunofluorescence data (Fig. 2B,

right panel). Neither pII nor YS1.2 cell lines appeared to be adversely affected by overnight incubation in acidic pH (data not shown), as judged by their ability to continue normal growth when returned back to a pH 7.4 environment.

Effect of extracellular pH on the actin cytoskeleton. Fig. 3A shows phalloidin staining of the actin cytoskeleton for YS1.2 and pII cells at pH 7.4. When YS1.2 cells were cultured in

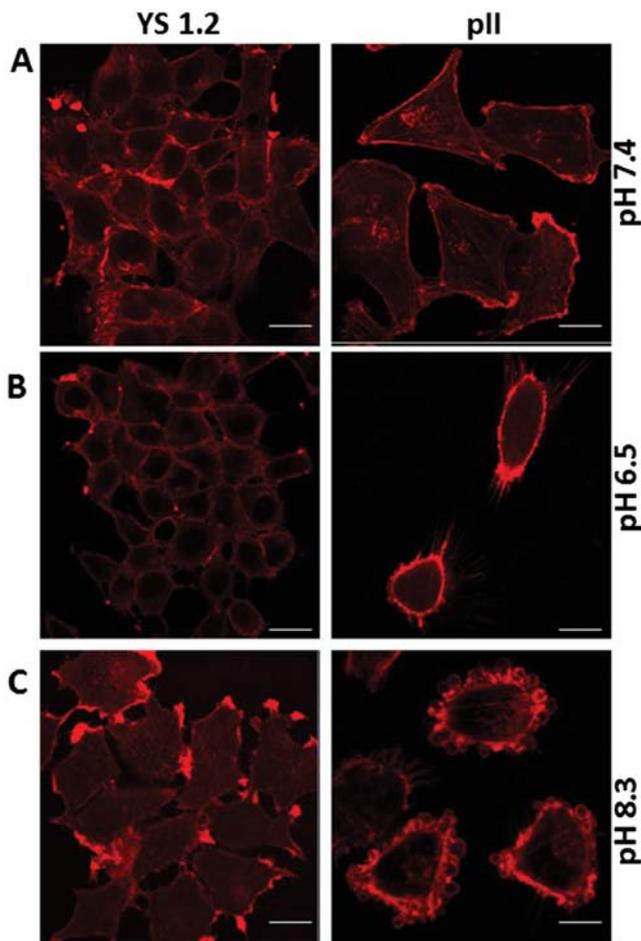


Figure 3. Staining of actin cytoskeleton after exposure to acidic or alkaline pH. YS1.2 and pII cells were cultured at pH 7.4 or exposed to acidic or alkaline pH for 1 h at 37°C by exchange with CO₂-independent medium, and stained with phalloidin. Scale bars represent 20 μ m.

either acidic or alkaline pH for 1 h, no marked difference in staining was observed (Fig. 3B and C, left panels). pII cells cultured in alkaline medium showed a re-distribution of phalloidin staining from the cytoplasm to the outer membranes with particularly intense staining of the membranes of the newly formed blebs (Figs. 2B, middle panel, and 3C, right panel). When pII cells were cultured in acidic pH medium, some cells did exhibit minor increased membrane staining and some rounding was observed in a few cells but without bleb formation (Fig. 3B, right panel). Fig. 4 shows the ultrastructural changes in the plasma membrane of pII cells upon exposure to alkaline pH as visualised by scanning electron microscopy. At neutral pH, there are few lamellipodia or other protruding structures apparent on the cell's outer membrane (Fig. 4, left panels). After brief exposure to alkaline pH, pII cells exhibited a rounded shape with numerous invaginations and prominent blebs covering the entire cell surface, confirming and further extending the live cell imaging and immunofluorescence data (Fig. 4, right panels). What appeared to be hair-like extensions under the light microscope are seen here as flowing cytoplasmic protrusions.

Effect of cytochalasin-D on phalloidin staining of pII cells. Fig. 5A shows actin distribution in pII cells using phalloidin

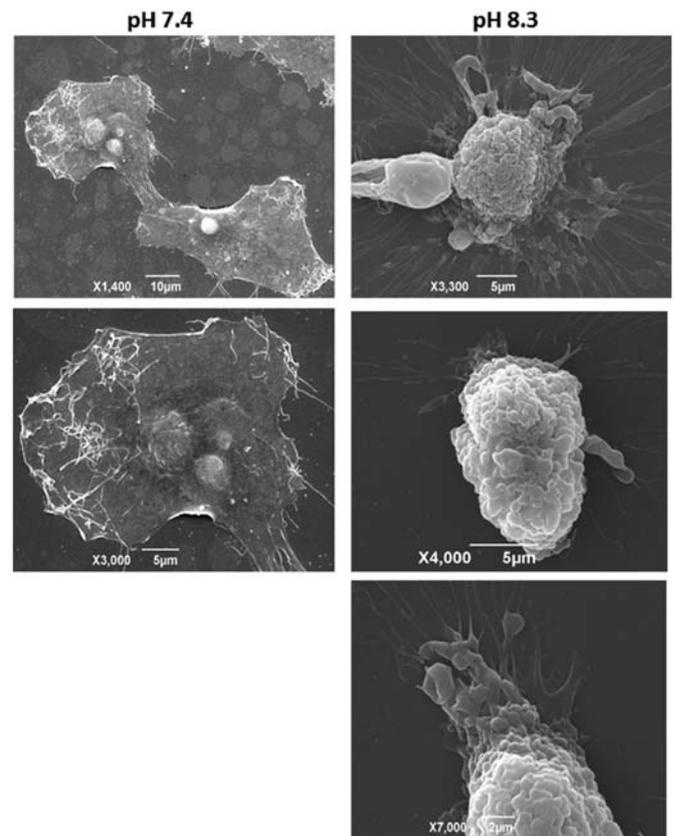


Figure 4. Visualisation of pII cells by scanning electron microscopy. pII cells grown on glass coverslips in DMEM media at either pH 7.4 (left panels), or after exposure to alkaline pH for 30 min (right panels) were fixed and processed as described in Materials and methods, and photographed at various magnifications as indicated using a SEM Carryscope JCM-5700 (Jeol).

staining after exposure to various concentrations of the potent actin polymerization inhibitor cytochalasin-D. At 1 μ M and higher, the staining pattern reflected a marked disruption of the membrane structure. We chose a lower concentration (200 nM) of the drug, at which it exhibited minimal effect on the actin structure at pH 7.4, to test its effect on the alkaline induced morphological changes in pII cells. At this concentration it completely inhibited or reversed the morphological changes and the re-distribution of actin associated with alkaline pH when it was added to the cells either before or after exposure to alkaline pH (Fig. 5B).

Effect of Rho kinase inhibitor on phalloidin staining of pII cells. Fig. 6A shows phalloidin staining of pII cells after treatment with various concentrations of the Rho kinase inhibitor. At 1 μ M and higher, there was a marked disruption of the membrane and actin cytoskeleton structure. At 100 nM the drug had minimal effect on the actin distribution, but completely inhibited the morphological changes associated with alkaline pH when it was added to the cells 1 h before culturing in alkaline pH (Fig. 6B).

