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Characteristics of multicellular tumor spheroids formed by pancreatic cells expressing different adhesion molecules

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ABSTRACT

Aims: Multicellular tumor spheroids (MCTS) produced by different methods vary in forms, sizes, and properties. The aim of this work was to characterize MCTS formed by six pancreatic cell lines on a non-adherent surface.
Materials and methods: Human pancreatic cells were grown in 2D and 3D conditions and compared for the expression of E- and desmosomal cadherins (PCR, confocal microscopy), growth, cell cycling, apoptosis (flow cytometry), and a response to antitumor drugs doxorubicin and gemcitabine (MTT-assay).
Key findings: Three types of MCTS were identified: BxPC-3, T_3M_4 formed small number of large and dense spheroids representing type I MCTS; COLO-357 and AsPC-1 generated type II multiple and loose MCTS of different sizes while MiaPaCa-2 and PANC-1 represented type III cultures which grew almost as floating monolayer films. Formation of type I MCTS depended on the simultaneous expression of DSG3 and several DSC proteins; II MCTS expressed solely DSG2-DSC2 but not DSG3, while type III cells either did not express E-cadherin or a pair of DSG and DSC proteins. Cells in type I MCTS but not in types II and III ones quickly became quiescent which correlated with a decrease in the proliferation, increased apoptosis, and a higher resistance to antitumor drugs doxorubicin and gemcitabine.
Significance: Taken collectively, pancreatic cells significantly vary in the expression of desmosomal cadherins, resulting in the formation of MCTS with different characteristics. The sensitivity of MCTS to various drugs depends on the type of cells and the method of spheroid preparation used.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most severe forms of cancer with high rate of mortality [1]. Most chemotherapeutic agents that successfully passed preclinical studies, are poorly effective in clinical trials both due to the specific features of PDAC and the lack of reliable predictive *in vitro* and *in vivo* tests. MCTS evidently better resemble tumors than flat cell monolayers (2D cultures) due to a different geometry of the culture leading to the change in nutrient and oxygen turnover and cell-to-cell cross talk. The development of a simple, high-throughput MCTS model of human PDAC is restrained by the methodological problems and poor understanding of MCTS physiology. There are several methods of MCTS production which can be divided in two types: i) conventional with the limitation of cell spreading from each other and ii) the formation of MCTS based on E-cadherin contacts [2–6]. First types of MCTS can be generated from any cells including heterogeneous populations [3–4] while the second one can be formed only by the cells expressing E-cadherin which mediates primary adhesion between epithelial cells [5–9]. Although E-cadherin mediates primary contacts between cells [10–11], the strength of adhesion is more dependent on the structure of desmosomes.

Human pancreatic tumor cell lines differ significantly in the phenotype and stage of differentiation that can affect forms, sizes and functions of cells in MCTS. Longati et al. generated MCTS from AsPC-1, BxPC-3, Capan-1, and PANC-1 using non-adherent surfaces technique [12]. AsPC-1, PANC-1, and Capan-1 formed irregular spheroids while BxPC-3 – round, regular, dense and compact ones. PANC-1, BxPC-3 and AsPC-1 are poorly differentiated, whereas Capan-1 is a well differentiated PDAC cell line [13]. AsPC-1 and Capan-1 originate from metastatic sites; PANC-1 is classified as a duct epiteloid carcinoma. BxPC-3 originated from primary PDAC [13]. McLeod et al. reported that AsPC-1, BxPC-3, and Capan-1 did not form MCTS when the spinner flasks

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Table 1
PCR primer sequences.

Gene	Size (bp)	Forward primer	Reverse primer
Desmoglein 1	554	GACAGGATTTGAACTAACAGAGG	GTGACATTACCATAGCCCAG
Desmoglein 2	475	TACAAGCCTACACCCATTCCCATC	TGTTTGGGTGTCCATCCAGGTC
Desmoglein 3	344	TCAGATTACCAAGCAACCCA	GTGGTTTGGTTCATCTGCATCTGTGG
Desmocollin 1	369	GAAGATGATAATGATAACGCCC	CTGTGAAAGATGGTGGATTGTC
Desmocollin 2	321	CTTGGATGAGAGGCAGGCG	TCGGCATCTAGTTTGGAGGGA
Desmocollin 3	560	GAACCGGACACAATGCATACGC	ACCAAGGCTCTGTTCAAGGCTGT
E-cadherin	567	CACATTTCCCAACTCCTCTC	GACCACACTGATGACTCCTG
β-actin	318	CACTCTTCCAGCCTTCCTTCC	CGGACTCGTCATACTCCTGCTT

were used [14]. The inconsistence in size, shape and growth parameters of MCTS was shown for pancreatic MCTS by other groups [15].

Spheroids larger than 200–300 μm are reported to contain a thin layer of proliferating cells surrounding an inner quiescent or necrotic core [16,17]. As soon as quiescent cells do not proliferate, the growth rate of MCTS must decrease with time. Several reports indeed demonstrated the absence of proliferation in MCTS [16] while others found constant increase in MCTS volumes [17–20]. The latter are usually produced by methods belonging to type I such as “hanging drops”, or agarose coating.

Most reports demonstrate a higher resistance of MCTS to chemo- and radiotherapies compared to cells cultured in monolayers [19,21,22]. However the sensitivity of MCTS to various drugs also can depend on the type of cells and the method of spheroid preparation used. In this study we have compared the expression of *E*- and desmosomal cadherins, growth, cell cycling, apoptosis, and a resistance to doxorubicin and gemcitabine in pancreatic cells grown in 2D and 3D conditions.

2. Methods

2.1. Cell cultures

Pancreatic cell lines ASPC-1 (CRL-1682); BxPC-3 (CRL-1687); MiaPaCa-2 (CRL-1420); and PANC-1 (CRL-1469) were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA); COLO -357 and T_3M_4 were a gift from R. Metzgar (Duke University, Durham, NC, USA). ASPC-1, BxPC-3, COLO-357, and T_3M_4 were grown in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin (all from PanEco, Moscow, Russian Federation) at 37 °C in a humidified 5% CO_2 atmosphere. PANC-1 and MiaPaCa-2 were grown in RPMI-1640 with the same supplements. Epithelial cells were passaged by trypsinization using Trypsin/EDTA solution (PanEco, Moscow, Russian Federation) twice a week. 24 h before assays cells were seeded in the appropriate plates (6- or 24-, or 96-well plates) adjusted to 2×10^5 cells/mL and incubated overnight to achieve standardized growth conditions.

