Comparison of cancer cells cultured in 2D vs 3D reveals differences in AKT/mTOR/S6-kinase signaling and drug response

Angelika Riedl1,§, Michaela Schlederer2,3, Karoline Pudelko1, Mira Stadler1, Stefanie Walter1, Daniela Unterleuthner1, Christine Unger1, Nina Kramer1, Markus Hengstschläger1, Lukas Kenner2,3,4, Dagmar Pfeiffer5, Georg Krupitza2 and Helmut Dolznig1#

1 Institute of Medical Genetics, Medical University of Vienna, Währinger Straße 10, A-1090 Vienna, Austria
2 Clinical Institute of Pathology, Medical University of Vienna, Währinger Gürtel 18-20, A-1090 Vienna, Austria
3 Ludwig Boltzmann Institute for Cancer Research, Währinger Strasse 13A, 1090 Vienna, Austria
4 Unit of Pathology of Laboratory Animals (UPLA), University of Veterinary Medicine Vienna, 1210 Vienna, Austria
5 Institute of Cell Biology, Histology and Embryology, Medical University of Graz, Harrachgasse 21, 8010 Graz, Austria

* This work is part of the PhD thesis of AW
# Corresponding author: email helmut.dolznig@meduniwien.ac.at
§ Present address: Boehringer Ingelheim RCV GmbH & Co KG, Dr. Boehringer Gasse 5-11, A-1121 Vienna, Austria

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Abstract

3D cancer models are used as preclinical systems to mimic physiologic drug response. We provide evidence for robust changes of proliferation and metabolic capacity in 3D by systematically analyzing spheroids of colon cancer cell lines. Spheroids showed relative lower AKT/mTOR/S6K activities compared to cells cultured in 2D. We identified spatial alterations in signaling, as the level of phospho-rpS6 decreased from the spheroid surface to the center, closely recapitulating the tumor areas around vessels \textit{in vivo}. These 3D-models displayed augmented anti-tumor response to AKT/mTOR/S6K- or MAPK-pathway inhibition compared to 2D. Inhibition of AKT/mTOR/S6K resulted in elevated ERK phosphorylation in 2D, whereas under these conditions MAPK-signaling was reduced in spheroids. Inhibition of the MAPK-pathway led to decreased AKT/mTOR/S6K-signaling in 3D but not in 2D. These data indicate a distinct rewiring of signaling in 3D and during treatment. Detached tumor cell clusters in vessels, in addition to circulating single tumor cells, play a putative role in metastasis in human cancers. Hence, the understanding of signaling in spheroids and the response in the 3D models upon drug treatment might be beneficial for anti-cancer therapies.
Introduction

Established cell lines were essential to understand the basic molecular principles of cancer (Bodnar et al., 1998; Hahn et al., 1999; Iyer et al., 1999). However, cancer cells were continuously cultivated in vitro for a long time and have been selected to adapt to the in vitro culture conditions. These concerns are often raised to question the relevance of cell lines as proper preclinical cancer models. Though, for colorectal cancer (CRC) the analysis of the mutational and gene expression status of about 150 available human CRC cell lines revealed that the entire variety of molecular and transcriptional subtypes, as previously described in CRC in vivo (Marisa et al., 2013), is represented in this cell collection (Medico et al., 2015). In addition, these cells display the full range of drug response to EGFR inhibition from sensitivity to resistance. Thus, CRC cell lines seem to fully cover the genetic, transcriptional and phenotypic makeup of cancer cells in vivo.

However, cells are often cultured as monolayers on flat surfaces and these conditions do not faithfully reflect the situation in vivo, since proper tissue architecture and cell-cell contacts are lost in such 2D systems. Three dimensional (3D) cell cultures are well documented to regain intrinsic properties and better mimic the in vivo situation than cells cultured as monolayers on plastic (Dolznig et al., 2011; Fischbach et al., 2009; Okawa et al., 2007; Pickl and Ries, 2009). Studies have shown that the gene expression profiles (Takagi et al., 2007) as well as the responses to treatment (Desoize and Jardillier, 2000) in the multicellular spheroid 3D models resemble more closely the in vivo situation. Similar to human tumors, proliferating, quiescent and dying cells coexist in normoxic, hypoxic or necrotic zones within spheroids (Hirschhaeuser et al., 2010). Therefore, 3D cancer models are increasingly recognized and biologically relevant for drug development and preclinical drug testing. In vivo, carcinoma cells not only form 3D structures but also interact with the surrounding ECM and cells of the tumor stroma. These interactions were shown to be essential for tumor development and progression, which prominently alter signaling pathways in both the tumor cells and the stromal cells.

Thus far, only a few comprehensive and systematic studies have been found which compare distinct signaling pathways in 2D versus 3D culture, in the presence or absence of ECM and/or stromal cells or treatment with inhibitors (Ekert et al., 2014; Luca et al., 2013; Pickl and Ries, 2009). Changes of signaling cascades in cancer cells or crosstalk with other cell types in 3D cultures are still poorly understood. How these pathways change in response to targeted therapy and whether there is a different response in 2D versus 3D remain rather undocumented so far.

In this study, we systematically analyze the signaling changes in 2D versus 3D culture without adding further levels of complexity such as the interaction of these cells with the ECM or stromal cells. We
focus on the AKT/mTOR and MAPK pathways because these two pathways are the most commonly mutated signaling routes in CRC and in many other cancers. The PI3K/AKT/mTOR pathway is a central regulator of cell growth, proliferation, survival, metabolism and ageing by integrating different environmental cues. Dysregulation of PI3K/AKT/mTOR has been implicated in various pathological conditions including cancer. Based on these findings, different efforts for modulating the PI3K/AKT/mTOR activity by pharmacologically targeting different molecules in the pathway are ongoing (Laplante and Sabatini, 2012).

We provide here some evidence of significant differences of PI3K/AKT/mTOR signaling in the 3D vs the 2D system, including spatial alterations in the signaling strength and responses upon treatment with AKT/mTOR/S6K- or MAPK-axis inhibitors.

Results

Colon cancer cells grown as spheroids display significantly reduced cell cycle progression

We used six colon cancer cell lines in this study. Caco-2 displays enterocyte-like differentiation and does not harbor known mutations in the PI3K/AKT/mTOR or Ras/Raf/MAPK pathways (Hidalgo et al., 1989; Pinto et al., 1983). LS174T, DLD-1 and HCT116 contain mutations in both PI3K and KRAS, HT29 has mutations in BRAF and PI3K and SW620 cells are driven by mutant KRAS (Suppl. Table S1).

All cell lines formed spheroids within 24-48 h after seeding. Spheroid morphology varied from compact appearance (DLD-1, HT29), less-condensed spheroids with smooth (HCT116) or irregular surfaces (LS174T) to loose aggregates (SW620) and adenomatous cell clusters (Caco-2) (Figure 1A). EdU-incorporation was used to precisely determine the cell cycle distribution of cells grown in 2D or 3D (Figure 1B). A significant reduction (-50%) of cells in S-phase in 3D comparing to those in 2D was observed in HT29, HCT116, Caco-2 and DLD-1. A minor reduction (-20%, Figure 1C) of S-phase was observed in LS174T and SW620 cells. Cell death, as determined by subG1 cells, was generally low in all cell lines cultured in 2D or 2D (Figure 1D). Nevertheless, there was a twofold increase of the apoptotic rate in 3D compared to the rate in 2D in DLD-1 (8% in 3D vs 4% in 2D). Low apoptosis was confirmed by cleaved-caspase-3 staining in 2D as well as in spheroid sections (Figure 1E). No spatial variations were detected in the spheroids: apoptotic cells appeared randomly without clustering in specific areas.
**Spheroid culture of CRC cells is associated with diminished AKT/mTOR/S6K-signaling**