Effect of myosin light chain kinase (MLCK) inhibitor on phalloidin staining of pII cells. Fig. 7A shows phalloidin staining of pII cells after treatment with various concentrations of the MLCK inhibitor peptide 18. At 5 μ M and higher, there was

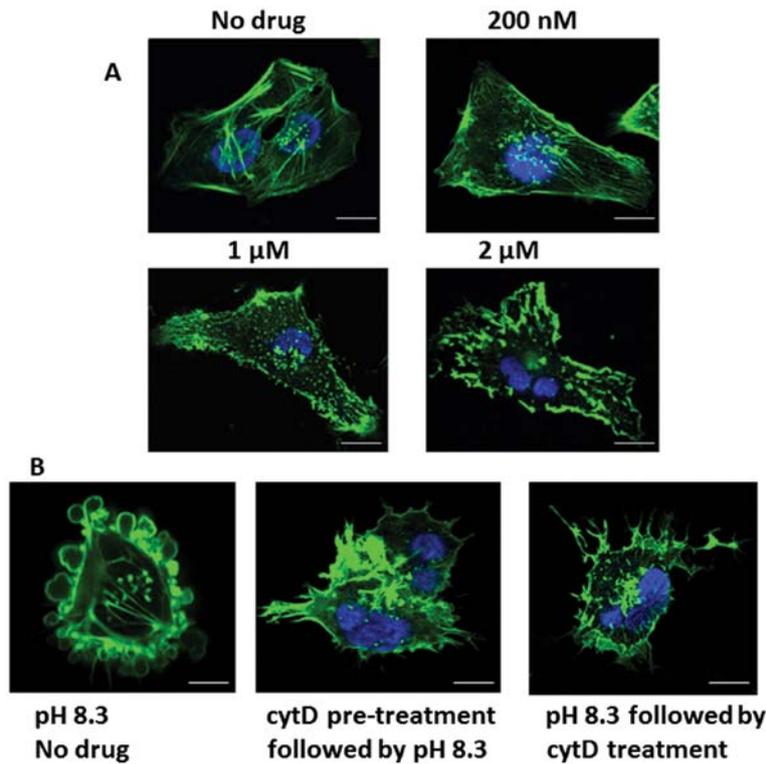


Figure 5. Effect of cytochalasin-D (cytD) on actin cytoskeleton of pII cells. (A) Cells cultured overnight at pH 7.4 stained with phalloidin (green) after treatment with various concentrations of cytD (200 nM-2 μ M) for 1 h. (B) Cells stained with phalloidin either after exposure to alkaline pH for 1 h, pre-treatment for 1 h with 200 nM cytD then exposure to alkaline pH for 1 h, or after exposure to alkaline pH for 1 h then treatment with 200 nM cytD. Nuclei are highlighted by the blue DAPI staining. Scale bar represents 10 μ m.

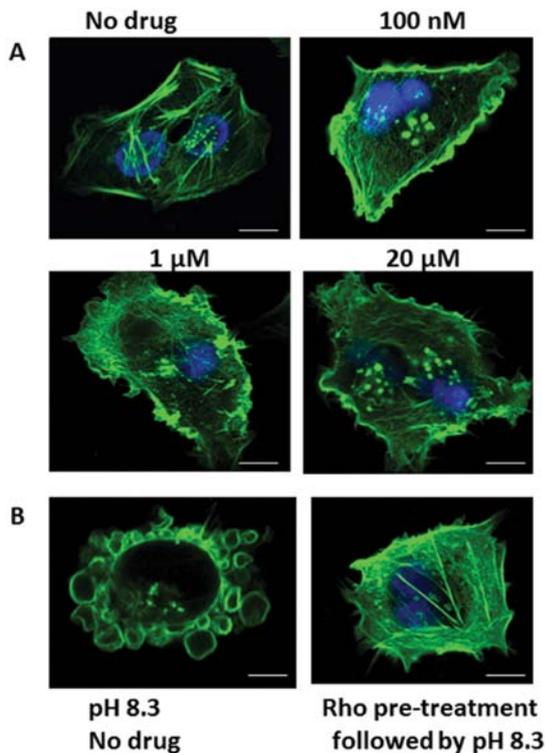


Figure 6. Effect of Rho kinase inhibitor on actin cytoskeleton of pII cells. (A) Cells cultured overnight at pH 7.4 stained with phalloidin (green) after treatment with various concentrations of Rho kinase inhibitor (100 nM-20 μ M) for 1 h. (B) Cells stained with phalloidin after exposure to alkaline pH for 1 h, or after 1-h pre-treatment with 100 nM of Rho kinase inhibitor followed by exposure to alkaline pH for 1 h. Nuclei are highlighted by the blue DAPI staining. Scale bar represents 10 μ m.

a marked disruption of the membrane and actin cytoskeleton structure. At 2-5 μ M the drug had minimal effect on the actin distribution. At this concentration it completely inhibited the morphological changes associated with alkaline pH when it was added to the cells 1 h before culturing in alkaline pH (Fig. 7B).

Effect of epidermal growth factor (EGF) on phalloidin staining of pII cells. Fig. 8A shows phalloidin staining of pII cells at neutral pH. Exposure to medium containing EGF (10 ng/ml) for 10 and 30 min at pH 7.4 (Fig. 8B and C respectively) resulted in more intense membrane staining, reflecting increased membrane ruffling, but there was no indication of formation of blebs. When cells were placed near a source of EGF (in a strip of agarose gel) they tended to move in that direction (Fig. 8E), showing polarization, reflected in the distribution of phalloidin staining. This was not seen with PBS-containing gel (Fig. 8D). pII cells exposed to alkaline pH (by transfer of the dish to normal atmosphere) for 1 h before fixation and staining, exhibited contractation as expected but with formation of blebs principally on the surface of the cell facing the EGF source (Fig. 8G), in marked contrast with the more random distribution of the blebs when the adjacent gel contained PBS only (Fig. 8F).

Effect of extracellular pH on expression of vimentin. Fig. 9A shows vimentin staining (green stain) in pII cells (which have undergone EMT and express mesenchymal markers) at neutral pH with a polarized peri-nuclear pattern. The distribution of

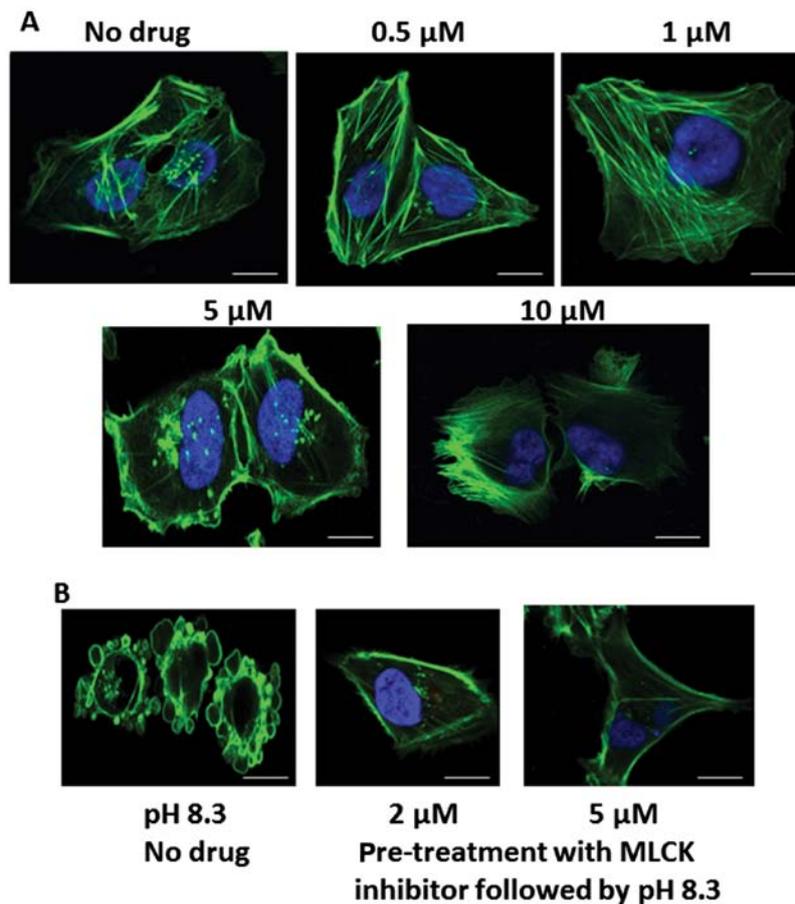


Figure 7. Effect of myosin light chain kinase (MLCK) inhibitor on actin cytoskeleton structure of pII cells. (A) Cells cultured at pH 7.4 stained with phalloidin (green) after treatment with various concentrations of MLCK inhibitor (0.5-10 μM) for 1 h at 37°C at pH 7.4. (B) Cells were stained with phalloidin after exposure to alkaline pH for 1 h, or after 1-h pretreatment with 2 or 5 μM of MLCK inhibitor followed by exposure to alkaline pH for 1 h. Nuclei are highlighted by blue DAPI staining. Scale bar represents 10 μm .

this protein was unaffected by exposure to either acidic or alkaline conditions for 1 h (Fig. 9B and C respectively) and it did not appear in the blebs.

