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## Extracellular protonation modulates cell-cell interaction mechanics and tissue invasion in human melanoma cells

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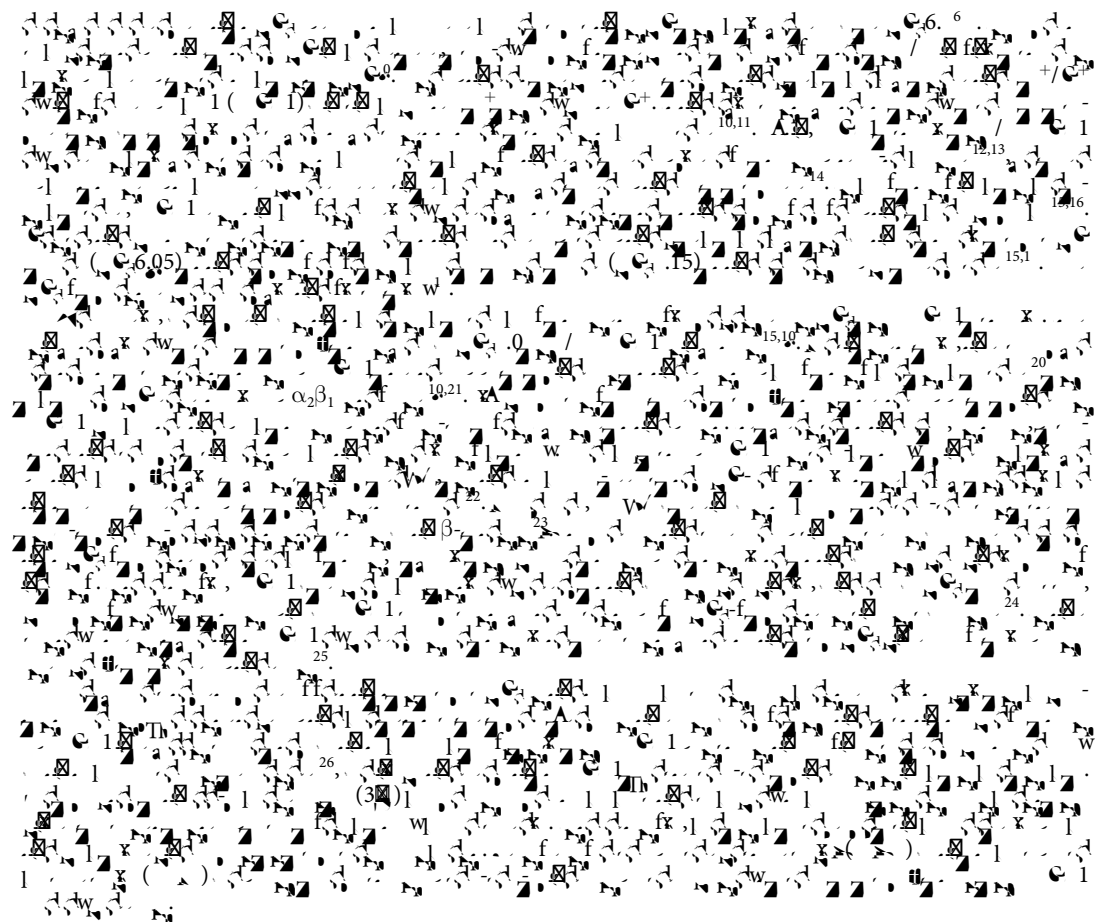
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Detachment of cells from the primary tumour precedes metastatic progression by facilitating cell release into the tissue. Solid tumours exhibit altered pH homeostasis with extracellular acidification. In human melanoma, the  $\text{Na}^+/\text{H}^+$  exchanger NHE1 is an important modifier of the tumour nanoenvironment. Here we tested the modulation of cell-cell-adhesion by extracellular pH and NHE1. MV3 tumour spheroids embedded in a collagen matrix unravelled the efficacy of cell-cell contact loosening and 3D emigration into an environment mimicking physiological confinement. Adhesive interaction strength between individual MV3 cells was quantified using atomic force microscopy and validated by multicellular aggregation assays. Extracellular acidification from  $\text{pH}_e 7.4$  to 6.4 decreases cell migration and invasion but increases single cell detachment from the spheroids. Acidification and NHE1 overexpression both reduce cell-cell adhesion strength, indicated by reduced maximum pulling forces and adhesion energies. Multicellular aggregation and spheroid formation are strongly impaired under acidification or NHE1 overexpression. We show a clear dependence of melanoma cell-cell adhesion on  $\text{pH}_e$  and NHE1 as a modulator. These effects are opposite to cell-matrix interactions that are strengthened by protons extruded via NHE1. We conclude that these opposite effects of NHE1 act synergistically during the metastatic cascade.