Next, we determined the molecular differences in signaling pathways in the CRC cells cultured in 2D or 3D using Western blot analysis (Figure 2A). The amount of (phospho)-proteins was quantified by the use of densitometric evaluation (Figure 2B) of three biological replicates (replicate #2/#3, Supporting Figure SF1). AKT-S473 phosphorylation was weak in SW620 compared to those in the other cell lines. Diminished phospho-AKT corresponded to low AKT activity as observed by the reduced phosphorylation of the direct AKT target PRAS40. Interestingly, in five cell lines (LS174T, HT29, HCT116, Caco-2, DLD-1), AKT phosphorylation was selectively reduced in 3D but not in 2D culture. This was associated with decreased phospho-PRAS40 in four of the five cell lines (i.e. cell lines LS174T, HT29, HCT116, DLD-1); in Caco-2 cells, PRAS40 was equally phosphorylated in 2D and 3D (Figure 2B upper panels). In all six lines tested, S6K1 and rpS6 phosphorylation were consistently and significantly reduced in spheroids, which indicated a reduced mTORC1 activity. Accordingly, another direct target of mTORC1, eukaryotic initiation factor 4 binding protein 1 (4E-BP1), was less phosphorylated in 3D. The level of hyperphosphorylated 4E-BP1 (i.e. 4E-BP1γ) was reduced to about 50% in LS174T, HT29, Caco-2 and DLD-1 cultured in 3D, or by 20% in SW620; no change in HCT116 cells was found (Figure 2B, middle panels). In contrast, phospho-ERK1/2 as an indicator for active RAS/RAF/MAPK-signaling was variably influenced by 3D culture conditions. In DLD-1, LS174T and HT29, ERK1/2 phosphorylation remained unchanged in 3D compared to 2D culture. Phospho-ERK1/2 was significantly induced in SW620 and Caco-2 in 3D, but decreased in HCT116. PKCβ–II phosphorylation was slightly (LS174T, HCT116, DLD-1, HT29) or strongly induced (SW620, Caco-2) in 3D, indicating that there was not a general decrease of phosphoproteins, thereby ruling out a handling, harvesting or cell lysis artifact only present in 3D cultures (Figure 2B, lower panel).

In summary, these experiments revealed a general, profound and highly specific decrease in AKT/mTOR/S6K signaling in cancer cells grown as spheroids as compared to those cultured in the respective conventional 2D system.

**Phenotypical analysis of CRC cells in 2D and 3D under treatment**

The global decrease in the AKT/mTOR/S6K axis in 3D cultures prompted further investigation of specific inhibitors targeting major hubs in this pathway and comparison of the phenotypic as well as molecular responses in signaling in 2D versus 3D. A MAPK-pathway inhibitor targeting MEK1 was also included. The inhibitors used in this study were Rapamycin, Torin1, PF4708671, MK2206 and AZD6244 to target mTORC1, mTOR, S6K1, AKT and MEK1 kinase activities, respectively (Suppl. Table S2). DLD-1, Caco-2 and HCT116 were selected for this in depth analysis since these cell lines belong
to the same transcriptional subtype of CRC cells (stem-cell-like, (Marisa et al., 2013; Medico et al., 2015)). DLD-1 and HCT116 harbor the same mutations in the PI3K/AKT and MAPK pathway, whereas for those pathways Caco-2 cells are wildtype.

Cells were grown in 2D or 3D culture for one day and were treated with compounds or solvent control (DMSO) for another 24h. Proper drug action was verified by Western blot analysis of key phospho-proteins of the interfered signaling pathway. Upon treatment with Rapamycin, PF4708671, MK2206 or AZD6244, cell morphology of DLD-1 cells (Figure 3A) in 2D was unchanged. In contrast, DLD-1 spheroids treated with Torin1, MK2206 and AZD6244 appeared darker in phase contrast (Figure 3A). Spheroid size was reduced to about 50% upon treatment with Torin1 and the Rapamycin-, MK2206- or AZD6244- treated spheroids displayed intermediate volume reduction (75% of controls), whereas PF4708671 had a small but significant impact on spheroid size (Figure 3A,B). The volume of HCT116 spheroid was reduced significantly under all treatment conditions. In Caco-2 spheroids treatment with Torin1 and MK2206 led to 80% the size of controls (Suppl. Figure S1A,B).

Cell cycle analysis revealed essential differences in treatment response between DLD-1 cells cultured in 2D vs 3D (Figure 3C, HCT116 and Caco-2, Suppl. Figure S1C). In the 2D system, Rapamycin, PF4708671 and AZD6244 had no effect on cell cycle progression, whereas Torin1 reduced the proportion of cells in S-phase to less than 15% as compared to 38% in DMSO controls. Inhibition of AKT had a small but significant effect on proliferation. In contrast, cell proliferation was affected by all compounds (except for the S6K inhibitor) in the 3D model. MK2206 displayed a more pronounced inhibitory effect by reducing the number the cells in S-phase by 50% (8% vs 16%). Unexpectedly, AZD6244 was even more effective resulting in only 4% replicating cells, whereas being ineffective in 2D. Interestingly, total inhibition of mTOR kinase activity led to a complete block in cell proliferation in 3D (0.3% cells in S-phase), whereas blocking mTORC1 activity by Rapamycin had a minor effect (10% vs 16% in controls). HCT116 cells were most effectively slowed down in cell proliferation by inhibition of S6K1 and MEK1 in 2D. However, in 3D culture AZD6244 and Torin1 displayed the most pronounced effect (Suppl. Figure S1C) similar to DLD-1 and Caco-2 spheroids (Suppl. Figure S1C). Detailed data are available in Supporting Figure SF2.

To further corroborate these findings, cellular metabolic capacity was determined (Figure 3D) which faithfully recapitulated the pattern of the cell cycle analysis in DLD-1 and HCT116. Interestingly, there was a general decrease in metabolic activity of cells cultured in 3D compared to 2D, such as 40% in DLD-1 (Figure 3D) and 60% in HCT116 and Caco-2 (Suppl. Figure S1D). Subtle but significant differences were detectable as compared to the cell cycle analysis. All treatments shown to have no effect on proliferation in 2D and in 3D culture (see Figure 3C, Suppl. Figure S1C) nevertheless
displayed small decreases in metabolic activity. Surprisingly, this was not the case in Caco-2. Here, the level of intracellular ATP was rather inversely correlated with the S-phase data.

As inhibition of the mTOR pathway results in reduced cellular volume (Fingar et al., 2002), we next determined whether a gross decrease of cell size was contributing to the effects of the inhibitors on spheroid size. Cell size was reduced by Torin1 in DLD-1 (Figure 3G), HCT116- (Suppl. Figure S2F) and Caco-2-spheroids (Suppl. Figure S2F) or in the 2D system only marginally.

Apoptosis remained low under treatment with compounds, as determined by cleaved-caspase-3 staining (DLD-1, Figure 3E; HCT116, Supporting Figure SF3) and subG1 DNA content (DLD-1, Figure 3F; HCT116, Caco-2, Suppl. Figure S1E). Single inhibitor treatment had no gross apoptotic effect on the CRC cells either in 2D or in 3D culture, which is indicated by the low percentage of cells in subG1. Interestingly, only inhibition of MEK (by AZD6244) induced low rates of apoptosis in 3D culture compared to the controls. Thus, the small differences in cell size and apoptosis led to the conclusion that reduced spheroid volume, which may serve as an approximation for impaired tumor growth, is predominantly due to a reduction of cellular proliferation capacity under effective treatment conditions. Taken together, there are sharp differences in drug response in 2D vs 3D culture.

Inhibition of mTOR(C1)/AKT displayed more severe anti-proliferative activity in 3D spheroids as compared to the 2D system.

**Altered signaling in 2D versus 3D culture upon AKT/mTOR and/or MAPK-pathway inhibition**

In addition, treated cells in 2D and 3D culture were analyzed for total and phosphorylated proteins involved in AKT/mTOR- and MAPK-signaling by Western blotting. Three biological replicates were analyzed (DLD-1, Figure 4A, Supporting Figure SF4, HCT116 and Caco-2, Supporting Figures SF5, SF6) and the mean response was calculated by densitometric evaluation (Figure 4B, Suppl. Figure S2). At first, this was conducted to demonstrate proper drug action, and secondly, to identify differences in signaling upon inhibitor treatment between 2D and 3D culture. In all three cell lines, treatment with Rapamycin and Torin1 resulted in complete abrogation of phospho-S6K1 in 2D and 3D, whereas Torin1 further inhibited AKT phosphorylation as a result of mTORC2 blockade. There was a consistent increase in phospho-AKT-S473 in the presence of Rapamycin or PF4708671, which is indicative for the discontinuation of the negative feedback loop to IRS1 by these two inhibitors (Tremblay et al., 2007). Treatment with PF4708671 led to an accumulation of p-S6K1 as shown previously found to be inactive (Pearce et al., 2010) and downstream to a decreased level of p-rpS6. Treatment with MK2206 abolished AKT phosphorylation at S473 and T308, and the phosphorylation of the downstream target PRAS40 was eliminated.
Of note, upon MK2206 or AZD6244 treatment, the levels of phospho-p70S6K1 and phospho-rpS6 were decreased in spheroids, but not in the 2D cultures, indicating a response of mTORC1 activity to AKT or MEK1 inhibition only in the 3D model. Furthermore, abrogating the AKT/mTOR/S6K-axis by any inhibitor increased ERK1/2 phosphorylation effectively in 2D, but not in the 3D model.