Effect of extracellular pH on expression of AKT. Fig. 10A shows AKT staining (red stain) in pII cells at neutral pH with a diffuse cytoplasmic staining pattern. The distribution of this protein was unaffected by exposure to acidic conditions for 1 h (Fig. 10B). At alkaline pH (Fig. 10C) the staining was greatly reduced and was conspicuously absent from the blebs.

Effect of extracellular pH on expression of adhesion molecules. Figs. 11-13 show distribution of integrin- $\alpha 2$, junctional adhesion molecule-1 (JAM-1), and focal adhesion kinase (FAK) (red stain), respectively, in pII cells, after 1-h exposure to neutral, acidic or alkaline pH. These molecules show diffuse cytoplasmic expression in neutral pH conditions (panels A); this pattern was not changed upon exposure to acidic pH (panels B). Under conditions of alkaline pH, these molecules were seen inside the newly formed blebs, but did not appear to be part of the membrane, which was stained with phalloidin (panels C). It was noted that the intensity of staining for FAK was markedly increased upon exposure to alkaline pH when compared to neutral or acidic conditions (Fig. 13) under which it was very poorly expressed.

Discussion

Acidic vs. alkaline pH. The majority of the data in the literature appears to support the idea that the tumor microenvironment is acidic in nature and aids in tumor development and progression through various mechanisms (reviewed in ref. 13). This has prompted exploration of alkalization-based therapeutic measures to nullify this acidity (30,31) and such proposals have already entered the popular press as a new type of cancer treatment. We have previously shown that alkaline conditions have a dramatic effect, though only on ER⁻ cells, and in particular on those that have acquired functional loss of ER as opposed to those that are *de novo* ER⁻ (29).

In the present study we observed that even prolonged (overnight) exposure to acidic conditions did not significantly affect either the ER⁺ or the ER⁻ cell lines with respect to either the type of morphological changes (Fig. 1) or enhancement of cell motility and invasion induced by very brief exposure to alkaline pH (data not shown). In contrast with this observation, both mouse B16-F1- melanoma (32) and human A-07, D-12, or T-22 melanoma (33) are reported to acquire enhanced invasive ability in acidic pH, suggesting that the pH effect might also be tissue specific. Indeed, consistent with our observations, exposure of human colonic adenoma and carcinoma cell lines to acidic pH significantly reduced their proliferative capacity

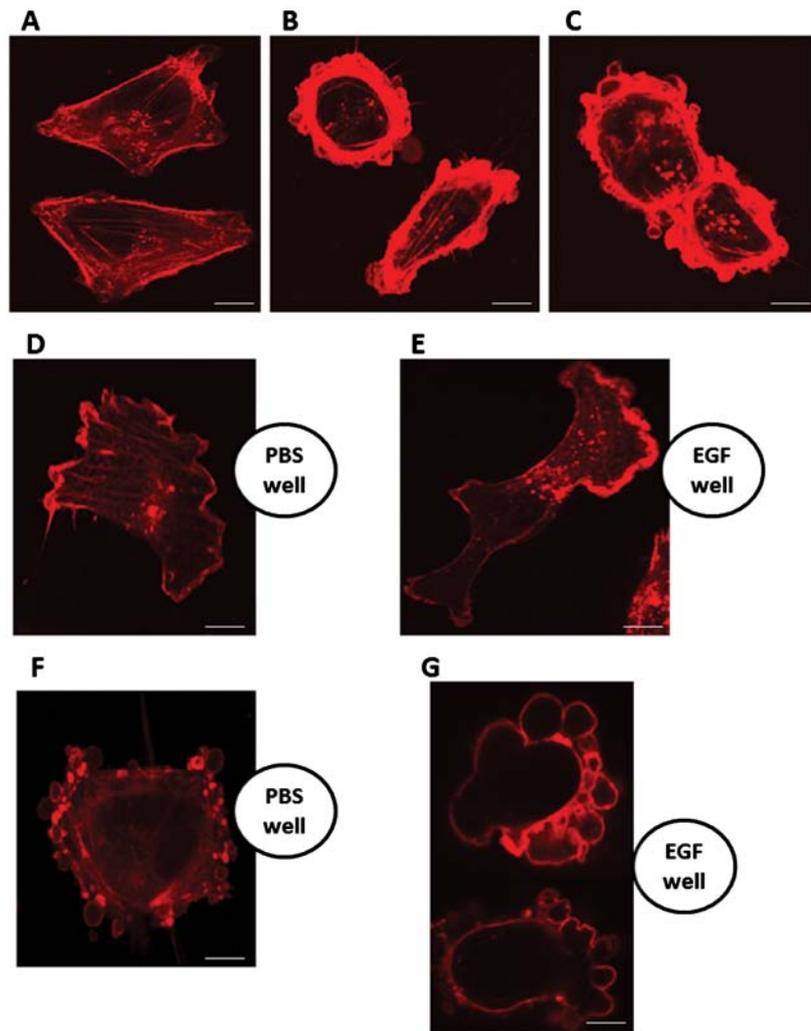


Figure 8. Effect of EGF (0.1 mg/ml) and pH on membrane ruffling and polarity of pII cells. (A-C) Cells cultured overnight at pH 7.4 and stained with phalloidin either directly (A), or after EGF treatment for 10 min (B) and 30 min (C). (D-G) Phalloidin staining of cells cultured overnight in wells adjacent to an agarose plug containing either PBS or EGF positioned to one side as indicated. Cells (F and G) were additionally exposed to pH 8.3 for 1 h (by moving to ungassed incubator) prior to fixation and staining. Scale bars represent 10 μ m.

(24). In addition, expression of the universal inhibitor of cell cycle dependent kinases WAF1 was increased by exposure of human glioblastoma cells to acidic pH and resulted in cell cycle arrest in G1 (34-36). With respect to the ability of cells to survive over longer periods under the stressful conditions of acid/alkaline pH (24), reported enhancement in proliferative capacity of human colonic adenoma and carcinoma derived cell lines that had previously been exposed to alkaline pH for 4 days (37) observed that exposure of their murine fibrosarcoma cell line Fsa-II to alkaline conditions caused cell death by generation of reactive oxygen species (within 90 min), DNA fragmentation (within 24 h) and mitochondrial damage (within 6 h). Whilst all our cell lines appear able to survive well in acidic conditions, alkaline pH starts to induce cell death of pII and YS2.5 [both ER silenced lines (3)] after ~4-h exposure. With exposures of <2 h they are able to revert to their original morphology and continue normal growth if restored to pH 7.4 medium. The ER⁺ MCF7 cells are able to survive for longer periods, with significant cell death evident from ~8-10 h onwards (data not shown). This might be due to the stronger cell-cell contact in ER⁺ cells compared to more

loose contacts in ER⁻ cells (due to the loss of adherent and tight junctions proteins during the EMT process), or perhaps to a higher threshold of resistance to alkaline-induced death in ER⁺ vs ER⁻ cells. Thus it seems brief exposure to alkaline pH confers advantages to endocrine resistant cancer cells, in terms of increased motility and invasive capacity, while a longer period is detrimental.

