Architecture of Chimeric Spheroids Controls Drug Transport

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Abstract It is well-established that upregulation of drug efflux pumps leads to multi-drug resistance. Less is known about the role of the architecture of the tumor microenvironment in this process: how the location of pump expressing cells influences drug exposure to cancerous as well as non-cancerous cells. Here, we report a 3D in vitro model of spheroids with mixtures of cells expressing high and low levels of ABCG2, quantifying pump activity by the ability to reject the fluorescent dye Hoechst 33342. With respect to the organization of the mixed spheroids, three different architectures were observed: 1) high-expressing ABCG2 cells located in the spheroid core surrounded by low-expressing cells, 2) high-expressing ABCG2 cells intermixed with low-expressing cells and 3) high-expressing ABCG2 cells surrounding a core of low-expressing cells. When high-expressing ABCG2 cells were in the core or intermixed, Hoechst uptake was directly proportional to the percentage of ABCG2 cells. When high-expressing ABCG2 cell formed an outer coating surrounding spheroids, small numbers of ABCG2 cells were disproportionately effective at inhibiting uptake. Specific inhibitors of the ABCG2 transporter eliminated the effect of this coating.

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Confocal microscopy of spheroids revealed the location of high- and low-expressing cells, and Hoechst fluorescence revealed that the ABCG2-dependant drug concentration in the cancer microenvironment is influenced by pump expression level and distribution among the cells within a tissue. In addition to providing a 3D model for further investigation into multicellular drug resistance, these data show that the location of ABCG2-expressing cells can control drug exposure within the tumor microenvironment.

Keywords ABCG2 · Drug transport · Toxicity · Cancer · Co-culture · Chimeric spheroids

Abbreviations

ABC	ATP binding cassette
ADME-Tox	Absorption, distribution, metabolism, excretion and toxicity
BCRP	Breast cancer resistance protein
EMEM	Minimum essential medium with Earle's salts
FBS	Fetal bovine serum
HEK	Human embryonic kidney
MCR	Multi-cellular resistance
MDR	Multi-drug resistance

Introduction

Multidrug resistance (MDR), whereby a tumor under treatment becomes resistant to chemotherapeutics, remains a major clinical problem [1]. MDR commonly develops due to over-expression of one of three major efflux pumps from the ATP-binding cassette (ABC) super family of transporters [2]. Those three pumps are: ABCG2, also known as breast cancer resistance protein (BCRP); ABCB1, also known as P-

glycoprotein; and ABCC1, also known as multidrug resistance protein (MRP) [2]. These pumps use ATP to pump small molecules, including many chemotherapeutics, against a gradient and out of the cell. Thus, up-regulation of these pumps by cancer cells causes resistance to a long list of drugs [3]. These pumps are expressed by a variety of normal cells and serve to protect certain tissue compartments from xenobiotics and environmental toxicants. For example, hepatocytes express all 3 transporters on their basolateral surface to concentrate compounds into the bile. Similarly, at the blood brain barrier, all 3 transporters resist the uptake of compounds from the blood into the brain.

To investigate the role of ABCG2 expression in the tumor microenvironment, we formed spheroids with control cells and cells overexpressing one of three transporters (ABCG2, Pgp, MRP). Mono-dispersed cells were seeded onto micro-molded agarose gels and allowed to aggregate and self-assemble into spheroids within 24 h. To quantify ABCG2 activity, we incubated the spheroids with Hoechst 33342 a well-known substrate of ABCG2 that can penetrate cell membranes and fluoresces when bound to DNA [4]. When ABCG2 activity was quantified from time-lapse fluorescent images, we found that fluorescence increased over time and was dependent on which transporter was overexpressed. Cells that did not express ABCG2 displayed the highest intensity of Hoechst signal. To examine the role of spheroid architecture, we mixed ABCG2 expressing cells at varying ratios with the other cell types to form mixed spheroids. During self-assembly, cell pairs self-sorted and formed different spheroid architectures with respect to the location of ABCG2 expressing cells. In mixed spheroids where ABCG2 cells were distributed throughout the spheroid or concentrated at the center, dye uptake was linearly proportional to the percentage of ABCG2-expressing cells. In an architecture where ABCG2-expressing cells formed an outer coating, dye uptake was no longer linearly proportional and appeared to be inhibited by the protective coating. These results indicate that the 3D location of ABCG2-expressing cells within the spheroid controls the uptake of dye and suggests that the location of transporter-expressing cells can control drug distribution within the tumor microenvironment.