In conclusion, these data revealed that mTOR activity and the crosstalk between AKT/mTOR/S6K signaling and the MAPK-pathway is altered in 3D compared to 2D.

Spatial differences of signaling strength account for reduced S6 phosphorylation in spheroids and faithfully recapitulate the in vivo situation

The prominent decrease of p-rpS6 in spheroids in Western blots could be due to two reasons. Either p-rpS6 is reduced in every cell to a similar extend or there are cells with variable expression levels from high to low or absent, which in sum give rise to the diminished p-rpS6-levels detected by immunoblotting.

Immunofluorescence (Figure 5A) confirmed the signaling response to treatment with inhibitors observed by Western blot analysis. In 2D treatment with Rapamycin, Torin1 and to a lesser extend PF4708671 led to a reduction of phospho-rpS6. Cross-sections of cells grown in 2D showed the same result (Supporting Figure SF7). DLD-1 cells treated with MK2206 or AZD6244 in 2D showed no decrease in phospho-rpS6. However, the spheroid sections stained for p-rpS6 revealed uneven intensities. A clear gradient of p-rpS6 staining intensity from the spheroid surface to the inner core was evident in the controls. This was also seen at reduced levels in PF4708671-, MK2206- and AZD6244- treated spheres. However, the staining inside displayed more variation than in DMSO controls (Figure 5A), whereas Torin1 and Rapamycin diminished phospho-rpS6 almost completely. Total rpS6 protein was not affected and was expressed at high levels in every cell (Suppl. Figure S3A,B).

As a more objective evaluation, image analysis was used (Schlederer et al., 2014). Automated spheroid recognition and zonation (Figure 5B, Suppl. Figure S3C) allowed to divide equatorial sphere sections into three zones: an outer-zone comprising the surface two cell layers followed by a middle-zone and the inner-core (Figure 5C). Nuclear-recognition and cytoplasmic-mask creation was used to quantitatively determine p-rpS6 levels in every single cell in the three distinct zones. In DMSO controls, the outer rim displayed a mean p-rpS6 level of 100 (Figure 5D). In the inner core, phospho-rpS6 was reduced to more than half; and in the middle zone, a mean value of 63 was determined, which indicated a non-linear, rapid decrease of S6 phosphorylation from the surface to the center (Figure 5D). In Rapamycin- or Torin1- treated spheroids, there was an almost complete loss of S6
phosphorylation in all zones. S6K1-, AKT- and MEK1-inhibition showed a gradient similar to the control but at reduced mean intensities. Interestingly, the range of the signals varied from 200 to 25 under MK2206 and AZD6244 in the outer zone, indicating that some cells still displayed maximal phospho-rpS6 levels in this area, comparable to DMSO, whereas the minimum was reduced to half. Inhibition of S6K1 reduced the range of phospho-rpS6 levels from 150 to 25 and the mean was smaller than with the AKT or MEK1 inhibitors, suggesting that rpS6 phosphorylation was reduced in all cells (Figure 5D).

Importantly, in vivo DLD-1 xenografted tumors phospho-rpS6 gradients were present as detected in the DMSO treated control spheroids (Figure 5E), underscoring the relevance of our model system to faithfully recapitulate the in vivo situation for AKT/mTOR/S6K-signaling. In vivo, highest levels of rpS6 phosphorylation were evident in the direct vicinity of cross-sectioned perpendicular tumor blood vessels. The levels of phospho-rpS6 were radially diminishing with increasing distance to the endothelium (Figure 5E). Total-rpS6 signals were invariably present in all cells (Suppl. Figure S4A). In the DLD-1 xenograft cellular p-rpS6 signals were quantified in vessel-proximal, intermediate and vessel-distal zones (Figure 5F). This analysis revealed an astonishingly similar pattern of intensity distributions, with highest levels of p-rpS6 in a three cell-layer thick vessel-proximal area, then a prominent drop in the intermediate layer and low to absent vessel-distal signals (Figure 5G). On the one hand, this qualifies DLD-1 spheroids as reliable model for physiologic mTOR/S6K-signaling in tumors. On the other hand, it revealed that growth factor, nutrient and/or oxygen gradients are a likely explanation for the formation of the gradient from the surface to the center of the spheroid.

However, this recapitulation of the in vivo data by the in vitro assay might be specific for DLD-1 cells and thus be of limited relevance. Hence, the experiments were repeated with HCT116 cells, which differ in their mutational status from DLD-1 cells (Suppl. Table S1). Indeed, the phospho-rpS6 gradient around blood vessels was confirmed in HCT116 xenografts (Figure 6A,B) and the gradual decrease of phospho-rpS6 from outside to inside was corroborated in HCT116 spheroid sections (Figure 6C), whereas total-rpS6 was unchanged (Suppl. Figure S4B). Moreover, phospho-rpS6 showed the same zonal pattern as for DLD-1 spheroids under the different treatments in 3D (Figure 6C, Suppl. Figure S3B). Phospho-rpS6 levels were quantified by image analysis (Figure 6D). Notably, the mean of all zones (Figure 6D, red dotted lines) closely recapitulated the pattern in Western blots (Suppl. Figure S2A). Ki67 and DAPI signal intensities in the three zones were also determined. The nuclei of the cells in the spheres were evenly stained with DAPI (Figure 6E). The proliferation marker Ki67 showed a well-described decrease of Ki67+ cells from the outside to the core of the spheroid (Grimes et al., 2014; Laurent et al., 2013). Ki67+ cells were significantly reduced in the treated samples as compared to the control (Figure 6F, Suppl. Table S4). Under treatment, Ki67+ cells did not
follow the strict pattern of p-rpS6 indicating that the mTOR/S6K-pathway is not solely or directly involved into cell cycle changes of HCT116 cells grown as spheres. Taken together, we show that the spatial differences of mTOR/S6K activities in human CRCs in vivo are faithfully reproduced in the spheroid model.

**Global overview of signaling differences in 2D versus 3D culture under normal and AKT/mTOR/S6K and MAPK pathway inhibitor treatment**

In order to visualize the results of this extensive study for simultaneous comparative evaluation, fold changes to the respective DMSO controls in the 2D and 3D systems were calculated and presented in heatmaps (Figure 7). First, 2D versus 3D culture was compared under normal growth conditions. The obtained data were arranged according to the mean change calculated for all cell lines (Figure 7A). This allowed assessing and identifying responsive phenotypes or signaling molecules and permitted general statements to be made. At first, a general significant downmodulation of AKT signaling was detectable in the 3D cultures (loss of S473 and T308 phosphorylation as well as reduced levels of p-PRAS40, one downstream target of AKT). Secondly, phosphorylation of S6K1 and rpS6 was significantly impaired in 3D in all cell lines. Together with the decrease of hyperphosphorylated 4E-BP1, these data clearly indicate a general decrease in mTORC1 activity in the 3D system. Interestingly, phospho-ERK1/2 was highly variable whereas phospho-PKC and E-cadherin expression was induced in the 3D spheroid culture. All the different responses could not be correlated to the mutational status (Figure 7A, right).

