

Piezo1 Knockdown Sensitizes Brain Cancer Cells to Focused Ultrasound-Induced Apoptosis

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Research Article

Keywords: Mechanotransduction, Mechanobiology, Brain tumors, Glioblastoma, Mechanosensitive ion channels, Piezo, Focused Ultrasound

Posted Date: August 10th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10569219/v1>

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Additional Declarations: No competing interests reported.

Abstract

Glioblastoma (GBM) is an aggressive, highly invasive adult brain tumor with a median survival of less than 15 months. As these tumors advance, they exhibit altered tissue mechanobiology, including the elevated expression of mechanosensitive ion channels, such as Piezo1. Focused ultrasound (FUS), used to open the blood-brain barrier for targeted drug delivery into GBM, exerts mechanical forces that could influence these aberrantly expressed channels. However, the specific role of Piezo1 in mediating GBM cellular response to FUS-induced mechanical stress remains unclear. We identified a 6.81-fold upregulation of Piezo1 in U-87 GBM cells compared to healthy brain tissue. Here we show that Piezo1 aids in GBM cellular resistance to FUS, and that targeted Piezo1 knockdown sensitizes cells to acoustic stress. Following sonication, Piezo1 deficiency increased susceptibility to early physical detachment. By 24 hours, control cells demonstrated reduced cell death (-70.2%) and increased proliferation (+ 45.5%), whereas Piezo1-deficient cells maintained significantly elevated dead cell number (+ 28.9%) and exhibited attenuated long-term growth (+ 14.9%). These *in vitro* results suggest that Piezo1 aids in GBM cell resistance to FUS by limiting physical detachment and promoting cell survival, which are required to resolve acute cytotoxicity and facilitate long-term GBM cell proliferation. Therefore, targeted inhibition of Piezo1 may enhance the cytotoxic efficacy of FUS in clinical therapeutic applications.

Introduction

Glioblastoma (GBM), a grade IV astrocytoma, is an aggressive primary malignant adult brain tumor with a median survival rate of 14.6 months^{1,2}. Current clinical intervention involves maximal surgical resection followed by radiotherapy and the chemotherapy drug temozolomide (TMZ), an alkylating agent that introduces methyl groups into DNA to disrupt tumor replication, yet these interventions have failed to significantly extend life. Treatment options for aggressive brain tumors, such as GBM, remain inadequate, largely as many systemic therapies, including chemotherapies, cannot cross the blood-brain barrier (BBB) to reach the tumor parenchyma³.

Focused ultrasound (FUS) has emerged as a promising non-invasive technology to overcome this highly selective, semipermeable membrane. FUS employs precisely targeted, highly focused ultrasound waves to exert thermal and/or mechanical effects on tissues. FUS has been used to induce localized thermal tissue ablation within oncology⁴, and within neuro-oncology utility has expanded to encompass sonodynamic therapy, immunomodulation, and enhanced liquid biopsies⁵. Most notably, FUS, paired with circulating microbubbles, enables transient disruption of the BBB to enhance drug delivery to brain tumors. The safety and efficacy of FUS-mediated BBB opening have been evaluated in mouse models⁶⁻⁹ and non-human primates¹⁰. Building on successful early-phase clinical trials that confirmed the safety of this technique for chemotherapy delivery in high-grade glioma patients¹¹⁻¹³, larger-scale studies are now further investigating its feasibility across diverse human brain tumors, including GBM and diffuse midline glioma (DMG)^{14,15}.

To optimize FUS as a therapeutic tool and evaluate its downstream effects, it is necessary to investigate the bioeffects of FUS and understand how acoustic radiation forces interact with the tumor's altered mechanobiology. GBM alterations compared to the surrounding brain parenchyma include aberrant expression of mechanosensitive ion channels. Compared to low-grade tumors and normal brain tissue, Piezo1 is more highly expressed in high-grade gliomas^{16–18}. Expression levels correlate with glioma grade; in GBM, Piezo1 is upregulated by a 2.43-fold change relative to normal brain tissue¹⁶. Because of this overexpression, high-grade gliomas may possess heightened mechanosensitivity, potentially amplifying downstream mechanotransductive signalling pathways, establishing Piezo1 as a potential therapeutic target and prognostic indicator for patients with GBM^{16–18}.

Mechanotransduction is the process by which cells detect and respond to mechanical forces¹⁹. This involves translating mechanical inputs into biochemical signals, which subsequently alter signaling pathways to dictate cellular fate such as survival and invasion^{19,20}. Cells express a variety of proteins activated in response to various mechanical stimuli such as pressure, stretch, or shear flow^{21–24}. Central to this process are mechanosensitive ion channels like Piezo1, a transmembrane protein activated by increases in membrane tension²⁵.

Several studies have explored the biological effects of FUS on GBM and other cancerous cells in culture²⁰. Few studies have explored the direct cellular effects of FUS, including its ability to reduce metabolic activity and spheroid size, and increase DNA double-strand breaks in GBM 2D and spheroidal cultures²⁶. Cell culture studies have shown that FUS can activate the mechanosensitive ion channel Piezo1 in various non-cancerous^{27,28} and cancerous cell lines (pancreatic²⁹ and breast cancer cells^{30,31}). Despite this, the direct effect of FUS on Piezo1 in brain cancer cells, and its broader role in brain cancer therapy, remains unexplored.

As a mechanosensitive ion channel with elevated expression in gliomas, Piezo1 represents an ideal candidate to elucidate how cancer cells broadly respond to FUS-induced mechanical stress. Because FUS has been shown to directly activate this channel, Piezo1 emerges as a promising candidate for a novel therapeutic target. Technologies such as FUS could be leveraged to non-invasively modulate these mechanotransduction pathways²⁰. Building upon this, the present study aims to investigate whether reducing the expression of Piezo1 impacts cell viability following FUS exposure. We hypothesized that reducing Piezo1 expression would disrupt this critical mechanotransduction pathway, potentially preventing GBM cells from detecting and adapting to dynamic mechanical stress exerted by FUS, thereby compromising their ability to tolerate the treatment. We employed siRNA-mediated knockdown in U-87 GBM cells to determine if disrupting this pathway increases sensitivity to clinically relevant FUS. By assessing cell viability, detachment, and apoptosis, we aim to establish whether targeted inhibition of Piezo1 can enhance the direct cytotoxic effects of FUS.

Materials and Methods

Cell Culture

U-87 MG (HTB-14; ATCC) cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% foetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin (Sigma-Aldrich) at 37°C and 5% CO₂.

RNA extraction

RNA extraction was carried out following the manufacturer's protocol (RNeasy kit; Qiagen). 1x10⁶ cells were harvested and lysed with Buffer RLT, homogenised in a QIAshredder spin column, and washed with buffers RW1, and RPE. 30 µl of RNase-free water was added and incubated for 10 minutes at room temperature before final centrifugation. RNA purity and concentration were measured using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific).

cDNA synthesis

Human brain reference RNA (QS0611; Invitrogen™, Thermo Fisher Scientific) was used as a control, as it represents pooled normal brain tissue with the heterogeneity of multiple neural cell types, providing a more physiologically relevant reference than single-cell-type RNA. U-87 and human reference brain cDNA were synthesized from RNA using SuperScript IV VILO (Thermo Fisher Scientific) per the manufacturer's instructions. Thermocycler conditions for cDNA synthesis are detailed in Table S1. All samples were plated and placed in a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific) following the thermocycler conditions for cDNA synthesis, as in Table S1. cDNA was stored at -20°C until use in quantitative polymerase chain reactions (qPCR). cDNA was diluted 1:10 to 0.1 µg/µl after the addition of 180 µl of water.