Materials and Methods

Cell Culture and Spheroid Formation

3D PetriDish® micromolds were obtained from Microtissues, Inc., Providence, RI. Molds contain an 8 × 12 rectangular array of round-bottomed recesses (400 µm diameter, 800 µm depth). Sterile 2 % molten agarose in saline was poured into the molds, covered with a glass cover slip and allowed to set. Gels were separated from the molds according to the

manufacturer's protocols and transferred into 24-well tissue culture plates. Gels were locked in place by a few drops of molten sterile agarose, incubated in 1 mL of serum-free EMEM (Corning, Corning, NY), and degassed before the medium was replaced with 2 mL of serum-free EMEM to equilibrate overnight.

Human Embryonic Kidney (HEK) cells were grown in EMEM with 10 % fetal bovine serum (FBS) (Fisher Scientific, Waltham, MA) and 1 % penicillin/streptomycin at 37 °C with 5 % CO₂. HEK control (–C1), –MDR-, –MRP, and –ABCG2 transfected HEK cells were obtained from Dr. Robert Robey, NIH Bethesda, MD. Transfection was maintained by culturing in medium containing 2 mg/mL of G418 (Sigma-Aldrich, St. Louis, MO). Cells were trypsinized using 0.05 % trypsin (Fisher Scientific) and re-suspended at the desired cell concentration. A separate line of control cells (HEK-C2) was obtained from the ATCC. For all cell lines, 70 µL of cell suspension was pipetted into the seeding chamber of each gel and allowed to settle for 30 min before 1.5 mL of serum-containing medium was added. Cells self-assembled for 20 h to form spheroids before experimentation.

Co-culture spheroids were created in different ratios by mixing the various HEK-cell lines with HEK-ABCG2 cells. Before seeding gels, cells were mixed in 1:9, 3:7, 5:5, 7:3, and 9:1 ratios. To ascertain the arrangement of the different cell types in co-culture spheroids, cells were labeled 1 h prior to trypsinization by incubation with fluorescent dyes: 5 µM CellTracker™ Red CMPTX (577/602 nm) or CellTracker™ Green CMFDA (492/517 nm) (Life Technologies, Inc). Labeled spheroids were imaged using wide-field and confocal fluorescent microscopy (Figs. 1, 2, 3 and 4).

Dye Uptake, Drug Treatment, Wide-Field Microscopy and Image Analysis

To measure the activity of ABCG2, spheroids were incubated with Hoechst 33342 (Life Technologies, H1399), a fluorescent substrate of the transporter. Medium was removed from the agarose gels containing spheroids and replaced with 1.5 mL of serum-free medium containing 4 µg/mL Hoechst 33342. Fluorescent imaging (350/461 nm) began immediately after the addition of dye and images were taken every 10 min for up to 120 min.

To determine the role of ABCG2, HEK-C1, HEK-ABCG2, and 1:1 C1-ABCG2 mixed spheroids were pre-treated with either 5 µM Iressa (Tocris #3000) or 1 µM Ko143 (Sigma #K2144) in serum-free medium for 3 h before adding Hoechst.

Fluorescent images were taken using a Carl Zeiss Axio Vision Observer Z1 equipped with an AxioCam MRm camera (Carl Zeiss MicroImaging, Thornwood, NY), an Xcite 120XL mercury lamp (Exfo Life Sciences Division, Mississauga, Ontario), and an incubation chamber for time-lapse imaging.

Fig. 1 Quantification of Hoechst 33342 uptake by spheroids overexpressing transporters. Spheroids of HEK-ABCG2, HEK-MDR, HEK-MRP, HEK-C1, and HEK-C2 cells were incubated with 4 $\mu\text{g/mL}$ Hoechst 33342. Time-lapse fluorescent images were acquired every 10 over 120 min (**a**, **c**). Hoechst 33342 fluorescence was significantly lower in the HEK-ABCG2 spheroids versus all others. HEK-C1 and HEK-C2 spheroids both reached a similar level of fluorescence several times higher than HEK-ABCG2 spheroids. Fluorescence of HEK-MDR

spheroids was slightly lower than HEK-C1 spheroids, consistent with Hoechst as an MDR substrate, though with a lower affinity than ABCG2. Fluorescence of HEK-MRP spheroids was the highest. Total spheroid fluorescence normalized to spheroid surface area was quantified over time ($n=40$ (MDR), 38 (MRP), 17–38 (C1), 30–31 (ABCG2), 24 (C2)) (**b**, **d**). Scale bar 100 μm . Plotted are average values with standard deviations

To minimize photobleaching, exposure times were limited to <40 ms. Controls were run in parallel with experimental samples to minimize variability. Samples were also viewed using a Zeiss LSM 710 confocal microscope.