The common/differential response to the treatment with inhibitors in 2D and 3D culture is summarized in Figure 7B. Most obviously, S6K1-, rpS6- and 4EBP1-phosphorylation were efficiently blocked in 2D and 3D culture with all tested inhibitors, except for PF4708671 treatment, which resulted in S6K-hyperphosphorylation both in 2D and 3D. It is well documented that binding of PF4708671 to S6K1 promotes its phosphorylation by mTORC1 but thereby remaining inactive (Pearce et al., 2010). All inhibitors were significantly decreasing spheroid size. However, the proportion of cells in S-phase was not altered by PF4708671 in DLD-1 in 3D and was induced in Caco-2 both in the 2D and the 3D models. Intracellular ATP levels were decreased in DLD-1 and HCT116 in 2D and 3D and in Caco-2 in 2D culture. However, treatment with Rapamycin, Torin1 and AZD6244 slightly induced ATP content in Caco-2 in 3D for unknown reasons. Most interestingly, there was an increase of p-ERK in 2D cultures when treated with AKT-, mTOR- or S6K-inhibitors whereas the opposite was true for HCT116 and DLD-1 3D spheroids (Figure 7B, bottom panel).
We identified spatial differences in rpS6 phosphorylation in spheroids under normal growth conditions. Phospho-rpS6 levels were high at the outer rim of DLD-1 and HCT116 spheroids and gradually decreased towards the inner core of the spheres. Interestingly, a similar p-rpS6 gradient was also detected in vivo in the same CRC cells grown as xenografted tumors in mice. Here, p-rpS6 was highest in close vicinity to blood vessels and diminished with increasing distance to the vessel (Figure 7C). The phospho-rpS6 signal was almost completely abolished in Rapamycin- or Torin1-treated samples, which fully blocked p-rpS6 in all three zones. On the contrary, p-rpS6 levels were reduced in each zone upon treatment with PF4708671, MK2206 or AZD6244 but the outer zone still displayed higher signals as compared to the respective middle and inner zones (Figure 7D).

**Identification of signaling pathways altered in 3D versus 2D cultures upstream of AKT/mTOR**

We used phospho-receptor tyrosine kinase (p-RTK) arrays as a first attempt to identify potentially altered signaling pathways upstream of AKT or mTOR/S6K and which might be responsible for the decreased AKT and/or mTOR/S6K activities in 3D culture. 28 different RTKs and 11 intracellular phosphoprotein-signaling molecules were evaluated in DLD-1 and HCT116 cells grown in 2D or 3D culture. The global phospho-RTK profile is shown in a heatmap (Figure 8A). As a general downregulation of AKT and mTOR/S6K activities was identified, we focused on consistently downregulated phospho-RTKs in 3D compared to 2D culture in DLD-1 and HCT116. Surprisingly, we detected a consistent loss of Ephrin receptor (EphA2, EphA3 and EphB4) phosphorylation as well as a decrease in c-Src phosphorylation (Figure 8B,C). Interestingly, ErbB2 and ErbB3 signaling was non-uniformly changed in 2D versus 3D culture. In DLD-1 spheroids ErbB2/3 phosphorylation was significantly increased, whereas there was a sharp decrease of ErbB2/3 phosphorylation in HCT116 spheroids compared to the 2D cultures. Importantly, AKT as well as rpS6 phosphorylation were significantly reduced in the 3D system (Figure 8C) as it was expected from our previous results.

**Discussion**

The overall survival rate of cancer patients undergoing systemic therapy was extended only marginally over the past 30 years. Cancer therapy for advanced cancers is far from being efficient to date (Scannell et al., 2012). Despite some key therapeutic success in targeted therapy, the current fail rate of anti-cancer compounds in clinic trials is 96% (Bhattacharjee, 2012). Clearly, there is urgent need for more innovative preclinical in vitro models to thoroughly test promising compounds before proceeding into clinical trials.
3D models might provide better predictive power by more faithfully recapitulating the response of human tumors to anti-cancer compounds *in vivo*. This study is not a complete and comprehensive assessment of every organotypic and/or 3D model currently available (Stadler et al., 2015; Unger et al., 2014), but intentionally focuses on the comparison of 2D monolayer with free-floating 3D spheroids. This relatively simple model lacks the interaction of cancer cells with their environment such as ECM and/or stromal cells and has been used for long time to better mimic chemotherapeutic and radiation therapy response *in vitro*.

As metastasis is concerned, the predominance and importance of single circulating tumor cells *in vivo* is well accepted. However, there is recent evidence that primary tumor cell spheroids might be also of *in vivo* relevance. Cancer cell clusters were identified to pass through lymphatic endothelial cell layers in human mammary carcinomas (Kerjaschki et al., 2011), whereas colon cancer clusters were found in blood vessels in human CRC. In CRC two types of clusters were identified, which differed in their polarity. On the one hand, they interacted with their environment and on the other hand, cancer cell spheres with inverse polarity lacked contact to host structures, closely resembling *in vitro* generated spheroids. Of note, these cancer tissue derived spheres were also shown to be critically involved in liver metastasis in mice (Okuyama et al., 2016). Thus, these results might qualify spheroids as proper models to recapitulate specific subsets of cancer metastasis. Nevertheless, comprehensive systematic investigations on phenotypic changes, potentially altered signaling pathways and the response to drug treatment in 2D versus 3D systems were only occasionally performed (Ekert et al., 2014; Luca et al., 2013; Pickl and Ries, 2009). We focused on colon carcinoma, being the third most deadly cancer type in the world, to thoroughly evaluate main signaling pathways in 2D versus 3D spheroid cultures under normal and treatment conditions.

Our findings, that AKT and mTOR signaling are drastically reduced in 3D, question the reason and the underlying mechanisms. In fact, it has been shown that receptor tyrosine kinase (RTK) signaling upstream of AKT/mTOR is specifically induced in 3D compared to 2D. For EGFR/ErbB signaling in HER2 overexpressing breast and ovarian cancer cells, it has been shown that 3D culture results in HER2 activation compared to 2D (Pickl and Ries, 2009; Weigelt et al., 2010). Another study using lung cancer cultures demonstrated elevated basal EGFR and c-Met phosphorylation levels upon 3D culture compared to the levels in 2D (Ekert et al., 2014). Counterintuitively, activation of HER2 signaling was associated with decreased phospho-AKT levels in the breast and ovarian cancer cells. In line with this discrepancy, RTK-profiling in HCT116 and DLD-1 cells revealed striking differences in EGFR signaling showing a significant increase in ErbB2/3 phosphorylation in DLD-1 spheroids, whereas HCT116 spheres displayed reduced phospho-ErbB2/3 levels as compared to the levels in 2D. However, consistent with the breast cancer study, AKT signaling was decreased in both cell lines.
in 3D culture. In fact, all six CRC cell lines used in our study depicted decreased AKT activity. Of note, reduction of AKT phosphorylation in 3D compared to 2D has been described in another study with CRC (Luca et al., 2013) and breast cancer cells (Weigelt et al., 2010), thus pointing to a general mechanism in 3D. Interestingly, we observed loss of Eph receptor and c-Src phosphorylation in 3D compared to 2D culture in HCT116 and DLD-1 cells as demonstrated by RTK profiling. However, phosphorylation levels of Eph receptors were low. Importantly, these data are only correlative and further functional experiments are needed to decipher the impact of Ephrin/Eph receptor signaling on AKT/mTOR activities in spheroids. One alternative explanation for the loss of AKT activation in 3D is that signaling is switching from the AKT/mTOR pathway in the 2D to the RAS/RAF/MAPK pathway in the 3D system. Indeed, phospho-MEK levels were increased in 3D in breast and ovarian cancer, however, p-ERK levels were not substantially increased (Pickl and Ries, 2009) or were not assessed (Weigelt et al., 2010). In colon cancer, expression profiling revealed an activation of MAPK-signaling and a slight increase of p-ERK levels in Lovo cells cultured in 3D (Luca et al., 2013), whereas six other CRC lines displayed the opposite regulation. Indeed, in our study ERK phosphorylation in 3D vs 2D culture was also highly variable in the six cell lines. Some of the above-described 3D models engage laminin rich extracellular matrix (lrECM) to induce three-dimensionality whereas others do not. Thus, the ECM adds another level of complexity (e.g. via integrin signaling) on top of the 2D vs 3D difference. Hence, further experiments are necessary to interpret decreased AKT-signaling in the 3D system. Another possibility for the loss of AKT activity in 3D might be simply due to the loss of integrin signaling, which is activated in 2D culture by attachment to the plastic surface (or an ECM substrate) and thus induces AKT activation (Persad and Dedhar, 2003). Unfortunately, phospho-AKT levels in CRC spheroids were so low that it was not possible to detect spatial differences by immunofluorescence analysis. However, AKT activity might be induced via integrin signaling by embedding the spheres into ECM (basement membrane extract or collagen I gel), which is readily testable for changes in signaling in future studies by using our comprehensive approach. Alternatively, diminished AKT activity in 3D might be mediated via a specific increase in PTEN activity, which was not tested in this study. Notably, all six cell lines tested harbor wildtype PTEN. Nevertheless, AKT inhibitor treatment, which abolished AKT kinase activity as demonstrated by the loss of downstream target phosphorylation, did not have such a profound effect on S6K activity in 2D as compared to 3D culture.

