ADVANCED HEALTHCARE MATERIALS

Supporting Information

Ultrasound-Induced Mechanical Compaction in Acoustically Responsive Scaffolds Promotes Spatiotemporally Modulated Signaling in Triple Negative Breast Cancer

Brock A. Humphries, Mitra Aliabouzar, Carole Quesada, Avinash Bevoor, Kenneth K. Y. Ho, Alex Farfel, Johanna M. Buschhaus, Shrila Rajendran, Mario L. Fabiilli* and Gary D. Luker*

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Supporting Information

Ultrasound-Induced Mechanical Stiffening in Acoustically Responsive Scaffolds Promotes Spatiotemporally Modulated Signaling in Triple Negative Breast Cancer

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Supplemental Table 1. Calculated *p*-values for the Akt KTR in MDA-MB-231 cells from data in Supplemental Fig S1 using Tukey's multiple comparisons test.

		Fibrin gel -ultrasound				ARS -ultrasound				Fibrin gel +ultrasound				ARS +ultrasound			
		Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
Fibrin gel –ultrasound	Day 0		n.s.	***	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.	n.s.	n.s.	***	*	n.s.	n.s.
	Day 1			**	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.	n.s.	n.s.	***	***	n.s.	n.s.
	Day 2				n.s.	***	**	n.s.	n.s.	***	**	n.s.	n.s.	***	***	***	***
	Day 3					**	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.	***	***	***	***
ARS -ultrasound	Day 0						n.s.	***	n.s.	n.s.	n.s.	n.s.	**	***	n.s.	n.s.	n.s.
	Day 1							***	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.
	Day 2								*	***	***	***	*	***	***	***	***
	Day 3									***	n.s.	n.s.	n.s.	***	***	**	***
Fibrin gel +ultrasound	Day 0										**	***	***	***	n.s.	n.s.	n.s.
	Day 1											n.s.	n.s.	***	***	n.s.	*
	Day 2												n.s.	***	***	***	***
	Day 3													***	***	***	***
ARS +ultrasound	Day 0														*	***	***
	Day 1															n.s.	n.s.
	Day 2																n.s.
	Day 3																

n.s. = not significant, * = *p* < 0.05, ** = *p* < 0.01, *** = *p* < 0.001.

Supplemental Table 2. Calculated *p*-values for the ERK KTR in MDA-MB-231 cells from data in Supplemental Fig S1 using Tukey's multiple comparisons test.

		Fibrin gel -ultrasound				ARS -ultrasound				Fibrin gel +ultrasound				ARS +ultrasound			
		Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
Fibrin gel -ultrasound	Day 0		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
	Day 1			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
	Day 2				n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Day 3					n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ARS -ultrasound	Day 0						n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
	Day 1							n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Day 2								n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s.
	Day 3									n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Fibrin gel +ultrasound	Day 0										n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.
	Day 1											n.s.	n.s.	***	n.s.	n.s.	n.s.
	Day 2												n.s.	***	n.s.	n.s.	n.s.
	Day 3													***	n.s.	n.s.	n.s.
ARS +ultrasound	Day 0														**	*	**
	Day 1															n.s.	n.s.
	Day 2																n.s.
	Day 3																

n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Supplemental Table 3. Calculated *p*-values for the Akt KTR in SUM159 cells from data in Supplemental Fig S2 using Tukey's multiple comparisons test.

		ARS -ultrasound				ARS +ultrasound			
		Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
ARS -ultrasound	Day 0		***	***	***	n.s.	*	n.s.	***
	Day 1			n.s.	***	***	n.s.	***	n.s.
	Day 2				**	***	n.s.	***	n.s.
	Day 3					***	***	***	*
ARS +ultrasound	Day 0						***	n.s.	***
	Day 1							n.s.	n.s.
	Day 2								***
	Day 3								

n.s. = not significant, * = *p* < 0.05, ** = *p* < 0.01, *** = *p* < 0.001.

Supplemental Table 4. Calculated *p*-values for the ERK KTR in SUM159 cells from data in Supplemental Fig S2 using Tukey's multiple comparisons test.

		ARS -ultrasound				ARS +ultrasound			
		Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
ARS -ultrasound	Day 0		n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
	Day 1			n.s.	n.s.	**	n.s.	***	n.s.
	Day 2				n.s.	n.s.	n.s.	**	n.s.
	Day 3					***	n.s.	***	n.s.
ARS +ultrasound	Day 0						n.s.	n.s.	n.s.
	Day 1							n.s.	n.s.
	Day 2								*
	Day 3								

n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Supplemental Table 5. Calculated p-values for the Akt KTR in SUM159 cells from data in Supplemental Fig S3 using Tukey's multiple comparisons test.

