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Supplementary Information

2D protein arrays induce 3D invivo-like assemblies of cells – cell types and cell densities

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The present studies reports on the capability of SbpA layers to induce the formation of multicellular spheroids (MCSs) using a standard tissue culture (TC) procedure. Though the communication mainly focusses on MCs made of HepG2, spheroid culture on SbpA-layer is not restricted to this cell line. Indeed, spheroids readily form out of carcinoma cell lines such as those of colorectal adenocarcinoma (Caco-2, figure S1a), breast carcinoma (MCF-7, figure S1b), as well as human embryonic kidney 293 cells (HEK293, figure S1c) or primary cells, such as HUVECs (figure S1d).

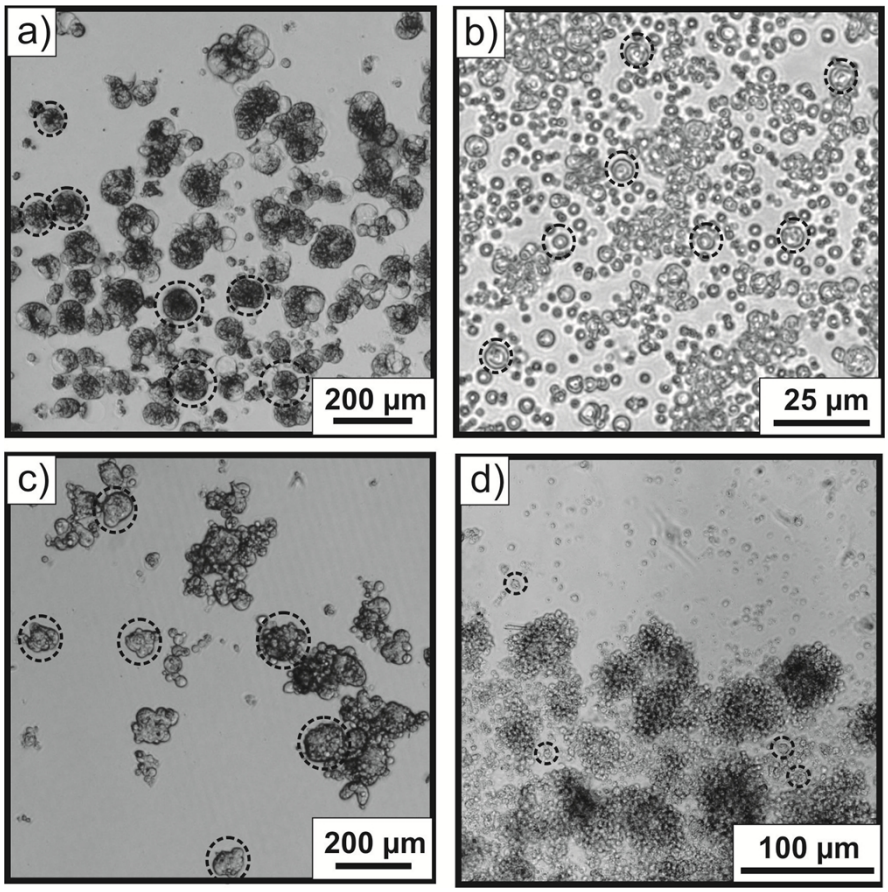

Figure S1. PhC optical micrographs showing large populations of four different homotypic MCSs, cultured on SbpA-layers. For the sake of clarity, some individual MCSs have been depicted in each case. (a) MCSs of Caco-2 cells; (b) MCSs of MCF-7 cells; (c) MCSs of HEK293 cells (d) MCSs of HUVECs. The bubble-like appearance of MCSs of MCF-7 cells is a known fact (http://www.scivax.com/usa/pdf/CellLine.pdf). Cells organized around an inside space or lumen as revealed by transmission electron microscopy (http://www.scivax.com/usa/application/spheroid_images.html)

