Supplementary figure legends:

Figure S1: FACS sorting gating criteria

(A) FACS histograms showing the sorting gates for the CD44v^{high} and CD44v^{low} from the CD44v-expressing cell line, H1650. **(B)** FACS histograms showing the sorting gates for pan CD44^{high} and pan CD44^{low} from the CD44v-non-expressing cell line, A549. **(C, D)** FACS dot blots showing the sorting gates for CD44v^{high}/ALDH^{high}, CD44v^{high}/ALDH^{low}, CD44v^{low}/ALDH^{high} and CD44v^{low}/ALDH^{low} cells from H1650 and HCC827 cell lines.

Figure S2: Xenograft tumor formation

(A, B) 100,000 CD44v^{high}/ALDH^{high}, CD44v^{high}/ALDH^{low}, CD44v^{low}/ALDH^{high}, CD44v^{low}/ALDH^{low} cells were sorted from the CD44v-expressing cell lines, H1650 and HCC827, and transplanted into immunocompromised mice, H1650: n=9 and HCC287: n=9.

Figure S3: Comparison of the ability of different populations sorted from H1650 cells to form anchorage-independent colonies (tumorspheres)

The sorted four populations from H1650 cells were seeded in suspension in 1000, 500, 100, 50, 10 and 1 cell/well and observed for the presence of >200 μm tumorspheres for 2-4 weeks.

(A) A cartoon showing number of wells containing at least one tumorsphere from each cell type in relation to number of seeded cells. **(B)** Graph presentation of the relation between number of cells/well and percentage of wells with at least one tumorsphere in each cell type. **(C)** Representative colony images are shown.

Figure S4: Comparison of the ability of different populations sorted from HCC827 cells to form anchorage-independent colonies (tumorspheres)

The sorted four populations from HCC827 cells were seeded in suspension in 1000, 500, 100, 50, 10 and 1 cell/well and observed for the presence of >200 μm tumorspheres for 2-4 weeks.

(A) A cartoon showing number of wells containing at least one tumorsphere from each cell type in relation to number of seeded cells. **(B)** Graph presentation of the relation between number of cells/well and percentage of wells with at least one tumorsphere in each cell type. **(C)** Representative colony images.

Figure S5: FACS sorting using a strict gating criteria

(A, B) FACS dot blots showing the sorting gates for CD44v^{high}/ALDH^{high}, CD44v^{high}/ALDH^{low}, CD44v^{low}/ALDH^{high} and CD44v^{low}/ALDH^{low} cells from H1650 and HCC827 cell lines. The gates were set to sort less than 5% for each population.

Figure S6: Comparison of the ability of different populations strictly sorted from H1650 cells to form anchorage-independent colonies (tumorspheres)

The strictly sorted four populations from H1650 cells were seeded in suspension in 1000, 500, 100, 50, 10 and 1 cell/well and observed for the presence of >200 μm tumorspheres for 2-4 weeks.

(A) A cartoon showing number of wells containing at least one tumorsphere from each cell type in relation to number of seeded cells. **(B)** Graph presentation of the relation between number of cells/well and percentage of wells with at least one tumorsphere in each cell type. **(C)** Representative colony images.

Figure S7: Comparison of the ability of different populations strictly sorted from HCC827 cells to form anchorage-independent colonies (tumorspheres)

The strictly sorted four populations from HCC827 cells were seeded in suspension in 1000, 500, 100, 50, 10 and 1 cell/well and observed for the presence of >200 μm tumorspheres for 2-4 weeks.

(A) A cartoon showing number of wells containing at least one tumorsphere from each cell type in relation to number of seeded cells. **(B)** Graph presentation of the relation between number of cells/well and percentage of wells with at least one tumorsphere in each cell type. **(C)** Representative colony images.

Figure S8: Association between xenograft necrotic area size and genotypes of initiating cells from the H1650 cell line

Tumor blocks were sectioned half way through the longest axis to expose the widest surface area possible. Sections were stained with H&E and imaged. **(A)** Representative tumors from the four populations of the H1650 cell line. **(B)** The necrotic area/total tumor area % from at least 5 tumors were measured.

Figure S9: Association between xenograft necrotic area size and genotypes of initiating cells from the HCC827 cell line
Tumor blocks were sectioned half way through the longest axis to expose the widest surface area possible. Sections were stained with H&E and imaged. **(A)** Representative tumors from the four populations of the HCC827 cell line. **(B)** The necrotic area/total tumor area % from at least 5 tumors were measured.

Figure S10: Association between stroma amount and genotypes of initiating cells
Sections from xenografts from all the four populations, from both H1650 and HCC827 cell lines, were immunostained for e-cadherin and CD34, which mark cancer and stromal cells, respectively. Percentages of stromal to tumor area were measured. No significant differences were detected among the four populations from either cell line. **(A)** Representative image from a $CD44v^{high}/ALDH^{high}$ tumor from HCC827. **(B)** Representative high power image. Scale bars: A=200 μm, B=50 μm.

Figure S11: EGFR expression is not associated with $CD44v^{high}$ expression
Freshly sorted HCC827 cells were cytospun and immunostained for EGFR (red) and pEGFR (green). Representative images from H1650: **(A)** $CD44v^{high}/ALDH^{high}$, **(B)** $CD44v^{high}/ALDH^{low}$, **(C)** $CD44v^{low}/ALDH^{high}$ and **(D)** $CD44v^{low}/ALDH^{low}$ cells. Nuclei were stained with DAPI. Scale bars: 20 μm. (**E)** shows quantification of fluorescence intensity. No significant differences were detected among the different populations.