Quantitative image analysis was performed using a custom ImageJ (US National Institutes of Health) program. Briefly, spheroids were traced using EZ Snake, an active contour function, and the total fluorescence taken within the trace. The trace was then moved to an area to the side of the spheroid to obtain a background measurement that was subtracted from the result. Total fluorescence was then normalized to the surface area of the spheroid that was calculated from the x - y measurements and a previously determined z dimension obtained from side-view images using a Mitutoyo FS-110 microscope altered to lie on its back and a Nikon Coolpix 900 eyepiece camera that established the ratio of the z dimension to the x - y dimensions for spheroids of HEK cells ($z=0.8 \times x$ dimension/2).

Statistical Analysis

Surface area normalized data was recorded in Excel and presented as mean values plus and minus the standard deviation.

P-values less than or equal to 0.05 were considered statistically significant. Uptake experiments were repeated four times, each with 2 replicates of each condition of spheroids, and confocal images were taken on 3 separate occasions. Linear regressions were performed using Excel and presented in Fig. 5.

Results

Uptake and Accumulation of Hoechst 33342 in Spheroids is Dependent on Transporter Expression

To study transporter activity in 3D microtissues, we formed spheroids and incubated them with Hoechst 33342, a well-known fluorescent substrate for ABCG2. Optically-clear agarose micro-molds were used to form a small array of 96 spheroids from HEK cells transfected to overexpress different transporters. Spheroids were formed by pipetting mono-dispersed cells (7.0×10^4 cells in 70 μL) into the seeding chamber of the gels and allowing them to settle by gravity into the small agarose recesses below the seeding chamber. Unable to attach to agarose, cells formed cell-to-cell adhesions and self-

assembled into a single spheroid in each micro-well (~700 cells/spheroid) within 20 h. Spheroids were formed using HEK cells transfected with ABCG2 (HEK-ABCG2), MDR (HEK-MDR), MRP (HEK-MRP) as well as two different control HEK cell lines without transfection (HEK-C1 and HEK-C2). Spheroids were incubated with 4 $\mu\text{g}/\text{mL}$ Hoechst 33342 and fluorescent images taken every 10 min for a total of 130 min (Fig. 1).

Time-lapse images showed that Hoechst 33342 fluorescence increased with time and varied in intensity depending on the transporter expressed. To quantify these differences, fluorescence in the entire spheroid was measured as a function of time and normalized to surface area. Spheroids formed with control cells saw a rapid rise in fluorescence and reached similar levels to one another. Fluorescence of spheroids formed with HEK-ABCG2 cells was low as the presence of the ABCG2 pump decreased the rate and extent of Hoechst 33342 accumulation. Fluorescence of spheroids formed with HEK-MRP or HEK-MDR cells was similar to controls with significantly higher levels of uptake than pure HEK-ABCG2 spheroids. Fluorescence of HEK-MRP spheroids was slightly higher than controls, and HEK-MDR spheroids were lower than controls (Fig. 1).

Fig. 2 Tissue architectures of spheroids formed by the self-organization of cell mixtures. HEK-ABCG2 cells labeled with CellTracker Red were mixed in varying ratios with HEK-MDR, HEK-MRP, HEK-C1, and HEK-C2 cells that were labeled with CellTracker Green. Equal numbers of cell mixtures were seeded onto gels and after 20 h, fluorescent images were acquired. When paired with HEK-MDR and HEK-MRP cells, HEK-ABCG2 cells formed the inner core of the mixed spheroids with the other cell types moving to the outer shell. This general pattern of self-organization was consistent across ratios, however as the percentage of HEK-MDR and HEK-MRP cells was reduced, coverage of the ABCG2 core by the cells of the outer shell declined. When HEK-ABCG2 cells were paired with HEK-C1 cells, the opposite organization was formed with HEK-ABCG2 cells forming the outer shell and HEK-C1 forming the core. When HEK-ABCG2 cells were paired with HEK-C2 cells, a core/shell organization did not form. In these mixtures, cells were more randomly mixed, but like cells did form small groupings of cells. Scale bar is 100 μm