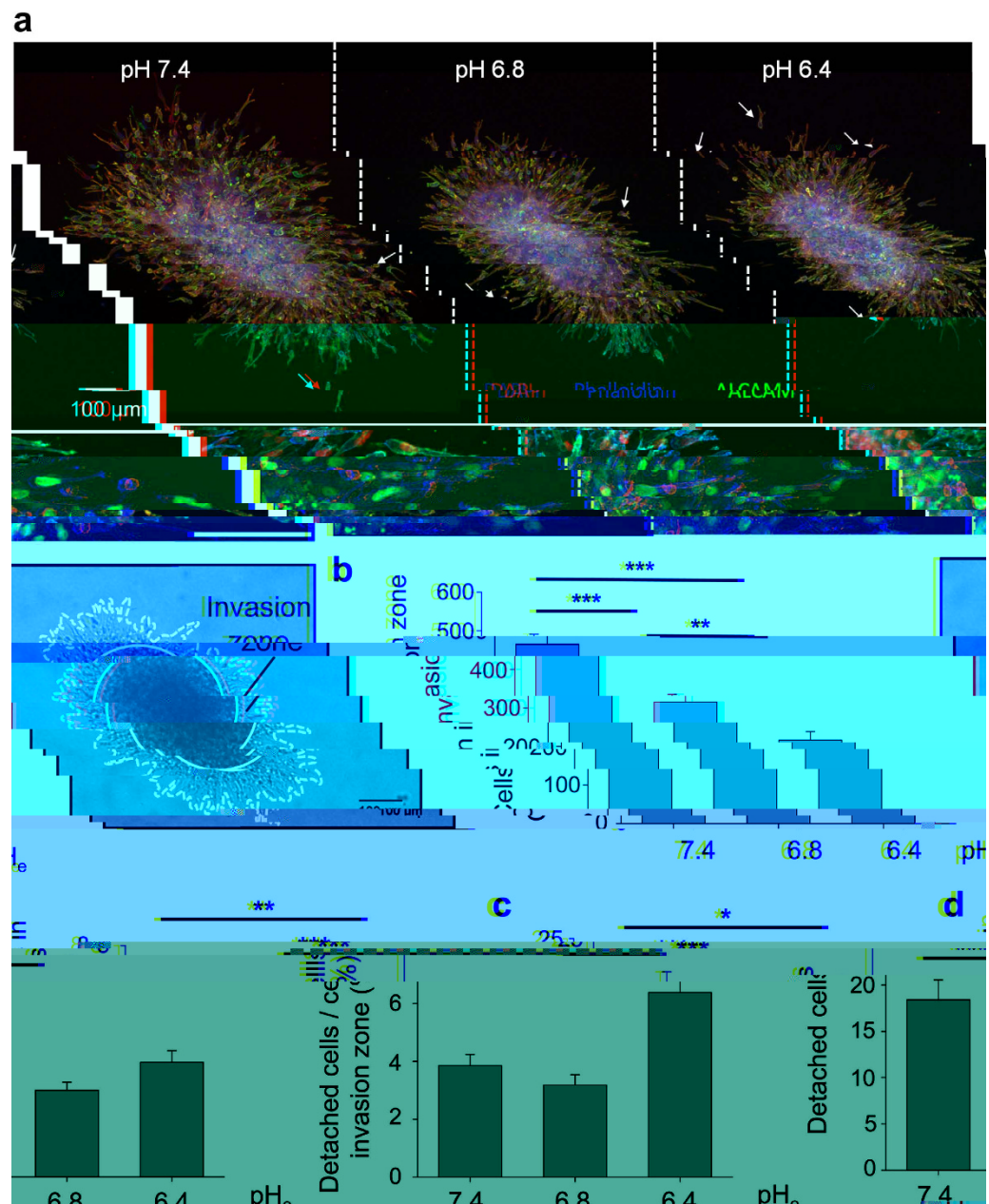


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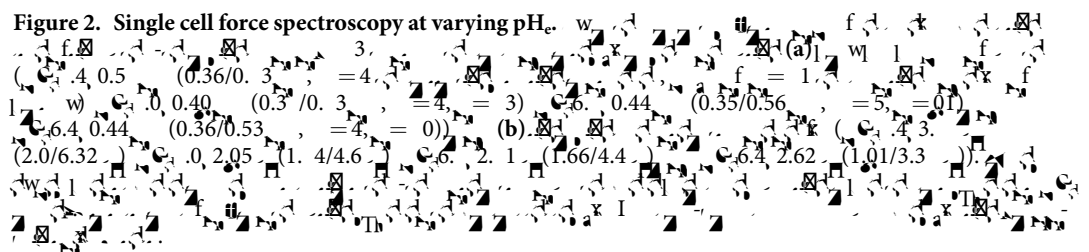
## Results

### In a 3D assay, acidificati



**Figure 1.** 3D emigration assays through a collagen I matrix. (a) Fluorescence microscopy images of cell spheroids at pH 7.4, 6.8, and 6.4. (b) Bar graph of invasion zone (μm) for pH 7.4, 6.8, and 6.4. (c) Bar graph of detached cells per invasion zone (%) for pH 7.4, 6.8, and 6.4. (d) Bar graph of detached cells (%) for pH 7.4.