2.2. MCTS

To generate MCTS, plates were coated with poly-2-hydroxyethyl methacrylate (polyHEMA) (Sigma), dried, washed with phosphate buffered saline (PBS) and used to seed cells. To analyze the cell growth in 2D or 3D cultures, all types of pancreatic cells were transferred in 24-well plates at 2×10^5 cells/well, and the number of cells was monitored at days 1, 2, 3, and 4 manually after trypsinization using hemacytometer chamber. The experiment was repeated five times and polled results were presented.

2.3. RT-PCR

Total RNA was extracted using Trizol reagent (Thermo Scientific). cDNA was synthesized using a set of synthesis kit #K1641 (Thermo Scientific). The PCR amplification mixture contained PCR buffer (EuroGen, Russia), 0.2 mM of each amplimer and 0.2 μg of the reverse transcription product. The PCR product was run on a 1.5% agarose gel with 1 μg/mL ethidium bromide. The size of the amplified products was evaluated using 100 bp DNA marker (EuroGen, Russia). Primer used is shown in the Table 1.

2.4. Cell cycle analysis

Analysis was conducted by flow cytometry according to the basic protocol [23]. Briefly, cells from 2D or 3D cultures were trypsinized, permeabilized with cold EtOH, treated with DNA-free RNAse, and stained with propidium iodide (PI). Gating was done to exclude dead and double cells basing on morphology.

2.5. MTT-assay

Effects of gemcitabine (Pliva, Germany) and doxorubicine (DOX) (Teva, Nederlands) on cell growth were studied in flat-bottom 96-well plates (Costar, USA). For 3D cultures plates were coated with PolyHEMA. Cells were seeded at 10^4 cells/well; incubated overnight to form either monolayers or MCTS, and the drugs in different concentrations were added for extra 72 h. Analysis was performed by a standard 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) test as described earlier [24].

2.6. Confocal analysis

E-cadherin, desmocollins 2, 3, desmogleins 2, 3 expression was analyzed by confocal microscopy in live MCTS using 2 μg/well of antibodies (all from SantaCruz, USA), followed by anti-mouse-AlexaFluor555 (Invitrogen). Hoechst 33342 (Sigma) was used to stain nuclei. After staining cells were fixed by 4% paraformaldehyde, washed and polymerized by Mowiol 4.88 (Calbiochem, Germany). Slides were analyzed using Eclipse TE2000 confocal microscope (Nikon, Japan).

Penetration of DOX into MCTS was studied in live cells incubated with 100 ng/mL of DOX for different intervals. Mitochondrial tracker $DiOC_6$ (Molecular Probes, USA) was used for counterstaining and was added for the last 1 h. Hoechst 33342 (Sigma) was used to stain nuclei.

3. Results

Formation of MCTS by pancreatic cells using anti-adhesive PolyHEMA films.

Among six pancreatic cell lines tested, BxPC-3 and T_3M_4 formed dense large spheroids from almost all seeded cells already after overnight incubation on anti-adhesive surface (Fig. 1a–b). BxPC-3 and T_3M_4 MCTS steadily increased in size with the time of incubation while their number stayed almost constant. COLO-357 and AsPC-1 primarily formed small clusters between abundant free cells (Fig. 1c–d) which steadily enlarged with time forming huge (COLO-357) or small (AsPC-1) irregular 3D cultures. Both COLO-357 and AsPC-1 constantly generated small growing clusters *de novo* from free cells separating from