We generated a gradient of 10 different initial cell densities with which we plated 30 TC wells coated with SbpA layers. The first well was filled with twice a fixed volume (100 µl) of cell dispersion (150000 cells/50µl). Half of the volume was then pipetted out and transferred to a second well. This procedure was repeated 8 times in order to produce 10 different cell densities in a row, $n_{well\ i}$, where $n_{well\ i}=n_{initial}/2^i$. Half a volume of TC medium was then added to all wells except for the first one, so that all wells contain 1x volume of fluid. Triplicates for each cell density were generated using the same procedure along columns of wells.
As a result, the initial cell density increases 2-fold from one well to the next one on the right. However, not all of these initial densities produced spheroids, at least not in a high yield. The cell densities reported in this study and denoted as n_1-n_6 are actually $n_{well\ 5}$ - $n_{well\ 10}$, which correspond to a range of 9400-300 cells/well. The larger cell densities $n_{well\ 1}$ –$n_{well\ 4}$ gave way to uncontrolled multicellular association. Figure S2 shows that large aggregates, which in some cases remind of chains of joint spheroids are visible after only three days of culture. At the highest densities (i=0,1), these aggregates associate to form even larger assemblies. For the purpose of the present study, only the five lowest densities (denoted as n_1-n_6) produce MCSs at an adequate concentration to follow single-spheroid formation and growth for a long period of time (16 days).

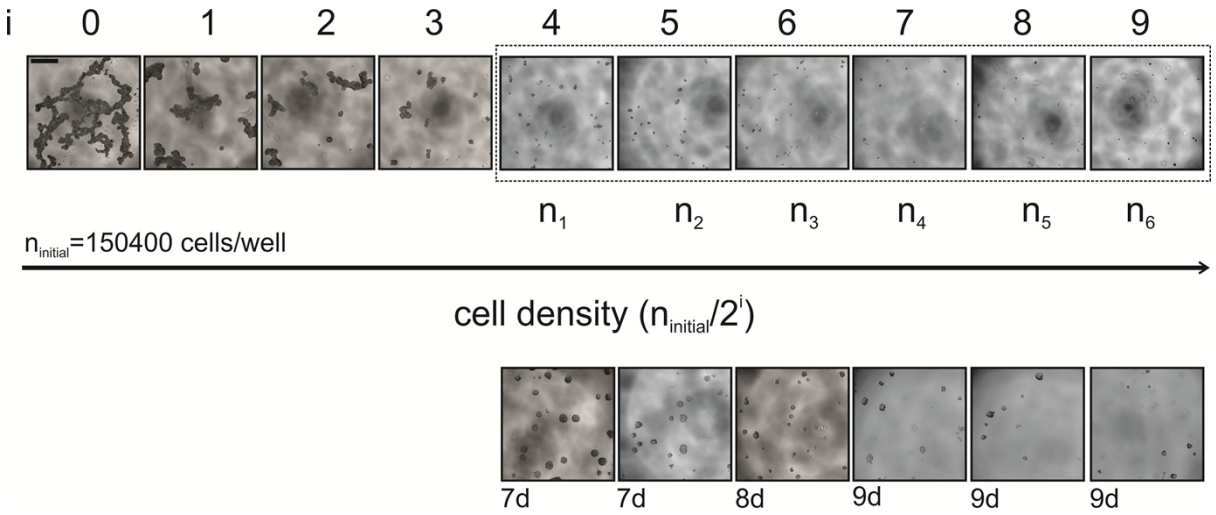

Figure S2. PhC optical micrographs showing the HepG2 assemblies after 3 days in culture on SbpA layers. The index i denotes the number of 1:2 dilutions of the initial cell concentration (150000 cells/100 µl medium ≡ well) to get a specific cell density. The lower row of optical micrographs show HepG2 spheroids after 7 days of incubation (for n_1 and n_2), 8 days in culture (for n_3) and 9 days in culture (for n_4, n_5 and n_6). Scale bar: 500 µm

Mathematical model of spheroid growth

There are several logistic models that describe tumour growth. We have selected the Gomp-ex model (Wheldon, Mathematical Models in Cancer Research 1988, Adam Hilger, Bristol), a modification of the classical Gompertz model (Laird, *British Journal of Cancer*, 1964, **18**, 490-502), since it is more suitable to describe the early phases of tumor growth (d'Onofrio, Physica D 2005, **208**, 220-235). Unlike the Gompertz model, the Gomp-ex model imposes a limit on the proliferation rate of the cellular population within the MSCs; in other words, it cannot be faster than the rate at which a cell divides. Since the competition for resources is *due to the increase in cellular population*, the Gomp-ex model sets the competition only after the cellular population reaches a threshold value: under the threshold, there is no competition and the growth is exponential, above the threshold, resources are no longer unlimited and the growth is gompertzian, reaching a plateau at sufficiently long times.