Quantitative polymerase chain reaction

qPCR evaluated Piezo1 gene expression across cell lines as well as post siRNA Piezo1 gene knockdown using SYBR green, Piezo1 RNA primers with human reference brain cDNA as the control. Forward and reverse human RNA primers used were Piezo1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Table S2; Merck Life Science UK Limited). 5 µl of cDNA was added to SYBR green and primer mix to a total volume of 20 µl per well. Reagents were plated and loaded into a QuantStudio™ 3 Thermal Cycler. Thermocycler conditions for qPCR, detailed in Table S3, were used to quantify target gene expression. The samples analyzed in biological triplicates were normalized to the reference gene GAPDH. The 2^{-ΔΔCT} method was utilized to evaluate Piezo1 gene expression in cell lines relative to the average expression in human reference brain samples.

siRNA Knockdown

siRNA-mediated knockdown of Piezo1 was performed in U-87 cells to evaluate its role in cellular responses following FUS exposure. This approach enabled us to isolate the specific contribution of Piezo1 suppression to post-sonication cell viability and gene expression in brain cancer cells, as well as to assess how FUS influences Piezo1-dependent mechanotransduction pathways. Three technical replicates per siRNA treatment condition were plated in 12-well plates at a density of 9×10^4 cells per well, in 2 mL of medium. The cells were left for 24 hours in the incubator to reach 60–70% confluence. Transfection reagent Dharmafect 1 (Horizon Discovery) and DMEM (antibiotic and FBS-free) were added to the wells following the manufacturer's instructions. siRNA pool (siControl, D-001810-10-50 and siPIEZO1, L-020870-03-0050; Horizon Discovery) were used at a final concentration of 25 nM.

Calcium Imaging

Calcium imaging was performed 72 hours after siRNA transfection. Experiments were conducted on U-87 Piezo1 siRNA KD cells and U-87 control non-targeting siRNA cells ($n = 3$). Cells grown on 12-well plates were loaded with 1 mL of Ca^{2+} sensitive dye Calbryte 520AM (4.5 μM , AAT Bioquest, in DMSO) and were incubated for 30 minutes at 37°C, 5% CO_2 , followed by a phosphate-buffered saline (PBS; Merck Life Science UK Limited) wash and 30 minutes of additional incubation. Cells were imaged under EVOS M5000 Microscope (Thermo Fisher Scientific) on time-lapse settings (5-second intervals), before and after 1 μL Yoda1 (10mM, Tocris) administration. Intracellular Ca^{2+} levels were imaged at room temperature using a 10 $\times$ objective lens and green fluorescent protein-compatible fluorescence settings (Ex: 482/25 nm; Em: 524/24 nm; exposure time: 20ms).

In Vitro FUS Stimulation

A single-element FUS transducer (part number H-195; Sonic Concepts Inc.) with an active diameter of 64 mm, geometric focus of 45 mm, focal depth of 30.5 mm, and focal width of 2.15 mm was used at its center frequency of 0.5 MHz. The transducer was driven by a PC-operated waveform generator (33500B series; Agilent Technologies) coupled with a 50 dB power amplifier (325LA; Electronics and Innovation). The entire apparatus was secured to a 3D positioning system (Velmex Inc.) as shown in Fig. 1A, which was used to reposition the transducer to each sonication target region separated by 10 mm to ensure no overlap of acoustic energy. This is a setup typical of transcranial FUS *in vivo* experiments³².

To accurately target the center of each chamber, a reflective metal cross was temporarily placed as a spatial reference. Alignment was conducted in pulse-echo mode using a 7.5 MHz transducer (Part No. U8423042, V320-SU, diameter: 12.7 mm; Evident Europe GmbH), which was co-aligned with the FUS transducer, with their foci overlapping, using a custom-made 3D printed holder. During this targeting process, a raster scan was performed to locate the metal cross, and the resulting acoustic signals were monitored and captured using a digital oscilloscope (Picoscope 5244D, Picotech; Fig. 1A).

siControl and siPiezo1KD cells were plated in individual collagen IV-coated flow chambers (Cat. no. 80172; Ibidi) with a channel height of 400 μm and a volume of 100 μL at a cell density of 2.75×10^5 cells/mL and incubated overnight to allow adhesion to the cell chamber. Cell chamber reservoirs were sealed with parafilm and placed in degassed and deionized water at 37°C to mimic *in vivo* conditions. The chambers were positioned within the focal volume of the FUS transducer to correctly target the cells along all three dimensions. Prior to sonication, the cell culture medium was replaced with 100 μL of microbubble suspension to improve the coupling of the acoustic energy to the cells and amplify the locally induced stress. SonoVue™-equivalent research-grade microbubbles (BR1, Bracco SpA) were suspended in 5 mL of DMEM and filled the channel. FUS exposures were performed with a fresh microbubble batch in the absence of flow.

Ultrasound parameters that did not cause significant immediate cell detachment or extensive apoptosis at 2 and 24 hours in either siControl or siPiezo1KD groups were chosen based on preliminary findings. This allowed us to isolate the specific effect of Piezo1 rather than just observing general FUS-induced cytotoxicity. While keeping all other parameters constant, we initially explored peak-negative pressures ranging from 30 kPa to 1 MPa. We found that pressures above 200 kPa immediately dislodged 70–90% of cells in the field of view, making them unsuitable for our aims. We therefore used the following FUS parameters: center frequency (f_c): 0.5 MHz, peak-negative pressure (PNP): 120 kPa, pulse length: 10 ms, pulse repetition frequency: 10 Hz (10% duty cycle), and treatment duration: 100 pulses (10 seconds). Three regions were sonicated per chamber as technical repeats at a spacing of 10 mm. Additionally, preliminary experiments showed that seeding cell concentrations above 3×10^5 cells/mL resulted in over-confluent monolayers. This high density prevented observable detachment at the tested pressure ranges and confounded the ability to accurately count individual cells.

Figure 1. In vitro FUS experimental setup. **A)** Ibidi cell chambers were placed in degassed and deionized water at 37°C. A 0.5 MHz single-element focused ultrasound transducer driven by a waveform generator through a 50 dB power amplifier was used for FUS exposure. Precise targeting was achieved using a 7.5 MHz transducer driven by a pulser-receiver in pulse-echo mode. Reflections were recorded with a digital oscilloscope. Cells were seeded in a collagen-coated flow chamber slide with a channel height of 400 μm and a volume of 100 μL . Three regions per flow chamber were sonicated, as indicated by the red circles. Tubing connectors were sealed with parafilm after the addition of microbubbles and during sonication. **B)** Experimental timeline **C)** FUS parameter table. Created with Biorender.com.