Figure S12: Effect of differentiation expression of CD44v and ALDH on ROS level
Freshly sorted $CD44v^{high}/ALDH^{high}$, $CD44v^{high}/ALDH^{low}$, $CD44v^{low}/ALDH^{high}$ and $CD44v^{low}/ALDH^{low}$ cells from H1650 and HCC827 cell lines were stained with H2DCFDA and their ROS levels were compared by flow cytometry.

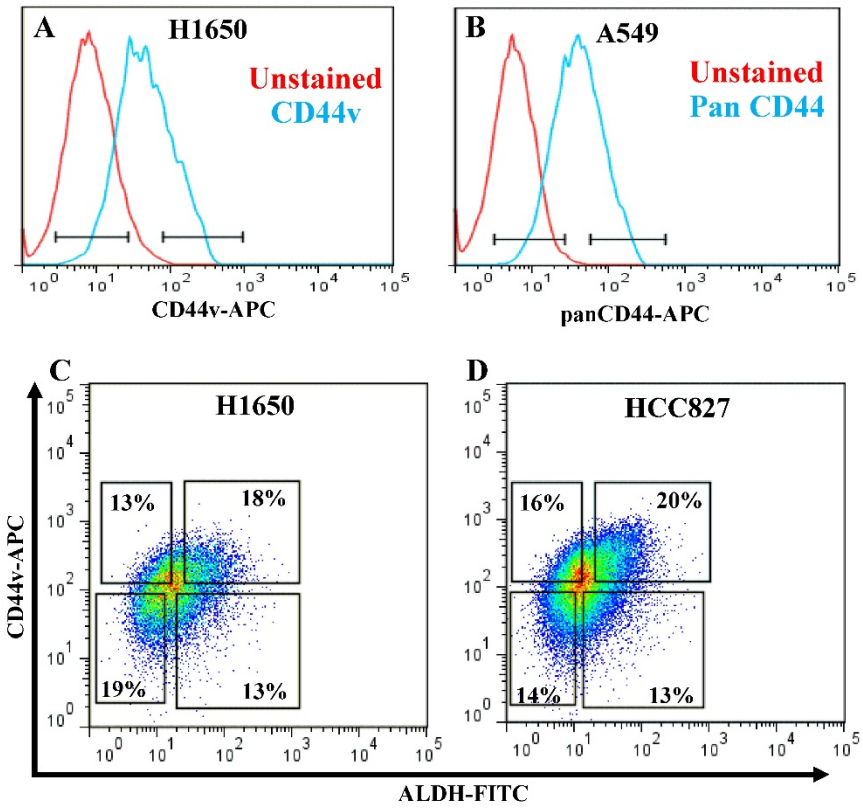

A
H1650
Unstained
CD44v
CD44v-APC
B
A549
Unstained
Pan CD44
panCD44-APC
C
H1650
13%
18%
19%
13%
D
HCC827
16%
20%
14%
13%
CD44v-APC
ALDH-FITC

Figure S1

Figure S2

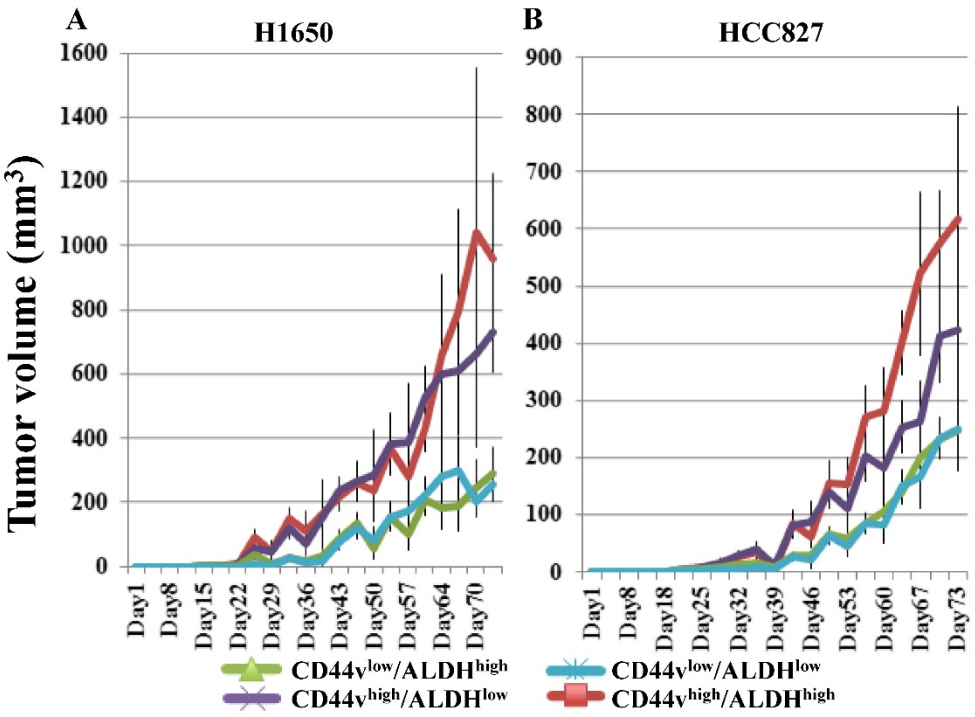
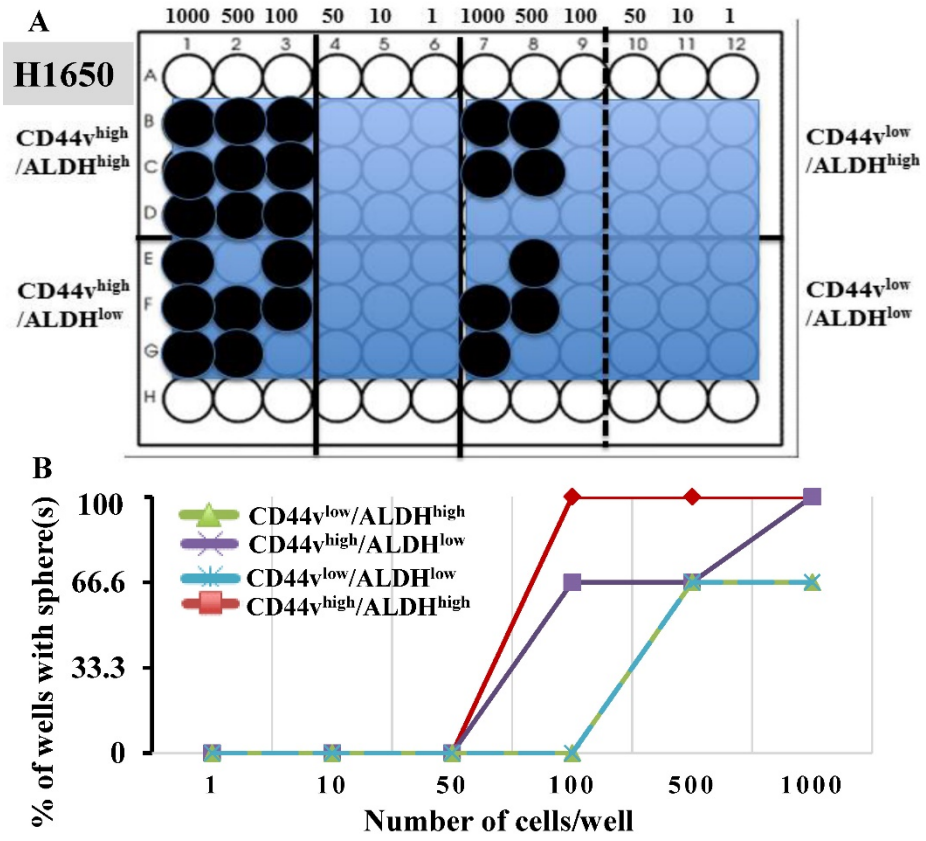