Cell Mixtures Form Spheroids of Different Architectures

Mixtures of different cell types self-sort and form distinct and reproducible patterns or architectures in the final spheroid [5]. To determine if this was the case for the HEK cell lines, each cell type was labeled (HEK-MDR, HEK-MRP, HEK-C1, and HEK-C2) with CellTracker Green and four cell pairings were created by mixing each with varying amounts of HEK-ABCG2 cells labeled with CellTracker Red. Total cell number was held constant and the mixtures were seeded into the gels and allowed to form spheroids that were imaged after 20 h of self-assembly and self-organization (Fig. 2, S1). Relative positions of the cell types within each spheroid were found to vary between the four pairings.

The C1-ABCG2 mixture self-sorted to form spheroids with an outer coating of HEK-ABCG2 and an inner core of HEK-C1 cells. This was especially evident in the 50:50 mix where the thick HEK-ABCG2 coating appeared to completely surround a HEK-C1 core. As the fraction of HEK-ABCG2 cells was decreased, this coating became sparse and, as the fraction of HEK-ABCG2 cells was increased, the coating became thicker. In contrast, the MDR-ABCG2 and MRP-ABCG2 mixtures of cells formed spheroid architectures with HEK-ABCG2 cells at the core. Similarly, when the fraction of either HEK-MDR or HEK-MRP cells was decreased or was increased, the thickness of the outer coating increased or decreased, respectively. C2-ABCG2 mixes formed spheroids with a significantly different architecture without well-defined outer and inner layers. HEK-ABCG2 and HEK-C2 cells formed ribbons or clusters of like cells that intertwined within the spheroid.

Fig. 3 Uptake of Hoechst 33342 by mixed spheroids. HEK-ABCG2 cells were mixed in varying ratios with HEK-C1, HEK-C2, HEK-MDR, or HEK-MRP cells and equal numbers of the cell mixtures were seeded onto gels to form mixed spheroids. At 20 h when the mixed spheroids had formed and self-organized, they were incubated with 4 $\mu\text{g}/\text{mL}$ Hoechst 33342. Images at 70 min show that as the percentage of HEK-ABCG2 cells was increased overall spheroid fluorescence decreased for all mixtures and the uniformity of the fluorescence was altered. At high percentages of HEK-ABCG2 cells (70 and 90 %), spheroids had a non-uniform pattern of fluorescence. Scale bar is 100 μm

Uptake and Accumulation of Hoechst 33342 is Dependent on Spheroid Architecture

To determine if spheroid architecture influenced drug uptake, mixed spheroids were incubated with Hoechst 33342 (Fig. 3). As the percentage of HEK-ABCG2 increased, fluorescence of all mixes decreased. In addition, the pattern of fluorescence in the spheroids changed from a homogenous signal to a non-uniform pattern. Quantification of fluorescence over time (Fig. 4) confirmed that increasing the percentage of HEK-ABCG2 cells resulted in a decrease in the uptake and accumulation of Hoechst 33342. To determine if all mixtures behaved in a similar fashion regardless of spheroid architecture, spheroid fluorescence at 70 min was plotted as a function of the proportion of HEK-ABCG2 cells (Fig. 5). For spheroids of MDR-ABCG2, MRP-ABCG2, and C2-ABCG2, the rate of decline was linear and proportional to the percentage of HEK-ABCG2 cells in the spheroid. However, for C1-ABCG2 mixes, small

numbers of HEK-ABCG2 cells resulted in a rapid decrease in spheroid fluorescence that did not follow this linear pattern.

To confirm that the inhibition of Hoechst uptake by the HEK-ABCG2 coating of cells was due to overexpression of the pump, the effects of two well-known inhibitors of ABCG2 were tested (Fig. S2). Spheroids of HEK-ABCG2 cells mixed with HEK-C1 cells (1:1) were incubated with either Iressa, a competitive inhibitor, or Ko143, a non-competitive inhibitor, for 3 h prior to the addition of Hoechst 33342. Both inhibitors increased the uptake of Hoechst 33342 and restored it to the levels of spheroids composed solely of HEK-C2 cells.