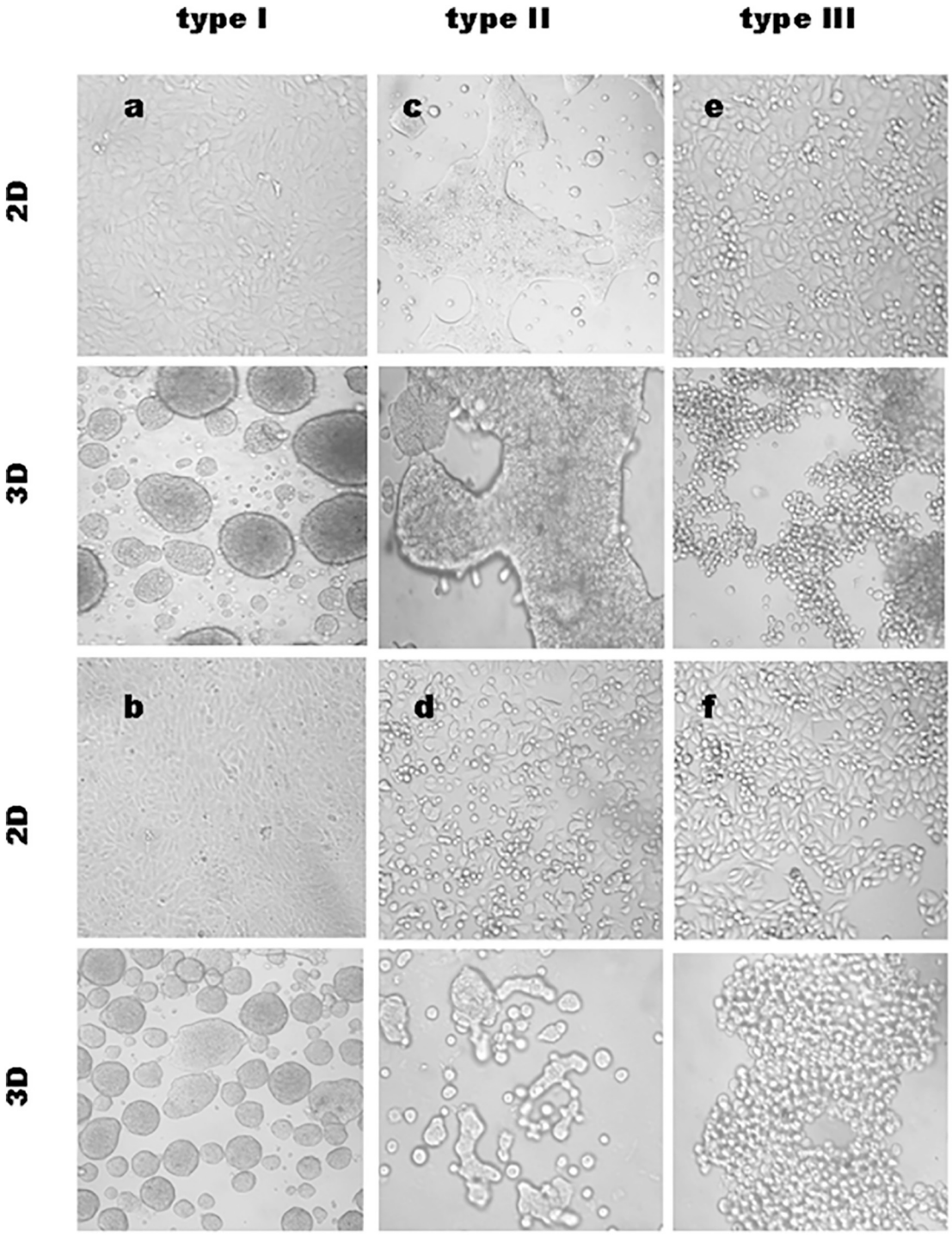

Fig. 1. Light microscopy of pancreatic MCTS cultures. Flat (2D) and multicellular tumor spheroids (3D) cultures formed by BxPC-3 (a), T_3M_4 (b), COLO-357 (c), AsPC-1 (d), PANC-1 (e), and MiaPaCa-2 (f). BxPC-3 and T_3M_4 form small number of dense spheroids; COLO-357 forms loose irregular spheroids which fuse in huge ones; AsPC-1 forms small irregular clusters only; PANC-1 and MiaPaCa-2 form flat monolayers only. Magnification 100×.

the main MCTS during all times of incubation. PANC-1 and MiaPaCa-2 started to grow in suspension (Fig. 1e–f) and later formed flat floating films (Table 2). Cultivation on adhesive plastic showed that cultures also differed: BxPC-3 and T_3M_4 formed dense monolayers where cells were in full contact with the adjacent ones; COLO-357 and AsPC-1 grew as sparse cultures with limited contacts between cell clusters; and PANC-1 and MiaPaCa-2 resembled fibroblast like cells (Fig. 1). Morphology of cells in 2D cultures predicts their behavior in 3D cultivation.

Expression of E-cadherin and desmogleins in pancreatic MCTS.

To understand the difference between the forms and density of 3D cultures, we studied the expression of E-cadherin and desmosomal cadherins desmogleins (DSG) in MCTS formed by pancreatic cells. Desmosomal proteins include cadherins, armadillo and plakin family proteins [25]. Among them only cadherins possess extracellular domains which form intercellular contacts between adjacent cells. There are four isoforms of DSG and three isoforms of DSC [25] all of which are expressed by epidermal keratinocytes.

Expression of cadherin genes was analyzed by PCR in 2D cultures. High level of E-cadherin, DSG3, DSC2 and 3 expression was found in BxPC-3 and a control keratinocyte cell line HaCaT (Fig. 2). None of

Table 2
Characteristics of multicellular tumor spheroids formed by pancreatic cell lines.

#	Cell line	Origin	MCTS	Type	Size, μm	Form
1	BxPC-3	PDAC	Yes	I	100–700	Dense, smooth
2	T_3M_4	Lymph node metastasis	Yes	I	100–300	Dense, smooth
3	COLO-357	Lymph node metastasis	Yes	IIa	50–1000	Loose, large
4	AsPC-1	Metastatic site ascites	Yes	IIb	50–200	Loose, small
5	PANC-1	PDAC	No	III	–	Films
6	MiaPaCa-2	PDAC	No	III	–	Films

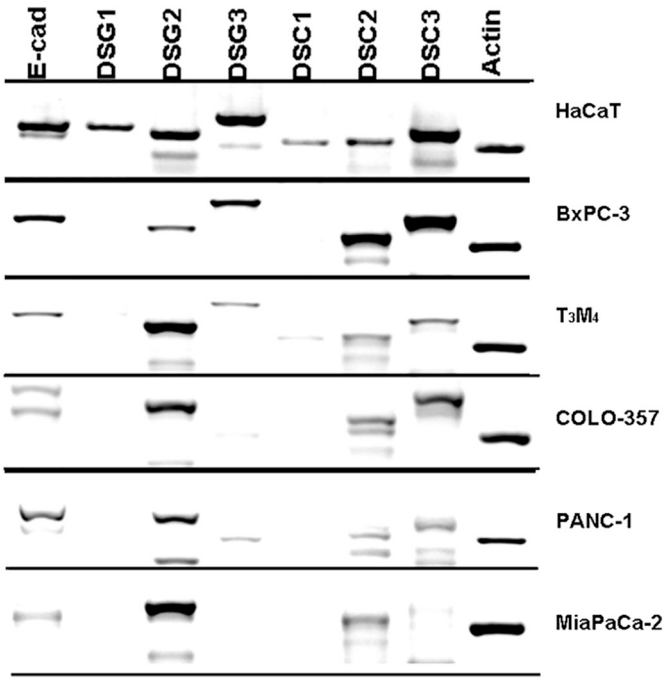

Fig. 2. Expression of cadherins by pancreatic cells grown in 2D cultures. PCR analysis of E-cadherin, desmogleins 1–3 (DSG), desmocollins 1–3 (DSC), and β-actin in monolayer cultures of human keratinocyte cell line HaCaT and pancreatic cells BxPC-3, T_3M_4, COLO-357, PANC-1, and MiaPaCa-2.

pancreatic cells expressed DSG1/DSC1; all cell lines expressed DSG2 at different level; T_3M_4 expressed DSG3/DSC3; COLO-357 expressed DSC3 but not DSG3, and PANC-1 and MiaPaCa-2 expressed neither DSG3 nor DSC3 (Fig. 2). BxPC-3 formed the largest spheroids and resembled HaCaT MCTS.

Expression of cadherins in MCTS was analyzed by confocal microscopy in BxPC-3, T3 M4, COLO-357, PANC-1, and MiaPaCa-2 using antibodies specific to DSG 1–3, DSC1–3, and E-cadherin visualized by secondary anti-mouse IgG-Alexa488 (Fig. 3, green) or anti-mouse IgG-Alexa555 (Fig. 3, red).