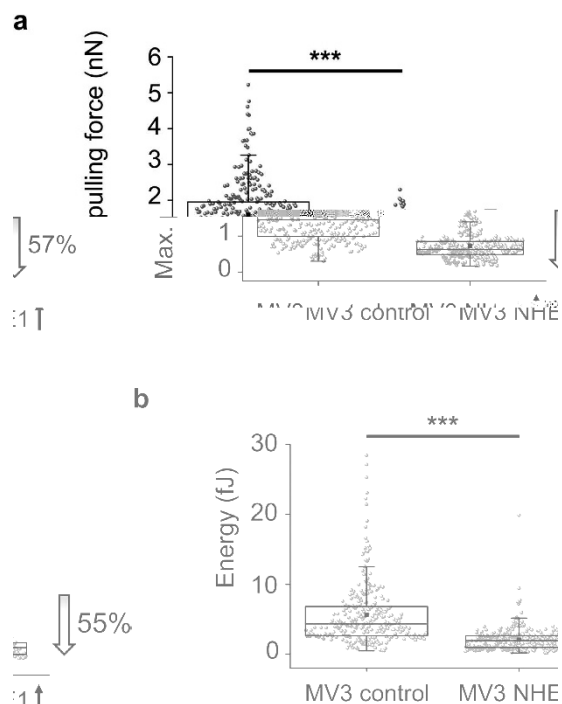
**NHE1-overexpressing cells do not form stable tumour spheroids in multicellular cell aggregation assays.**



### Extracellular acidification increases adhesion between NHE1-overexpressing MV3 cells.

### NHE1 expression correlates with the expression of melanoma cell adhesion molecule.

Figure 10. The effect of the initial concentration of the monomer on the polymerization of  $\alpha$ -methylstyrene initiated by  $\text{C}_6\text{H}_5\text{MgBr}$  in THF at  $-78^\circ\text{C}$  for 1 h. The concentration of the initiator was  $0.01 \text{ mol/L}$ . The concentration of the monomer was  $0.01 \text{ mol/L}$  (a),  $0.02 \text{ mol/L}$  (b),  $0.03 \text{ mol/L}$  (c),  $0.04 \text{ mol/L}$  (d),  $0.05 \text{ mol/L}$  (e),  $0.06 \text{ mol/L}$  (f),  $0.07 \text{ mol/L}$  (g),  $0.08 \text{ mol/L}$  (h),  $0.09 \text{ mol/L}$  (i),  $0.10 \text{ mol/L}$  (j),  $0.11 \text{ mol/L}$  (k),  $0.12 \text{ mol/L}$  (l),  $0.13 \text{ mol/L}$  (m),  $0.14 \text{ mol/L}$  (n),  $0.15 \text{ mol/L}$  (o),  $0.16 \text{ mol/L}$  (p),  $0.17 \text{ mol/L}$  (q),  $0.18 \text{ mol/L}$  (r),  $0.19 \text{ mol/L}$  (s),  $0.20 \text{ mol/L}$  (t),  $0.21 \text{ mol/L}$  (u),  $0.22 \text{ mol/L}$  (v),  $0.23 \text{ mol/L}$  (w),  $0.24 \text{ mol/L}$  (x),  $0.25 \text{ mol/L}$  (y),  $0.26 \text{ mol/L}$  (z),  $0.27 \text{ mol/L}$  (aa),  $0.28 \text{ mol/L}$  (ab),  $0.29 \text{ mol/L}$  (ac),  $0.30 \text{ mol/L}$  (ad),  $0.31 \text{ mol/L}$  (ae),  $0.32 \text{ mol/L}$  (af),  $0.33 \text{ mol/L}$  (ag),  $0.34 \text{ mol/L}$  (ah),  $0.35 \text{ mol/L}$  (ai),  $0.36 \text{ mol/L}$  (aj),  $0.37 \text{ mol/L}$  (ak),  $0.38 \text{ mol/L}$  (al),  $0.39 \text{ mol/L}$  (am),  $0.40 \text{ mol/L}$  (an),  $0.41 \text{ mol/L}$  (ao),  $0.42 \text{ mol/L}$  (ap),  $0.43 \text{ mol/L}$  (aq),  $0.44 \text{ mol/L}$  (ar),  $0.45 \text{ mol/L}$  (as),  $0.46 \text{ mol/L}$  (at),  $0.47 \text{ mol/L}$  (au),  $0.48 \text{ mol/L}$  (av),  $0.49 \text{ mol/L}$  (aw),  $0.50 \text{ mol/L}$  (ax),  $0.51 \text{ mol/L}$  (ay),  $0.52 \text{ mol/L}$  (az),  $0.53 \text{ mol/L}$  (ba),  $0.54 \text{ mol/L}$  (bb),  $0.55 \text{ mol/L}$  (bc),  $0.56 \text{ mol/L}$  (bd),  $0.57 \text{ mol/L}$  (be),  $0.58 \text{ mol/L}$  (bf),  $0.59 \text{ mol/L}$  (bg),  $0.60 \text{ mol/L}$  (bh),  $0.61 \text{ mol/L}$  (bi),  $0.62 \text{ mol/L}$  (bj),  $0.63 \text{ mol/L}$  (bk),  $0.64 \text{ mol/L}$  (bl),  $0.65 \text{ mol/L}$  (bm),  $0.66 \text{ mol/L}$  (bn),  $0.67 \text{ mol/L}$  (bo),  $0.68 \text{ mol/L}$  (bp),  $0.69 \text{ mol/L}$  (bq),  $0.70 \text{ mol/L}$  (br),  $0.71 \text{ mol/L}$  (bs),  $0.72 \text{ mol/L}$  (bt),  $0.73 \text{ mol/L}$  (bu),  $0.74 \text{ mol/L}$  (bv),  $0.75 \text{ mol/L}$  (bw),  $0.76 \text{ mol/L}$  (bx),  $0.77 \text{ mol/L}$  (by),  $0.78 \text{ mol/L}$  (bz),  $0.79 \text{ mol/L}$  (ca),  $0.80 \text{ mol/L}$  (cb),  $0.81 \text{ mol/L}$  (cc),  $0.82 \text{ mol/L}$  (cd),  $0.83 \text{ mol/L}$  (ce),  $0.84 \text{ mol/L}$  (cf),  $0.85 \text{ mol/L}$  (cg),  $0.86 \text{ mol/L}$  (ch),  $0.87 \text{ mol/L}$  (ci),  $0.88 \text{ mol/L}$  (cj),  $0.89 \text{ mol/L}$  (ck),  $0.90 \text{ mol/L}$  (cl),  $0.91 \text{ mol/L}$  (cm),  $0.92 \text{ mol/L}$  (cn),  $0.93 \text{ mol/L}$  (co),  $0.94 \text{ mol/L}$  (cp),  $0.95 \text{ mol/L}$  (cq),  $0.96 \text{ mol/L}$  (cr),  $0.97 \text{ mol/L}$  (cs),  $0.98 \text{ mol/L}$  (ct),  $0.99 \text{ mol/L}$  (cu),  $1.00 \text{ mol/L}$  (cv),  $1.01 \text{ mol/L}$  (cw),  $1.02 \text{ mol/L}$  (cx),  $1.03 \text{ mol/L}$  (cy), 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**Figure 3.** Single cell force spectroscopy using MV3 cells with different NHE1 expression levels.