To the best of our knowledge we are the first to demonstrate that spheroid culture is associated with profoundly reduced mTORC1 signaling activity. This is manifested by diminished levels of active S6K1 and S6 phosphorylation and by the decrease of hyperphosphorylated 4E-BP1, another target of mTORC1. Whether the reduced AKT activity is the reason for the downstream impairment of
mTORC1 activity is not known. However, the decrease in phospho-rpS6 levels in 3D in Western blots might be due to a gradual decrease of rpS6 phosphorylation in the inner regions of the spheroids, whereas in the surface area (two cell layers) rpS6 phosphorylation remained high. This opens an alternative possibility for the diminishing mTORC1 activity inside the sphere. The inner regions of spheroids experience decreased nutrient availability (such as amino acids) translating it into diminished nutrient signaling, which is mandatory for mTOR activation (Jewell et al., 2013). Indeed intracellular amino acid concentrations rapidly drop to the inside of spheroids (Kasinskas et al., 2014). This might explain the drastic decrease of rpS6 phosphorylation from high levels in the outer two-cell-layer thick zone to low levels in the deeper areas. Of note, cell proliferation also decreased gradually in the inner zones of the spheroids, as demonstrated earlier (Grimes et al., 2014; Laurent et al., 2013). However, when mTOR activity was inhibited rpS6 phosphorylation was lost but the Ki67 remained, indicating that there is no direct correlation of rpS6 phosphorylation with cell proliferation. Importantly, the drop of rpS6 phosphorylation in the DLD-1 and HCT116 spheroids was also seen in vivo in viable cell clusters around blood vessels in tumor xenografts in mice. These results clearly qualify our spheroids as faithful models of the in vivo situation at least for rpS6 phosphorylation and most probably for the upstream AKT/mTOR signaling pathway.

It is well known that the RAS/MAPK and the PI3K/AKT pathways can either positively or negatively regulate each other (Mendoza et al., 2011). Inhibition of MEK can induce RTK mediated activation of AKT via ERK mediated inhibitory phosphorylation of GAB1 (Yu et al., 2002). On the contrary, AKT can negatively regulate ERK activation by inhibitory phosphorylation of RAF (Guan et al., 2000). This might explain why ERK phosphorylation is induced in all our cell lines in 2D upon inhibition of AKT. However, inhibiting mTOR or S6K1 also results in profound MAPK pathway activation in the 2D system. To the best of our knowledge, there are no reports on this obvious cross inhibition, which is released by AKT/mTOR pathway inhibitors. Even more puzzling is the fact that induction of ERK phosphorylation only occurs in 2D cultures, whereas in 3D the ERK phosphorylation was diminished upon inhibition of AKT/mTOR/S6K suggesting a substantial rewiring of the two pathways in 3D. Intriguingly, differential responses to inhibitor treatment of the three cell lines in 2D were abolished in 3D, and all cell lines behaved similar, indicating a more robust response in the 3D system.

Taken together, our results demonstrate a general theme in spheroid culture of colon cancer cells, namely the reduction of the AKT/mTOR/S6K pathway and a substantial rewiring of signaling in 3D compared to 2D culture. Our comprehensive study on colon cancer cells identifies common mechanisms being active in 3D and significantly different to 2D cultures. Despite being a simple and well-known model for many years, many unknown mechanisms are yet to be discovered in spheroids as a 3D model. Notably, the identification of sphere-like cancer cell clusters (Kerjaschki et
al., 2011; Okuyama et al., 2016) entering, traveling in and evading vessel structures with or without interacting with their environment may open novel perspectives for metastasis intervention. Such findings also strengthen the importance of spheroids as valid model for mimicking certain aspects of human cancer biology. Moreover, here we demonstrate that mTOR/S6K signaling gradients present in vivo are reliably reproduced in spheroid models. Thus, better understanding of 3D cancer biology in vitro - such as AKT/mTOR and MAPK signaling in spheroids - might help to better comprehend cancer biology in vivo, predict the response of cancer cells to targeted therapy as well as develop novel therapeutic concepts.

Materials and Methods

Cell culture

Human colon cancer cell lines LS174T (ATCC® #CL-188™), HT29 (ATCC® #HTB-38™), SW620 (ATCC® #CCL-227™), HCT116 (ATCC® #CCL-247™), Caco-2 (ATCC® #HTB-37™) and DLD-1 (ATCC® #CCL-221™) were obtained from the American Type Culture Collection (ATCC®) and used from early passages derived from our master cell bank. STR profiling was performed for cell authentication. The cells were cultivated in DMEM high glucose (4.5 g/l) supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics (60 mg/l penicillin, 100 mg/l streptomycin sulfate) at 37°C and 5% CO₂.

Spheroid formation (3D) with 3,000 cells per spheroid was induced as described (Korff and Augustin, 1998; Walzl et al., 2014). In brief, cells were detached, counted and seeded in 100 µl of DMEM/5% FCS/Gln/PenStrep containing 0.3% methylcellulose into 96-well plates (round bottom, untreated) for 24h. In parallel, cells were seeded on cell culture plates (2D) and cultivated in the same medium. Cells were cultured at 80% humidity, 5% CO₂ and 20% O₂ (Walzl et al., 2012). Cultures were treated with different inhibitors (see Suppl. Table S2) for additional 24h. Spheroid formation time and/or the presence of methylcellulose did not alter drug response (Supporting Figures SF8, SF9, respectively).

Cellular/spheroid morphology was evaluated microscopically and was analyzed (Cell^F, Olympus, Tokio, JPN) to determine spheroid volume by measuring projected areas, followed by radius and volume (µm³) determination.
**Inhibitor treatment**

For inhibitor treatment in 2D and 3D culture, 100 nM of rapamycin (Grabiner et al., 2014; Kang et al., 2013; Sarbassov et al., 2006), 250 nM of Torin1 (Cheng et al., 2016; Grabiner et al., 2014; Kang et al., 2013; Thoreen et al., 2012), 10 µM of PF47068671 (Pearce et al., 2010; Schipany et al., 2015; Zhang et al., 2015), 1 µM of MK2206 (Devery et al., 2015; Shen et al., 2015; Sung et al., 2016) and 1 µM of AZD6244 ((Devery et al., 2015; Ewald et al., 2015) were employed as these concentrations have been most frequently used in cell-based assays and displayed full response at their respective targets (phospho-protein analysis in Western blots (see Figure 4 and Westerns in accompanying Figures).

**Cell cycle, cell size and metabolic activity assays**

Cell cycle analysis was done with the Click-iT® EdU Alexa Fluor® 488 Kit (Invitrogen, Thermo Scientific Inc., Waltham, MA). Cells and spheroids were exposed to EdU (5-ethynyl-2'-deoxyuridine, 10 µM) at 37°C for 20 min. Thereafter, spheroids were trypsinized and cells were analyzed. 7AAD was used to stain total DNA and forward scatter to determine cell size. The metabolic capacity of cells was determined with CellTiter-Glo® (Promega, Madison, WI) according to the manufacturer’s protocol and luminescence measurement (Synergy HT, Biotek®).

**Immunoblot analysis**

Whole cell lysates were prepared from 2D or 3D cultures. Cells and spheroids were washed in PBS and extracted in RIPA lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1% TritonX-100, 0.1% SDS, 0.5% sodium-deoxycholate, 1 mM PMSF, 4 µg/ml aprotinin, 4 µg/ml leupeptin, 0.6 µg/ml benzamidinchloride, 20 µg/ml trypsin-inhibitor). Supernatants were collected after centrifugation (14,000rpm/4°C/20 min). Equal amounts of protein (10-15 µg) were mixed with loading dye (200 mM Tris pH 6.8, 400 mM DTT, 8% SDS, 0.4% bromphenol blue, 40% glycerol), denatured, subjected to SDS-PAGE and transferred to a nitrocellulose membranes. Blocked membranes were probed with primary antibodies at 4°C overnight and with horseradish-peroxidase-conjugated secondary antibodies (anti-mouse-IgG-heavy-and-light-chain and anti-rabbit-IgG-heavy-and-light-chain, Bethyl Laboratories Inc., Montgomery, TX) at RT for 1h. Signals were detected using chemiluminescence and x-ray films. The mean integrated density of each band was determined with ImageJ (National Institutes of Health, Bethesda, MD). For antibodies see Suppl. Table S3.
**Immunohistochemistry and Immunofluorescence**

Cells and spheroids were fixed in 4% PFA at RT for 30 min. Fixed spheroids were molded into agarose-gels (1%), embedded into paraffin, sectioned (5 µm) and subjected for antigen-retrieval in citrate buffer pH6 (DAKO, CA) at 120°C for 10 min.