A
H1650
B
HCC827
Tumor volume (mm3)
1600
1400
1200
1000
800
600
400
200
0
900
800
700
600
500
400
300
200
100
0
Day1
Day8
Day15
Day22
Day29
Day36
Day43
Day50
Day57
Day64
Day70
Day1
Day8
Day18
Day25
Day32
Day39
Day46
Day53
Day60
Day67
Day73
CD44v low/ALDH high
CD44v high/ALDH low
CD44v low/ALDH low
CD44v high/ALDH high

Figure S3


A
H1650
1000 500 100 50 10 1 1000 500 100 50 10 1
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
CD44v^high /ALDH^high
CD44v^high /ALDH^low
CD44v^low /ALDH^high
CD44v^low /ALDH^low
B
100
66.6
33.3
0
% of wells with sphere(s)
CD44v^low/ALDH^high
CD44v^high/ALDH^low
CD44v^low/ALDH^low
CD44v^high/ALDH^high
1
10
50
100
500
1000
Number of cells/well

C

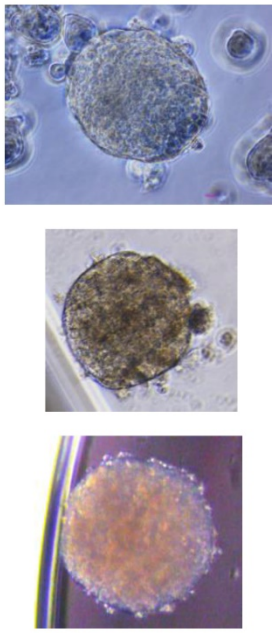
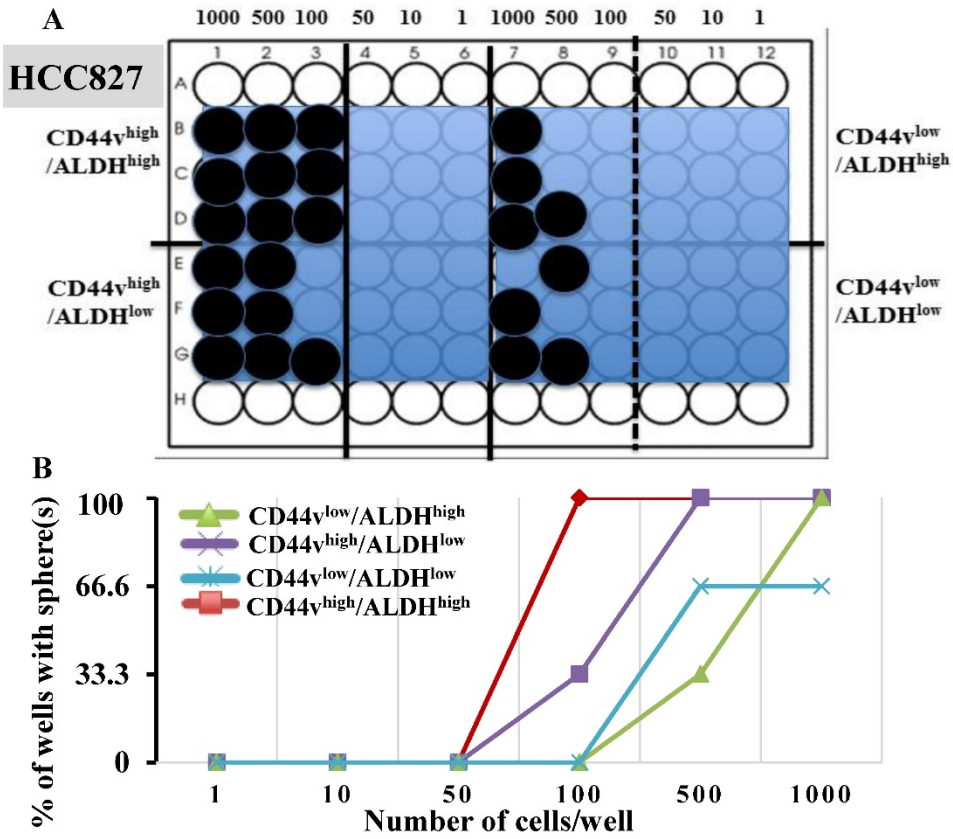
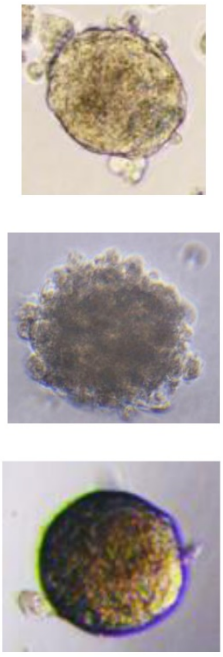