		2.5 mg/mL fibrin				10 mg/mL fibrin				20 mg/mL fibrin			
		Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
2.5 mg/mL fibrin	Day 0		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Day 1			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Day 2				n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Day 3					n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
10 mg/mL fibrin	Day 0						n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Day 1							n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Day 2								n.s.	n.s.	n.s.	n.s.	n.s.
	Day 3									n.s.	n.s.	n.s.	n.s.
20 mg/mL fibrin	Day 0										n.s.	n.s.	n.s.
	Day 1											n.s.	n.s.
	Day 2												n.s.
	Day 3												

n.s. = not significant.

Supplemental Table 6. Calculated p-values for the ERK KTR in SUM159 cells from data in Supplemental Fig S3 using Tukey's multiple comparisons test.

		2.5 mg/mL fibrin				10 mg/mL fibrin				20 mg/mL fibrin			
		Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
2.5 mg/mL fibrin	Day 0		n.s.	*	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Day 1			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Day 2				n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	*	n.s.
	Day 3					***	n.s.	**	n.s.	***	***	***	**
10 mg/mL fibrin	Day 0						n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Day 1							n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Day 2								n.s.	n.s.	n.s.	n.s.	n.s.
	Day 3									n.s.	n.s.	n.s.	n.s.
20 mg/mL fibrin	Day 0										n.s.	n.s.	n.s.
	Day 1											n.s.	n.s.
	Day 2												n.s.
	Day 3												

n.s. = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

Supplemental Table 7. Calculated p-values for the Akt KTR in MDA-MB-231 cells from data in Supplemental Fig S4 using Tukey's multiple comparisons test.

		2.5 mg/mL fibrin				10 mg/mL fibrin				20 mg/mL fibrin			
		Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
2.5 mg/mL fibrin	Day 0		***	***	***	n.s.	***	***	***	n.s.	**	**	n.s.
	Day 1			n.s.	n.s.	***	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.
	Day 2				n.s.	***	*	n.s.	n.s.	***	**	*	***
	Day 3					***	**	n.s.	n.s.	***	***	**	***
10 mg/mL fibrin	Day 0						***	***	***	n.s.	*	**	n.s.
	Day 1							n.s.	n.s.	***	n.s.	n.s.	n.s.
	Day 2								n.s.	n.s.	n.s.	n.s.	**
	Day 3									***	n.s.	n.s.	**
20 mg/mL fibrin	Day 0										*	**	n.s.
	Day 1											n.s.	n.s.
	Day 2												n.s.
	Day 3												

n.s. = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

Supplemental Table 8. Calculated p-values for the ERK KTR in MDA-MB-231 cells from data in Supplemental Fig S4 using Tukey's multiple comparisons test.

		2.5 mg/mL fibrin				10 mg/mL fibrin				20 mg/mL fibrin			
		Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
2.5 mg/mL fibrin	Day 0		***	***	***	n.s.	**	***	***	n.s.	*	***	***
	Day 1			n.s.	n.s.	**	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.
	Day 2				n.s.	***	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.
	Day 3					***	**	n.s.	n.s.	***	***	n.s.	n.s.
10 mg/mL fibrin	Day 0						*	***	***	n.s.	n.s.	**	***
	Day 1							n.s.	n.s.	**	n.s.	n.s.	n.s.
	Day 2								n.s.	***	n.s.	n.s.	n.s.
	Day 3									***	n.s.	n.s.	n.s.
20 mg/mL fibrin	Day 0										**	***	***
	Day 1											n.s.	n.s.
	Day 2												n.s.
	Day 3												

n.s. = not significant, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

	Day 0	**Day 1**	**Day 2**	**Day 3**
Supplemental Table 9. Ninety five percent (95%) confidence intervals for linear regression of Akt KTR vs. distance from bubble for Figure 3B.				
Equation	y = -0.0005935x + 0.2037	y = -0.0004201x + 0.1099	y = -0.001566x + 0.1790	y = -0.0005983x + 0.09662
Slope	-0.0008264 to -0.0003606	-0.0006227 to -0.0002175	-0.001977 to -0.001155	-0.0009211 to -0.0002756
Y-intercept	0.1632 to 0.2441	0.08359 to 0.1363	0.1352 to 0.2227	0.06068 to 0.1326
X-intercept	280.0 to 477.6	203.3 to 413.6	100.6 to 131.1	128.3 to 246.9

