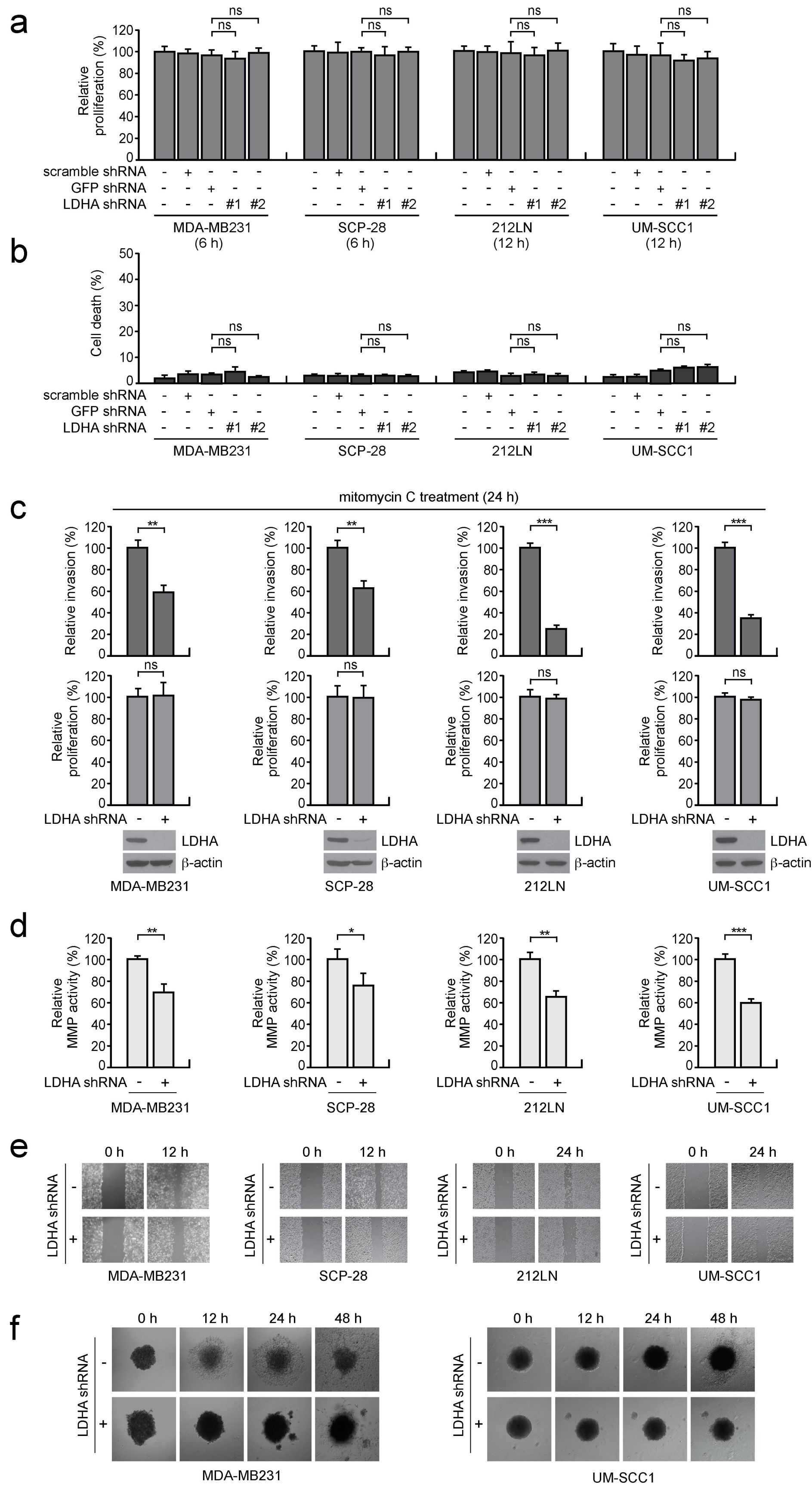
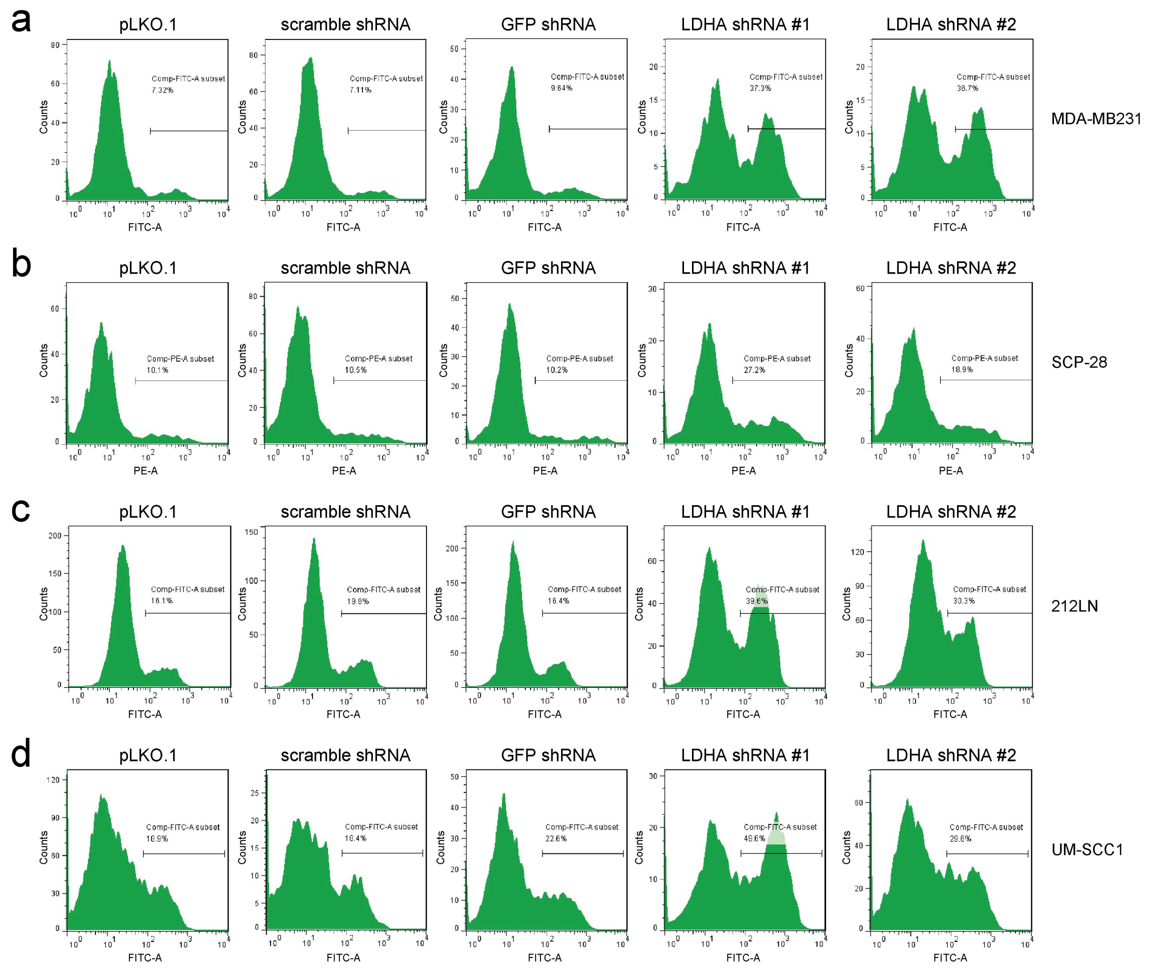
Phosphorylation-mediated activation of LDHA promotes cancer cell invasion and tumor metastasis