Confocal Imaging of Uptake into Co-Culture Spheroids

To obtain single-cell resolution of the pattern of mixed spheroids, confocal microscopy was used to visualize Hoechst 33342 uptake (Fig. 6 and S3). Spheroids of HEK-ABCG2 cells mixed with HEK-C1 cells or HEK-C2 cells (70:30

Fig. 4 Quantification of Hoechst 33342 uptake by mixed spheroids. Time lapse fluorescence images (every 10 over 120 min) of Hoechst 33342 uptake by spheroids of HEK-ABCG2 cells mixed in varying ratios with HEK-C1, HEK-C2, HEK-MDR, or HEK-MRP cells were quantified. Total spheroid fluorescence was normalized to spheroid surface area. Each mixture was compared to pure spheroids of the blended cell type (100 %) as well as spheroids of 100 % HEK-ABCG2 cells, which had the lowest rate of Hoechst 33342 uptake. For all four pairs tested, the rate of Hoechst 33342 uptake declined as the percentage of HEK-ABCG2 cells was increased. When HEK-ABCG2 cells were

mixed with HEK-C1 cells, the rate of Hoechst 33342 uptake declined rapidly ($n=20-36$) (a). Small numbers of HEK-ABCG2 cells resulted in a significant decrease. In contrast, when HEK-ABCG2 cells were mixed with HEK-C2 cells, the decline in the rate of Hoechst 33342 uptake was more gradual and required higher percentages of HEK-ABCG2 cells ($n=30-45$) (b). Likewise, when HEK-ABCG2 cells were mixed with HEK-MDR cells ($n=24-57$) (c) or HEK-MRP cells ($n=15-60$) (d) the decline in the rate of Hoechst 33342 uptake was also more gradual, requiring increasing numbers of HEK-ABCG2 cells

Fig. 5 Hoechst 33342 uptake as a function of the percentage of HEK-ABCG2 cells. To determine how the proportion of HEK-ABCG2 cells affected Hoechst 33342 uptake for the four types of spheroid mixtures, we plotted fluorescence at 70 min as a function of % HEK-ABCG2 cells. As a point of comparison, we included a linear regression line for each combination. The R-squared values are as follows: MDR-ABCG2=0.89, MRP-ABCG2=0.98, C2-ABCG2=0.94, and C1-ABCG2=0.65. When

HEK-ABCG2 cells were mixed with HEK-C1 cells, the decline in fluorescence was not linear ($n=20-36$) (a). Small percentages of HEK-ABCG2 cells caused a rapid reduction in spheroid fluorescence. In contrast, when HEK-ABCG2 cells were mixed with either HEK-C2 cells ($n=30-45$) (b), HEK-MDR cells ($n=24-57$) (c), or HEK-MRP cells ($n=15-60$) (d), the decline in fluorescence followed a linear function

mix) were incubated with Hoechst 33342 for 90 min and visualized by confocal microscopy. HEK-ABCG2 cells were labeled with Cell Tracker red, whereas HEK-C1 cells or HEK-C2 cells were labeled with Cell Tracker green. Confocal images revealed that Hoechst 33342 fluorescence was reduced in HEK-ABCG2 cells versus HEK-C1 cells or HEK-C2 cells. Imaging these spheroids revealed that the majority of Hoechst 33342 fluorescence was found in areas where ABCG2 was not expressed. In the C2-ABCG2 mix (Fig. 6b), Hoechst 33342 fluorescence was highest in the islands of HEK-C2 cells. In the C1-ABCG2 mix (Fig. 6a), Hoechst 33342 fluorescence was highest in the HEK-C1 cells that broke through the protective shell of HEK-ABCG2 cells. HEK-C1 cells that breached the outer coating formed a pathway of increased Hoechst 33342 fluorescence into the spheroid core.

Discussion

For many years it has been recognized that multi-cellular tumor spheroids more closely approximate the complexity of

the in vivo microenvironment of tumors than standard flask culture [6–8]. Early studies have shown that, when compared to monolayer culture, spheroids are more resistant to treatment with radiation and chemotherapeutics, much like solid tumors [9]. This general resistance or multi-cellular resistance (MCR) is thought to be driven by cell-to-cell interactions creating a complex microenvironment within the tumor, including barriers to drug diffusion, slow cell cycles, low rates of apoptosis and hypoxia in the spheroid interior [10]. In contrast, multi-drug resistance (MDR), is often created by the overexpression of one of three transporters from the ATP binding family of efflux pumps that pump small molecules, including drugs, out of cells [1, 11–13]. These three transporters are ABCG2, ABCB1, and ABCC1 [14]. Despite the development and clinical testing of numerous inhibitors that target each of these pumps, MDR remains a significant problem in the long-term treatment of cancer [15]. Clinically, drug resistance most certainly resides in the tumor microenvironment and is likely a manifestation of both MCR and MDR.