	Day 0	Day 1	Day 2	Day 3
Supplemental Table 10. Ninety five percent (95%) confidence intervals for linear regression of ERK KTR vs. distance from bubble for Figure 3B.				
Equation	y = -0.0007124x + 0.3955	y = -0.0006304x + 0.2849	y = -0.001362x + 0.3461	y = -0.0009457x + 0.3018
Slope	-0.001027 to -0.0003978	-0.0009266 to -0.0003342	-0.001889 to -0.0008353	-0.001383 to -0.0005080
Y-intercept	0.3408 to 0.4501	0.2464 to 0.3235	0.2901 to 0.4022	0.2531 to 0.3506
X-intercept	425.8 to 882.0	338.1 to 761.3	206.0 to 358.8	246.2 to 512.9

	Day 0	Day 1	Day 2	Day 3
Supplemental Table 11. Ninety five percent (95%) confidence intervals for linear regression of Akt KTR vs. distance from bubble for Supplemental Figure S5.				
Equation	y = -0.0002381x + 0.2693	y = -0.0009537x + 0.1982	y = -0.001898x + 0.3603	y = -0.001101x + 0.2443
Slope	-0.001010 to 0.0005335	-0.0009537 to 0.0005257	-0.002375 to -0.001421	-0.001378 to -0.0008246
Y-intercept	0.2109 to 0.3278	0.1252 to 0.2711	0.3117 to 0.4089	0.1987 to 0.2898
X-intercept	311.9 to infinity	262.5 to infinity	162.9 to 231.8	188.6 to 268.7

Supplemental Table 12. Ninety five percent (95%) confidence intervals for linear regression of ERK KTR vs. distance from bubble for Supplemental Figure S5.

	Day 0	Day 1	Day 2	Day 3
Equation	y = -0.0002821x + 0.1691	y = -0.0008171x + 0.1280	y = -0.0008090x + 0.1800	y = -0.0003123x + 0.09255
Slope	-0.001075 to 0.0005114	-0.001374 to -0.0002602	-0.001194 to -0.0004243	-0.0005058 to -0.0001189
Y-intercept	0.1089 to 0.2292	0.07308 to 0.1829	0.1408 to 0.2192	0.06072 to 0.1244
X-intercept	198.6 to infinity	111.4 to 335.7	169.2 to 360.3	207.8 to 604.6

| **Supplemental Table 13. Ninety five percent (95%) confidence intervals for linear regression of Akt and ERK KTR vs. distance from bubble for Figure 4C.**		
	Akt	**ERK**
Equation	y = -0.03214x + 4.422	y = -0.05444x + 5.076
Slope	-0.05417 to -0.01011	-0.08944 to -0.01944
Y-intercept	2.057 to 6.786	1.319 to 8.833
X-intercept	113.9 to 223.8	57.81 to 115.9

Supplemental Table 14. Calculated p-values for the Akt KTR in MDA-MB-231 cells in mice from data in Figure 5B using Tukey's multiple comparisons test.

	Day 0 -ultrasound	Day 0 +ultrasound	Day 3 -ultrasound	Day 3 +ultrasound
Day 0 -ultrasound		*	*	n.s.
Day 0 +ultrasound			***	n.s.
Day 3 -ultrasound				**
Day 3 +ultrasound				

n.s. = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Supplemental Table 15. Calculated p-values for the ERK KTR in MDA-MB-231 cells in mice from data in Figure 5B using Tukey's multiple comparisons test.

	Day 0 -ultrasound	Day 0 +ultrasound	Day 3 -ultrasound	Day 3 +ultrasound
Day 0 -ultrasound		***	n.s.	***
Day 0 +ultrasound			***	**
Day 3 -ultrasound				***
Day 3 +ultrasound				

n.s. = not significant, ** = $p < 0.01$, *** = $p < 0.001$.

Supplemental Table 16. Ninety five percent (95%) confidence intervals for linear regression of Akt and ERK KTR vs. distance from bubble for Figure 5C.