Confocal microscopy analysis demonstrated that E-cadherin expression was essential for spheroid formation. E-cadherin in type I BxPC-3 and T_3M_4 and type II COLO-357 cells MCTS was found mostly in the surface cells (Fig. 3). At the same time type III PANC-1 but not MiaPaCa-2 cells also expressed E-cadherin to some level in 3D conditions however neither PANC-1 nor MiaPaCa-2 formed stable spheroids. Analysis of desmosomal cadherins demonstrated that for the formation of type I and II MCTS expression of at least one pair of DSG and DSC was essential. BxPC-3 expressed high level of DSG3 and all DSC proteins (Fig. 3, a). T_3M_4 expressed all types of DSG and DSC (Fig. 3b). COLO-357 expressed DSG2 and DSC2 (Fig. 3c). PANC-1 demonstrated low level of DSG2 and DSG3 but not of desmocollins (Fig. 3d) while MiaPaCa-2 expressed mostly DSC2 (Fig. 3e). In contrast to surface restricted E-cadherin expression, desmosomal cadherins were expressed by cells throughout MCTS such as found for DSG3 and DSC2–3 in BxPC-3; for DSG1, DSG3, DSC1–3 in T_3M_4; for DSG2 and DSC2 in COLO-357; for DSG2–3 in PANC-1, and for DSC2 in MiaPaCa-2. These results show that MCTS formation is initiated by E-cadherin binding followed by stable DSG-DSC bonding.

To our opinion it is desmosomal DSG-DSC paired contacts that mediate MCTS formation. Among desmosomal cadherins DSG3 pairing either with DSC2 (BxPC-3) or DSC3 (T_3M_4) was responsible for the type I MCTS formation. The absence of DSG3 expression resulted in the formation of type II MCTS characteristic to COLO-357.

3.1. Growth of cells in different types of MCTS

To compare the growth of pancreatic cells in flat 2D cultures or MCTS, the cells were seeded in 24-well plates in equal quantity and cultivated either on anti-adhesive polyHEMA surface (3D cultures) or tissue culture grade plastic (2D cultures) for 24, 48, 72, or 96 h. At each time point cultures were trypsinized and cell numbers were counted manually. Pancreatic cell lines grew in 2D conditions with different rates: AsPC-1 = COLO-357 = MiaPaCa-2 > T_3M_4 = PANC-1 > BxPC-3 (Fig. 4). The growth of cells in MCTS depended on the type of cultures. BxPC-3 and T_3M_4, which formed dense 3D cultures (type I), quickly became quiescent and did not proliferate; COLO-357 and AsPC-1 forming loose MCTS (type II) proliferated slower than in 2D cultures; PANC-1 and MiaPaCa-2 (type III), grew at a comparable rate in both conditions (Fig. 4a–c).

The numbers of live cells in MCTS were determined by flow cytometry and confocal microscopy. Percentage of apoptotic cells in type I MCTS increased tremendously (3–5 times) while it was comparable in 2D and 3D conditions for type II and III cells (Fig. 4d). To understand the difference between viability of type I and II cells, MCTS were stained with a vital mitochondrial dye $DiOC_6$. Confocal microscopy analysis demonstrated that both in type I and II 3D cultures functionally active cells localized to the surface of the spheroids (Fig. 4e, f, green staining). The center cells in these cultures are likely to be in a quiescent (COLO-357) or quiescent and apoptotic states (BxPC-3 and T_3M_4). Type III cells in MCTS were viable and functionally active throughout MCTS which correlates well with the MTT results.

3.2. Cell cycling, quiescence, and apoptosis in MCTS

A decrease in cell proliferation in types I and II cultures can be explained either by an increased apoptosis or a decreased cell cycle progression. Cell cycle analysis was conducted for the representative type I-III cells BxPC-3, T_3M_4, COLO-357, and PANC-1 at days 3 and 5 of cultures. The percentage of dividing G2/M phase cells (Fig. 5a, M1) in type II and III cells was comparable at 3 and 5 days of cultivation in 2D cultures and decreased to day 5 in MCTS. On the contrary, type I cells BxPC-3 and T_3M_4 decreased proliferation in 3D conditions already to day 3 and both 2D and 3D cells divided slower with time (Fig. 5a). Summary on the percentage of cells in different cycle phases is shown in the Table 3 averaged for 5 experiments.

At day 5 type I but not II and III cells demonstrated a decrease in DNA staining of cells in G1 fraction in 3D in comparison with 2D cultures (Fig. 5a, arrows). It is likely that pancreatic cells forming type I MCTS become quiescent and rest in G0 phase instead of G1 which is found in 2D proliferating cells. Of note, flow cytometry protocol of DNA staining and then live cell gating does not count for the late phase apoptosis and necrotic cells, the percentage of which was also significantly higher in type I BxPC-3 and T_3M_4 MCTS. To visualize dead and apoptotic cells, MCTS were stained by PI and analyzed by confocal microscopy. Low numbers of apoptotic cells was found at day1 of cultivation in all types of MCTS while at day 5 significant staining was registered only in type I cultures (Fig. 5b).