Figure S4


A
HCC827
1000 500 100 50 10 1 1000 500 100 50 10 1
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
CD44v^high /ALDH^high
CD44v^low /ALDH^high
CD44v^high /ALDH^low
CD44v^low /ALDH^low
B
% of wells with sphere(s)
100
66.6
33.3
0
CD44v^low/ALDH^high
CD44v^high/ALDH^low
CD44v^low/ALDH^low
CD44v^high/ALDH^high
1
10
50
100
500
1000
Number of cells/well

C



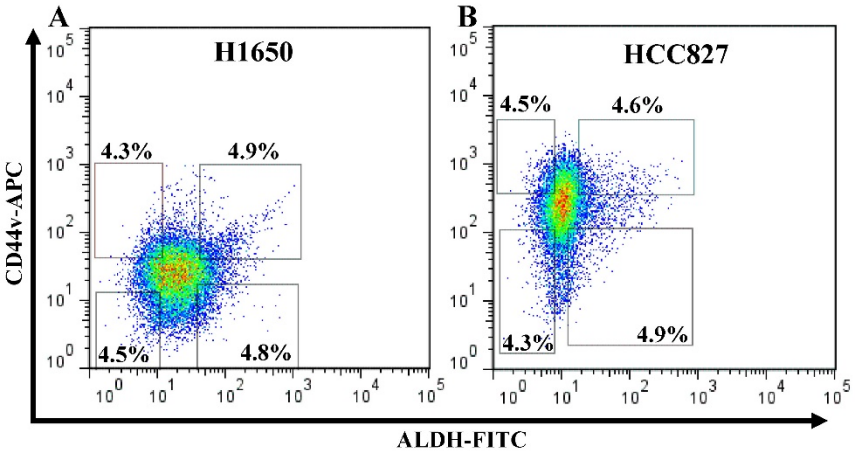


Figure S5

Figure S6

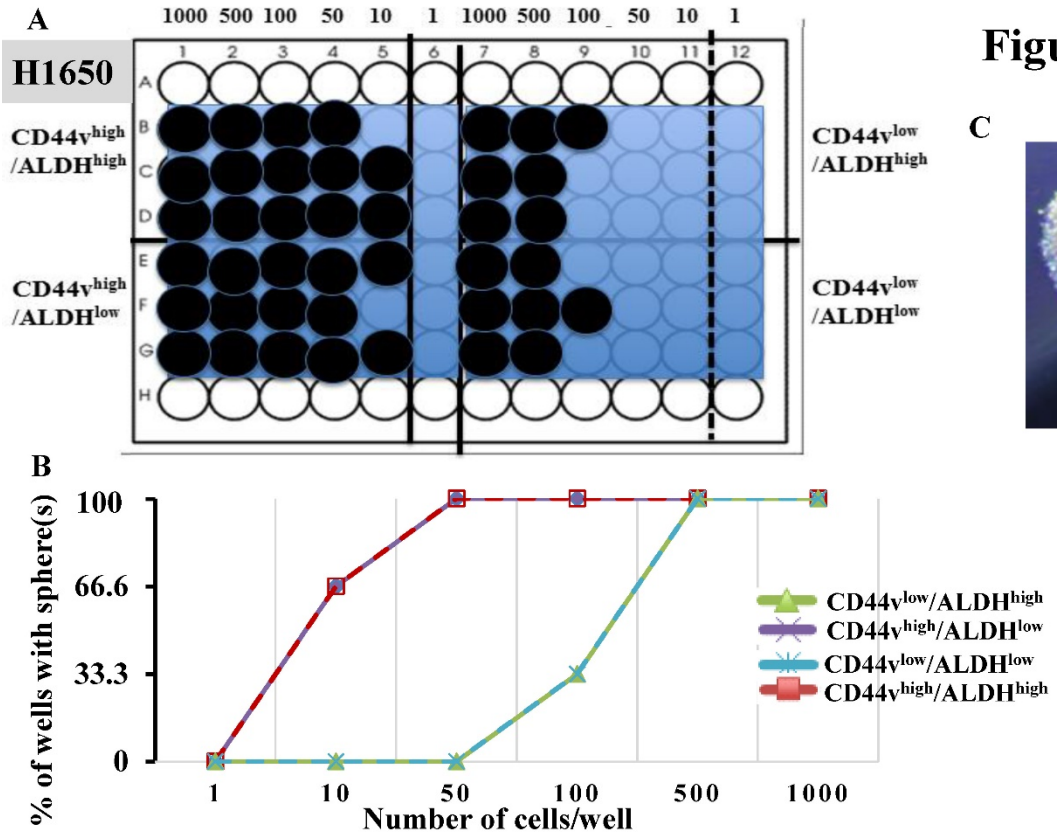
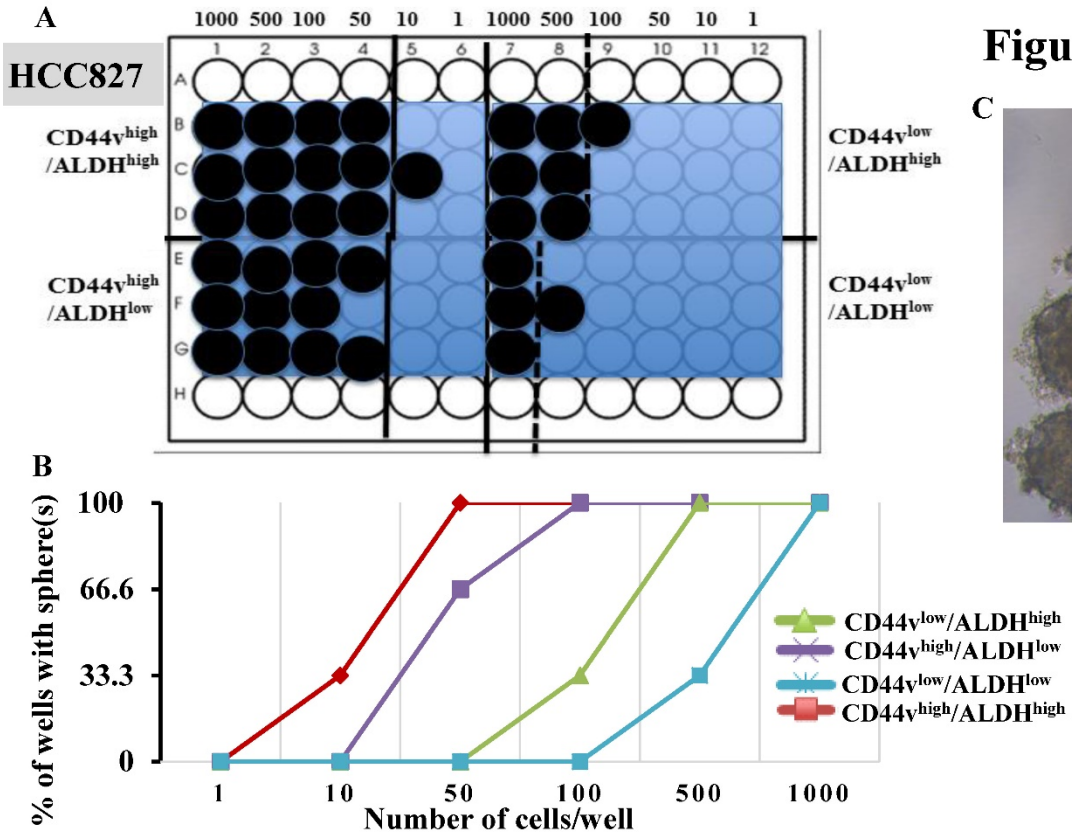

A
H1650
1000 500 100 50 10 1 1000 500 100 50 10 1
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
CD44v^high /ALDH^high
CD44v^low /ALDH^high
CD44v^high /ALDH^low
CD44v^low /ALDH^low
B
% of wells with sphere(s)
100
66.6
33.3
0
1
10
50
100
500
1000
Number of cells/well
CD44v^low/ALDH^high
CD44v^high/ALDH^low
CD44v^low/ALDH^low
CD44v^high/ALDH^high
C

Figure S7


A
HCC827
1000 500 100 50 10 1 1000 500 100 50 10 1
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
CD44v^high /ALDH^high
CD44v^low /ALDH^high
CD44v^high /ALDH^low
CD44v^low /ALDH^low
B
% of wells with sphere(s)
100
66.6
33.3
0
1
10
50
100
500
1000
Number of cells/well
CD44v^low/ALDH^high
CD44v^high/ALDH^low
CD44v^low/ALDH^low
CD44v^high/ALDH^high