	Akt Day 3	ERK Day 3
Equation	y = -0.001024x + 0.09879	y = -0.001083x + 0.2895
Slope	-0.001358 to -0.0006900	-0.001450 to -0.0007164
Y-intercept	0.04527 to 0.1523	0.2308 to 0.3482
X-intercept	58.18 to 126.4	223.3 to 346.6
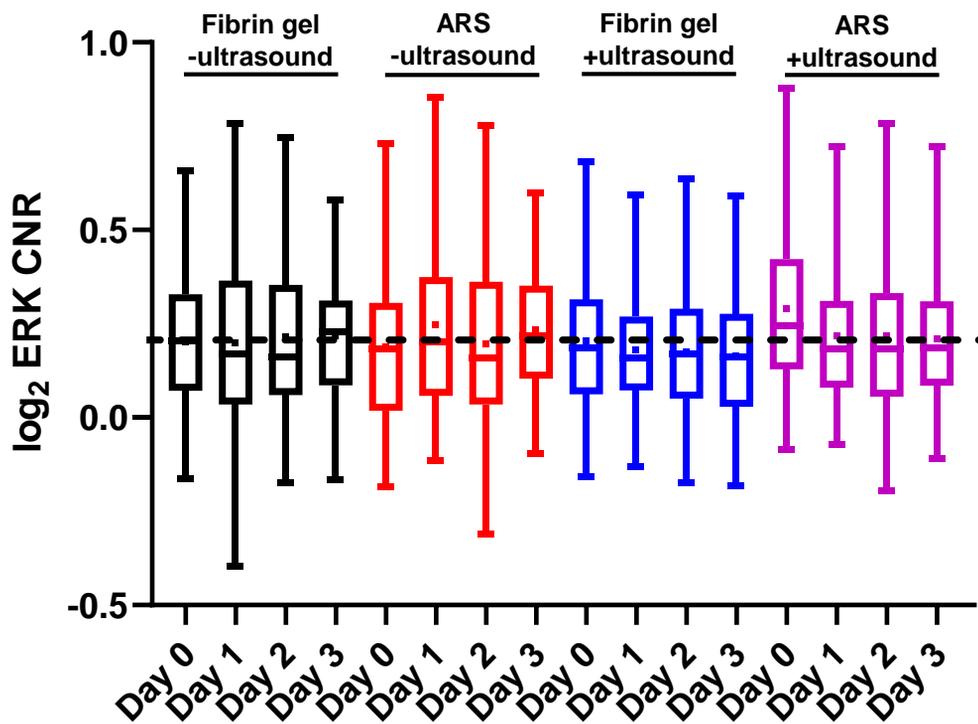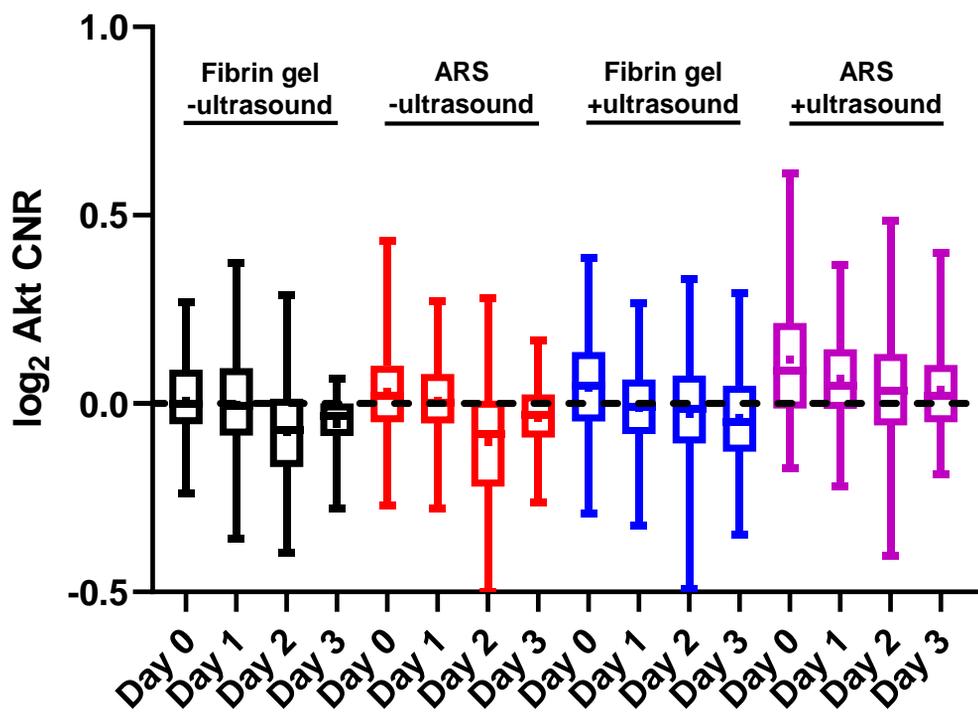
Supplemental Figure S1. ADV results in elevated Akt and ERK signaling in MDA-MB-231 cells. Box plot and whiskers for quantified $\log_2$ cytoplasmic/nuclear fluorescence intensities for Akt (top) and ERK (bottom) activities based on the imaging from **Fig 3**. Line within the box denotes the median, and the "+" symbol denotes the mean. Dashed line represents the median of the control group at the initial time point.