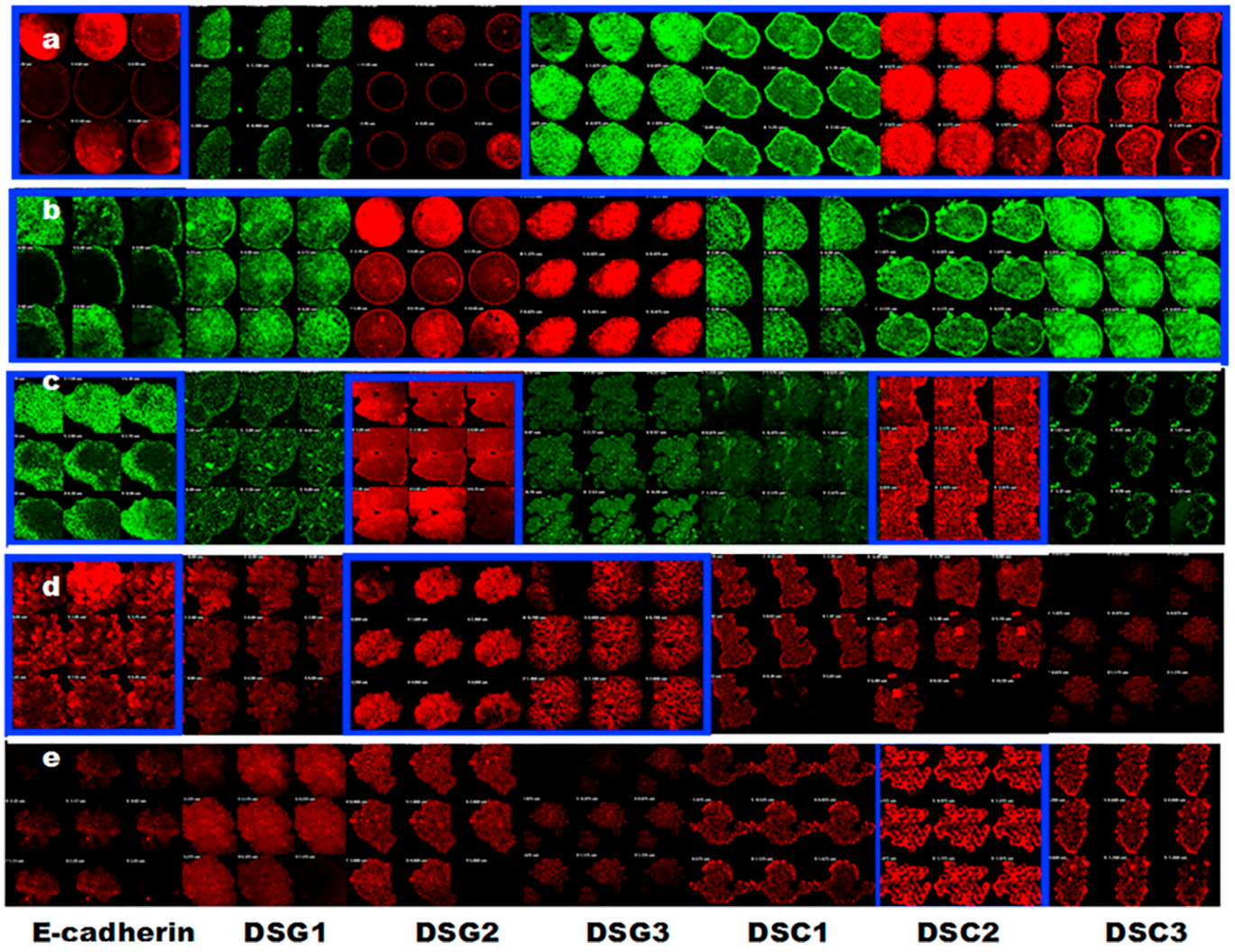

Fig. 3. Expression of cadherins by pancreatic cells grown in 3D cultures. Zet-stack confocal microscopy of E-cadherin and desmosomal cadherins expressed by BxPC-3 (a), T_3M_4 (b), COLO-357 (c), PANC-1 (d), and MiaPaCa-2 (e) 3 days MCTS. Secondary antibodies were labeled either by AlexaFluor488 (green) or AlexaFluor555 (red). Blue frames mark significant expression of different proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Sensitivity of pancreatic 2D and 3D cultures to antitumor drugs

Gemcitabine is a first-line medication for chemotherapy of pancreatic tumors. Effect of gemcitabine on cell proliferation was dramatically (2 order) reduced in 3D cultures of type I cell lines such as BxPC-3 and T_3M_4 (Fig. 6a) and was comparable in type II and III types of cells (Fig. 6b, c). The same dependencies were registered using doxorubicin (Fig. 6d–f).

3.4. Penetration of antitumor drugs into MCTS

Doxorubicin is a fluorescent molecule and can be used to monitor MCTS penetration. Fig. 7 demonstrates an example of time dependent penetration of doxorubicin into type I BxPC-3 and type II COLO-357 MCTS. Penetration of doxorubicin into type I spheroids was 1–2 h delayed in comparison with type II MCTS (Fig. 6a, b) while later after 10 h of incubation equal staining was found through the full depth of both BxPC-2 and COLO-357 spheroids (Fig. 7c–f).

4. Discussion

Development of reliable and predictive test systems for the *in vitro* analysis of chemotherapeutic drugs is still unresolved problem [26]. MCTS may demonstrate a higher potential however the discrepancy in the results obtained by different groups must be first explained. The choice of how to develop MCTS from epithelial cell lines should depend on the aim of the study, as at least two major mechanisms of cell adhesion affect the results. Epithelial cells express intercellular adhesion molecules cadherins and extracellular matrix (ECM) adhesion molecules integrins [10,27]. MCTS can be generated basing on the cadherin or cadherin/integrin contacts. Application of cadherin/integrin based MCTS for drug sensitivity analysis will result in dual effects both on the growth of tumor cells and on the indirect effect of cell cooperation with normal ECM or integrin expressing cells [27,28]. Cadherin based MCTS can be used to estimate the sensitivity of solely tumor cells to the drugs. Formation of MCTS in collagen, agarose, matrigel, hanging drops, and other ECM scaffolds cannot separate the effects of integrin and cadherin adhesion. Cultivation on anti-adhesive films, like polyHEMA or methylcellulose, relies exclusively on primary cadherin contacts [5–10]. The difference between these two types of MCTS resulted in 3D cultures formed by MiaPaCa-2 and PANC-1 but not by BxPC-3 or Capan-2 in spinner cultures [14]; formation of MCTS by Capan-2, AsPC-1, and PANC-1 on agarose gels [16,21,29,30]; by AsPC-1, PANC-1, MiaPaCa-2, and Capan-2 on concave microwell plates [15]; by BxPC-3 - on type I collagen scaffolds [30]. In accordance with our results, Longati *et al* demonstrated the formation of dense spheroids by BxPC-3 and irregular