Contractolation. The alkaline induced shrinking and rounding phenomenon of the cell that we documented previously, when examined more closely at higher magnification, indicates thickening of the plasma membrane (often also referred to as ruffling) and formation of vesicular invaginations closely resembling cytoplasmic 'blebbing' described during early embryogenic migration (38). In a more general context, and as distinct from the more extensively studied membrane protrusions enabling motile functions such as lamellipodia and filopodia, blebs are associated with motility (39), cell spreading (40), cytokinesis (41) and mitosis (42-45).

Membrane blebbing (46,47) is also seen during the execution phase of apoptosis which subsequently leads to DNA

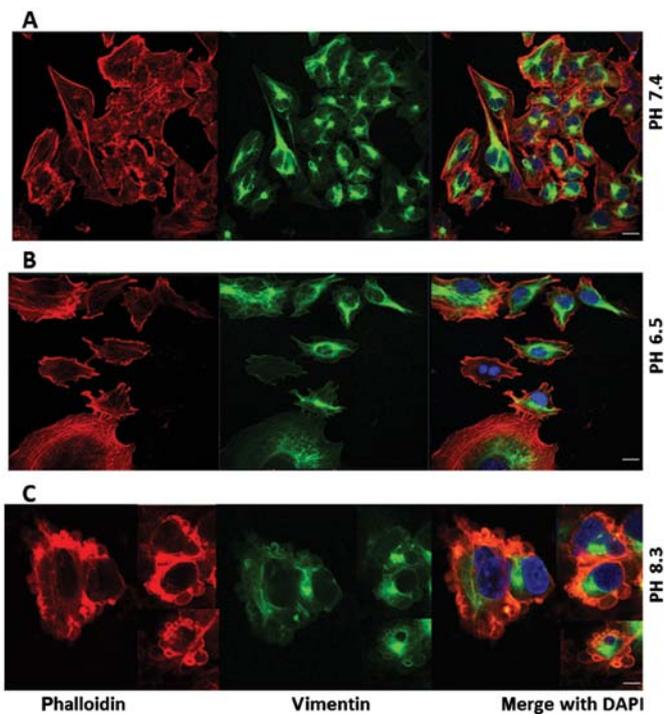


Figure 9. Effect of pH on localization of vimentin in pII cells. Cells stained with phalloidin (red), anti-vimentin antibody (green), or DAPI (blue) after culturing in pH 7.4 (A), or after exposure to acidic (B) or alkaline (C) pH for 1 h at 37°C. Scale bar represents 20 μ m.

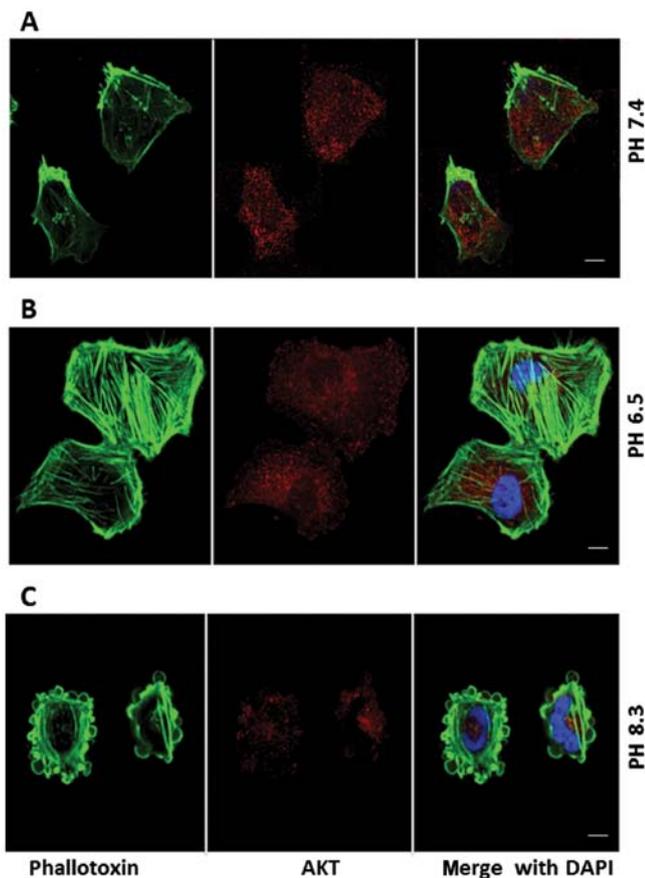


Figure 10. Effect of pH on localization of AKT in pII cells. Cells stained with phalloitin (green) and anti-AKT (red) after culturing in pH 7.4 (A), or after exposure to acidic (B) or alkaline (C) pH for 1 h at 37°C. Scale bar represents 20 μ m.

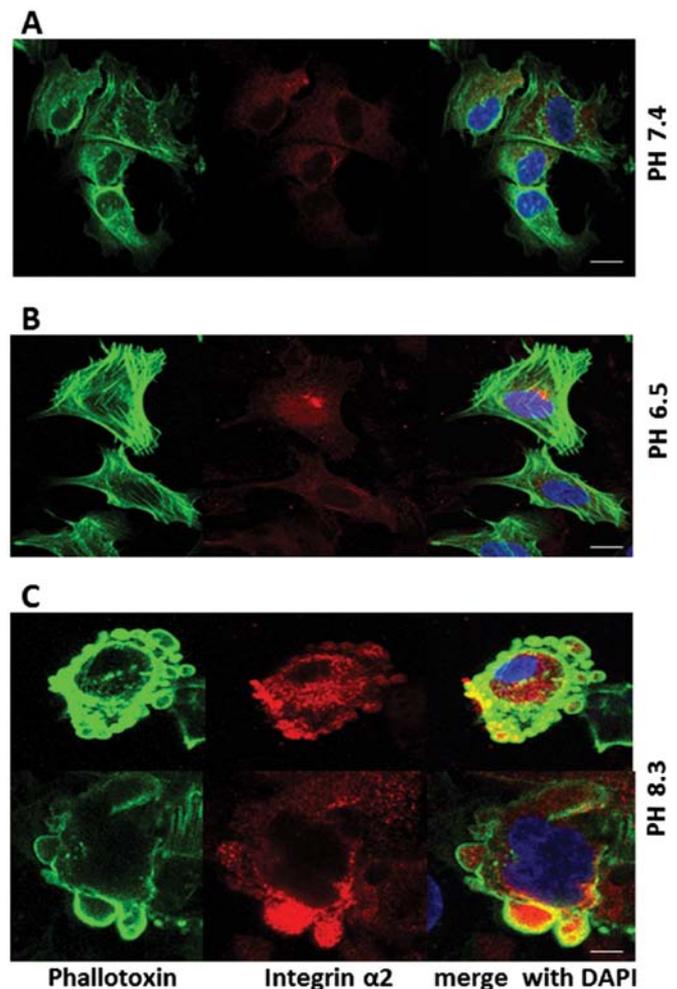


Figure 11. Effect of pH on localization of integrin α 2 in pII cells. Cells stained with phalloitin (green) and anti-integrin α 2 antibody (red) after culturing in pH 7.4 (A), or after exposure to acidic (B) or alkaline (C) pH for 1 h at 37°C. Scale bar represents 20 μ m.

fragmentation, chromatin condensation and apoptotic cell death (48-50). Treatment of PC6-3 cells with the caspase inhibitor z-VAD-FMK for 24 h generated similar blebs (51), and these were also correlated with cell death. The time needed to form blebs in PC6-3 cells was significantly longer compared to what we have observed in pII cells upon exposure to alkaline pH (24 h vs \leq 10 min). In general however, bleb dynamics are quite rapid (52).