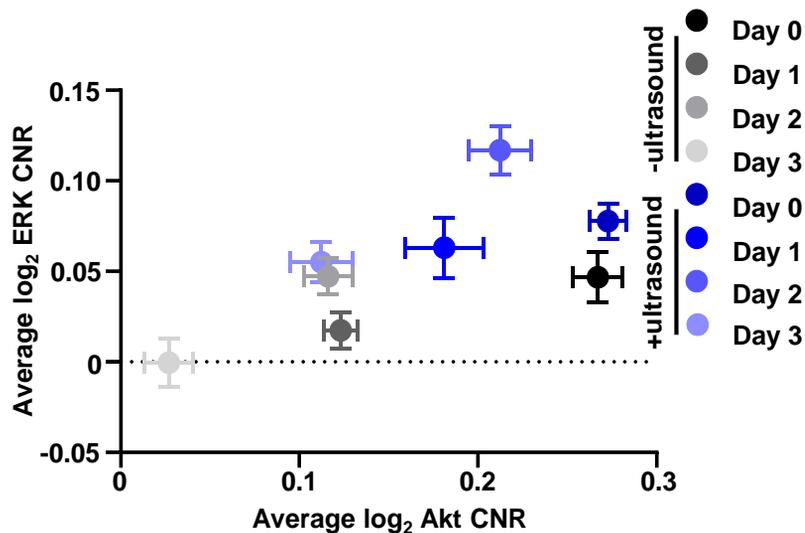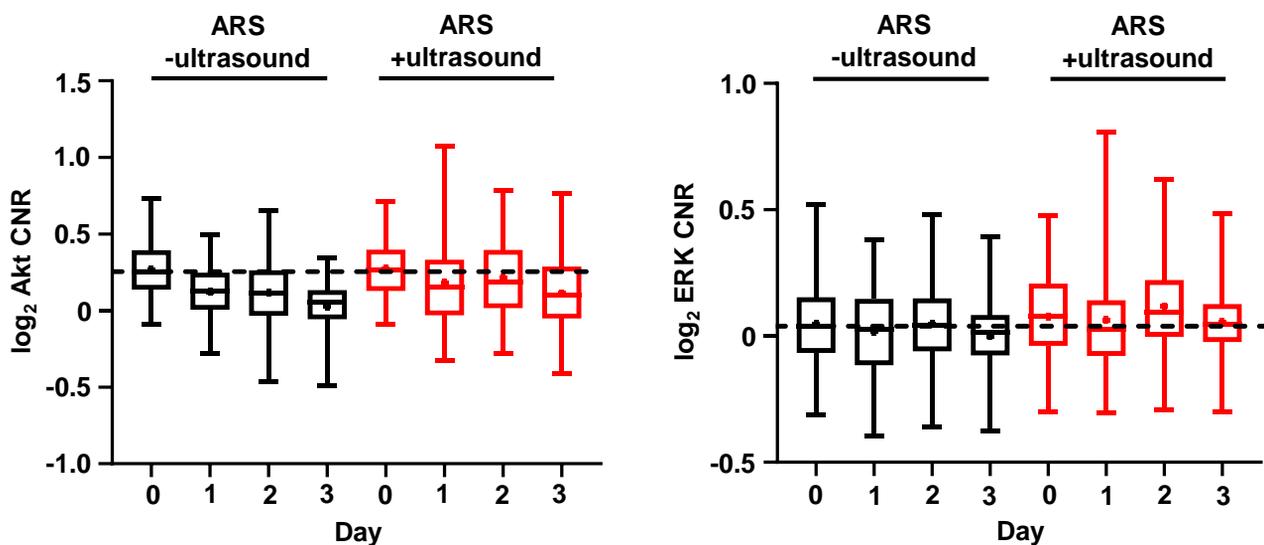
Supplemental Figure S2. ADV results in elevated Akt and ERK signaling in SUM159 cells. A. Graphs show mean ± SEM Akt vs. ERK KTR in SUM159 breast cancer cells treated with or without targeted ultrasound cultured in ARSs with the phase-shift double emulsion (n ≥ 186 cells per group). Cells were imaged immediately after targeted ultrasound (Day 0) and for 3 subsequent days. **B.** Box plot and whiskers for quantified $\log_2$ cytoplasmic/nuclear fluorescence intensities for Akt (left) and ERK (right) activities based on the imaging from (**A**). Line within the box denotes the median, and the "+" symbol denotes the mean. Dashed line represents the median of the control group at the initial time point.