Mathematically, the Gomp-ex model can be expressed as follows:

$$S(t) = \begin{cases} S_0 exp(\alpha t), & t < t_c \\ S_c exp\left(\frac{\alpha}{\beta}\right) exp\left[-\frac{\alpha}{\beta} e^{-\beta(t-t_c)}\right], & t \geq t_c \end{cases}$$

Where S_0=S(0), S_c=S(t_c), the critical size of the spheroid that defines the transition between the exponential and gompertzian phases of growth, α and β are constants related to the ability of the cells to proliferate throughout time at the exponential and gompertzian phases, respectively. $S_c exp\left(\frac{\alpha}{\beta}\right)$ is thus the maximum spheroid´s size that can be reached under the existing environmental conditions, also called *carrying capacity* of the environment.

Figure S3 shows the predictions of the Gomp-ex model (lines) of the experimental data shown in figure 2 (symbols) and figure S4 shows the fitting parameters as a function of the initial cell density.

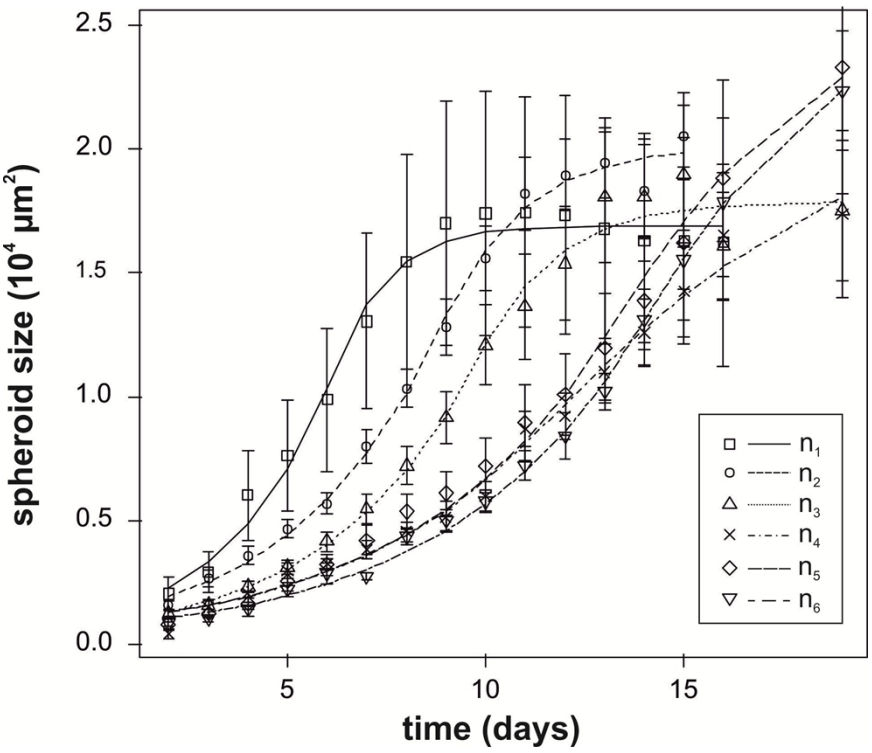

Figure S3. Gomp-ex fits (lines) to the experimental data (symbols) (Weighted non-linear least squares fit. Weights are the experimental errors)