Live-Dead Cell Imaging

Viability of siControl and Piezo1KD cells pre- and post-sonication was assessed using live-dead staining. To evaluate the effects of the knockdown and FUS, staining was performed at pre-sonication and at 0 (~ 5 minutes post-treatment), 2, and 24 hours post-sonication. At each designated time point, DMEM was aspirated, and Ibidi chambers were loaded with 100 μL of live-dead dye solution (Sigma Aldrich, Cat no: CBA415). The staining mixture contained 5 μL Calcein-AM, 20 μL Propidium Iodide, and 8 μL Hoechst

33342 added to 24 mL of a 1:1 mixture of DMEM and PBS. Cells were incubated with the dye solution for 30 minutes at 37°C. Following incubation, cells were imaged using an EVOS M5000 Microscope at 4× objective lens (exposure time: 20 ms) with filter sets to detect live cells (Calcein-AM; Ex/Em: 490/515 nm), dead cells (Propidium Iodide; Ex/Em: 535/617 nm), and total nuclei (Hoechst 33342; Ex/Em: 361/486 nm).

Automated cell counting

Total (Hoechst 33342), live (Calcein-AM), and dead (Propidium Iodide) cell numbers were quantified using a custom automated MATLAB script. Individual cells exhibited a variety of shapes, but generally contained a bulk, globular nucleus often from which long thin extensions of the cell body would protrude. The cell counting algorithm targeted the globular nucleus for identification of the cells³³. Raw fluorescence images were contrast-enhanced with a gamma filter, and image noise was reduced with a non-local means filter. Cells were segmented using adaptive thresholding using MATLAB's *adapthres* function, followed by morphological filtering to exclude non-cellular debris. The cell segmentation maps were analyzed using principal component analysis, computing the first principal component of the vector space containing the cell size, area, eccentricity, and maximum intensity³⁴. All principal component values were sorted in magnitude, and the top 25 maps were averaged to obtain an estimate of the nucleus of the cell. The averaged cell nucleus maps were fitted to a 2D Gaussian distribution using non-linear least squares fit, and a 2D cross-correlation of the Gaussian was computed with the cell image. Overlapping cells were separated using a distance transform watershed segmentation algorithm seeded with the maxima from the correlation image^{35,36}. Markers where the distance transform value was lower than the nucleus size estimate were discounted to filter any selection on any long thin cell bodies extending from the nucleus. Final counts were determined by evaluating these segmented regions; for contiguous cell clusters, the total area was divided by the average pixel area of a single U-87 cell.

Data Analysis

Statistical analysis was performed in GraphPad Prism version 9.0 (GraphPad Software, California, USA). For Piezo1 gene expression analysis, Cq values were produced using DataConnect™ software (Thermo Fisher Scientific) and exported to Microsoft Excel to perform $2^{-\Delta\Delta CT}$ method calculations as a relative quantification strategy for quantitative real-time qPCR data analysis. Statistical significance between two independent groups, including relative gene expression, area under the curve (AUC) for calcium imaging, dead cell number changes, and direct pairwise comparisons of total cell numbers or detachment between siControl and Piezo1KD groups at specific timepoints, was evaluated using an unpaired, two-tailed Student's t-test. For comparisons across multiple timepoints within the same experimental group relative to a pre-sonication baseline, a one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test was utilized. Statistical significance was assumed at $p < 0.05$.

Results

The GBM cell line U-87 expresses high levels of Piezo1

To establish a clinically relevant *in vitro* model for evaluating FUS bioeffects, we first sought to confirm whether the GBM cell line U-87 expresses high levels of Piezo1, as previous studies observed Piezo1 upregulation in patient GBM samples^{16–18}. Therefore, we quantified Piezo1 expression in the U-87 GBM cell line. We observed a significant 6.81-fold upregulation in Piezo1 expression ($p = 0.0092$; Fig. 2A) in U-87 cells compared to a human reference brain control. To explore the effect of Piezo1 on FUS cell viability, we generated a transient Piezo1 knockdown (Piezo1KD) using siRNA. The efficacy of this knockdown was confirmed transcriptionally via RT-qPCR, which showed a significant 84.5% reduction in Piezo1 mRNA levels ($p = 0.0108$; Fig. 2B) compared to non-targeting siControl cells. We further confirmed this knockdown functionally utilizing a Calbryte calcium imaging assay, observing changes in fluorescence as a readout for internal calcium concentrations, which leads to downstream signaling and biological consequences. Following the administration of the Piezo1-specific agonist Yoda1, Piezo1KD cells exhibited a diminished increase in fluorescence intensity, indicating a lower calcium influx compared to the higher-expressing siControl cells (Fig. 2C). Piezo1KD cells displayed a significantly reduced total area under the curve by 81.4% ($p = 0.0017$; Fig. 2D). This attenuated overall fluorescence in the Piezo1KD cells is consistent with depleted Piezo1 expression in these cells. We observed a transcriptional reduction and attenuated calcium response, confirming the efficacy of Piezo1 knockdown, providing a model that allows exploration of how this reduced expression of Piezo1 influences cell tolerance to FUS.

Figure 2. Piezo1 gene expression in glioblastoma (GBM) cells and confirmation of Piezo1 knockdown.

A) Increased Piezo1 gene expression in U-87 GBM cells compared to human brain reference control. Real-time quantitative polymerase chain reaction (RT-qPCR) comparing total RNA levels of Piezo1 in human brain reference RNA samples and GBM cell line U-87. Housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference for normalization. **B)** Transcriptional confirmation of Piezo1 knockdown. GBM cells were transfected with either non-targeting siRNA (siControl) or siRNA targeting Piezo1 to generate transient Piezo1 knockdown (Piezo1KD) cells. RT-qPCR analysis comparing Piezo1 mRNA levels in siControl and siPiezo1 knockdown (Piezo1KD) cells. Expression levels were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data shown as mean $\pm$ standard error of the mean (SEM) from three biological experiments, each with three technical replicates. Significance was determined using an unpaired t-test. **C)** Functional validation of Piezo1 knockdown. The graph shows calcium influx measured as normalized fluorescence (F/F_0 , where F is the fluorescence at a given time point and F_0 is the mean baseline fluorescence), in response to the Piezo1-specific agonist Yoda1, which was reduced in Piezo1KD cells (red line) compared with siControl cells (blue line). Yoda1 was added at 3 minutes (grey band), and fluorescence images were captured every 5 seconds for a further 3 minutes. The inset shows representative images of siControl (top) and Piezo1KD (bottom) cells at maximum fluorescence following Yoda1 administration. Scale bar = 350 μm . **D)** Area under the curve (AUC) analysis of the fluorescence traces shown in **C)**, quantifying the total Yoda1-induced calcium response in siControl and

Piezo1KD cells. Statistical comparison was performed using an unpaired, two-tailed Student's t-test. Data are presented as mean $\pm$ SEM from three biological experiments. Statistical significance was set at $p < 0.05$. *: $p < 0.05$, **: $p < 0.01$.