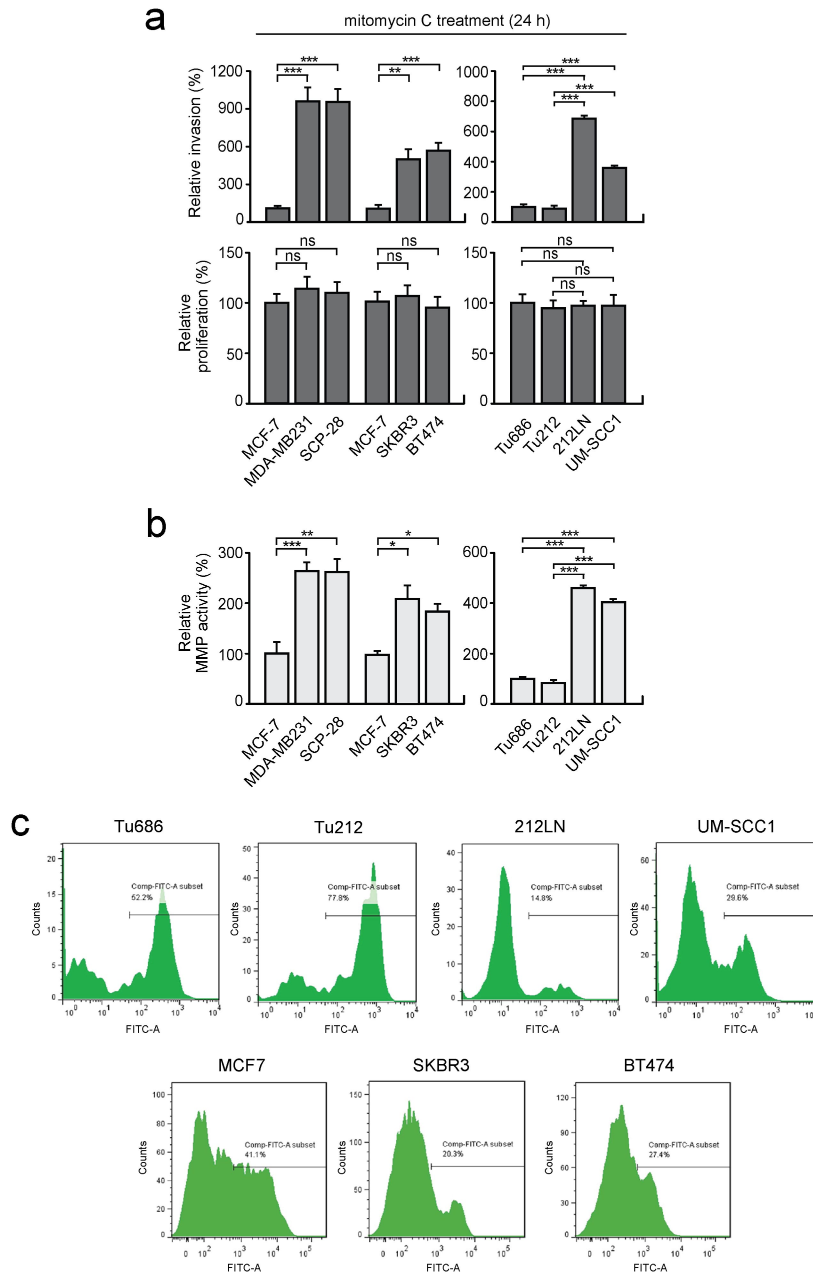
Jin et al. - Supplementary Figures



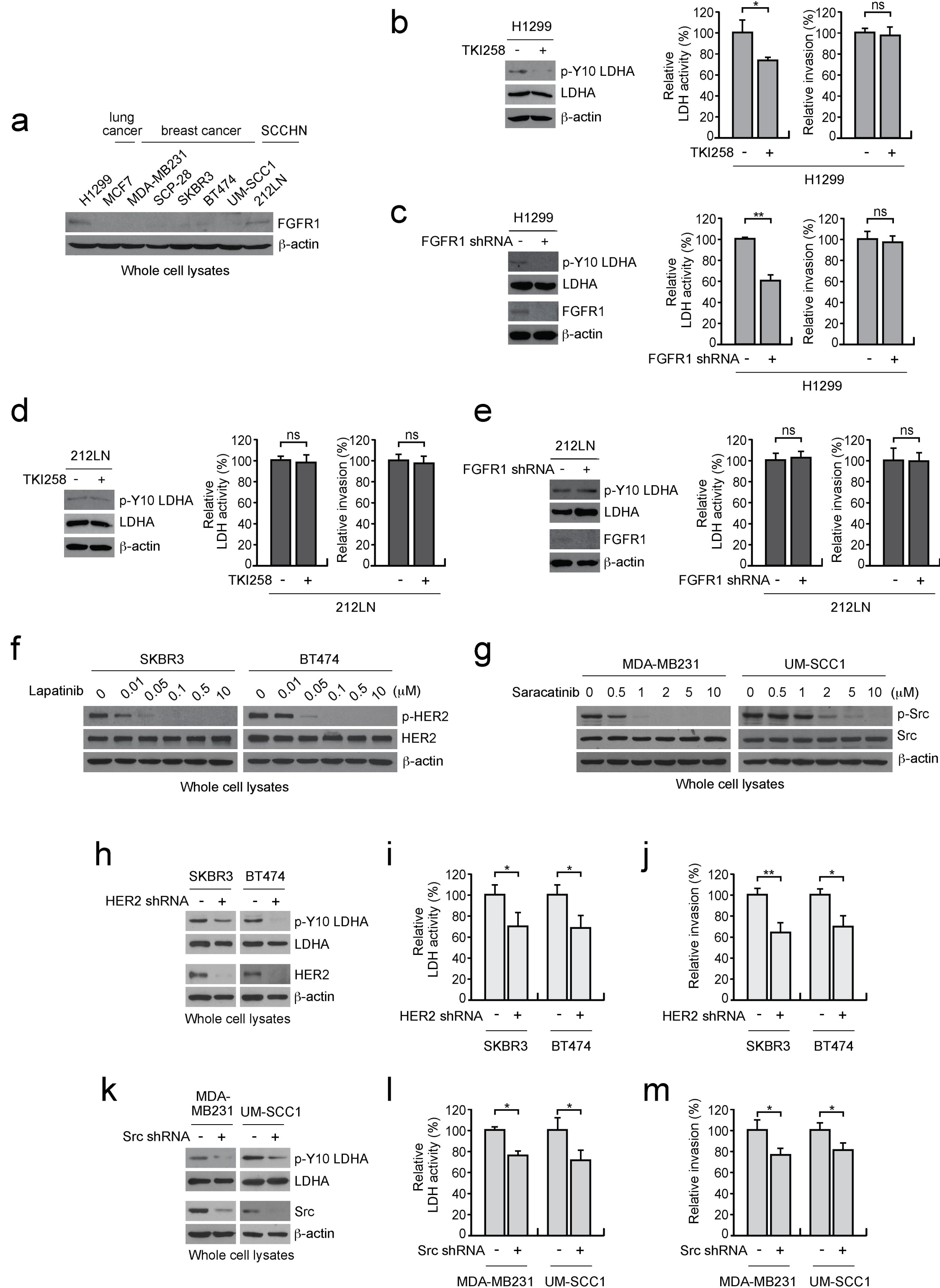
Supplementary Fig. 1. **(a-b)** Cell proliferation (a) and cell death (b) were determined in MDA-MB231, SCP-28, 212LN and UM-SCC1 cells with LDHA knockdown using two shRNA clones. pLKO.1 vector, scramble and GFP shRNA were used as controls. **(c)** MDA-MB231, SCP-28, 212LN and UM-SCC1 cells with empty vector or LDHA stable knockdown were treated with mitomycin C for 2 hours, followed by invasion assay and proliferation assay as described in the Materials and Methods. Western blot analyses of LDHA expression are shown. **(d-e)** MMP activity assay (d) and scratch assay (e) were performed in MDA-MB231, SCP-28, 212LN and UM-SCC1 cells with empty vector or LDHA stable knockdown. **(f)** Spheroid invasion assay was performed at different time points in MDA-MB231 and UM-SCC1 cells with empty vector or LDHA stable knockdown. Data are mean ± SD from three technical replicates of each sample. *P* values were determined using two-tailed Student's *t* test (ns: not significant, *: $0.01 < p < 0.05$, **: $0.001 < p < 0.01$, ***: $p < 0.001$).