It has been well documented that actin is the main component of most of these membrane blebs (53-57), with other known constituents being myosin and ezrin (52). Formation of blebs, such as in Walker 256 carcinosarcoma cells, have also been described as areas where cortical actin is initially depolymerized and constitutes a boundary separating the blebs from the rest of the cell (58); this would generally be followed by reconstitution of an actin cortex. It is considered that the major role of the actin cytoskeleton in formation of membrane blebs is to increase the intracellular hydrostatic pressure by cortical contraction, which results in protrusions at sites where the elastic resistance is reduced. Myosin also plays a critical role in the formation of membrane blebs. The myosin II contractile activity is stimulated through phosphorylation of myosin light

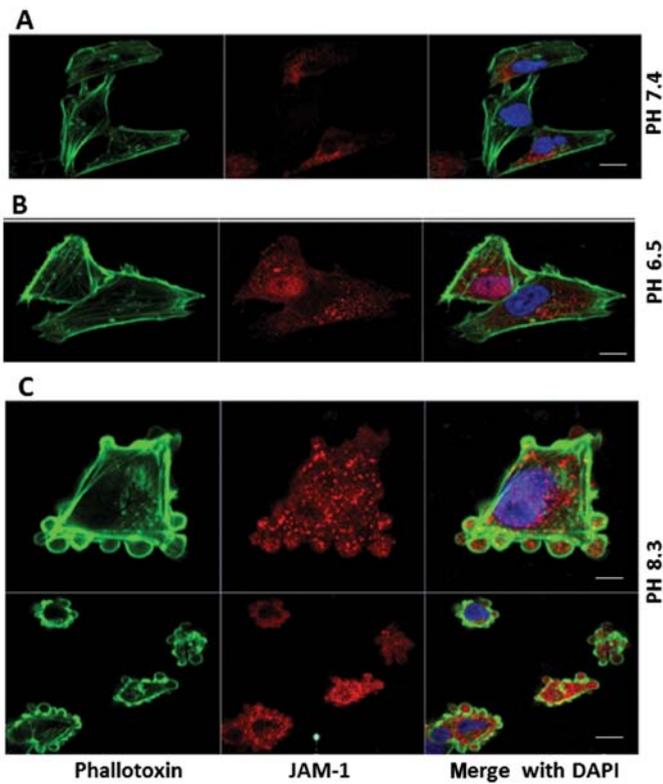


Figure 12. Effect of pH on distribution of junctional adhesion molecule-1 (JAM-1) in pII cells. Cells were stained with phallotoxin (green), anti-JAM-1 antibody (red), or DAPI (blue) after culturing in pH 7.4 (A), or after exposure to acidic (B) or alkaline (C) pH for 1 h at 37°C. Scale bar represents 20 μ m.

chain on serine 19 by MLCK which catalyzes the interaction between myosin and actin needed to produce sliding forces for cell contraction and movement (59,60). Microinjection of catalytically active MLCK can induce bleb formation (41). In addition, the small GTPases Rho, Rac and CDC42 [which are elevated in pII cells (3)] play an important role in cytoskeletal rearrangement and actin stress fiber formation (61,62); treatment with the actin de-polymerizer cytochalasin-D, or inhibitors of MLCK and Rho kinase activity, all inhibited bleb formation in serum-deprived z-VAD-FMK treated PC6-3 cells (51). We found that F-actin is the main component of the membranes of the blebs formed in response to exposure to alkaline pH. Molecules critical for adhesion and motility, such as JAM-1, FAK and integrin- α 2, were seen to flow from their diffuse cytoplasmic locations into the newly formed blebs, but were not part of the membrane. This shows that the blebs are continuous with the rest of the cell; however, other molecules such as vimentin, exemplifying the mesenchymal phenotype (in place of the keratin found only in the ER⁺ cells), did not alter their perinuclear distribution and were not seen inside the blebs (Fig. 9). Cytochalasin-D, MLCK and Rho kinase inhibitors all completely disrupted the plasma membrane of all our cells. Pre-treatment of pII cells, prior to exposure to alkaline pH, with lower concentrations, at which the integrity of plasma membranes was maintained (reflected in the continuity of actin staining), prevented bleb formation. Moreover, these drugs also caused retraction of already formed blebs when added to pII cultures that had been previously shifted to pH 8.3 (Figs. 5-7) (51) had previously found that MLCK inhibitors were able to

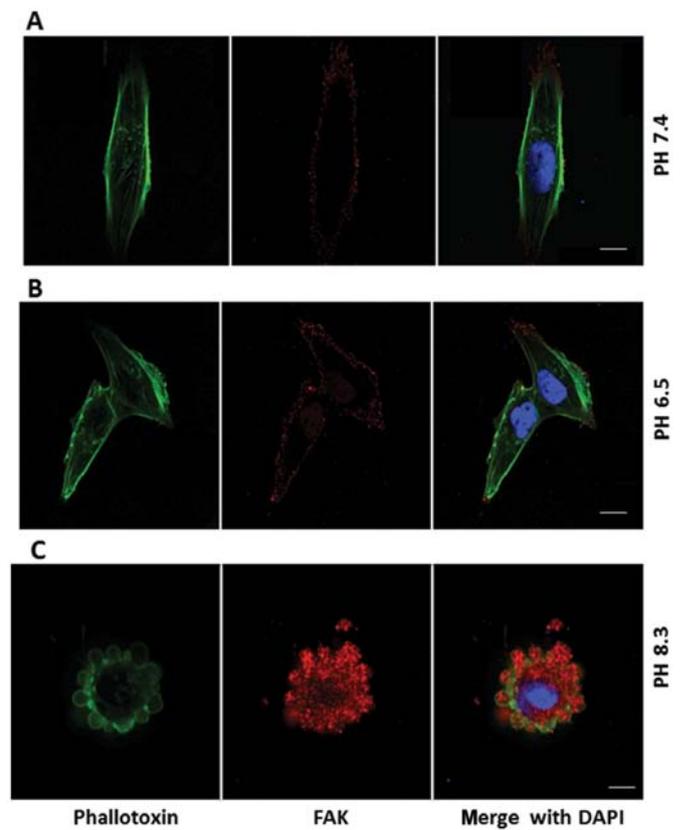


Figure 13. Effect of pH on distribution of focal adhesion kinase (FAK) in pII cells. Cells were stained with phallotoxin (green), anti-FAK antibody (red), or DAPI (blue) after culturing in pH 7.4 (A), or after exposure to acidic (B) or alkaline (C) pH for 1 h at 37°C. Scale bar represents 20 μ m.

decrease blebbing. Involvement of RhoA-ROCK and myosin in bleb formation has also been previously documented (46,63), with (64) suggesting that bleb-associated motility could be reflective of reduced substratum adhesion, as indeed would be true of our pII cells that lack many critical adhesion proteins such as E-cadherin (65) previously showed that blebs can be either stationary or move to the leading edge or backward over the cell body. We observed that the formation of blebs, in response to alkaline pH, is relatively uniform along the entire cell body but they can re-distribute towards a chemoattractant source (in this case EGF) in a polarized manner (Fig. 8D-G). It is interesting that while the chemoattractant is also clearly able to cause cell polarization in its direction at pH 7.4, with increased mobilization of actin into a thickened membrane, it did not induce any blebbing.