Piezo1 knockdown exacerbates acute FUS-induced cell loss and attenuates population expansion

Representative fluorescence images captured within the focal volume of FUS sonication (Fig. 3A) qualitatively showed a reduction in total adherent cells in Piezo1KD conditions at 2 hours post-sonication, while siControl cells were largely maintained. To quantify these visual observations, relative changes in total cell number were assessed (Fig. 3B). Following treatment, siControl cells exhibited non-significant reductions at 0 hours (-13.98%; $p = 0.3162$; Fig. 3B) and 2 hours (-16.09%; $p = 0.2197$; Fig. 3B) timepoints. Piezo1KD cells showed an initial non-significant decline at 0 hours post-sonication (-19.84%; $p = 0.1074$; Fig. 3B) followed by a significant reduction at 2 hours (-34.90%; $p = 0.0039$; Fig. 3B). Direct comparison between groups at 2 hours did not reach statistical significance ($p = 0.3354$; Fig. 3C). By 24 hours post-sonication, siControl cells demonstrated robust proliferation above their pre-sonication baseline (+ 45.50%; $p = 0.0004$; Fig. 3B). Conversely, Piezo1KD cells exhibited a blunted, non-significant increase in growth (+ 14.89%; $p = 0.2713$; Fig. 3B). Direct comparison at 24 hours revealed Piezo1KD cell numbers were significantly reduced compared to siControl ($p = 0.0419$; Fig. 3C). These results suggest that Piezo1KD increases early cell loss and attenuates the net growth of GBM cells.

Piezo1 knockdown results in higher cell death counts at 24 hours

To investigate sonication-induced cytotoxicity, the percentage change in dead cell numbers relative to pre-sonication baselines was evaluated (Fig. 3D). At 2 hours post-sonication, both groups exhibited substantial acute increases in dead cell populations relative to their respective baselines, with no significant difference observed between Piezo1KD and siControl cells (+ 91.67% vs + 140.10%; $p = 0.6563$; Fig. 3D). This indicates that Piezo1 knockdown does not change the immediate cell death caused by FUS. However, by 24 hours, their post-FUS viability profiles diverged. The siControl group demonstrated a pronounced reduction in dead cells, reducing below their initial pre-sonication baseline (-70.20%; Fig. 3D). This decrease is potentially due to dead cells physically detaching over time. In contrast, the Piezo1KD cells failed to resolve the cytotoxicity, maintaining an elevated dead cell number above baseline (+ 28.89%). As a result, the Piezo1KD group had a significantly higher number of dead cells compared to the control at 24 hours ($p = 0.0150$; Fig. 3D). The observed higher dead cell count suggests either that the Piezo1KD cells may fail to detach after the initial disruption caused by sonication or that these cells may be undergoing delayed cell death. Overall, these findings indicate that Piezo1 plays a crucial role in reducing cell death after FUS exposure in GBM cells.

Figure 3. Piezo1 knockdown alters U-87 GBM cell recovery and proliferation dynamics following FUS treatment. A) Representative fluorescence microscopy images of U-87 GBM cells captured within the focal volume of FUS sonication. Columns compare non-targeting control (siControl) and Piezo1

knockdown (Piezo1KD) cells at pre-FUS baseline, 2 h, and 24 h post-FUS. Rows display individual fluorescence channels: total nuclei (Hoechst 33342, blue), live cells (Calcein AM, green), and dead cells (Propidium Iodide, PI, red). Arrows highlight PI staining. Images selected are representative of the mean population for each experimental condition. Scale bars = 750 μ m. **B)** Relative change in total cell number at each post-sonication time point compared to the pre-sonication baseline (%). **C)** Direct comparison of the relative percentage change in total cell numbers between siControl and Piezo1KD cells at corresponding time points. **D)** Percentage change in dead cell numbers post-sonication relative to the pre-sonication baseline. Data are presented as mean $\pm$ SEM from three independent biological experiments, comprising 3–6 technical replicates per condition. Statistical significance across timepoints was either determined using a one-way ANOVA followed by Dunnett's post-hoc test comparing timepoints to the pre-sonication baseline or via direct pairwise comparisons between siControl and Piezo1KD at specific timepoints, which were performed using a Student's t-test. Statistical significance was defined as $p < 0.05$. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant.

2-hour post-FUS cell loss is characterized by physical detachment rather than acute cytotoxicity

To investigate the discrepancy between the acute changes observed in total cell numbers (Fig. 3B) and the relative number of dead cells (Fig. 3D), and determine if this early cell loss was due to physical membrane damage or a loss of cell adhesion, cell fate analysis was conducted (Fig. 4A). Evaluation of the total cell fate model indicated that the initial reduction in cell numbers at 2 hours post-sonication was primarily driven by physical cell detachment rather than immediate cell death. To quantify this immediate cell loss, the percentage of cell detachment was assessed at 2 hours post-sonication (Fig. 4B). Piezo1 knockdown cells exhibited a mean detachment rate greater than 2-fold compared to the control group ($34.90 \pm 3.69\%$ vs. $16.09 \pm 8.76\%$, respectively). Although an unpaired t-test revealed this difference did not reach statistical significance ($p = 0.119$; Fig. 4B) due to high intra-group variance within the siControl replicates, this pronounced trend suggests that Piezo1 knockdown exacerbates acute physical cell loss. While not statistically significant, these observations suggest that Piezo1 may influence cell adhesion post-FUS, where its depletion potentially exacerbates physical detachment.

Piezo1 knockdown attenuates net post-FUS cellular proliferation

While both siControl and Piezo1KD cells maintain the ability to divide after FUS, their long-term population expansion depended on the acute response to physical disruption. To determine if Piezo1 knockdown impacted long-term cell viability following this acute detachment phase, cell expansion and net proliferation were evaluated distinctly at 24 hours post-sonication (Fig. 4C). When assessing immediate expansion, defined as the percentage increase in cells relative to the 2 hours post-sonication total cell count, both groups demonstrated a statistically similar rebound. Control cells (siControl) populations grew by $73.4 \pm 6.1\%$, while Piezo1KD cells exhibited a comparable increase of $76.5 \pm 2.6\%$ ($p = 0.668$; Fig. 4C). However, despite this equivalent capacity for acute growth, the overall net proliferation of the population relative to the initial pre-sonication baseline was severely impaired by Piezo1

knockdown. At 24 hours, siControl cells achieved a net proliferation of $45.5 \pm 5.2\%$ over their starting baseline. In contrast, net proliferation in Piezo1KD cells was significantly blunted, reaching only $14.9 \pm 1.7\%$ ($p = 0.0048$; Fig. 4C). Taken together, these data indicate that while Piezo1KD cells retain the ability to proliferate following the immediate physical disturbance of FUS, the initial loss of cells combined with the elevated number of dead cells results in a significantly reduced final cell population. This suggests that the attenuated net proliferation observed in Piezo1KD cells is driven by detachment and sustained elevated cell death rather than an impaired ability to divide.