(a) (b)

(0.62 (0.4 / 0.5), = 0.354 (1.45 (0.00/1.05), = 326 4.35 (2.6 / 6.1), = 2.15)

## Discussion

1. The results of this study show that NHE1 expression levels significantly affect the mechanical properties of MV3 cells. Specifically, the NHE1 group showed a significant decrease in pulling force and energy compared to the control group. This suggests that NHE1 plays a role in maintaining the mechanical integrity of the cell membrane.

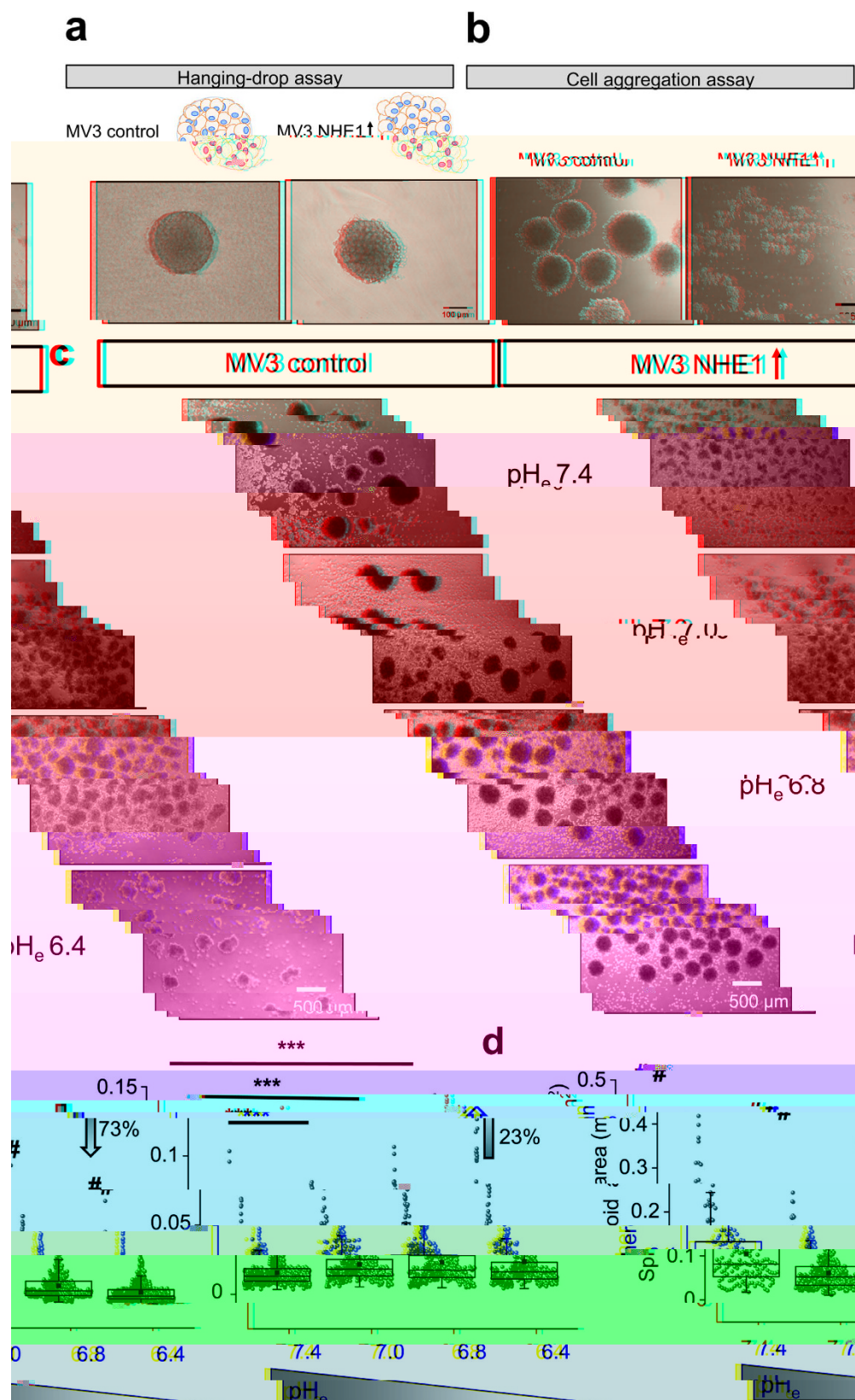
2. The decrease in pulling force and energy in the NHE1 group may be due to the loss of NHE1-mediated ion transport and osmotic balance. NHE1 is a Na<sup>+</sup>/H<sup>+</sup> exchanger that helps maintain intracellular pH and osmotic pressure. When NHE1 expression is reduced, the cell's ability to regulate its internal environment is compromised, leading to changes in its mechanical properties.