C

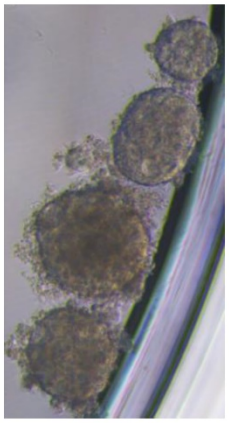


Figure S8

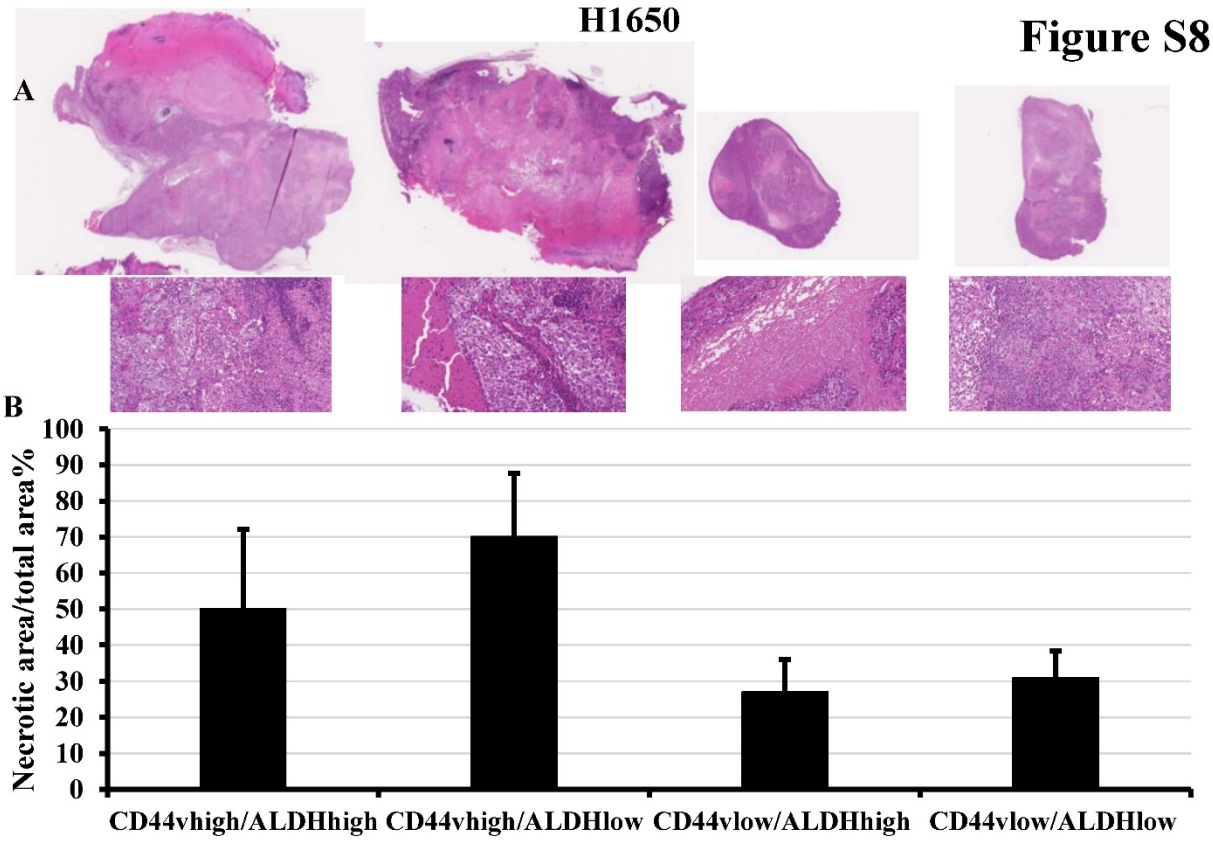
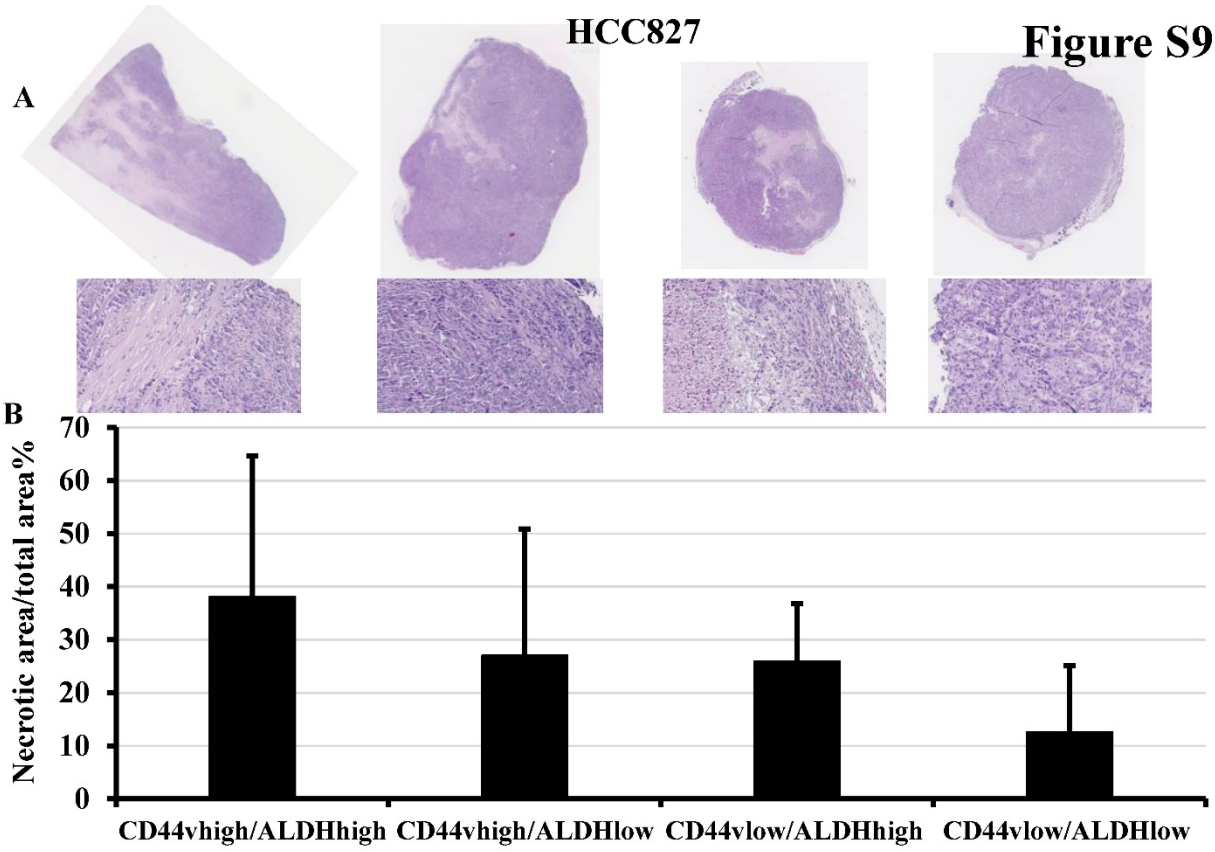