Supplementary Fig. 2. Representative flow cytometry data for detachment-induced apoptotic cells in Figure 1c are shown.

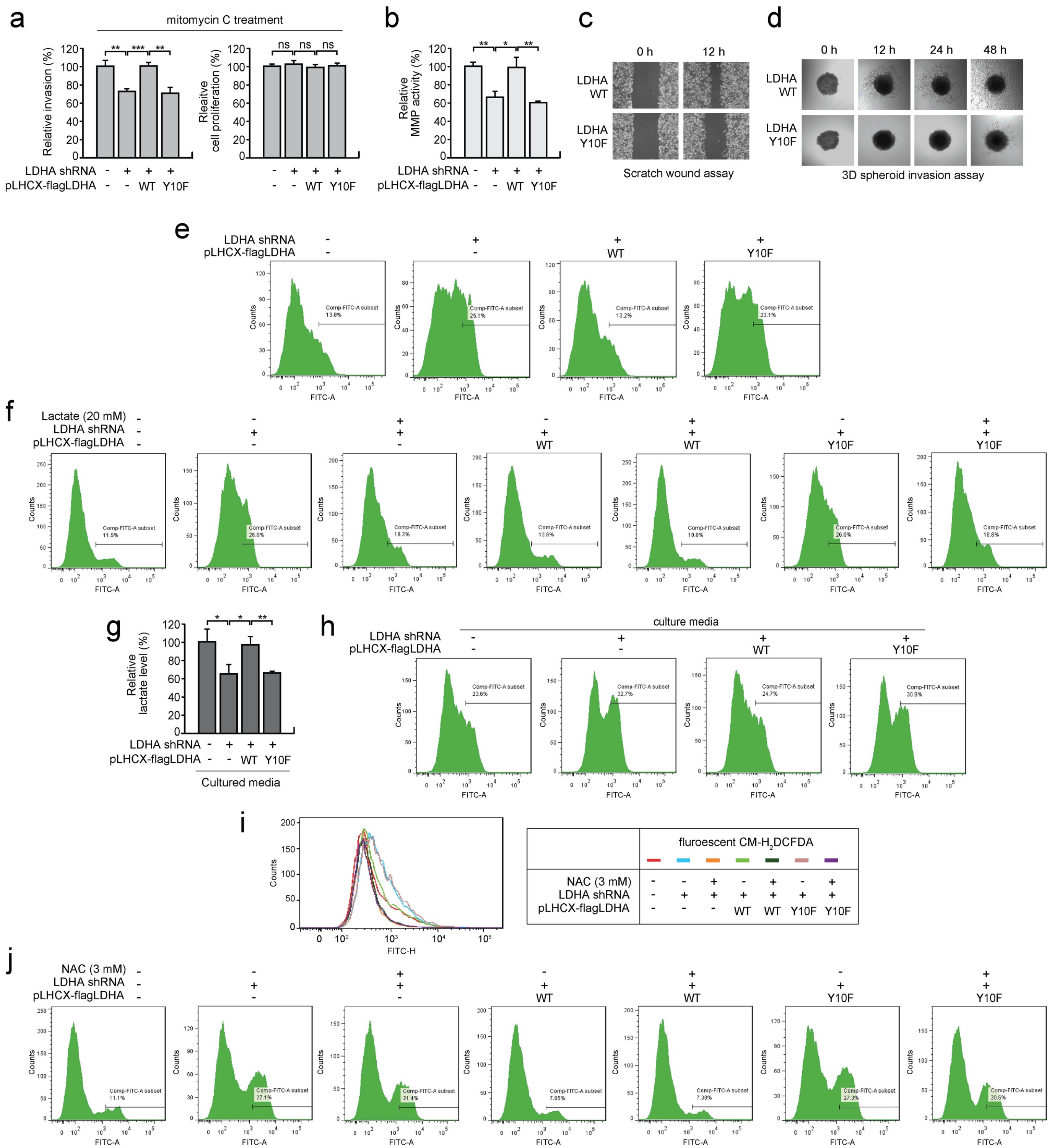


Supplementary Fig. 3. (a) Human breast cancer cells (MCF-7, MDA-MB231, SCP-28, SKBR3, BT474) and SCCHN cells (Tu686, Tu212, 212LN, UM-SCC1) were treated with 10 μg/ml of mitomycin C for 2 hours, followed by invasion assay and proliferation assay as described in the Materials and Methods. **(b)** MMP activity was measured in cells described in (a). **(c)** Representative flow cytometry data for Figure 2d. Data are mean ± SD from three technical replicates of each sample. *P* values were determined using two-tailed Student's *t* test (ns: not significant, *: 0.01< *p*< 0.05, **: 0.001< *p*<0.01, ***: *p*<0.001).



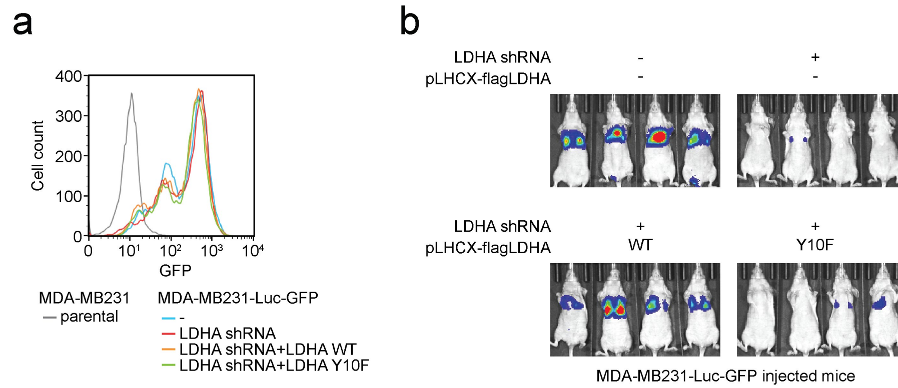
Supplementary Fig. 4. (a) The expression levels of FGFR1 were determined by western blot in diverse breast cancer cells and SCCHN cells. The lung cancer H1299 cell line was used as a positive control. **(b-c)** Inhibition of FGFR1 by 500 nM TKI258 (b) or FGFR1 shRNA (c) in H1299 cells leads to decreased LDHA Y10 phosphorylation and LDH activity, but shows no impact on cell invasion. **(d-e)** Inhibition of FGFR1 by 500 nM TKI258 (d) or FGFR1 shRNA (e) in 212LN cells does not affect LDHA Y10 phosphorylation,

activity or cell invasion. **(f)** SKBR3 and BT474 cells were treated with different concentrations of lapatinib and HER2 phosphorylation at Y1248 was determined by western blot analysis. **(g)** MDA-MB231 and UM-SCC1 cells were treated with different concentrations of saracatinib and Src phosphorylation at Y418 was determined by western blot analysis. **(h-j)** LDHA Y10 phosphorylation (h), LDH activity (i) and invasiveness (j) were determined in SKBR3 and BT474 cells with empty vector or HER2 stable knockdown. **(k-m)** LDHA Y10 phosphorylation (k), LDH activity (l) and invasiveness (m) were determined in MDA-MB231 and UM-SCC1 cells with empty vector or Src stable knockdown. Data are mean ± SD from three technical replicates of each sample. *P* values were determined using two-tailed Student's *t* test (ns: not significant, *: $0.01 < p < 0.05$, **: $0.001 < p < 0.01$).