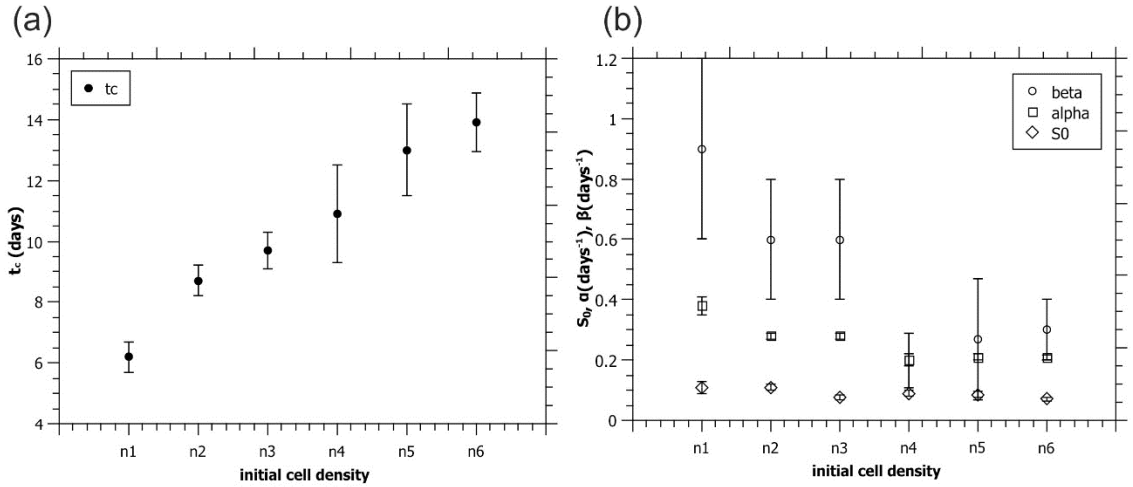

Figure S4. Best fit parameters of the Gomp ex model as a function of the initial cell density

Cell medium aging and peripheral cell death

The micrographs of figure S5 show spheroids of HepG2s after 21 days in culture at an initial cell density of n_4. Cores appear dark in the phase contrast image (figure S5a), and are necrotic, according to the dead-live assay (red fluorescence image, figure S5b). Most cells at the periphery remain viable (overlay figure S5c and green fluorescence image, figure S5d). A distinctive feature of peripheral viability in old spheroids is the smooth edges as shown in phase contrast micrographs (figure S5a). The spheroids of figure 2b (here shown in figure S5e for the sake of comparison) are surrounded by loosely attached (dead) cells.

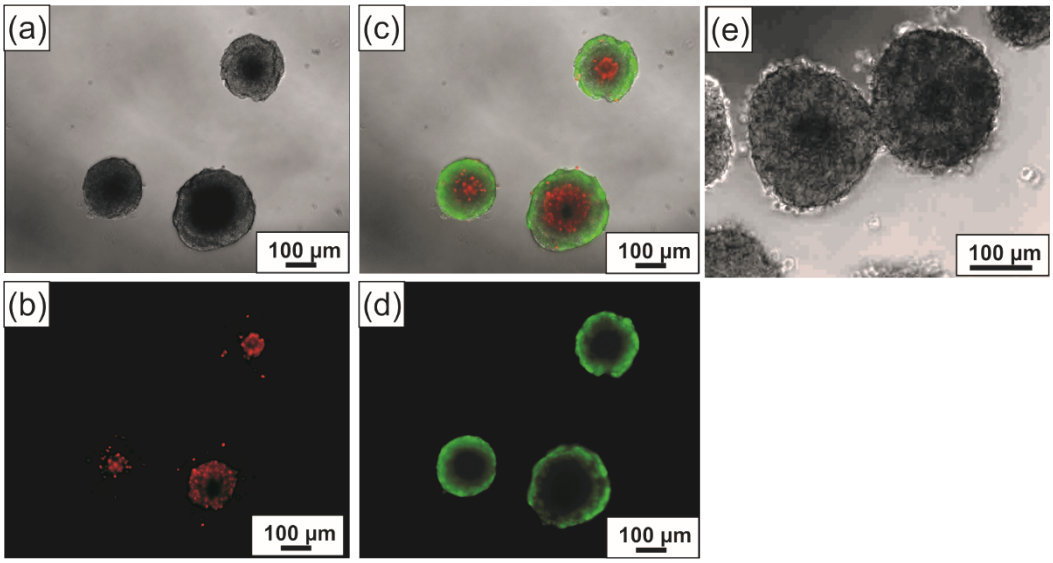

Figure S5. HepG2 spheroids after 21 days in culture at the initial cell density n_4. (a) Phase contrast image; (b-c) live (green) - dead (red) assays; (d) overlay; (e) HepG2 spheroids after 14 days in culture at the initial cell density n_1 (phase contrast micrograph, inset of figure 2b)

Necrotic cores

Cell death in the core of HepG2 MCSs cannot be prevented and eventually occurs when the spheroid reaches a certain size (> 100 µm in diameter).