H1650
A
B
Necrotic area/total area%
100
90
80
70
60
50
40
30
20
10
0
CD44vhigh/ALDHhigh
CD44vhigh/ALDHlow
CD44vlow/ALDHhigh
CD44vlow/ALDHlow

Figure S9


HCC827
A
B
Necrotic area/total area%
70
60
50
40
30
20
10
0
CD44vhigh/ALDHhigh
CD44vhigh/ALDHlow
CD44vlow/ALDHhigh
CD44vlow/ALDHlow

E-cadherin/CD34/DAPI

HCC827

Figure S10

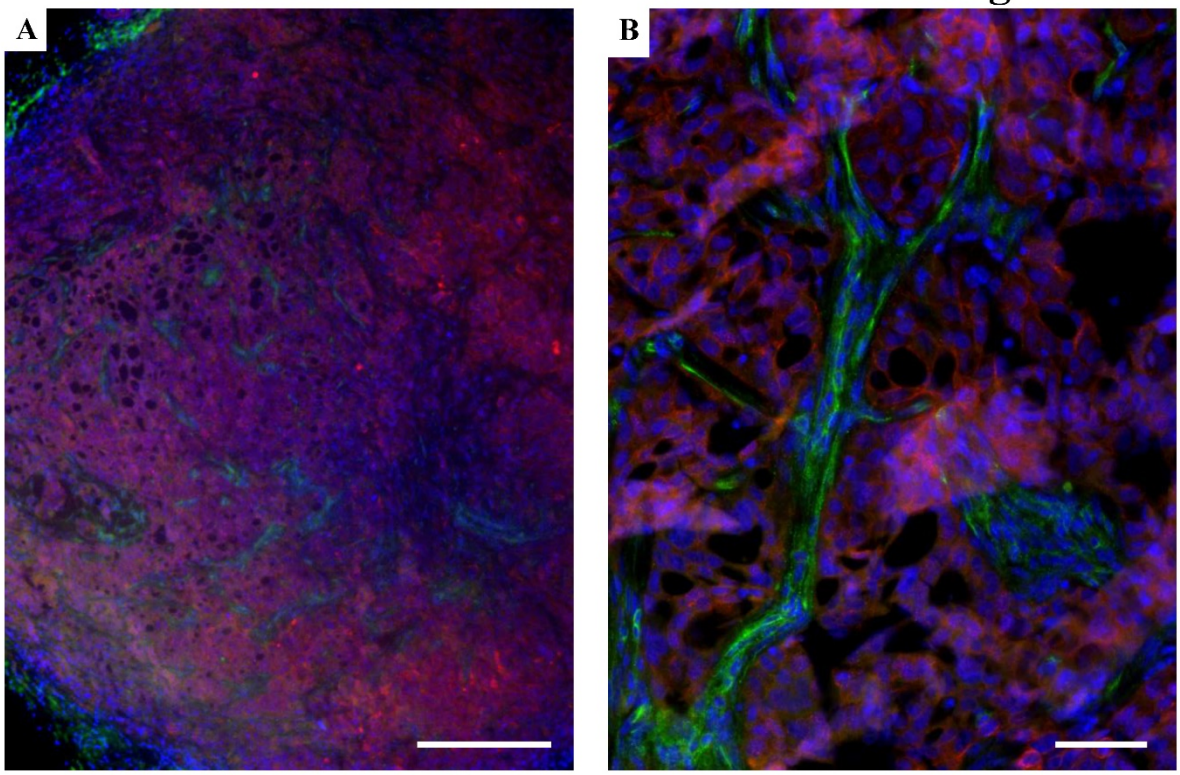


Figure S11