Discussion

As brain tumors advance, they exhibit altered mechanobiology, characterized by increased extracellular matrix stiffness^{37,38} and upregulated Piezo1 expression^{16–18,39}. FUS is rapidly expanding within neuro-oncology as a non-invasive intervention⁵ capable of eliciting targeted mechanical and thermal effects, with demonstrated efficacy in reducing GBM metabolic activity and inducing DNA damage²⁶ in cultured cells. While prolonged activation of these channels drives tumor progression and tissue stiffness^{16–18,40–48}, acute dynamic stimulation via FUS has been shown to trigger apoptotic pathways and induce cell death in other non-central nervous system cancer models^{29–31}. Our findings indicate that Piezo1 may have a mechanoprotective role against acoustic stress in GBM. Reducing Piezo1 expression attenuates cellular resistance to FUS, sensitizes GBM cells to early physical detachment, and restricts long-term growth.

In GBM patients, Piezo1 is upregulated 2.43-fold compared to healthy brain tissue¹⁶. We have previously proposed that this overexpression may play a role in mediating tumor progression by creating a pathological positive feedback cycle: the confined, stiffening tumor microenvironment exerts continuous mechanical stress that hyperactivates Piezo1. This activation subsequently triggers downstream mechanotransduction signaling that promotes further extracellular matrix remodeling, tumor proliferation, and tissue stiffening, thereby perpetuating the cycle²⁰. Due to this overexpression, Piezo1 has been proposed as a potential prognostic indicator and therapeutic target in patients with GBM^{16–18}. Here, U-87 cells showed a 6.81-fold upregulation compared to healthy brain tissue. We hypothesize that this amplified expression is influenced by the standard 2D substrates utilized in this study (tissue culture plastics and Ibidi chambers) being orders of magnitude stiffer than the *in vivo* microenvironment (gigapascal [GPa] vs. kilopascal [kPa], respectively)⁴⁹.

Given the increasing clinical interest in FUS as a non-invasive therapeutic modality, we investigated the specific role of Piezo1 in mediating GBM cellular responses to acute acoustic mechanical stress. Following acute FUS exposure, Piezo1-deficient cells exhibited a trend towards increased physical detachment and a subsequent significantly attenuated net proliferation at 24 hours, accompanied by a persistently elevated number of dead cells. This indicates that while cells were able to divide post-FUS disruption, Piezo1 contributes to maintaining adhesion during acoustic stress and preventing sustained cytotoxicity. In a clinical context, FUS-induced cellular detachment presents a challenge. Because non-

adherent cells are removed during post-treatment solution replacement to allow for staining in our *in vitro* model, it remains unclear whether these detached Piezo1-deficient cells sustained lethal mechanical damage or if they remained viable but lost adhesion. If they do not undergo necrosis, FUS-induced detachment *in vivo* could theoretically pose a risk for migration or secondary metastasis. Future studies must evaluate the viability of the detached population and profile downstream mechanotransduction components, such as integrin expression, focal adhesion kinase (FAK) activation, and cytoskeletal remodeling, to understand how Piezo1 anchors these cells under acoustic shear. We hypothesize that this restricted long-term expansion may be linked to downstream Piezo1 mechanotransduction signaling pathways, such as the Yes-associated protein/transcriptional coactivator with PDZ-binding motif (YAP/TAZ) or FAK pathways, which are critical for survival and proliferation⁵⁰. Our results provide some evidence that Piezo1 aids in mitigating acute sonication-induced cell loss and facilitates long-term net growth. Furthermore, utilizing Piezo1 overexpression plasmids in future models could be helpful to determine if these vulnerabilities reverse, confirming whether elevated Piezo1 levels inherently increase cellular resistance to FUS.

Targeting Piezo1 represents a potential strategy to sensitize GBM cells to ultrasound-based therapies. As an antagonist for specific Piezo1 inhibition is currently not available, siRNA knockdown or short hairpin RNA (shRNA) knockout are potential avenues to explore. However, as Piezo1 is expressed in other cell types in the central nervous system, delivering these therapeutics systemically would likely affect Piezo1 in non-brain cancer cells. For example, microglia⁵¹, the resident immune cell in the CNS, also highly express Piezo1⁵¹. Our data also show that Piezo1 is highly expressed in healthy human astrocytes (Supplementary Fig. 3); therefore, systemic non-specific downregulation could potentially induce severe neurological damage. The translational feasibility of overcoming this barrier is strongly supported by recent preclinical evidence; low-intensity FUS combined with circulating microbubbles has been successfully utilized to deliver nanoparticle- and liposome-encapsulated siRNA and shRNA across the BBB, achieving efficient target gene knockdown in both neurodegenerative⁵² and glioma models^{53,54}. Building on these established delivery platforms, a dual-stage combinatorial strategy could be employed: utilizing FUS to facilitate the highly localized BBB opening and subsequent delivery of tumor-targeted Piezo1 siRNA alongside standard-of-care chemotherapeutics, followed by a secondary application of therapeutic FUS to mechanically stress the newly sensitized tumor cells.

The use of the Ibidi μ -Slide I Luer (400 μ m channel) used here for FUS exposure is supported by previous research demonstrating the acoustic compatibility of these chambers for ultrasound applications, establishing both its fundamental acoustic transparency⁵⁵ and its functional efficacy in *in vitro* therapeutic ultrasound models⁵⁶. While exact *in situ* pressures differ under our specific parameters (0.5 MHz, modified incident angles), prior validation⁵⁵ confirms that the chamber material introduces minimal acoustic attenuation. Despite this controlled approach, the use of a 2D *in vitro* model inherently lacks the 3D spatial architecture, extracellular matrix constraints, and complex cell-cell interactions present in the *in vivo* tumor microenvironment. Additionally, we used microbubbles here to couple the acoustic energy to the cells; however, *in vivo*, microbubbles would likely not be in the vicinity of the GBM cells. It should

also be noted that the setup used does not model the physical barriers of clinical transcranial FUS, including skull-induced acoustic aberrations, scattering, and energy absorption by surrounding tissue and bone. However, this approach was favorable for our requirements to isolate direct acoustic mechanotransduction, enabling precise *in situ* live/dead quantification and gene modification without the confounding variables of a complex tissue matrix.

Quantification of cell death immediately following FUS exposure was intentionally excluded as acoustic waves can induce transient membrane permeabilization or sonoporation, which carries a high risk of false-positive cell death staining⁵⁷. Instead, to confirm that the initial acoustic stress did not induce immediate, significant cell loss, we verified overall monolayer integrity and immediate viability. As demonstrated in Supplementary Fig. 2, there was no statistically significant reduction in either total cell counts (Hoechst 33342) or live cells (Calcein AM) immediately (0 h) following sonication in both the siControl and Piezo1KD groups. Use of propidium iodide serves as a marker for definitively tracking terminal membrane compromise at later time points (pre-sonication, 2 hours, and 24 hours). Despite its limitations in detecting early apoptosis, *in situ* spatial imaging with PI allowed evaluation of cytotoxicity within the targeted sonicated regions.

The scope of this investigation is constrained by the use of a single GBM cell line (U-87) and a single set of FUS parameters. However, these specific sonication conditions were carefully selected following rigorous initial parameter optimization. To provide greater confidence in these findings, future studies utilizing broader FUS parameters, additional adult and pediatric brain cancer cell lines, 3D spheroid models, and specific molecular profiling of downstream signaling pathways (such as YAP/FAK) will be necessary to fully validate these foundational mechanobiological responses for complex *in vivo* applications. Notably, our supplementary qPCR analysis (Supplementary Fig. 1) shows that Piezo1 is also significantly upregulated in pediatric brain tumor diffuse midline glioma (DMG). This shared Piezo1 expression profile suggests that the protective role of Piezo1, and its potential as a therapeutic target alongside FUS, potentially extends beyond GBM and warrants validation in future *in vitro* and *in vivo* models.