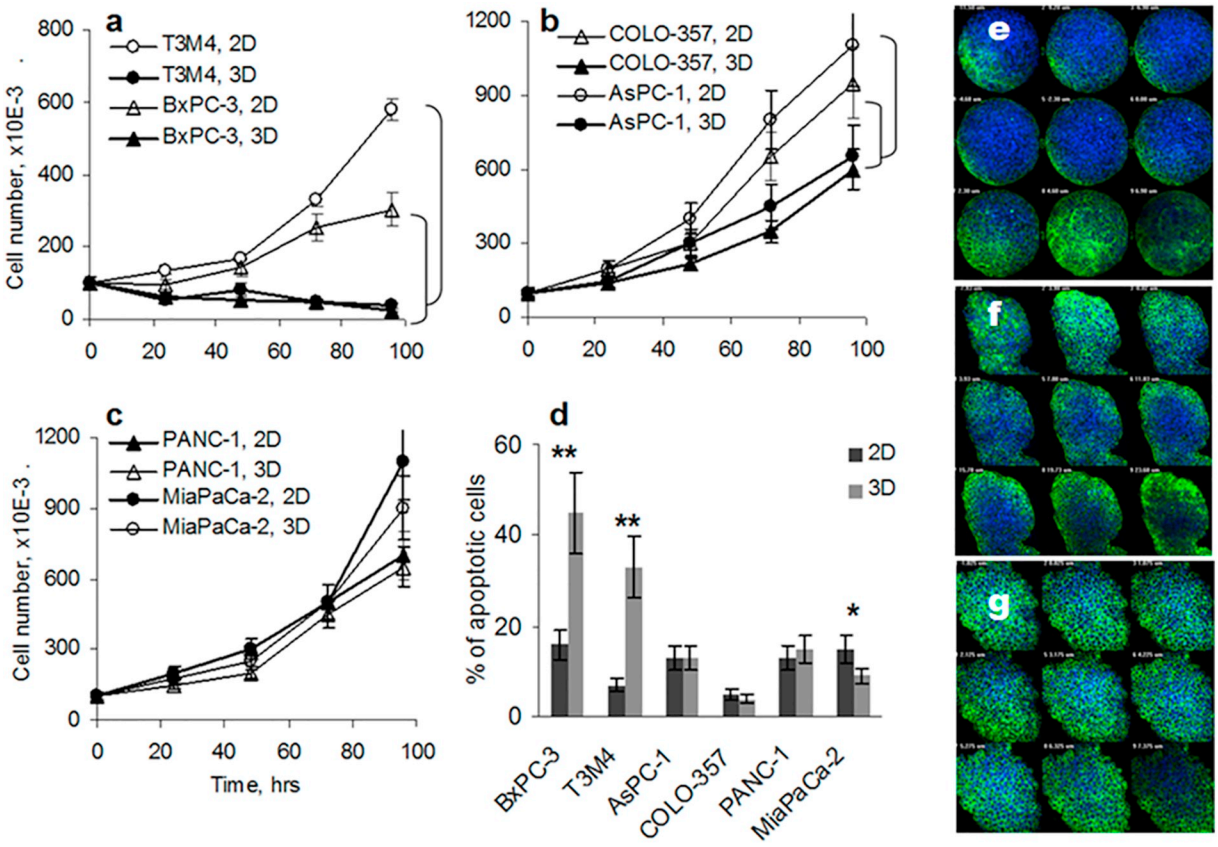

Fig. 4. Growth of pancreatic cells in 2D and 3D conditions. Dynamics of cell growth in type I (a), type II (b), and type III (c) cultures of pancreatic cells. Parenthesis show significant difference ($p < 0.05$) between 2D and 3D cell growth. Percentage of apoptotic cells in 3 day cultures estimated by flow cytometry using propidium iodide staining (d). Significant apoptosis is shown with the asterisks ($p < 0.01$ and < 0.05). Distribution of live cells (green) in 3 day MCTS formed by BxPC-3 (e), COLO-357 (f), and PANC-1 (g). Confocal zet-stacks of MCTS stained with mitochondrial dye $DiOC_6$ (green) and nuclear stain Hoecst 33,342 (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and loose ones by AsPC-1 and PANC-1 using methylcellulose coating [12].

It is relatively easy to predict the type of the MCTS by the appearance of cell monolayers. The type I cells have a cobble stone-like appearance in 2D cultures and they form well shaped smooth and round large spheroids resistant to pipetting in 3D conditions. Such MCTS contain live quiescent, as well as apoptotic and necrotic cells in the center with small number of proliferating cells on their surfaces. Consequently the cells in the type I MCTS are 1–2 order more resistant to antitumor drugs than 2D cultures due to a low number of proliferating cells. Quiescent cells in type I MCTS do not express E-cadherin however they do express desmosomal cadherins, in particular DSG3 paired with one or more DSC molecules. The increase in S-phase and decrease in G2/M MCTS cells was found in PANC-1 cells cultivated in rotationary cultures [32]. Contrary to this, a decrease both in S-phase and G2/M phase cells was found in 6 day Capan-2 MCTS [29]. We have demonstrated that the cells in the type I MCTS not only rested in G1 phase of cell cycle, they transited to G0 phase and had more condensed DNA. Indeed, it was shown earlier that quiescent cells have a more condensed DNA loop structures [31]. HaCaT, A431, BxPC-1, T_3M_4 are examples of type I cells.

Type II cells grow as sparse 2D cultures; they form irregular loose MCTS without smooth boundaries and with evident single cells separating from the spheroid surfaces. Proliferation of cells in type II MCTS decreased with time however the cells did not become quiescent, continued to express E-cadherin for at least 3–5 days. Expression of desmosomal cadherins DSG2 and DSC2 was characteristics to these MCTS and their expression increased with time which was associated with the loss of E-cadherin expression (data not shown).

The sensitivity of the type II 2D and 3D cultures to antitumor drugs was comparable at short incubation time (3 days) possibly due to a sufficient amount of live cells in the spheroids. Analysis of cell viability in type II MCTS demonstrated that functionally active proliferating cells were found on the surface of the spheroids which corresponds well to multiple studies [33]. At the same time percentage of apoptotic cells in 2D and 3D type II cultures was comparable which shows that center cells transit to G0 cell cycle phase and become quiescent. Most known tumor cell lines belong to the type II cells. There are two subtypes of the type II cells forming either large (COLO-357) or small (AsPC-1) MCTS differing in the high and low level of DSG2/DSC2 expression. COLO-357 and HEK293 represent the type IIa cells; AsPC-1, HepG2, MDCK, HBL100, A549, HeLa – the type IIb.

Type III cells have mesenchymal-like phenotype in 2D cultures and are unable to form MCTS. They grow almost as floating monolayer films or suspension on anti-adhesive surface and are easily dispersed into small clusters of cells. They express low level of E-cadherin in 2D conditions and can lose it when grown in 3D cultures. Type III cells can express some desmosomal cadherins however they usually do not express a counter partner protein. The loss or low expression of E-cadherin and the paired desmosomal cadherins in the type III 3D cells prevents the formation of stable spheroids. PANC-1, MiaPaCa-2, A375, SkBr-3 cell lines are the examples of the type III cells.