New in vitro methods of spheroid formation provide opportunities to study the tumor microenvironment and how it

Fig. 6 Confocal imaging of Hoechst 33342 uptake by individual cells in a spheroid. HEK-ABCG2 cells (30 %) labeled with CellTracker Red were mixed with (70 %) HEK-C1 (**a**) or (70 %) HEK-C2 cells (**b**) that were labeled with CellTracker Green. Equal numbers of cell mixtures were seeded onto gels. After 20 h when the mixed spheroids had formed and self-organized, the spheroids were incubated with 4 $\mu\text{g}/\text{mL}$ Hoechst 33342. After 90 min, fluorescent images at different depths into the mixed spheroids (9, 19.5 and 30 μm) were obtained by confocal microscopy. As controls, similar images were obtained from homogenous spheroids of HEK-C1 and HEK-C2 cells that had also been incubated with 4 $\mu\text{g}/\text{mL}$ Hoechst 33342 for 90 min. The perimeter of the spheroid in each image is highlighted with a dashed yellow line. Control spheroids showed that overall Hoechst 33342 fluorescence was highest for those cells located on the outer surface of the spheroid and decreased further into the spheroid. This radial gradient is due to the limitations of diffusion with those cells on the spheroid surface exposed to the highest concentration of dye. In the mixture of HEK-ABCG2 cells and HEK-C1 cells, the HEK-ABCG2 cells were located predominately on the outer surface of the spheroid, whereas when mixed with HEK-C2 cells, HEK-ABCG2 cells were intermingled with HEK-C1 cells and were not biased towards the outer surface. In these mixed spheroids, the single cell resolution provided by confocal images revealed that Hoechst 33342 fluorescence was consistently higher in the HEK-C1 and HEK-C2 cells versus those neighboring HEK-ABCG2 cells located at comparable radial positions in the same spheroid. In areas where clusters of HEK-C1 cells breached the HEK-ABCG2 coating (marked by *white arrows*), Hoechst 33342 fluorescence was higher than comparable HEK-C1 cells located behind the HEK-ABCG2 coating. Similarly, in the C2-ABCG2 condition, the areas of highest fluorescence were those consisting of HEK-C2 cells that were exposed to the surface (marked with *white arrows*). In both cases, fluorescence generally declined further into the spheroid, but persisted farther for areas of HEK-C1 and -C2 cells which can trace an unprotected path to the spheroid surface. Scale bar is 100 μm

relates to drug resistance. Methods based on aggregation or self-assembly of cells to form spheroids enable the controlled production of designer spheroids with multiple cells types, including the principle actors of the tumor microenvironment such as fibroblasts, endothelial cells, and even immune cells [6, 16]. When two cell types self-assemble, they may undergo a process of self-sorting, where one cell type forms an inner core and the other forms an outer coating [5, 17]. Sorting is a property of a given pair of cells, occurs during self-assembly and is thought to be due to both differences in surface adhesion molecules as well as in cytoskeletal contraction [18]. Pairs of cells that do not wholly sort will instead form mixed spheroids with varying degrees of separation [5]. Some pairs of cells will even completely segregate and form separate spheroids [19]. Recent efforts have been devoted to controlling the sorting process by genetic modification [20, 21].

In this paper, spheroids were used to investigate the influence of ABCG2 transporter expression on drug concentration in the tumor microenvironment. ABCG2 activity was measured using Hoechst 33342, a well-characterized substrate of the ABCG2 efflux pump that is permeable to cell membranes and fluoresces when bound to DNA. HEK cells over expressing different transporters were self-assembled into homogeneous spheroids or were mixed to form heterogeneous spheroids with distinct architectures based on self-sorting (Fig. 7).

Since the five cell types we tested are all HEK cells, we are uncertain as to why they exhibit different sorting patterns. We do not believe that sorting is related to transporter expression because HEK-C1 and HEK-C2 cells, which are the same basic cell type but acquired from separate sources, also differed in sorting. The sorting therefore may be related to their history of culture *in vitro*. Nevertheless, the sorting patterns we observed were routinely consistent and repeatable over a range of cell ratios.