3. The results of this study have implications for understanding the role of NHE1 in various cellular processes. For example, NHE1 is involved in cell migration, proliferation, and differentiation. The mechanical properties of the cell membrane are known to influence these processes, so the changes observed in the NHE1 group may have downstream effects on cellular function.

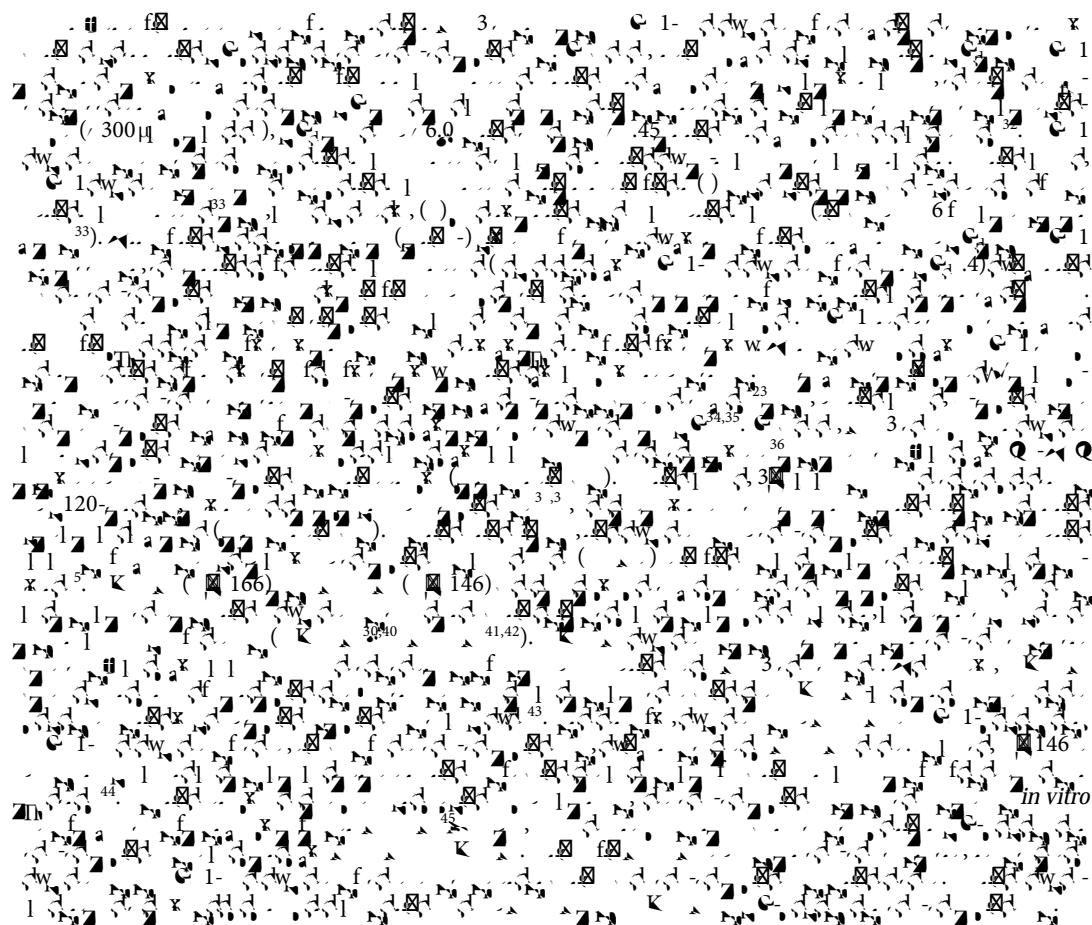
4. Further studies are needed to explore the mechanisms underlying the changes in mechanical properties observed in the NHE1 group. This could involve investigating the role of NHE1 in the cytoskeleton and the cell membrane, as well as the effects of NHE1-mediated ion transport on the cell's mechanical properties.

5. The results of this study also have potential applications in the field of cancer research. MV3 cells are a model for studying cancer cell invasion and metastasis. The mechanical properties of the cell membrane are known to play a role in these processes, so understanding the role of NHE1 in these properties could provide insights into the mechanisms of cancer progression.