Conclusion

Piezo1 expression is significantly elevated in GBM cells, supporting its role in tumor mechanobiology. This study provides evidence suggesting that reducing Piezo1 expression compromises cellular resistance to FUS-induced mechanical stress. This vulnerability is characterized by acute cell loss driven primarily by an increased susceptibility to physical detachment, sustained cytotoxicity, and restricted long-term growth. Modulating Piezo1 expression using siRNA represents a potential strategy to sensitize GBM cells to acoustic stress and enhance the efficacy of ultrasound-based therapies. Further work is required to explore downstream signaling pathways and to validate this approach in more complex models.

Declarations

Author contributions

LG: Conceptualisation, data collection, data analysis, writing – original draft, writing – review and editing; CPP: Conceptualisation, experimental support, writing – original draft, writing – review and editing. DB: Experimental support, writing – review and editing. DL: Data analysis, writing – original draft, writing – review and editing. RB: Experimental setup design and production, writing – review and editing. JR: Conceptualisation, funding acquisition, resources, supervision, writing – review and editing. ANP: Conceptualisation, funding acquisition, resources, supervision, writing – review and editing. All authors have read and approved the final version of the manuscript.

Declaration of interests

All authors declare no competing interests.

Acknowledgements

The authors would like to acknowledge funding support from the Children's Cancer and Leukaemia Group (CCLG)/Little Princess Trust (CCLGA 2022 25), Action Medical Research/LifeArc (GN3017), the Focused Ultrasound Foundation (FUS1050R1), and Abbie's Army/Children's Brain Tumour Drug Delivery Consortium (KCL/G12/22). L.G. is a recipient of a Leverhulme Doctoral Scholarship (DS-2020-023). C.P.P. is the recipient of a Long-Term Fellowship (LT000654/2019- L) from the Human Frontier Science Program organization and a Marie Skłodowska-Curie Fellowship (898067) from the European Union's Horizon 2020 research and innovation program. J.R. is the recipient of a Wellcome Trust Investigator Award (221908/Z/20/Z), a Cancer Research UK grant (DRCNPG-May21\100007), an Academy of Medical Sciences Professorship (APR2\1007), and a King's College London start-up grant. We acknowledge BioRender.com as a platform for our figure design. Our funding bodies did not have any role in the study design, data collection and analysis, or writing of this manuscript.

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Figures

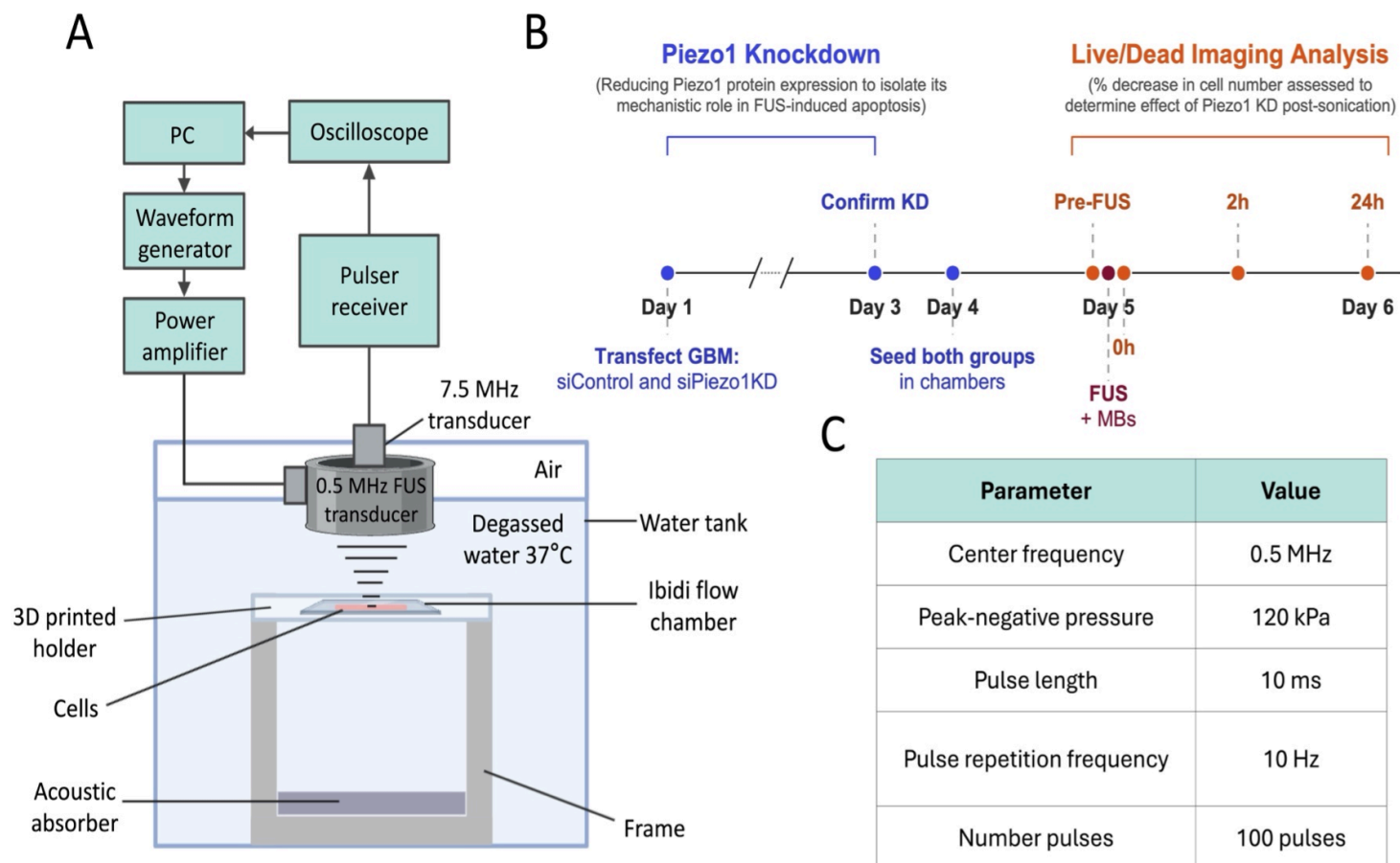


Figure 1

***In vitro* FUS experimental setup.** **A)** Ibbidi cell chambers were placed in degassed and deionized water at 37°C. A 0.5 MHz single-element focused ultrasound transducer driven by a waveform generator through a 50 dB power amplifier was used for FUS exposure. Precise targeting was achieved using a 7.5 MHz transducer driven by a pulser-receiver in pulse-echo mode. Reflections were recorded with a digital oscilloscope. Cells were seeded in a collagen-coated flow chamber slide with a channel height of 400 μm and a volume of 100 μL . Three regions per flow chamber were sonicated, as indicated by the red circles. Tubing connectors were sealed with parafilm after the addition of microbubbles and during sonication. **B)** Experimental timeline **C)** FUS parameter table. Created with Biorender.com.