These observations are consistent with our previous data that suggest that the blebs may enhance the directional invasive ability of endocrine resistant breast cancer cells (29). It would appear that in fact, blebs are normal protrusions of locomoting cells both *in vitro* and *in vivo* (66-68) and they can transform into lamellipodia and vice versa (38,69,70). Migrating *dictyostelium* cells can switch from pseudopods to blebs (71). In addition, the mesenchymal type of tumor cells (such as pII) have characteristics of fibroblast-like motility with elongated spindle-like shape and obvious leading edge towards the source of the chemotactic agent (72). On the other hand, an amoeboid-like movement was observed in various leukocytes

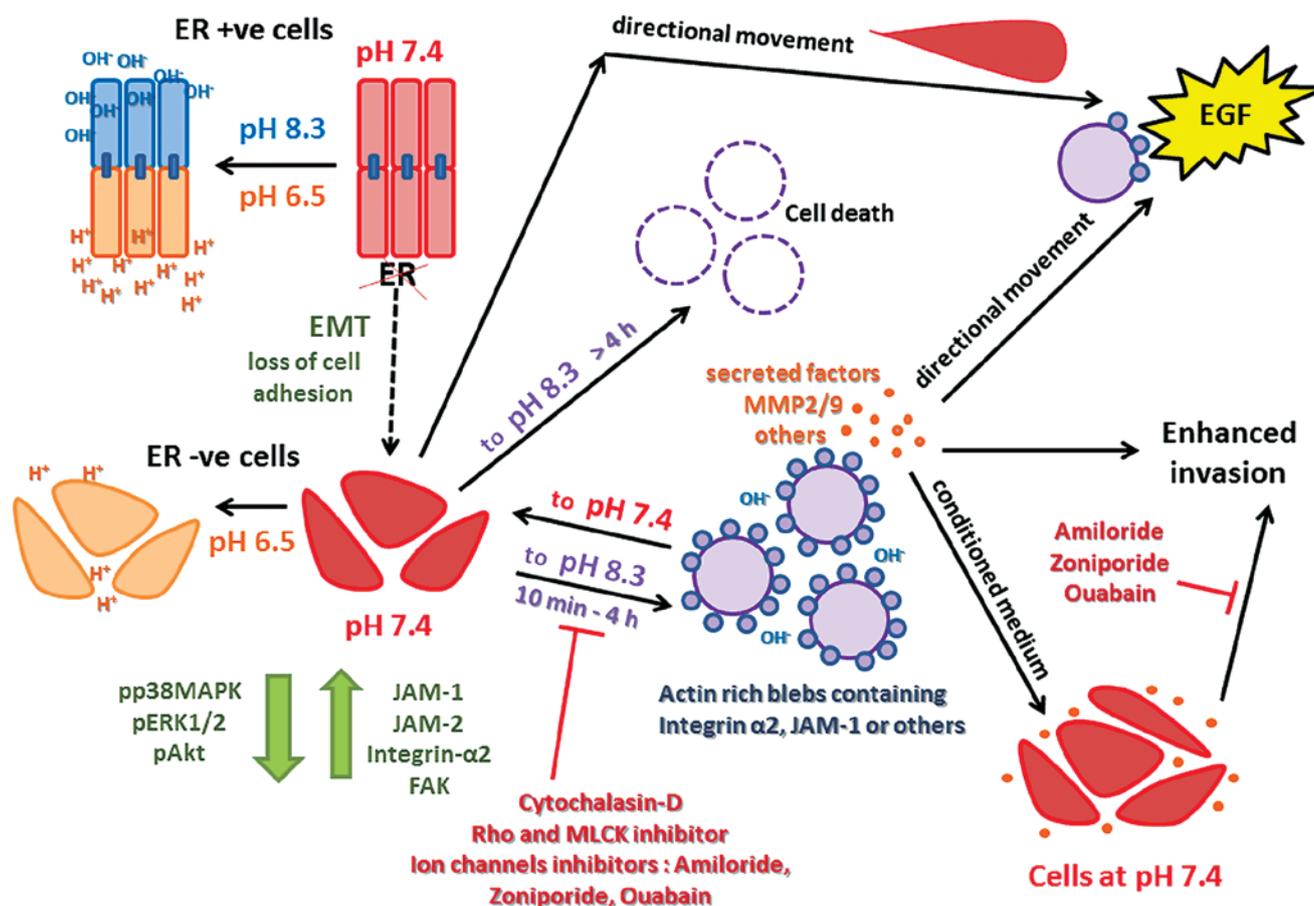


Figure 14. A model for pH regulation of breast cancer cells. ER⁺ cells remain morphologically unchanged after short exposure to either acidic or alkaline conditions, maintaining cellular adhesion. Loss of functional ER pushes cells towards a mesenchymal phenotype (EMT) characterized by loss of cell adhesion proteins and decreased signaling activity. These show little response to acidic pH but undergo dramatic but reversible morphological change with short exposure to alkaline pH; contractation and formation of blebs. This transformation, which can be blocked by small GTPase associated molecules and ion channel inhibitors, further increases motility towards chemotactic factors such as EGF. Factors released into alkaline medium can also confer increased (ion-channel sensitive) invasive ability to cells maintained at pH 7.4. Longer term exposure to alkaline pH leads to cell death.

(73,74) and tumor cells (75-78) and is characterized by rounded shape of the cell during its motility. Cells with amoeboid type of movement have enhanced contractility and invasive capacity due to their rounded shape, which would conceivably enable them to squeeze through the small spaces in the extracellular matrix (ECM) fibers and exert higher force to deform the surrounding ECM (73-77). Furthermore, supra-stimulation of pancreatic acinar cells with CCK, induced rapid membrane actin cytoskeleton-rich blebs within 2-3 min, and the cells exhibited amoeboid shape (79). The formed blebs were rapidly absorbed when the agonist level was reduced, indicating that blebbing is a reversible, not necessarily a terminal event, which is in agreement with our data. Indeed, formation and retraction of blebs is not an uncommonly reported phenomenon. In view of these data a possible explanation of the behaviour of pII cells is that alkaline pH induces an amoeboid-like spherical shape (Figs. 3C and 4), enabling enhanced invasive potential, mediated in part through elevated MMP2/9 activity (29) or possibly other secreted factors (currently under investigation).

In conclusion, we have demonstrated that ER silenced breast cancer cells, when shifted for brief periods from neutral to alkaline, but not acidic conditions, markedly change their morphological and functional properties. Cells tend to become

spherical (amoeboid-like) and segregate further from each other and form dynamic and reversible blebs and other long cytoplasmic protrusions which are made up of actin/myosin filaments on the outer membrane, with molecules critical for cell adhesion and motility in the cytoplasmic compartment of the formed blebs. These are uniformly distributed but can polarize in the direction of movement towards EGF, which by itself cannot elicit formation of blebs though it can induce polarization and membrane thickening through actin aggregation. Interruption of formation, as well as disruption of pre-formed blebs, can be achieved with drugs that interfere with several GTPase associated effector proteins. The blebs also appear to be dependent upon ion channel activity and confer increased invasive capacity. The differential longer term detrimental effects of alkaline pH are being further investigated as a possible means of selective cell killing. Fig. 14 shows a summary schematic representation of the events described in this study.

Acknowledgements

This study was supported by Kuwait University Research Sector grant PT02/11. Parts of this study were supported

by grant SRUL02/13 to the Research Unit for Genomics, Proteomics and Cellomics Studies (OMICS), Kuwait University. We also thank the Electron Microscopy Unit of the Faculty of Medicine and the Nanoscopy Science Centre of Faculty of Science, Kuwait University for assistance with the scanning electron microscopy.