For immunohistochemical staining endogenous peroxidase activity was blocked in 3% H₂O₂ for 10 min. Blocking was performed in horse serum (2.5% in PBS, Vector Laboratories, Burlingame, CA) for 20 min at RT. Primary antibody incubation at 4°C overnight and secondary antibody (biotin-labeled, Vector Laboratories) at RT for 1h, followed by streptavidin-HRP (Leica Microsystems, Germany) incubation for 30 min at 4°C and chromogenic development (AEC, DAKO). Nuclei were counterstained with hematoxylin, and slides were cover-slipped (Aquatex®, Merck-Millipore, Billerica, MA). 20x (3D) and 40x (2D) objectives (NA = 1.3) were used.

For immunofluorescence spheroids were handled as described above. In 2D, PFA (4%) fixed cells were permeabilized in methanol at -20°C for 10 min. 2D and 3D samples were blocked in PBS/1% BSA at RT for 1h. Primary antibody incubation at 4°C overnight. AlexaFluor®488- and AlexaFluor®546-conjugated secondary antibodies (Thermo Scientific Inc., Waltham, MA) were used at RT for 1h. Nuclei were counterstained with 2 µg/ml DAPI (Sigma, St. Louis, MO). Slides were mounted with Vectashield® (Vector Laboratories). Confocal fluorescence images were recorded on a Leica-SP8 with a 20x immersion objective (NA = 1.3).

**Automated spheroid zonation and image analysis**

Image analysis of spheroid sections was performed with StrataQuest software (TissueGnostics, Vienna, Austria). For automated spheroid detection a virtual channel was created followed by threshold segmentation, generation of a background mask followed by distance transformation. Three intervals were set on this distance-transformed image to correspond to three zones of interest in the spheroid. The outer zone was set to be two-cell-layer thick; the remaining zones were equally divided. Nuclear detection was performed in the DAPI channel and cellular masks were computed. For each cell the intensities for the DAPI, phospho-rpS6, Ki67 channels were calculated and the belonging to a certain zones was determined. For phospho-rpS6 quantification of the areas around cross-sectioned blood vessels in HCT116 and DLD-1 xenografted tumors the same image analysis was performed except that the zonation was performed by hand. The vessel-proximal zone was defined by a three-cell-layer thickness immediately around the vessel, followed by an intermediate and a vessel-proximal area approximating the size of the analyzed spheroids.
**RTK Signaling Antibody Array**

Phosphorylation levels of receptor tyrosine kinases (RTKs) and other signaling nodes were determined (Cell Signaling Technology). DLD-1 and HCT116 colon cancer cells cultivated in 2D and 3D in normal growth medium (DMEM 5% FCS) harvested 48h after seeding. The assay was done as described in the manufacturer’s protocol. The arrays were developed using x-ray films, spot intensities were quantified and background corrected using ImageJ.

**Statistical analysis**

Bar graphs are presented as mean ±standard deviation (SD) or standard error of mean (SEM). For statistical analysis Student’s t-test (unpaired, two-tailed) was performed. P-values are indicated as * (p ≤ 0.05), ** (p ≤ 0.01), *** (p ≤ 0.001) or **** (p ≤ 0.0001). For exact p-values of all experiments see Suppl. Table S4.
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Competing interests

The authors declare no competing interests.

Author contributions

AW and HD designed the research; AW, MSch, KP, MS, SW, DU, CU, NK performed the research; AW and HD analyzed the data; DS, LK, MH, GK were involved in manuscript preparation; AW and HD wrote the manuscript; all authors approved the manuscript for publication.