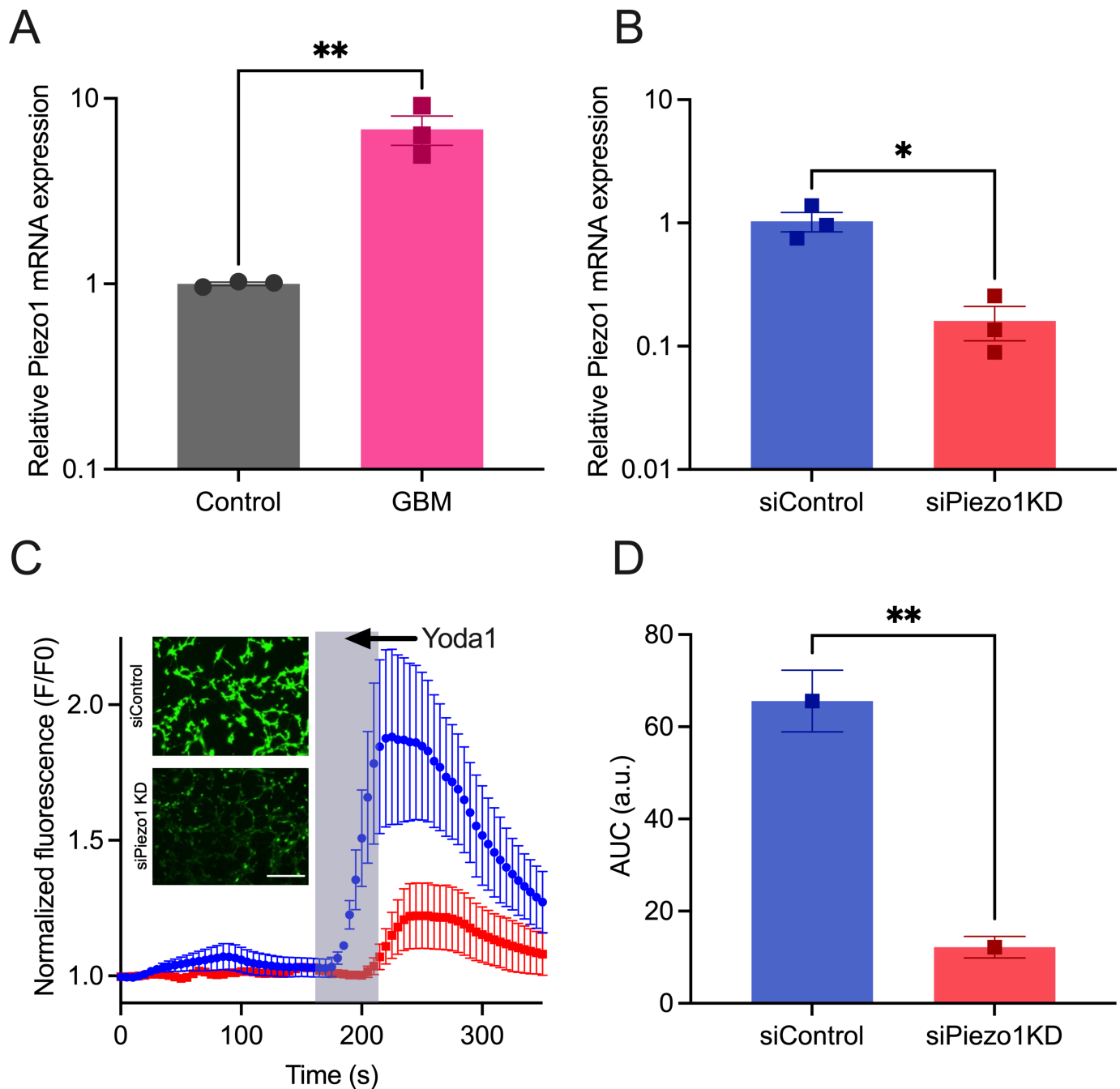


Figure 2

Piezo1 gene expression in glioblastoma (GBM) cells and confirmation of Piezo1 knockdown. A)

Increased Piezo1 gene expression in U-87 GBM cells compared to human brain reference control. Real-time quantitative polymerase chain reaction (RT-qPCR) comparing total RNA levels of Piezo1 in human brain reference RNA samples and GBM cell line U-87. Housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference for normalization. **B)** Transcriptional confirmation of Piezo1 knockdown. GBM cells were transfected with either non-targeting siRNA (siControl) or siRNA targeting Piezo1 to generate transient Piezo1 knockdown (Piezo1KD) cells. RT-qPCR analysis comparing Piezo1 mRNA levels in siControl and siPiezo1 knockdown (Piezo1KD) cells. Expression levels were

normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data shown as mean $\pm$ standard error of the mean (SEM) from three biological experiments, each with three technical replicates. Significance was determined using an unpaired t-test. **C)** Functional validation of Piezo1 knockdown. The graph shows calcium influx measured as normalized fluorescence (F/F_0 , where F is the fluorescence at a given time point and F_0 is the mean baseline fluorescence), in response to the Piezo1-specific agonist Yoda1, which was reduced in Piezo1KD cells (red line) compared with siControl cells (blue line). Yoda1 was added at 3 minutes (grey band), and fluorescence images were captured every 5 seconds for a further 3 minutes. The inset shows representative images of siControl (top) and Piezo1KD (bottom) cells at maximum fluorescence following Yoda1 administration. Scale bar = 350 μ m. **D)** Area under the curve (AUC) analysis of the fluorescence traces shown in **C)**, quantifying the total Yoda1-induced calcium response in siControl and Piezo1KD cells. Statistical comparison was performed using an unpaired, two-tailed Student's t-test. Data are presented as mean $\pm$ SEM from three biological experiments. Statistical significance was set at $p < 0.05$. *: $p < 0.05$, **: $p < 0.01$.