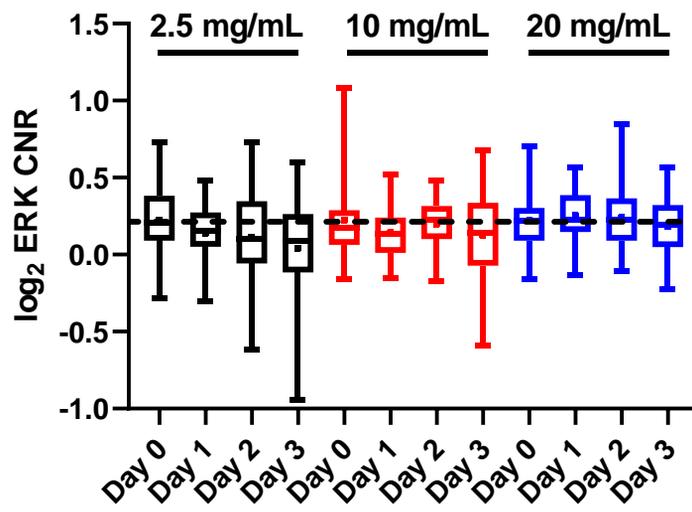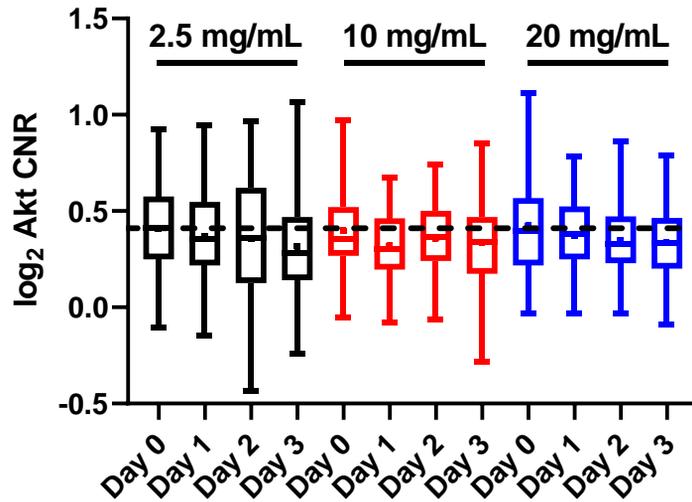
Supplemental Figure S3. Enhanced fibrin stiffness increased Akt and ERK signaling in SUM159 cells. Box plot and whiskers for quantified $\log_2$cytoplasmic/nuclear fluorescence intensities for Akt (top) and ERK (bottom) activities in gels with different stiffnesses driven by different fibrin concentrations (n ≥ 67 cells per group). Line within the box denotes the median, and the "+" symbol denotes the mean. Dashed line represents the median of the control group at the initial time point.
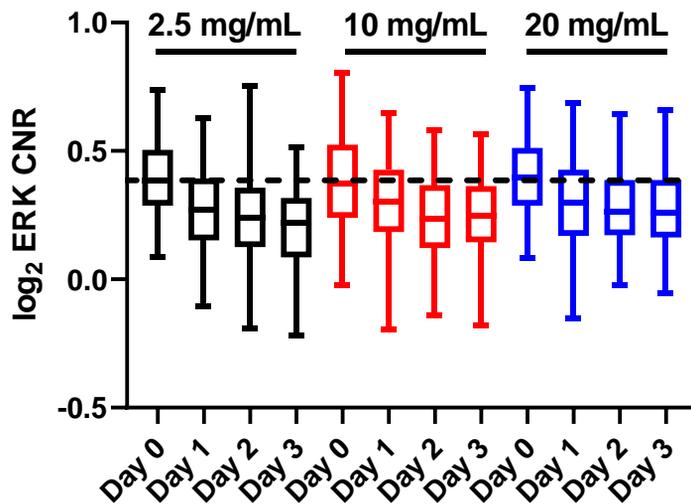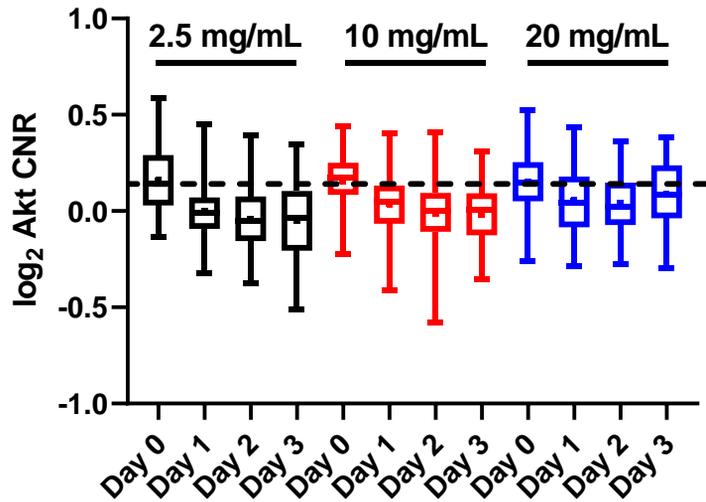