The role of the desmosomal cadherins in the formation of the MCTS was never studied. Earlier DSG2 expression was shown in BxPC-3 cells but not in PANC-1 and AsPC-1 ones [34,35]. Expression of DSG3 in some pancreatic cells is shown for the first time. Of note, DSG1 and DSG3 are autoantigens in pemphigus [36]. Expression of DSG1 in pancreas was shown by Romani *et al* [34]. DSG1 and 3 are the main

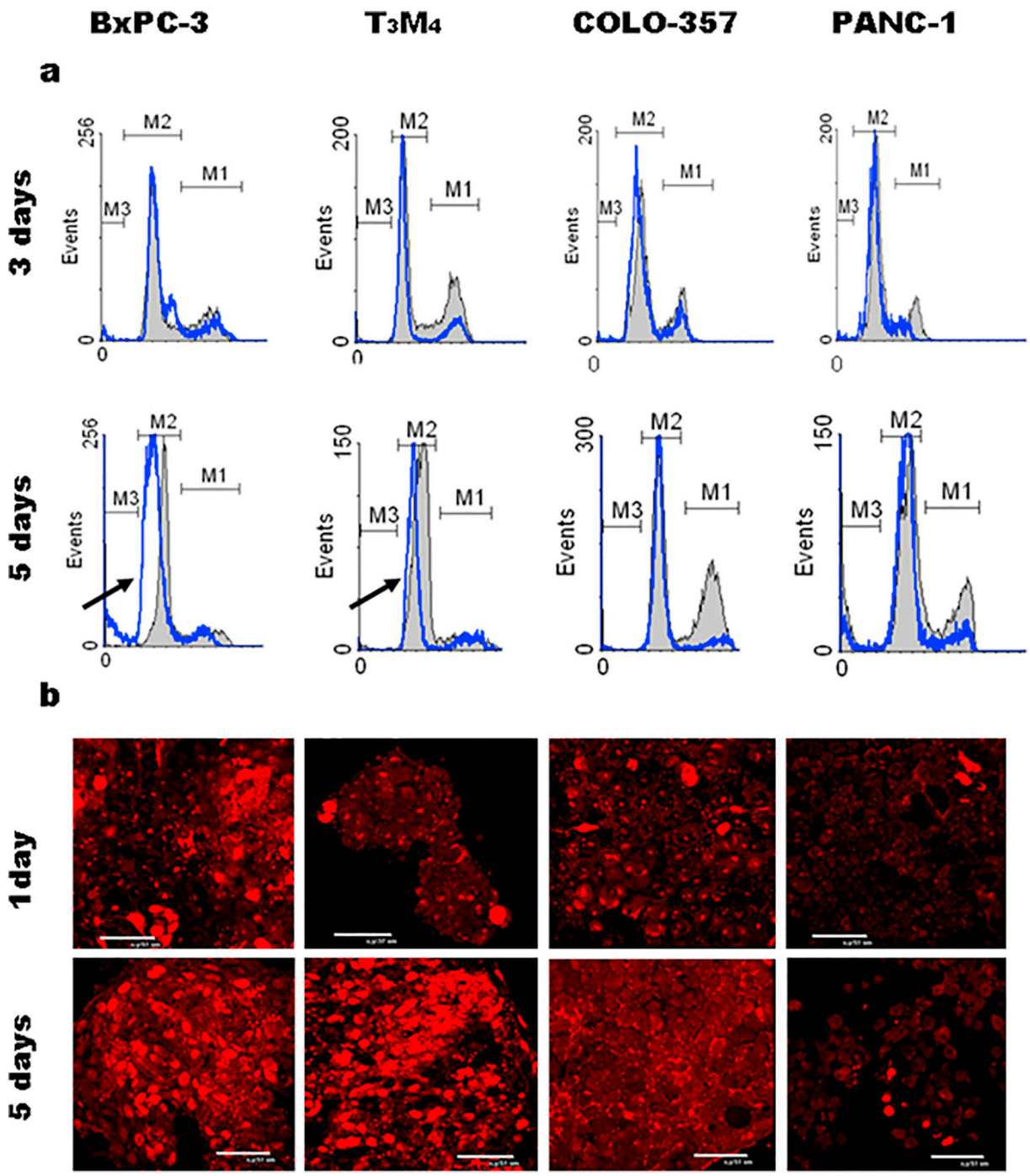

Fig. 5. Proliferation of pancreatic cells in 2D and 3D cultures. a. Cell cycle analysis of BxPC-3, T_3M_4, COLO-357, and PANC-1 in 2D (grey shedding) and 3D (blue lines) cultures at 3 and 5 days of cultivation. b. Apoptosis in the corresponding MCTS at 1 and 5 days analyzed by propidium iodide staining. Scale bars correspond to 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

desmosomal cadherins expressed in stratified epithelia while DSG2 is highly expressed in simple epithelia [36]. Expression of DSG1 and DSG3 in pancreas explains why the prevalence of type 1 diabetes is significantly increased in patients with pemphigus compared with the general population [37]. It is likely that DSG1 or DSG3 can be expressed by epithelial cells in other organs as autoimmune thyroid disease and rheumatoid arthritis are also found more often in pemphigus patients [37]. Loss of desmosomal adhesion is a necessary condition for epithelial-mesenchymal transition involved in the transformation of cells in invasive cancer [38]. However, the role of different isoforms of desmosomal cadherins in cell transformation is not well known.

Multiple papers demonstrated a higher resistance of cells in MCTS to antitumor drugs. This includes PANC-1 cultivated in rotationary cultures; HeLa and HT29 forming MCTS on non-adherent Sumilon PrimeSurface plates; HT29, HCT116, and SW480 MCTS generated in round bottom Ultra Low Attachment plates, and many others [28,32,39,40]. In most cases a decrease in the sensitivity to drugs was only marginal (2–5 times). To the best of our knowledge HeLa and HT29 belong to the type IIb cells while PANC-1 - to the type III. Our results show that both the type II and III cells in 2D and 3D cultures are equally sensitive to antitumor drugs. The difference found in multiple studies can be a result of the test-systems used to compare the cultures.

Table 3
Percentage of cells in different cell cycle phases in 2D and 3D cultures.