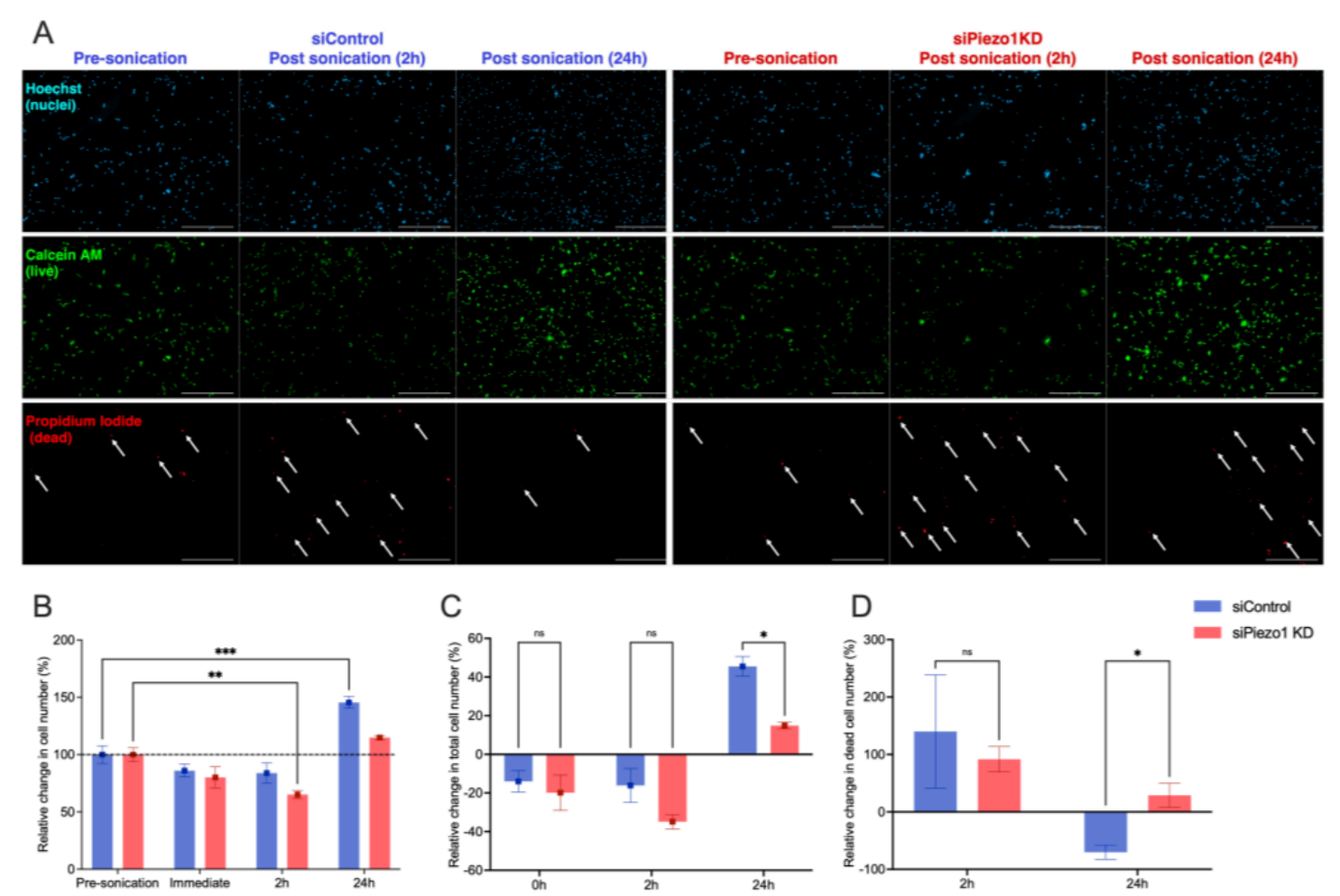


Figure 3

Piezo1 knockdown alters U-87 GBM cell recovery and proliferation dynamics following FUS treatment.

A) Representative fluorescence microscopy images of U-87 GBM cells captured within the focal volume

of FUS sonication. Columns compare non-targeting control (siControl) and Piezo1 knockdown (Piezo1KD) cells at pre-FUS baseline, 2 h, and 24 h post-FUS. Rows display individual fluorescence channels: total nuclei (Hoechst 33342, blue), live cells (Calcein AM, green), and dead cells (Propidium iodide, PI, red). Arrows highlight PI staining. Images selected are representative of the mean population for each experimental condition. Scale bars = 750 μ m. **B)** Relative change in total cell number at each post-sonication time point compared to the pre-sonication baseline (%). **C)** Direct comparison of the relative percentage change in total cell numbers between siControl and Piezo1KD cells at corresponding time points. **D)** Percentage change in dead cell numbers post-sonication relative to the pre-sonication baseline. Data are presented as mean $\pm$ SEM from three independent biological experiments, comprising 3–6 technical replicates per condition. Statistical significance across timepoints was either determined using a one-way ANOVA followed by Dunnett’s post-hoc test comparing timepoints to the pre-sonication baseline or via direct pairwise comparisons between siControl and Piezo1KD at specific timepoints, which were performed using a Student’s t-test. Statistical significance was defined as $p < 0.05$. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ns: not significant.

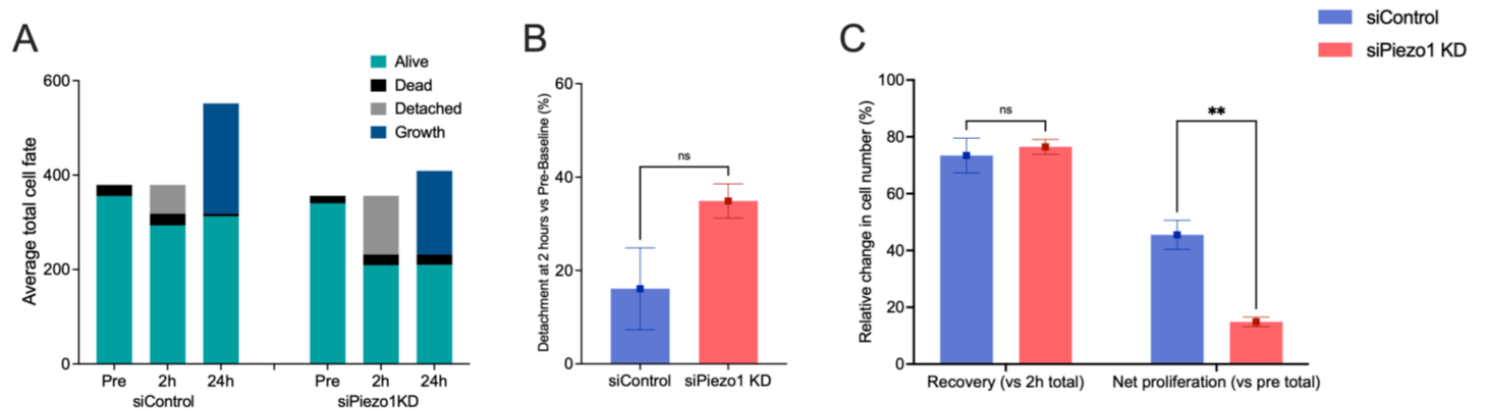


Figure 4

Piezo1 knockdown increases acute detachment and restricts net proliferation following focused ultrasound. **A)** Average total cell fate modeling over 24 h. Stacked bars represent the absolute mean distribution of cell outcomes (alive, dead, proliferated, and dislodged). Dislodgment is calculated as the net loss in total cells (live + dead) at 2 h post-FUS relative to the pre-sonication baseline. Proliferation is calculated as the net growth at 24 h relative to the 2 h post-FUS total cell count. **B)** Percentage detachment of cells at 2 h post-sonication in siControl and Piezo1KD cells. **C)** Relative change in cellular expansion (%). Left: 24 h recovery, calculated as the percentage increase in cell number at 24 h relative to the 2 h post-sonication total cell count. Right: Net proliferation, calculated as the total percentage increase in cell number at 24 h relative to the initial pre-sonication baseline. Data are presented as mean $\pm$ SEM from three independent biological experiments, comprising 3–6 technical replicates per condition. Statistical significance between siControl and Piezo1KD groups at specific timepoints was determined using an unpaired Student’s t-test. Statistical significance was defined as $p < 0.05$. **: $p < 0.01$, ns: not significant.

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