References

- Al Saleh S, Sharaf LH and Luqmani YA: Signalling pathways involved in endocrine resistance in breast cancer and associations with epithelial to mesenchymal transition (Review). *Int J Oncol* 38: 1197-1217, 2011.
- Luqmani YA, Al Azmi A, Al Bader M, Abraham G and El Zawahri M: Modification of gene expression induced by siRNA targeting of estrogen receptor alpha in MCF7 human breast cancer cells. *Int J Oncol* 34: 231-242, 2009.
- Al Saleh S, Al Mulla F and Luqmani YA: Estrogen receptor silencing induces epithelial to mesenchymal transition in human breast cancer cells. *PLoS One* 6: 21, 2011.
- Roxanis I: Occurrence and significance of epithelial-mesenchymal transition in breast cancer. *J Clin Pathol* 66: 517-521, 2013.
- Giuliano M, Schiff R, Osborne CK and Trivedi MV: Biological mechanisms and clinical implications of endocrine resistance in breast cancer. *Breast* 9776: 70293-70294, 2011.
- Khajah MA, Al Saleh S, Mathew PM and Luqmani YA: Differential effect of growth factors on invasion and proliferation of endocrine resistant breast cancer cells. *PLoS One* 7: 30, 2012.
- Vaupel P, Kallinowski F and Okunieff P: Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res* 49: 6449-6465, 1989.
- Gatenby RA and Gillies RJ: Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* 4: 891-899, 2004.
- Hashim AI, Zhang X, Wojtkowiak JW, Martinez GV and Gillies RJ: Imaging pH and metastasis. *NMR Biomed* 24: 582-591, 2011.
- Cairns R, Papandreou I and Denko N: Overcoming physiologic barriers to cancer treatment by molecularly targeting the tumor microenvironment. *Mol Cancer Res* 4: 61-70, 2006.
- Izumi H, Torigoe T, Ishiguchi H, *et al*: Cellular pH regulators: potentially promising molecular targets for cancer chemotherapy. *Cancer Treat Rev* 29: 541-549, 2003.
- Boedtker E, Moreira JM, Mele M, *et al*: Contribution of Na⁺/HCO₃⁻-cotransport to cellular pH control in human breast cancer: a role for the breast cancer susceptibility locus NBCn1 (SLC4A7). *Int J Cancer* 132: 1288-1299, 2013.
- Abaza M and Luqmani YA: The influence of pH and hypoxia on tumor metastasis. *Expert Rev Anticancer Ther* 13: 1229-1242, 2013.
- Rozhin J, Sameni M, Ziegler G and Sloane BF: Pericellular pH affects distribution and secretion of cathepsin B in malignant cells. *Cancer Res* 54: 6517-6525, 1994.
- Brisson L, Reshkin SJ, Gore J and Roger S: pH regulators in invadosomal functioning: proton delivery for matrix tasting. *Eur J Cell Biol* 91: 847-860, 2012.
- Xiao H, Li TK, Yang JM and Liu LF: Acidic pH induces topoisomerase II-mediated DNA damage. *Proc Natl Acad Sci USA* 100: 5205-5210, 2003.
- Morita T, Nagaki T, Fukuda I and Okumura K: Clastogenicity of low pH to various cultured mammalian cells. *Mutat Res* 268: 297-305, 1992.
- Matsubara T, Diresta GR, Kakunaga S, Li D and Healey JH: Additive influence of extracellular pH, oxygen tension, and pressure on invasiveness and survival of human osteosarcoma cells. *Front Oncol* 3: 2013, 2013.
- Raghunand N, Altbach MI, van Sluis R, *et al*: Plasmalemmal pH-gradients in drug-sensitive and drug-resistant MCF-7 human breast carcinoma xenografts measured by 31P magnetic resonance spectroscopy. *Biochem Pharmacol* 57: 309-312, 1999.
- Robey IF, Baggett BK, Kirkpatrick ND, *et al*: Bicarbonate increases tumor pH and inhibits spontaneous metastases. *Cancer Res* 69: 2260-2268, 2009.
- Ibrahim-Hashim A, Cornnell HH, Abrahams D, *et al*: Systemic buffers inhibit carcinogenesis in TRAMP mice. *J Urol* 188: 624-631, 2012.
- Morimura T, Fujita K, Akita M, Nagashima M and Satomi A: The proton pump inhibitor inhibits cell growth and induces apoptosis in human hepatoblastoma. *Pediatr Surg Int* 24: 1087-1094, 2008.
- Kastelein F, Spaander MC, Steyerberg EW, *et al*: Proton pump inhibitors reduce the risk of neoplastic progression in patients with Barrett's esophagus. *Clin Gastroenterol Hepatol* 11: 382-388, 2013.
- Williams AC, Collard TJ and Paraskeva C: An acidic environment leads to p53 dependent induction of apoptosis in human adenoma and carcinoma cell lines: implications for clonal selection during colorectal carcinogenesis. *Oncogene* 18: 3199-3204, 1999.
- Putney LK and Barber DL: Na-H exchange-dependent increase in intracellular pH times G2/M entry and transition. *J Biol Chem* 278: 44645-44649, 2003.
- Ohtsubo T, Wang X, Takahashi A, *et al*: p53-dependent induction of WAF1 by a low-pH culture condition in human glioblastoma cells. *Cancer Res* 57: 3910-3913, 1997.
- Smallbone K, Maini PK and Gatenby RA: Episodic, transient systemic acidosis delays evolution of the malignant phenotype: Possible mechanism for cancer prevention by increased physical activity. *Biol Direct* 5: 1745-6150, 2010.
- Chen JL, Lucas JE, Schroeder T, *et al*: The genomic analysis of lactic acidosis and acidosis response in human cancers. *PLoS Genet* 4: 5, 2008.
- Khajah MA, Almohri I, Mathew PM and Luqmani YA: Extracellular alkaline pH leads to increased metastatic potential of estrogen receptor silenced endocrine resistant breast cancer cells. *PLoS One* 8: 2013, 2013.
- Calorini L, Peppicelli S and Bianchini F: Extracellular acidity as favouring factor of tumor progression and metastatic dissemination. *Exp Oncol* 34: 79-84, 2012.
- McCarty MF and Whitaker J: Manipulating tumor acidification as a cancer treatment strategy. *Altern Med Rev* 15: 264-272, 2010.
- Kato Y, Nakayama Y, Umeda M and Miyazaki K: Induction of 103-kDa gelatinase/type IV collagenase by acidic culture conditions in mouse metastatic melanoma cell lines. *J Biol Chem* 267: 11424-11430, 1992.
- Rofstad EK, Mathiesen B, Kindem K and Galappathi K: Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. *Cancer Res* 66: 6699-6707, 2006.
- el-Deiry WS, Tokino T, Velculescu VE, *et al*: WAF1, a potential mediator of p53 tumor suppression. *Cell* 75: 817-825, 1993.
- Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R and Beach D: p21 is a universal inhibitor of cyclin kinases. *Nature* 366: 701-704, 1993.
- Noda A, Ning Y, Venable SF, Pereira-Smith OM and Smith JR: Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp Cell Res* 211: 90-98, 1994.
- Majima HJ, Oberley TD, Furukawa K, *et al*: Prevention of mitochondrial injury by manganese superoxide dismutase reveals a primary mechanism for alkaline-induced cell death. *J Biol Chem* 273: 8217-8224, 1998.
- Trinkaus JP: Surface activity and locomotion of *Fundulus* deep cells during blastula and gastrula stages. *Dev Biol* 30: 69-103, 1973.
- Bergert M, Chandradoss SD, Desai RA and Paluch E: Cell mechanics control rapid transitions between blebs and lamellipodia during migration. *Proc Natl Acad Sci USA* 109: 14434-14439, 2012.
- Norman LL, Brugues J, Sengupta K, Sens P and Aranda-Espinoza H: Cell blebbing and membrane area homeostasis in spreading and retracting cells. *Biophys J* 99: 1726-1733, 2010.
- Fishkind DJ, Cao LG and Wang YL: Microinjection of the catalytic fragment of myosin light chain kinase into dividing cells: effects on mitosis and cytokinesis. *J Cell Biol* 114: 967-975, 1991.
- Boss J: Mitosis in cultures of newt tissues. IV. The cell surface in late anaphase and the movements of ribonucleoprotein. *Exp Cell Res* 8: 181-187, 1955.
- Boss J: Mitosis in cultures of newt tissues. III. Cleavage and chromosome movements in anaphase. *Exp Cell Res* 7: 443-456, 1954.
- Boss J: Mitosis in cultures of newt tissues. I. A critical study of the methods and material. *Exp Cell Res* 7: 215-231, 1954.
- Moes MJ, Bijvelt JJ and Boonstra J: Attachment of HeLa cells during early G1 phase. *Histochem Cell Biol* 136: 399-411, 2011.
- Charras G and Paluch E: Blebs lead the way: how to migrate without lamellipodia. *Nat Rev Mol Cell Biol* 9: 730-736, 2008.