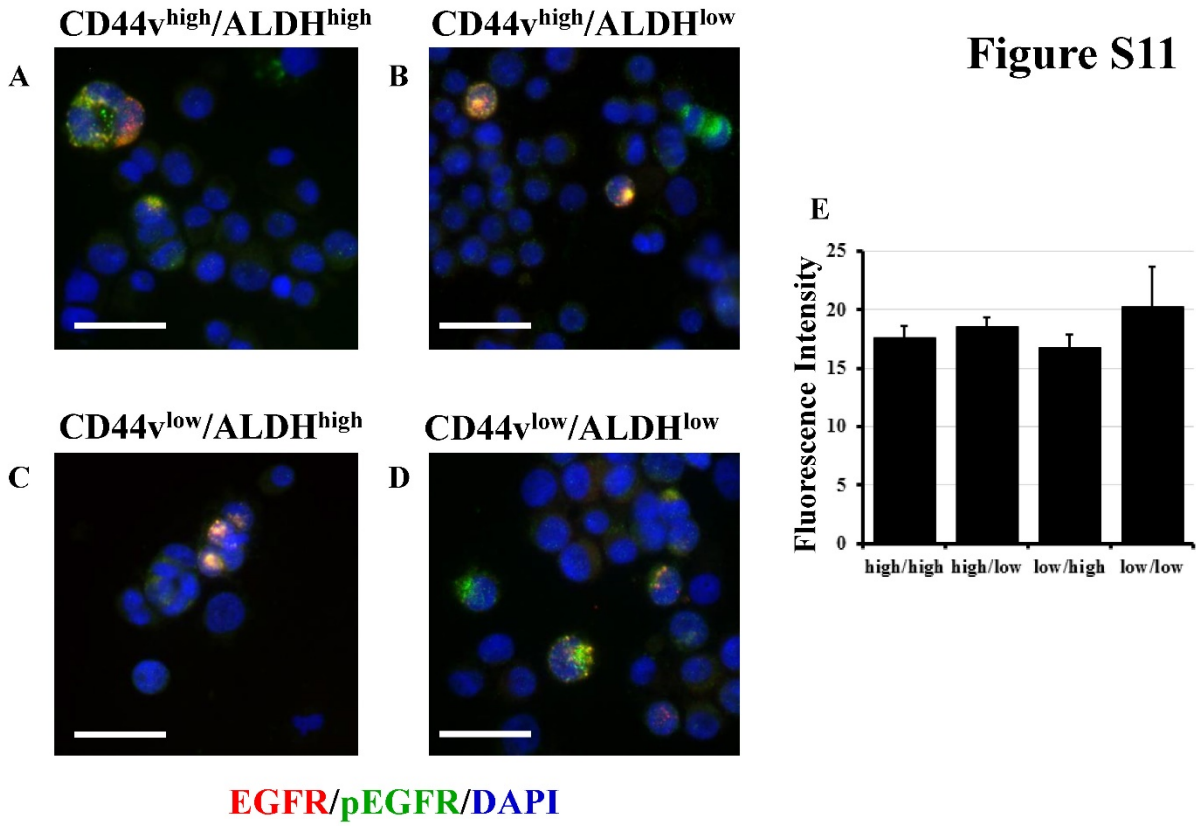
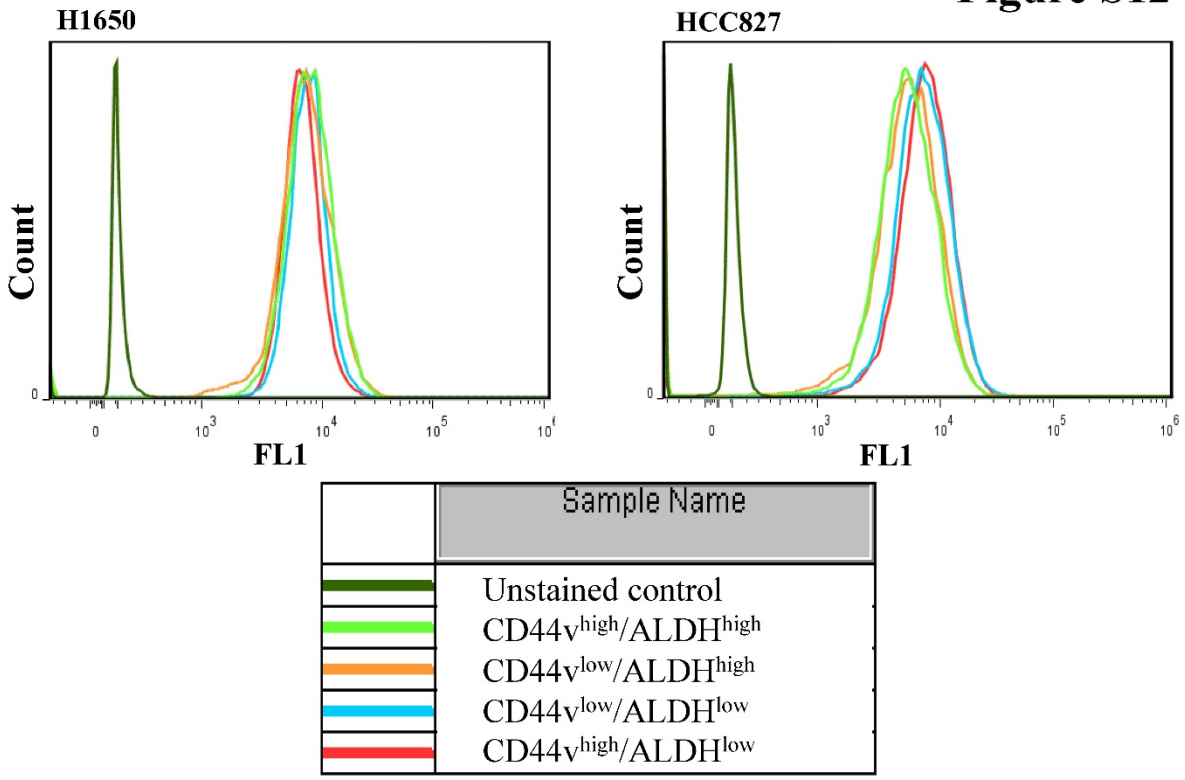

A
CD44v^high/ALDH^high
B
CD44v^high/ALDH^low
C
CD44v^low/ALDH^high
D
CD44v^low/ALDH^low
EGFR/pEGFR/DAPI
E
Fluorescence Intensity
25
20
15
10
5
0
high/high
high/low
low/high
low/low

Figure S12


H1650
HCC827
Count
FL1
Sample Name
Unstained control
CD44v^high/ALDH^high
CD44v^low/ALDH^high
CD44v^low/ALDH^low
CD44v^high/ALDH^low