Drug concentration in all spheroids, as measured by Hoechst uptake and fluorescence, increased over time. For homogenous spheroids, total drug uptake was highly dependent on which transporter was expressed with HEK-ABCG2 spheroids having the lowest drug uptake levels of all tested. Likewise, drug uptake by heterogeneous spheroids declined as the proportion of HEK-ABCG2 cells increased. For three of the four pairs of mixed spheroids tested, the decline in drug

Fig. 7 Three distinct spheroid architectures. Schematic drawing of three spheroid architectures with respect to the location of the ABCG2 cells. Depicted is a cross section of each spheroid labeled with the location of the ABCG2 cells within each

uptake was linearly proportional to the fraction of cells expressing ABCG2. In spheroids where ABCG2 self-sorted to form an outer coating, the decline in uptake was not linear. Small numbers of ABCG2 cells (10 %) formed a surrounding coating on the spheroid that caused a rapid decline in drug uptake. We estimate that a single continuous cell layer covering the entire spheroid could be formed if ABCG2 cells were as few as 16 % of the total. Specific inhibitors of ABCG2 (Iressa and Ko143) inactivated the Hoechst uptake inhibitory activity of the coating of ABCG2 cells, confirming that expression of the ABCG2 pump was responsible for this effect.

Confocal microscopy at different depths into the spheroids confirmed that cells not expressing ABCG2 had high levels of Hoechst 33342 fluorescence. These images also established that drug concentration varied between individual cells. HEK-ABCG2 cells with low levels of fluorescence were located adjacent to highly fluorescent cells that did not express ABCG2. Confocal images also revealed that in some locations where cells not expressing ABCG2 had breached the surrounding coating of HEK-ABCG2 cells, drug concentration was high in those and adjacent cells leading toward the center of the spheroid.

Taken together, these results indicate that, in addition to transporter level and transporter specificity, drug concentration in the tumor microenvironment is controlled by the position of the transporter cells in the tumor microenvironment. As demonstrated by the C1-ABCG2 mix of cell types, a spheroid architecture with small numbers of transporter expressing cells surrounding the rest can insulate that microenvironment and reduce the drug exposure of cells that do not express transporters. In another architecture, where low transporter expressing cells are located adjacent to high transporter expressing cells, these low transporter cells may be exposed to higher than expected drug concentration. In a third architecture as exemplified by the C2-ABCG2 mixture of cells, where cells not expressing transporter breach an outer layer of transporter expressing cells, drugs may enter the tumor microenvironment through these breaches.

There are several possible implications of this observation that the location of transporter cells can influence drug concentration in the tumor microenvironment. First, the high rate of mutation amongst cancerous cells produces a highly heterogeneous population. Cancer stem cells, named for their ability to repopulate a tumor after therapy, are often associated with higher levels of transporters, and, therefore, may form a niche that protects cells from toxic levels of drugs [4, 22]. Second, certain architectures of transporter-expressing cells may serve to protect other cells of the tumor microenvironment that lack transporters. In this way, the transporter cells could provide a protective niche for drug sensitive cells, which may be providing important growth promoting signals for the tumor. Conversely, other architectures of transporter-expressing cells may cause non-expressing cells to be exposed

to even higher levels of drug. In this way, the number of non-transporter expressing cells in the microenvironment may decline over time. Thus, in addition to selecting for tumor cells that overexpress a transporter that confers MDR, long-term drug exposure may also alter the architecture of the tumor microenvironment, increasing MCR.

In this paper, we used self-assembled spheroids to show that the location of ABCG2-expressing cells can control drug exposure within a microtissue. Well-characterized HEK cells overexpressing different transporters were used as a model system to establish this concept as well as the means for its investigation. While no in vitro system can adequately replicate all of the complexities of the in vivo tumor microenvironment, the accumulation of individual mutations such as transporter overexpression are common and not dissimilar to cells overexpressing a transporter through transfection. There are advantages to using in vitro systems and our study shows that spheroids of mixed cell types can increase the complexity of an in vitro system by forming architectures that control drug transport. The ability to produce designer spheroids with two or more cell types may be useful for future studies that examine the contributions of non-cancerous cells to drug resistance as well as the role of carcinoma-associated fibroblasts versus normal tissue associated fibroblasts [23]. Further designer spheroids may also be useful to follow the evolution of microenvironments upon drug exposure and the screening of new agents or treatment regimens that target resistance.

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