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Figure 1 – Analysis of morphology and cell cycle in 2D vs 3D cell culture. LS174T, HT29, SW620, HCT116, Caco-2 and DLD-1 colon cancer cells were grown as monolayers or spheroids (3,000 cells/spheroid). After 48h, cells and spheroids were photographed and cell cycle analysis was performed. (A) Representative microscopic images of colon cancer cells in 2D and 3D. Scale bars: 100 µm. (B) Cell cycle analysis of monolayer cells and spheroids using EdU/7AAD. Percentages of S-phase (C) and subG1 (D) of cell cycle profiles (boxed in red for LS174T in B) were determined (LS174T: n=4, HT29: n=3, SW620: n=6, HCT116: n=8, Caco-2: n=4, DLD-1: n=9 per condition). Bars are mean ±SD and p-values are indicated. Detailed cell cycle analysis is available in Supporting Figure SF2 (figshare). (E) Cleaved-caspase-3 (green) staining in 2D and in spheroid sections. White arrowheads indicate apoptotic cells, DAPI (blue).
Figure 2 – PI3K/AKT/mTOR- and RAS/RAF/MAPK-signaling in 3D spheroids compared to 2D. The cells were cultured in 2D and 3D for 48h. Cells and spheroids (3,000 cells/spheroid) were harvested and (A) immunoblot analysis of different proteins of the PI3K/AKT/mTOR- and RAS/RAF/MAPK-pathway was performed. Detection of phospho-proteins is indicated by a prefixed “p-“. α-tubulin
and GAPDH are loading controls. (B) The protein levels in 2D and 3D of Western blots shown in A were evaluated densitometrically (n=6-12 per condition, three independent experiments, additional blots in Supporting Figure SF1 (figshare). Bars are mean-integrated-density ±SD.
Figure 3 – Morphologic and phenotypic response of colon cancer cells to inhibitor treatment in 2D vs 3D. DLD-1 cells were grown as monolayer or spheroids (3,000 cells/spheroid) for 24h and further cultured in the presence or absence of Rapamycin (100 nM), Torin1 (250 nM), PF4708671 (10 µM), MK2206 (1 µM) and AZD6244 (1 µM) for 24h. DMSO served as control. (A) Representative microscopic images of control and treated DLD-1 cells in 2D and 3D are shown. Scale bars: 100 µm. (B) The volume of spheroids was calculated and normalized to DMSO controls (four independent experiments, n=18 per condition). (C) Cell cycle analysis was performed using EdU/7AAD and %S-phase is shown (n=3 per condition). (D) Cellular metabolic capacity (intracellular ATP) was evaluated in DLD-1 (n=6 per condition). (E) Cleaved-caspase-3 staining (green) in DLD-1 cells in 2D/3D. White arrowheads indicate apoptotic cells. Nuclear stain (DAPI, blue). (F) Quantification of dead cells by subG1 content analysis; n=3. (G) Cell size was determined by forward scatter (FSC) and normalized to the 2D-DMSO controls (n=3 per condition). Bars are mean ±SD. The box ranges from the lower (Q1) to the upper quartile (Q3) in whisker-box-plots; horizontal line, median; whiskers extend to the minimum/maximum.
Figure 4 –PI3K/AKT/mTOR- and RAS/RAF/MAPK-signaling in the presence or absence of inhibitory compounds. DLD-1 colon cancer cells pre-grown in 2D and 3D (3,000 cells/spheroid) for 24h, were treated with DMSO, 100 nM Rapamycin, 250 nM Torin1, 10 µM PF4708671, 1 µM MK2206 and 1 µM AZD6244 for 24h. 2D and 3D cultures were harvested and subjected to (A) Immunoblot analysis.
Detection of phospho-proteins is indicated by a prefixed “p-“. α-tubulin and GAPDH served as loading control. (B) The bands in Western blots from 3 independent experiments (Supporting Figure SF4) were evaluated densitometrically (n=2-8 per condition). Bars are mean-integrated-density ±SEM.
Figure 5 – Gradual decrease of rpS6 phosphorylation from the outside to the inner core of DLD-1 spheroids and from vessel proximal to vessel distal areas of DLD-1 tumors grown in vivo. DLD-1 colon cancer cells were grown in 2D and 3D (3,000 cells per spheroid) conditions for 24h and treated with DMSO, 100 nM Rapamycin, 250 nM Torin1, 10 µM PF4708671, 1 µM MK2206 and 1 µM AZD6244 for another 24h. (A) Confocal images of DLD-1 cells in 2D or 3D (5 µm sections) stained for p-rpS6 S240/244 (white, green in merge). Nuclei (blue, DAPI). Representative images are shown. Scale bars: 20 µm. See also Suppl. Figure S3A. (B) Image analysis by segmentation and spheroid recognition with the StrataQuest software allowed for automated spheroid zonation in three areas. (C) Spheroids were separated into an outer (o), middle (m) and inner (i) zone and cell-fluorescence signals were measured (minimum 5 sections from different spheroids, cell number: n=157-803 per condition). (D) The mean p-rpS6 S240/244 fluorescence intensity of all individual DLD-1 cells in spheroids was quantified using StrataQuest software. Scatter blots are shown; individual data points (grey dots), the horizontal black lines indicate means. A red-dotted line depicts the mean calculated from all three areas. (E) p-rpS6 S240/244 (green), CD31 (red) and DAPI (blue) staining of a DLD-1 xenograft tumor (5 µm sections). (F) For quantification, xenograft tumors were separated into vessel proximal (vp), intermediate (i) and vessel distal (vd) zones. (G) Quantification of mean p-rpS6S240/244 and DAPI intensity in DLD-1 xenograft tumors (3 animals, 15 tumor regions, cell numbers analyzed: n=3128-3372) using StrataQuest. Representative images are shown in Suppl. Figure S4A.
Figure 6 – Phospho-rpS6 levels and Ki67 positivity in HCT116 spheroids compared to 2D and HCT116 xenografts in mice. (A) Phospho-rpS6 S240/244, total rpS6 and DAPI staining of a HCT116 xenograft tumor (5 µm sections). For quantification, xenograft tumors were separated into a vessel proximal (vp), an intermediate (i) and a distal vessel (dv) zone (white dotted circles). The position of the vessel is indicated by a white *. (B) Quantification of mean p-rpS6S240/244 and DAPI intensity in HCT116 xenograft tumors (3 animals, 23 tumor regions, individual cells analyzed n=2714-3484) using the StrataQuest software in vessel proximal (vp), intermediate (i) and vessel distal (vd) zones. Representative images are shown in Suppl. Figure S4B. (C) HCT116 cells were grown in 2D and 3D...
(3,000 cells per spheroid) conditions for 24h and treated with DMSO, 100 nM Rapamycin, 250 nM Torin1, 10 µM PF4708671, 1 µM MK2206 and 1 µM AZD6244 for another 24h. Immunofluorescence staining of p-rpS6 S240/244 and Ki67 of HCT116 cells in 2D or 3D (5 µm sections). Nuclei were counterstained with DAPI. Representative confocal images are shown. Scale bars: 25 µm (2D), 50 µm (3D). (D,E) The mean p-rpS6 S240/244 and DAPI fluorescence intensity of all individual HCT116 cells in 3D was quantified using the StrataQuest software. Spheroids were separated into an outer, middle and inner zone and fluorescence signals were measured (minimum 5 sections from different spheroids analyzed, cell number: n=170-1161 per condition). Scatter blots are shown; individual data points (grey dots), the horizontal lines indicate means. The mean calculated from all three areas is indicated by a red dotted line. Significant differences of these means compared to DMSO controls are indicated by red stars. (F) Whisker box plots represent % of Ki67 positive (Ki67+) cells in HCT116 spheroids, which were separated into an outer, middle and inner zone as in D. The box ranges from the lower (Q1) to the upper quartile (Q3) in whisker-box-blots; horizontal line, median; whiskers extend to the minimum/maximum.
Figure 7 – Schematic overview of all results obtained in this study. Heatmaps indicate mean fold changes. Downregulation is indicated in blue, upregulation in red. (A) Regulation (fold change to 2D) of cell cycle distribution, PI3K/AKT/mTOR- and RAS/RAF/MAPK-signaling and an overview of mutations are given for the six colon cancer cell lines (LS174T, HT29, SW620, HCT116, Caco-2, DLD-1) in 3D compared to 2D. The cancer cell transcriptional subtype is indicated: G, goblet like, TA, transit amplifying like, S, stem cell like. (B) For Caco-2, HCT116 and DLD-1 differences in the regulation (fold change to respective DMSO controls) of signaling molecules, cell cycle distribution, cell size, metabolic capacity and spheroid size in 2D and 3D cell culture upon inhibition of different targets of the pathways are shown. (C) Similar regulation (fold change to outer zone or vessel proximal mean intensity) of p-rpS6 S240/244 in DMSO treated DLD-1 and HCT116 spheroids or in DLD-1 and HCT116 xenograft tumors separated into an outer, middle and inner zone or in a vessel proximal, intermediate and distal zone, respectively. (D) Regulation (fold change to DMSO controls, mean intensity) of p-rpS6 S240/244 in control and treated (R, Rapamycin; T, Torin1; P, PF4708671; M, MK2206; A, AZD6244) DLD-1 and HCT116 spheroids separated into an outer, middle and inner zone.
**Figure 8 – Analysis of phosphorylation levels of RTKs.** DLD-1 and HCT116 cells were grown as monolayer and spheroids (3,000 cells per spheroid) for 48h. 2D and 3D samples were harvested and subjected to phospho-RTK analysis using the PathScan® Array. Spot intensities were quantified by densitometry using ImageJ. 

(A) Heatmaps show the distinct expression levels of 18 phospho-RTKs.
and 11 signaling nodes in DLD-1 (two biological replicates) and HCT116 cells in 2D vs 3D culture. Absent/very low signals are indicated in white; high expression (red); intermediate signals (yellow). (B) Selected spots are shown. (C) Different expression patterns of particular phospho-RTKs and other signaling molecules. Bars represent means (DLD-1, n=4; HCT, n=2); error bars indicate the range.
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Supplementary Figure S1 – Morphologic and phenotypic analysis of colon cancer spheroids compared to monolayer cultures. HCT116 and Caco2 were grown in 2D and 3D for 24 h and treated with Rapamycin (100 nM), the mTOR inhibitor Torin1 (250 nM), the S6K1 inhibitor PF4708671 (10 μM), the AKT inhibitor MK2206 (1 μM) and the MEK1 inhibitor AZD6244 (1 μM) for another 24 h. DMSO served as control. Representative brightfield microscopic images of HCT116 and Caco2 (A) are shown. Scale bars represent 100 μm. For HCT116 (n = 15 per condition) and Caco2 (n = 15 per condition), the spheroid volume and the spheroid area (B), respectively, were calculated. The percentage of HCT116 and Caco2 cells in 2D and 3D cultures in S-phase (C) and sub-G1-phase (E) was determined using the Click-it® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (Invitrogen, Thermo Scientific Inc., Waltham, MA). Metabolic activity (D) of HCT116 and Caco2 cells cultured as monolayers and spheroids ± inhibitors was evaluated using the CellTiter-Glo luminescence assay (Promega, Madison, WI) by analyzing the level of ATP present within the cells (n = 6 per condition). Evaluation of the forward scatter (FSC) was used for determining cell size of HCT116 and Caco2 (F) 2D and 3D cultures. Bar graphs represent the mean ± standard deviation (SD). Boxplots were created in GraphPad Prism 4. The box represents the middle 50% of the data ranging from the lower quartile (Q1) to the upper quartile (Q3). The line within the box indicates the median (Q2) and the whiskers represent the lower and upper 25% of the data - the minimum and maximum values respectively.
Supplementary Figure S2 – PI3K/AKT/mTOR- and RAS/RAF/MAPK-signaling in the presence or absence of inhibitory compounds. HCT116 and Caco2 colon cancer cells, grown in untreated conditions in 2D and 3D (3,000 cells per spheroid) for 24h, were treated with DMSO, 100 nM Rapamycin, 250 nM Torin1, 10 μM PF4708671, 1 μM MK2206 and 1 μM AZD6244 for another 24h. 2D and 3D cultures were harvested and subjected to Western blot analysis. The bands in Western blots from two to three independent experiments were evaluated densitometrically with ImageJ (n=2-8 per condition). Quantification is shown for (A) HCT116 and (B) for Caco2 cells. Bars are mean integrated density ±SEM. For statistical analysis Student’s t-test (unpaired, two-tailed) was performed. p-values are indicated. Detection of phospho-proteins is indicated by a prefixed “p-”. α-tubulin and GAPDH served as loading control. Western blots used for this quantification can be viewed in Supporting Figure SFS for HCT116 and in Supporting Figure SF6 on figshare.
Supplementary Figure S3 – Phospho-rpS6 and total rpS6 expression in human colon cancer spheroids. A DLD-1 and B HCT116 colon cancer cell spheroids were formed for 24h and subsequently treated with DMSO, 100 nM Rapamycin, 250 nM Torin1, 10 μM PF4708671, 1 μM MK2206 and 1 μM AZD6244 for another 24h. Spheroids were subjected to FFPE and sections stained with antibodies against phospho-rpS6 (green) and total rpS6 (red). Representative samples are shown. C StrataQuest software was used for automated spheroid recognition and subsequent zonation by generating a mask followed by distance transformation. This allowed automatic generation of three zones. Nuclear segmentation was performed to measure cytoplasmic p-rpS6 fluorescence intensity around individual DAPI stained nuclei in the different zones.
Supplementary Figure S4 – Phospho-rpS6 and total rpS6 expression in human colon cancer xenografts.
A DLD-1 and B HCT116 colon cancer cells were subcutaneously injected into SCID mice and xenograft tumors were removed after reaching a size of 1cm diameter. Tumors were subjected to FFPE and sections stained with antibodies against phospho-rpS6 (green) and total rpS6 (red). Nuclei are counterstained with DAPI (blue). Analyzed areas for phospho-rpS6 intensity quantification are shown (white dotted circles). The position of cut blood vessels perpendicular to the section plane are indicated (white stars). Scale bars (black): 50 µm.
Supporting Figure legends for Figures on figshare (please click on the hyperlinks to open the figures on figshare)