Supplemental Figure S4. Enhanced fibrin stiffness increased Akt and ERK signaling in MDA-MB-231 cells. Box plot and whiskers for quantified $\log_2$ cytoplasmic/nuclear fluorescence intensities for Akt (top) and ERK (bottom) activities in gels with different stiffnesses driven by different fibrin concentrations (n ≥ 80 cells per group). Line within the box denotes the median, and the "+" symbol denotes the mean. Dashed line represents the median of the control group at the initial time point.
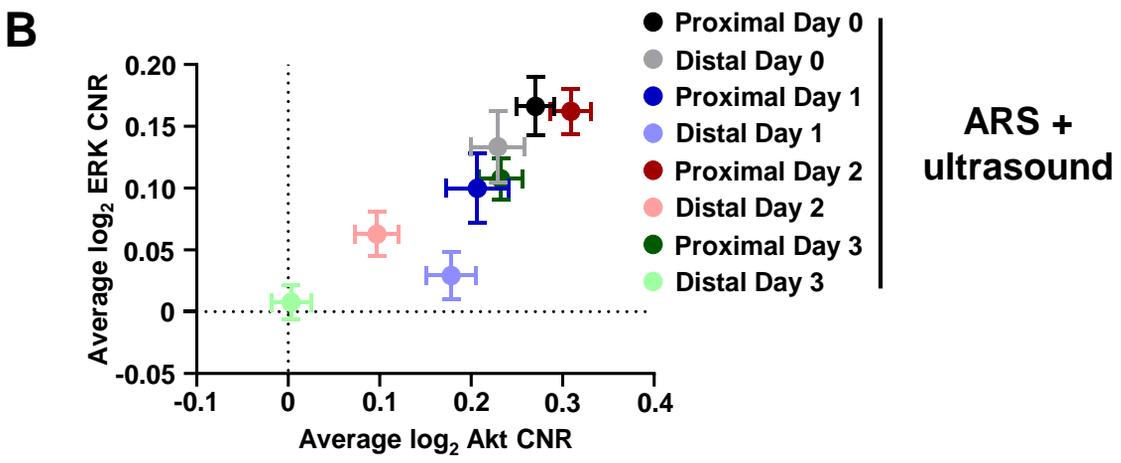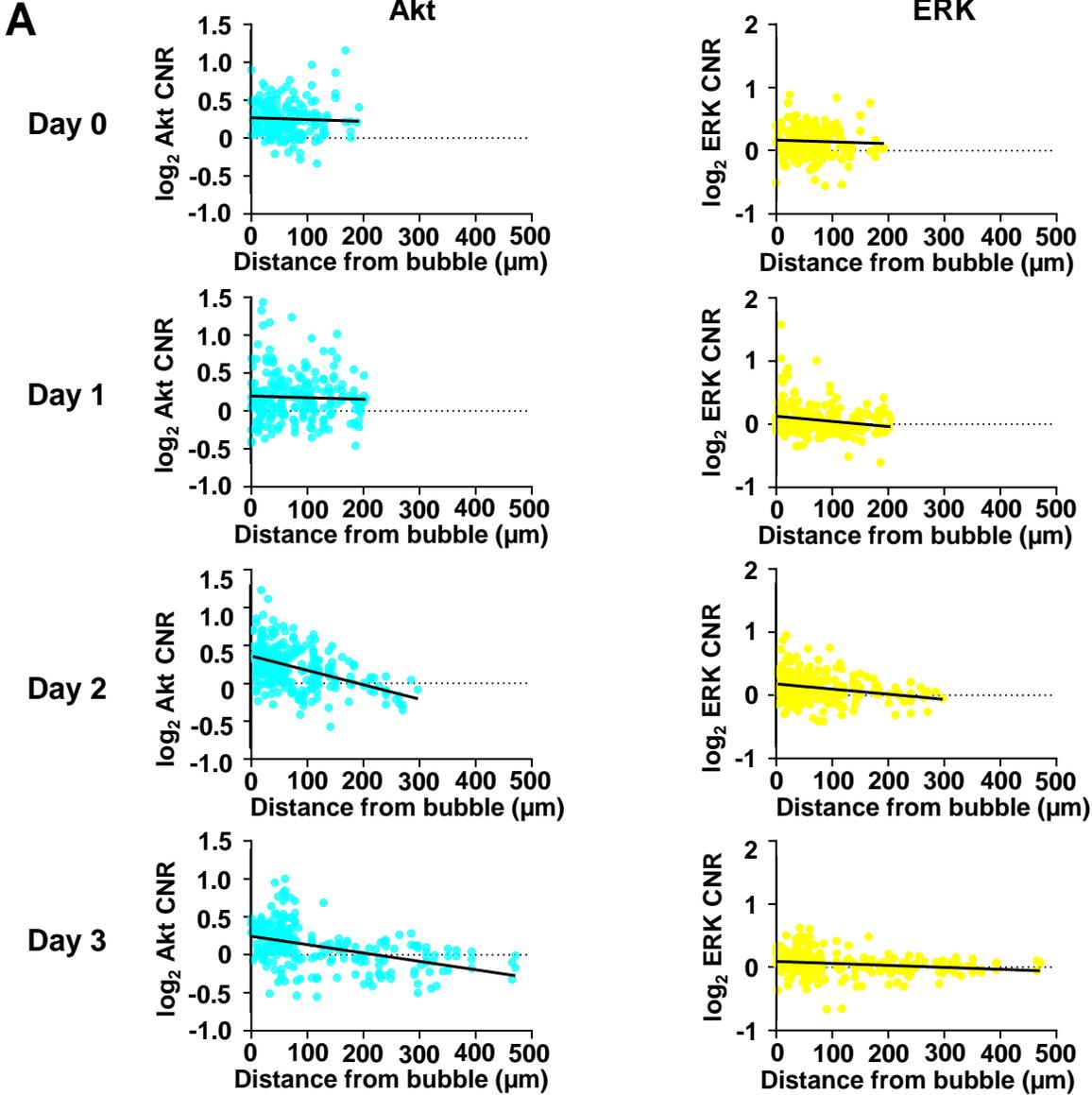
Supplemental Figure S5. Enhanced signaling in SUM159 breast cancer cells closer to the ADV-induced bubble. A. We quantified activation of Akt (left) and ERK (right) signaling