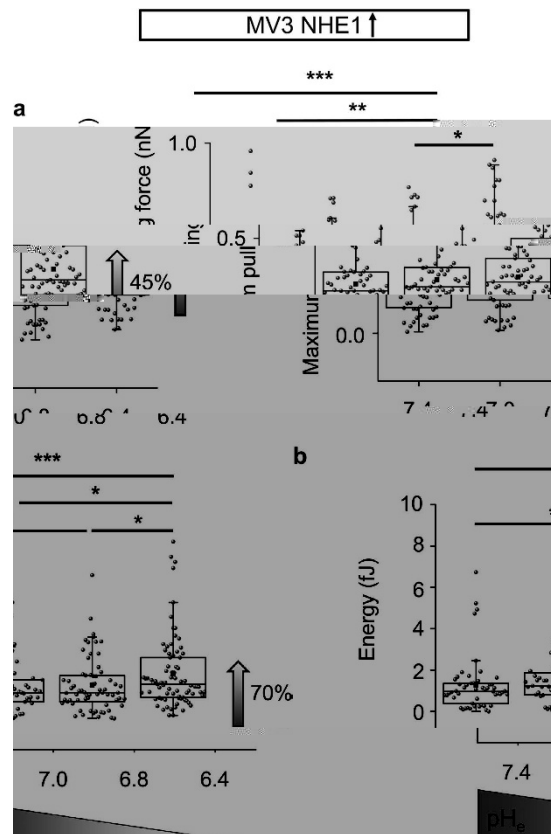




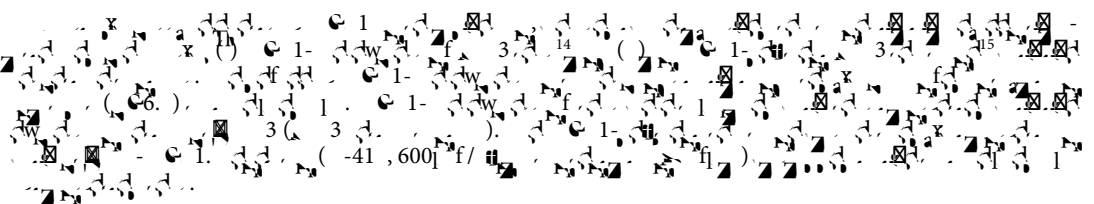
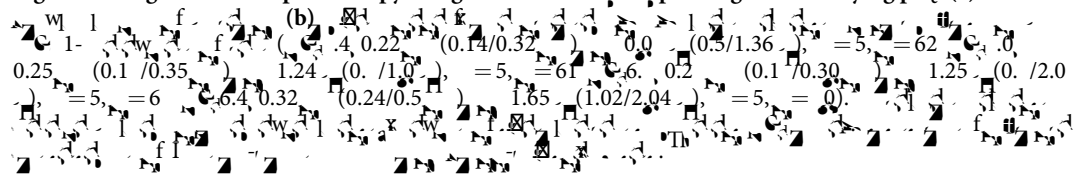
**Figure 4. Multicellular adhesion assays.** (a) A



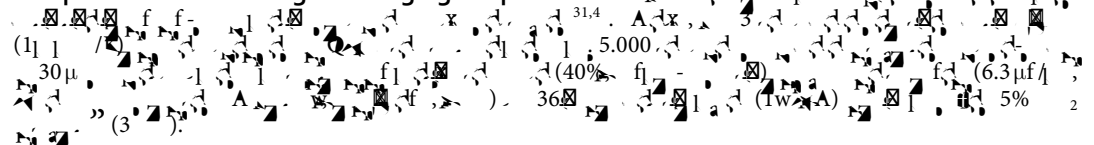
**Cell culture.** Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin (100 U/ml), streptomycin (100 U/ml), and nystatin (40 U/ml) in 25 cm<sup>2</sup> tissue culture flasks (Corning Costar, High Wycombe, UK). Cells were grown to confluence and then washed with serum-free DMEM. Cells were then grown in serum-free DMEM for 24 h before being treated with 10% FBS for 24 h. Cells were then harvested and total RNA was extracted using RNeasy spin columns (Qiagen, Crawley, UK). Total RNA (10 µg/lane) was separated on 1% agarose formaldehyde gels and transferred to Gene-Screen Plus membrane (NEN, Boston, MA, USA). Blots were probed sequentially with <sup>32</sup>P-labelled cDNA probes for *IL-1* and *IL-6* (see above) and then exposed to a PhosphorImager Screen (Molecular Dynamics, Little Chalfont, UK). The results were quantified using a PhosphorImager (Beckman LS 5000TD, High Wycombe, UK).