47. Charras GT: A short history of blebbing. *J Microsc* 231: 466-478, 2008.
48. Wyllie AH, Kerr JF and Currie AR: Cell death: the significance of apoptosis. *Int Rev Cytol* 68: 251-306, 1980.
49. Earnshaw WC: Nuclear changes in apoptosis. *Curr Opin Cell Biol* 7: 337-343, 1995.
50. Jacobson MD, Weil M and Raff MC: Programmed cell death in animal development. *Cell* 88: 347-354, 1997.
51. Mills JC, Stone NL, Erhardt J and Pittman RN: Apoptotic membrane blebbing is regulated by myosin light chain phosphorylation. *J Cell Biol* 140: 627-636, 1998.
52. Bovellan M, Fritzsche M, Stevens C and Charras G: Death-associated protein kinase (DAPK) and signal transduction: blebbing in programmed cell death. *FEBS J* 277: 58-65, 2010.
53. Cotter TG, Lennon SV, Glynn JM and Green DR: Microfilament-disrupting agents prevent the formation of apoptotic bodies in tumor cells undergoing apoptosis. *Cancer Res* 52: 997-1005, 1992.
54. Cunningham CC: Actin polymerization and intracellular solvent flow in cell surface blebbing. *J Cell Biol* 129: 1589-1599, 1995.
55. Laster SM and Mackenzie JM Jr: Bleb formation and F-actin distribution during mitosis and tumor necrosis factor-induced apoptosis. *Microsc Res Tech* 34: 272-280, 1996.
56. Pitzer F, Dantes A, Fuchs T, Baumeister W and Amsterdam A: Removal of proteasomes from the nucleus and their accumulation in apoptotic blebs during programmed cell death. *FEBS Lett* 394: 47-50, 1996.
57. Vemuri GS, Zhang J, Huang R, Keen JH and Rittenhouse SE: Thrombin stimulates wortmannin-inhibitable phosphoinositide 3-kinase and membrane blebbing in CHR-288 cells. *Biochem J* 314: 805-810, 1996.
58. Keller H and Eggli P: Protrusive activity, cytoplasmic compartmentalization, and restriction rings in locomoting blebbing Walker carcinosarcoma cells are related to detachment of cortical actin from the plasma membrane. *Cell Motil Cytoskeleton* 41: 181-193, 1998.
59. Kohama K, Ye LH, Hayakawa K and Okagaki T: Myosin light chain kinase: an actin-binding protein that regulates an ATP-dependent interaction with myosin. *Trends Pharmacol Sci* 17: 284-287, 1996.
60. Gallagher PJ, Herring BP and Stull JT: Myosin light chain kinases. *J Muscle Res Cell Motil* 18: 1-16, 1997.
61. Ridley AJ and Hall A: The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70: 389-399, 1992.
62. Chrzanowska-Wodnicka M and Burridge K: Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J Cell Biol* 133: 1403-1415, 1996.
63. Jia Z, Vadnais J, Lu ML, Noel J and Nabi IR: Rho/ROCK-dependent pseudopodial protrusion and cellular blebbing are regulated by p38 MAPK in tumour cells exhibiting autocrine c-Met activation. *Biol Cell* 98: 337-351, 2006.
64. Fackler OT and Grosse R: Cell motility through plasma membrane blebbing. *J Cell Biol* 181: 879-884, 2008.
65. Keller HU and Cottier H: Crawling-like movements and polarisation in non-adherent leucocytes. *Cell Biol Int Rep* 5: 3-7, 1981.
66. Johnson KE: Circus movements and blebbing locomotion in dissociated embryonic cells of an amphibian, *Xenopus laevis*. *J Cell Sci* 22: 575-583, 1976.
67. Haston WS and Shields JM: Contraction waves in lymphocyte locomotion. *J Cell Sci* 68: 227-241, 1984.
68. Grinnell F: Migration of human neutrophils in hydrated collagen lattices. *J Cell Sci* 58: 95-108, 1982.
69. Keller HU, Naef A and Zimmermann A: Effects of colchicine, vinblastine and nocodazole on polarity, motility, chemotaxis and cAMP levels of human polymorphonuclear leukocytes. *Exp Cell Res* 153: 173-185, 1984.
70. Keller HU and Zimmermann A: Shape changes and chemokinesis of Walker 256 carcinosarcoma cells in response to colchicine, vinblastine, nocodazole and taxol. *Invasion Metastasis* 6: 33-43, 1986.
71. Zatulovskiy E, Tyson R, Bretschneider T and Kay RR: Bleb-driven chemotaxis of *Dictyostelium* cells. *J Cell Biol* 204: 1027-1044, 2014.
72. Pankova K, Rosel D, Novotny M and Brabek J: The molecular mechanisms of transition between mesenchymal and amoeboid invasiveness in tumor cells. *Cell Mol Life Sci* 67: 63-71, 2010.
73. Mandeville JT, Lawson MA and Maxfield FR: Dynamic imaging of neutrophil migration in three dimensions: mechanical interactions between cells and matrix. *J Leukoc Biol* 61: 188-200, 1997.
74. Friedl P, Borgmann S and Brocker EB: Amoeboid leukocyte crawling through extracellular matrix: lessons from the *Dictyostelium* paradigm of cell movement. *J Leukoc Biol* 70: 491-509, 2001.
75. Sahai E and Marshall CJ: Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. *Nat Cell Biol* 5: 711-719, 2003.
76. Wolf K, Mazo I, Leung H, *et al*: Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *J Cell Biol* 160: 267-277, 2003.
77. Wyckoff JB, Pinner SE, Gschmeissner S, Condeelis JS and Sahai E: ROCK- and myosin-dependent matrix deformation enables protease-independent tumor-cell invasion in vivo. *Curr Biol* 16: 1515-1523, 2006.
78. Rosel D, Brabek J, Tolde O, *et al*: Up-regulation of Rho/ROCK signaling in sarcoma cells drives invasion and increased generation of protrusive forces. *Mol Cancer Res* 6: 1410-1420, 2008.
79. Torgerson RR and McNiven MA: The actin-myosin cytoskeleton mediates reversible agonist-induced membrane blebbing. *J Cell Sci* 111: 2911-2922, 1998.