relative to distance of the cell from the bubble surface (n ≥ 189 cells per group), demonstrating that cells closest to the bubble surface signal more. Day 1 ERK ($p < 0.01$), Day 2 Akt and ERK ($p < 0.001$), and Day 3 Akt and ERK graphs ($p < 0.01$) show a significant non-zero linear regression. **B.** Graph shows mean ± SEM Akt vs. ERK KTR of cells close (< 100μm) or far (> 100μm) from the bubble at each time point (n ≥ 75 cells per group).
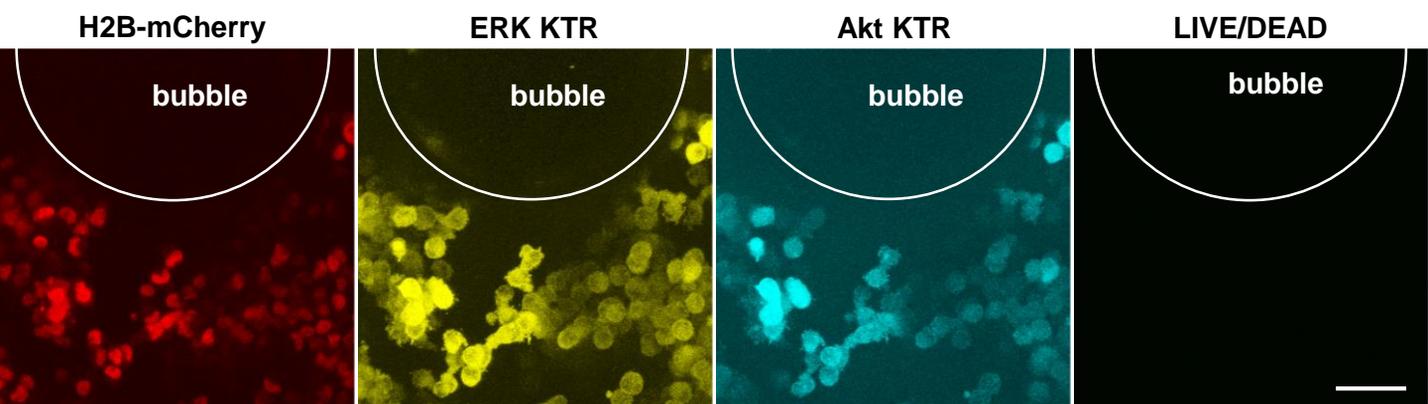
Supplemental Figure S6. LIVE/DEAD staining of MDA-MB-231 cells after ligand treated time-lapse imaging. Representative image of LIVE/DEAD staining of cells demonstrated that all cells imaged were alive after time-lapse imaging. Scale bar is 50 μm.