**Supporting Figure SF1 (figshare)** - Analysis of PI3K/AKT/mTOR- and RAS/RAF/MAPK-signaling in 2D vs 3D cell culture. The colon cancer cells (LS174T, HT29, SW620, HCT116, Caco2 and DLD-1) were cultured in 2D and 3D for 48h. Cells and spheroids were harvested and Western blot analysis of different proteins of the PI3K/AKT/mTOR- and RAS/RAF/MAPK-pathway was carried out. Detection of phosphorylated proteins is indicated by p- prior to the protein name and the phosphorylated amino acid is indicated behind the protein name. α-tubulin and GAPDH served as loading control. Two independent biological replicates are shown (AB).

**Supporting Figure SF2 (figshare)** - Analysis of cell cycle and PI3K/AKT/mTOR- and RAS/RAF/MAPK-signaling in 2D vs 3D cell culture. The colon cancer cells (LS174T, HT29, SW620, HCT116, Caco2 and DLD-1) were cultured in 2D and 3D for 48h. Cells and spheroids were harvested and cell cycle analysis (A) was performed using the Click-iT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (Invitrogen, Thermo Scientific Inc., Waltham, MA) (LS174T: n = 4, HT29: n = 3, SW620: n = 6, HCT116: n = 8, Caco2: n = 4, DLD-1: n = 9 per condition).

**Supporting Figure SF3 (figshare)** - Apoptosis detection in HCT116 cells to inhibitor treatment in 2D vs 3D. HCT116 cells were grown as monolayer and spheroids (3,000 cells per spheroid) for 24h. 2D and 3D samples were cultured in the presence or absence of Rapamycin, Torin1, PF4708671, MK2206 and AZD6244 for another 24h. DMSO served as control. Apoptotic cells were identified by cleaved caspase 3 staining (green) in 2D and in spheroid sections. Apoptotic cells are indicated by white arrowheads. Nuclei are counterstained with DAPI (blue).

**Supporting Figure SF4 (figshare)** - Molecular evaluation of PI3K/AKT/mTOR- and RAS/RAF/MAPK-signaling in the presence or absence of inhibitory compounds. DLD-1 colon cancer cells, grown in 2D and 3D for 24h, were treated with DMSO, which served as control, Rapamycin, the pan mTOR inhibitor Torin1, the S6K1 inhibitor PF4708671, the AKT inhibitor MK2206 and the MEK1 inhibitor AZD4266 for another 24h. Afterwards, 2D and 3D cultures were harvested and whole protein extracts were subjected for Western blot analysis of different proteins of the PI3K/AKT/mTOR- and RAS/RAF/MAPK-pathway. A biological replicate 2, B biological replicate 3. Detection of phospho-proteins is indicated by p- prior to and the phosphorylated amino acid is indicated behind the protein name. α-tubulin and GAPDH served as loading control.

**Supporting Figure SF5 (figshare)** - Molecular evaluation of PI3K/AKT/mTOR- and RAS/RAF/MAPK-signaling in the presence or absence of inhibitory compounds.
HCT116 colon cancer cells, grown in 2D and 3D for 24h, were treated with DMSO, which served as control, Rapamycin, the pan mTOR inhibitor Torin1, the S6K1 inhibitor PF4708671, the AKT inhibitor MK2206 and the MEK1 inhibitor AZD4266 for another 24h. Afterwards, 2D and 3D cultures were harvested and whole protein extracts were subjected for Western blot analysis of different proteins of the PI3K/AKT/mTOR- and RAS/RAF/MAPK-pathway. A biological replicate 1, B biological replicate 2. Detection of phospho-proteins is indicated by p- prior to and the phosphorylated amino acid is indicated behind the protein name. α-tubulin and GAPDH served as loading control.

**Supporting Figure SF6 (figshare)** - Molecular evaluation of PI3K/AKT/mTOR- and RAS/RAF/MAPK-signaling in the presence or absence of inhibitory compounds. Caco-2 colon cancer cells, grown in 2D and 3D for 24h, were treated with DMSO, which served as control, Rapamycin, the pan mTOR inhibitor Torin1, the S6K1 inhibitor PF4708671, the AKT inhibitor MK2206 and the MEK1 inhibitor AZD4266 for another 24h. Afterwards, 2D and 3D cultures were harvested and whole protein extracts were subjected for Western blot analysis of different proteins of the PI3K/AKT/mTOR- and RAS/RAF/MAPK-pathway. A biological replicate 1, B biological replicate 2. Detection of phospho-proteins is indicated by p- prior to and the phosphorylated amino acid is indicated behind the protein name. α-tubulin and GAPDH served as loading control.

**Supporting Figure SF7 (figshare)** - Gradual decrease of rpS6 phosphorylation from the outside to the inner core of DLD-1 spheroids. DLD-1 colon cancer cells were grown in 2D and 3D conditions for 24h and treated with DMSO, Rapamycin, Torin1, PF4708671, MK2206 and AZD6244 for another 24h. Representative images of p-rpS6 S240/244 immunohistochemistry staining (chromogen: diaminobenzidine, brown) of DLD-1 cells cultured on filter membranes and as spheroids (cross sections, 5 µm). Scale bars: 20 µm.

**Supporting Figure SF8 (figshare)** - Different spheroid formation time does not alter phenotypes in 3D. A DLD-1 colon cancer cells were seeded at different cell numbers for spheroid formation (1000, 1500, 2000, 3000) and these spheroids were for different time periods in order to reach the same spheroid size prior to experimentation (e.g. 3000 cells were incubated for 24 hrs, whereas 1000 cells were incubated for 96 hrs to reach the same sphere size). The initial differences of spheroid sizes are shown below in A after 24 hrs. After 24, 48 and 72 hrs of further incubation spheroids formed by 2000, 1500 and 1000 cells, respectively, reached the same size as 3000 cells after 24 hrs. B These spheroids of the same size but with different spheroid formation periods were then subjected to cell cycle analysis (EdU incorporation) and DNA staining (7-AAD) and displayed no apparent differences in cell cycle distribution. C To generalize these observations the same experimental approach was repeated with HCT116 cells and showed the same results.

**Supporting Figure SF9 (figshare)** - The presence of methylcellulose does not affect treatment response. DLD-1 colon cancer cells, grown in 2D and 3D for 24h, were treated with DMSO, which served as control, Rapamycin, the pan mTOR inhibitor Torin
and the AKT inhibitor MK2206 for further 24h in the presence or absence of 0.3% methylcellulose. Spheroid formation was carried out in ultra low attachment plates (96 well U-shaped, Thermo Scientific) to avoid cell attachment in the absence of methylcellulose. 2D and 3D cultures were harvested and whole protein extracts were subjected to Western blot analysis of different proteins of the PI3K/AKT/mTOR pathway. No difference in Akt and S6 phosphorylation was detected in methylcellulose containing culture conditions versus methylcellulose free incubation in DMSO controls and in Torin1 or MK2206 treated cells.