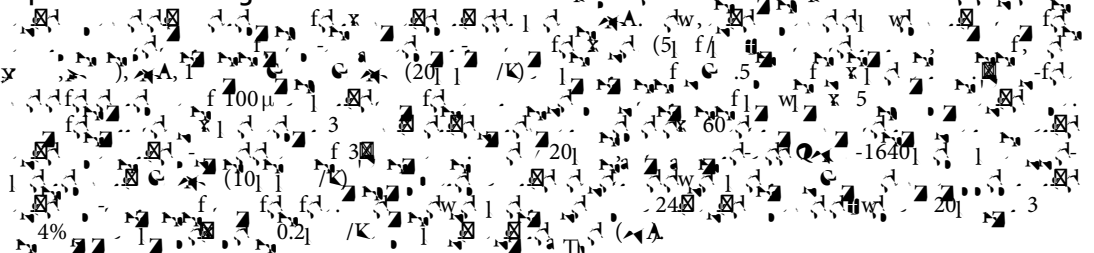
**Figure 5.** Single cell force spectroscopy using MV3 NHE1-overexpressing cells at varying  $pH_e$ . (a)



### 3D spheroid culture using the hanging-drop method.

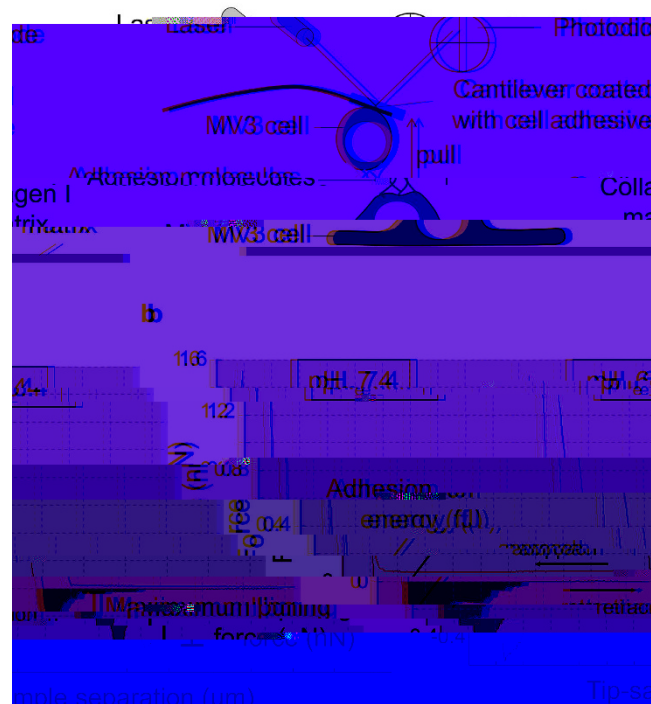


### Spheroid embedding in 3D matrix and fixation.

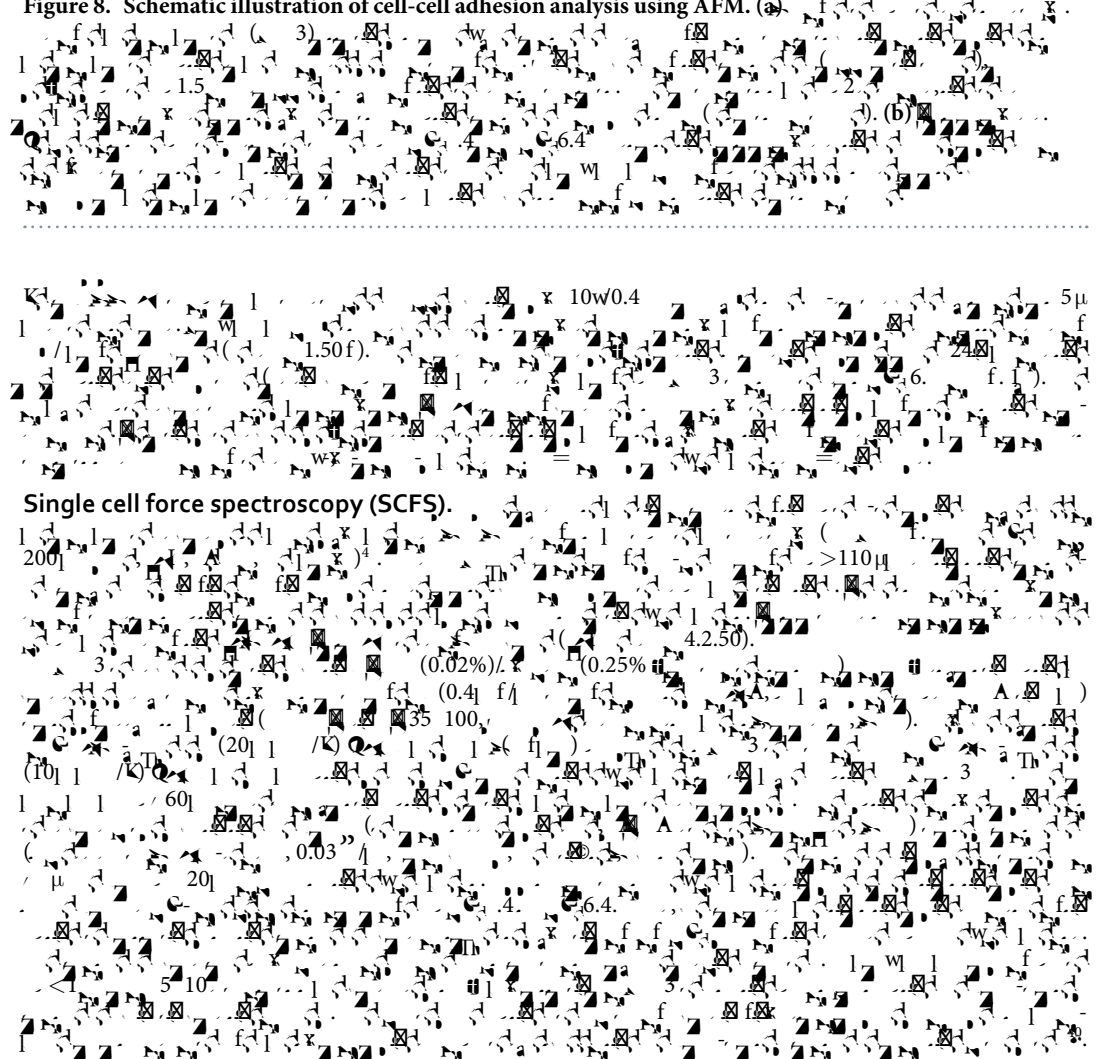


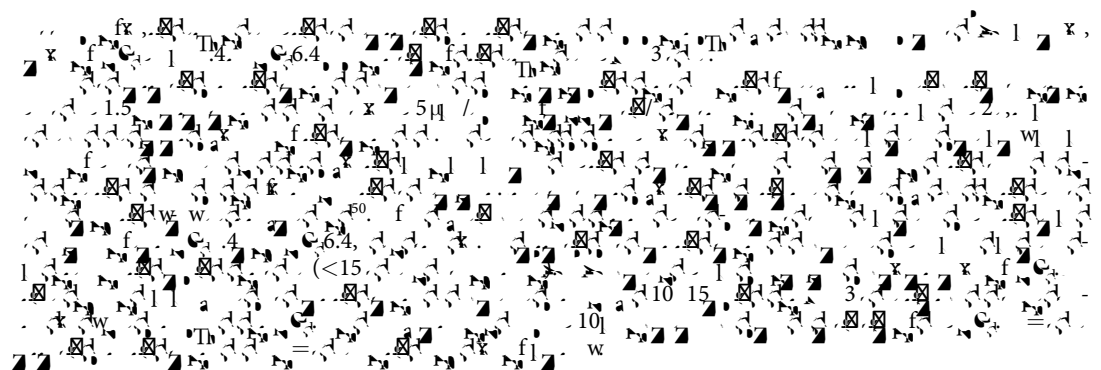




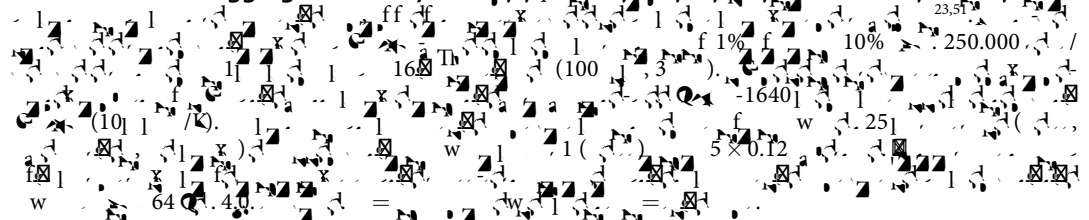
**a**

**Figure 8.** Schematic illustration of cell-cell adhesion analysis using AFM. (a)





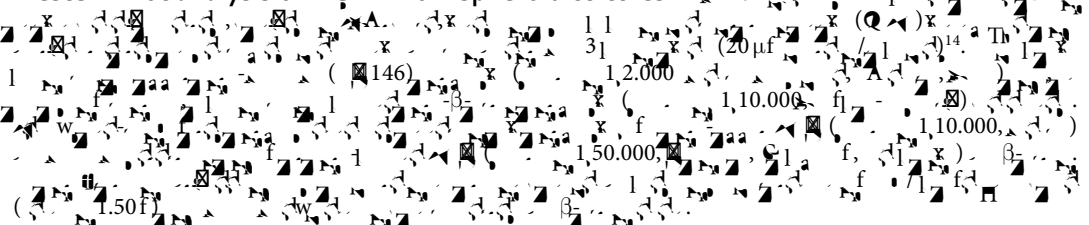
### Multicellular cell aggregation assay.



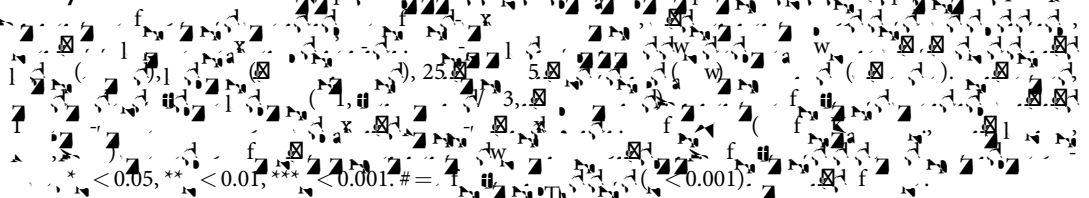
### Immunofluorescence staining of ALCAM in 2D cultures.



### Western Blot analysis of MAM from spheroid cultures.



### Analysis and statistics.



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## Author Contributions

The authors declare that they have read and approved the final manuscript. ...

## Additional Information

**Supplementary information** ...

**Competing financial interests:** ...

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