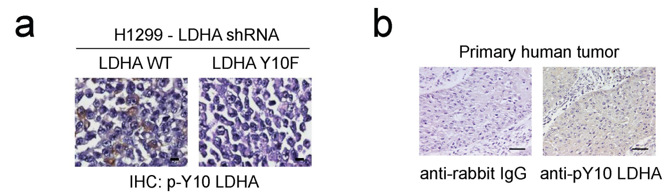


Supplementary Fig. 5. (a) MDA-MB231 cells with empty vector or stable LDHA knockdown were stably transduced with different retrovirus harboring LDHA variants and treated with mitomycin C for 2 hours, followed by invasion assay and cell proliferation assay as described in the Materials and Methods. **(b)** MMP

activity was determined in cells described in (a). **(c-d)** Scratch assay (c) and spheroid invasion assay (d) were performed in MDA-MB231 LDHA knockdown cells expressing LDHA WT or Y10F mutant. **(e)** Representative flow cytometry data for Figure 5d. **(f)** Representative flow cytometry data for Figure 5f. **(g)** Lactate levels were determined in MDA-MB231 cells incubated for 48 hours with culture media from MDA-MB231 cells with empty vector or stable LDHA knockdown and stably expressing different LDHA variants. **(h)** Representative flow cytometry data for Figure 5g right. **(i)** Representative flow cytometry data for Figure 5h. **(j)** Representative flow cytometry data for Figure 5j. Data are mean ± SD from three technical replicates of each sample. *P* values were determined using two-tailed Student's *t* test (ns: not significant, *: $0.01 < p < 0.05$, **: $0.001 < p < 0.01$, ***: $p < 0.001$).



Supplementary Fig. 6. (a) Comparable levels of luciferase-GFP among cell lines used in animal study were confirmed by flow cytometric analysis of GFP level. **(b)** Representative images of mice injected with MDA-MB231 cells with empty vector or LDHA knockdown and different LDHA variants at day 42.



Supplementary Fig. 7. The specificity of phopho-Y10 LDHA antibody used in immunohistochemistry (IHC) analysis was evaluated using paraffin-embedded H1299 cancer cells with expression of LDHA wild type (WT) or Y10F with stable knockdown of endogenous LDHA (a) and primary tumor tissue samples from breast cancer patients (b). Scale bars indicate 10 μm for (a) and 50 μm for (b).

Reagents

pLKO.1-puro lentiviral vector-based shRNA construct for FGFR1, HER2, or Src knockdown was purchased from Dharmacon, GE Healthcare Life Sciences (Lafayette, CO). The sense strand of the FGFR1 shRNA were clone was 5'- TTGAGTCCGCCATTGGCAAGC-3' (TRCN0000121308). The sense strands of the HER shRNA and Src shRNA were 5'-ATTGTCTTCAATGAGCCGAGC-3' (TRCN0000038149) and 5'-TACAAAGCCTGGATACTGACA-3' (TRCN0000039878), respectively.

MMP activity assay, scratch assay and spheroid invasion assay

MMP activity was determined using MMP Activity Assay Kit (ab112146, Abcam, UK) according to the manufacturer's protocol. Briefly, 1×10^5 cells were seeded into 12-well plates and incubated for 24 hours. 25 µl of culture media was mixed with 25 µl of 2 mM APMA working solution and incubated for 15 min followed by addition of 50 µl of the MMP green substrate solution. MMP activity was determined by recording the fluorescence at 490nm/525 nm.

Scratch wound assay

Cells were seeded into 6-well plates and grown to 80–90% confluence. The cell monolayer was scratched with a 200 µL sterile pipette tip and cell migration was monitored using microscope at 12 or 24-hour time point.

3D spheroid invasion assay

5000 cells per well were seeded into Corning Costar ultra-low attachment multi-well plates (Sigma Aldrich, St. Louis, MO), and allowed to grow for 5 days. The spheroids were then embedded into 4 mg/ml of matrigel in 1 u-slide 8 well plate (iBidi, Martinsried, Germany), and supplied with culture media. Spheroids were monitored at different time points with Leica SP8 (Leica Microsystems, Mannheim, Germany).