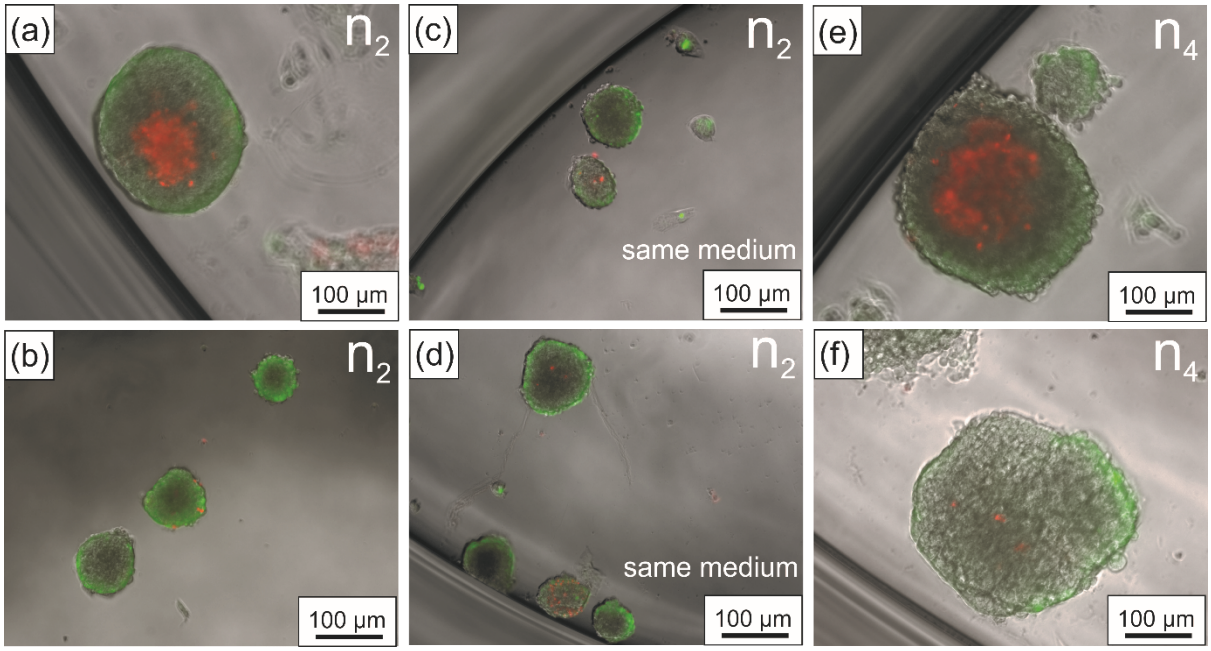

Figure S6. Spheroids of HepG2s after 14 days in culture. Each image is an overlay of a phase contrast, live-(green), and dead-(red) micrographs. (a-b) initial cell density = n_2, and medium exchanged every 2 days; (c-d) initial cell density = n_2, same medium; (e-f) initial cell density= n_4, and medium exchanged every 2 days. Seeding with a lower number of cells may delay cell death at the core in some cases (f).

Viability of heterotypic spheroids: HepG2+NHDF

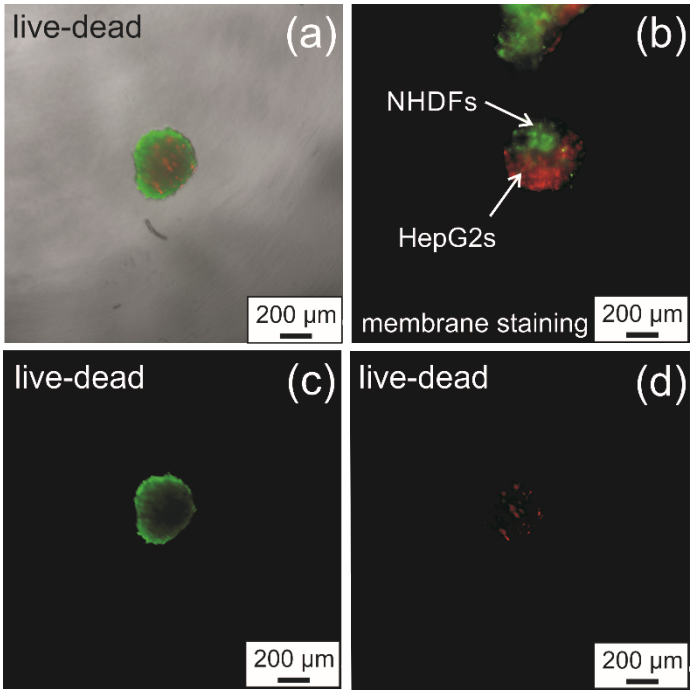

Figure S7. Heterotypic MCS of HepG2s and fibroblasts (NHDFs) grown on SbpA layers after 4 days of incubation. (a) overlay of a phase contrast image, the live-image (c), and the dead-image (d); (b) distribution of HepG2s and NHDFs in a replicate sample under the same conditions.