	M1, G2/S, %		M2, G0/G1, %		M3, Apoptosis, %	
	2D	3D	2D	3D	2D	3D
BxPC-3	28 ± 10	11 ± 18	70 ± 10	62 ± 8	1 ± 0.3	9 ± 2
T_3M_4	22 ± 8	9 ± 11	78 ± 12	61 ± 12	1 ± 0.3	10 ± 02
COLO-357	16 ± 20	8 ± 12	84 ± 13	89 ± 6	1 ± 0.3	1 ± 0.4
PANC-1	21 ± 12	11 ± 7	72 ± 8	70 ± 12	6 ± 1	7 ± 1
MiaPaCa-2	17 ± 12	11 ± 10	77 ± 12	85 ± 5	3 ± 1	5 ± 1

	3D/2D ratio		
BxPC-3	0.3↓	0.9	8.0↑
T_3M_4	0.4↓	0.9	14.0↑
COLO-357	0.5↓	1.1	1.0
PANC-1	0.8	1.0	1.2
MiaPaCa-2	0.8	1.0	1.7

↓↑ - significant decrease or increase in 3D/2D ratio.

In our hands, the cells in the MCTS have different metabolism and MTT reduction to formazan was less efficient in the cells cultivated in 3D than in 2D cultures. Other tests used to estimate the number of live cells in 3D cultures such as CellTiter-Glo, trypsinization and counting, as well as some others can also be less efficient in comparison with 2D cell analysis.

The difference between pancreatic cell lines in the morphology of both 2D and 3D cultures presumes that they originate from the different sites or the different types of tumors. BxPC-3 obtained from PDAC (Table 1) may correspond to the authentic PDAC still without metastatic potential while MiaPaCa-2 and PANC-1, also derived from PDAC, evidently demonstrate the predisposition to metastasis (low adhesion between cells). COLO-357 and AsPC-1 are obtained form the metastatic site while T_3M_4 cells, derived from the lymph node metastatic mass, resemble BxPC-3 however they form smaller MCTS. It can be speculated that epithelial-mesenchymal transition occurs in three steps: i) a decrease in the expression of desmosomal cadherins; ii) an increase in the expression of E-cadherin by cells which earlier have not expressed it, this leads to an increased cell proliferation and cell separation from the parental tumor; and iii) a final loss of E-cadherin expression in cells which started to grow in a different site without sufficient E-cadherin cell-to-cell contacts.

Recent research in tissue engineering is trying to take into account the tumor microenvironment, which is thought to be important for metastatic progression and vascularization. It is easier to model such complex systems knowing the behavior of different MCTS and critical molecules expressed by these cells. Moreover, cells which express different types of desmosomal cadherins segregate (Fig. S1). Consequently, pancreatic as well as other cell lines originated from different tumor stages can be used to study epithelial-mesenchymal transition as well as for the analysis of interaction with tumor microenvironment cells.

5. Conclusions

Many compounds entering clinical trials have low efficacy, leading to a waste of time and money. Even today after 30 years of studies, implementation of 3D cell cultures into cell-based research programs is limited due to the inconsistency of data obtained by different groups. To obtain predictable test systems it is imperative to characterize MCTS. Here we studied MCTS formed by six pancreatic cell lines on anti-adhesive surface polyHEMA which predetermined solely cadherin dependent adhesion. Three types of MCTS were identified basing both on the morphology of 2D and 3D cultures. Only the cells expressing

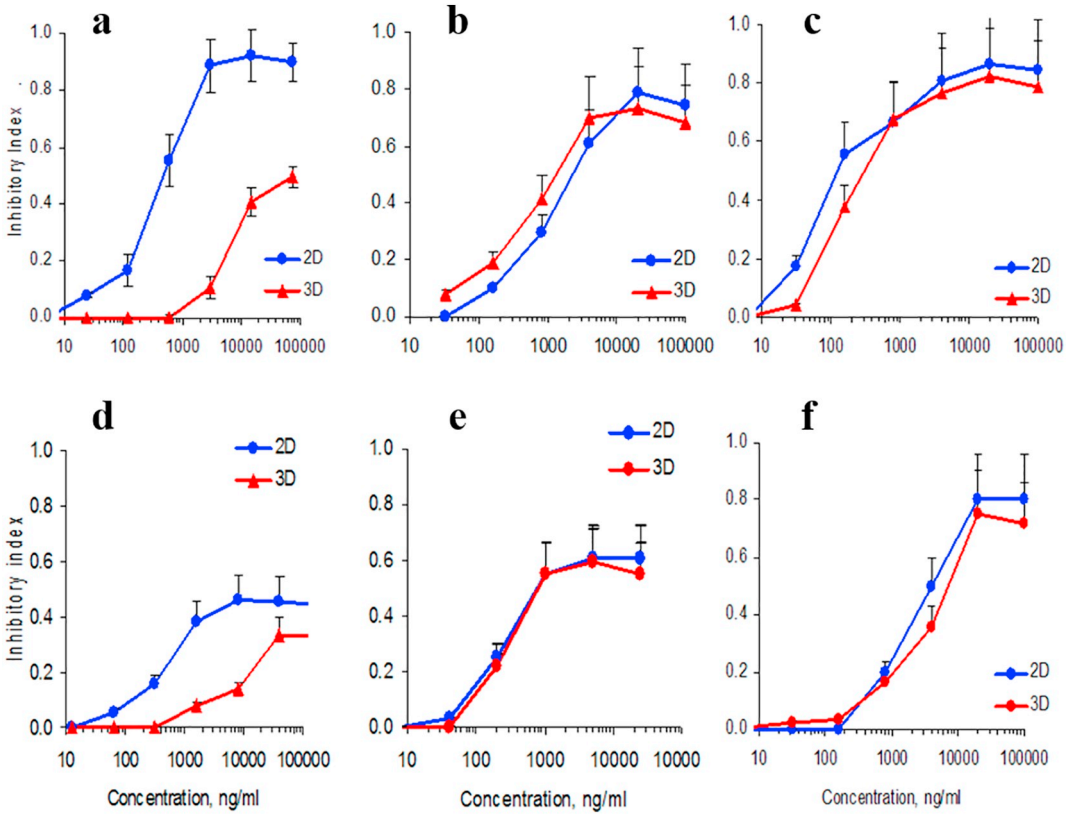

Fig. 6. Sensitivity of pancreatic 2D and 3D cultures to antitumor drugs in MTT-assay. Inhibition of proliferation by gemcitabine (a–c) and doxorubicin (d–f) of BxPC-3 (a, d), COLO-357 (b, e) or PANC-1 (c, f) cells incubated in 2D (blue lines) or 3D (red lines) conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

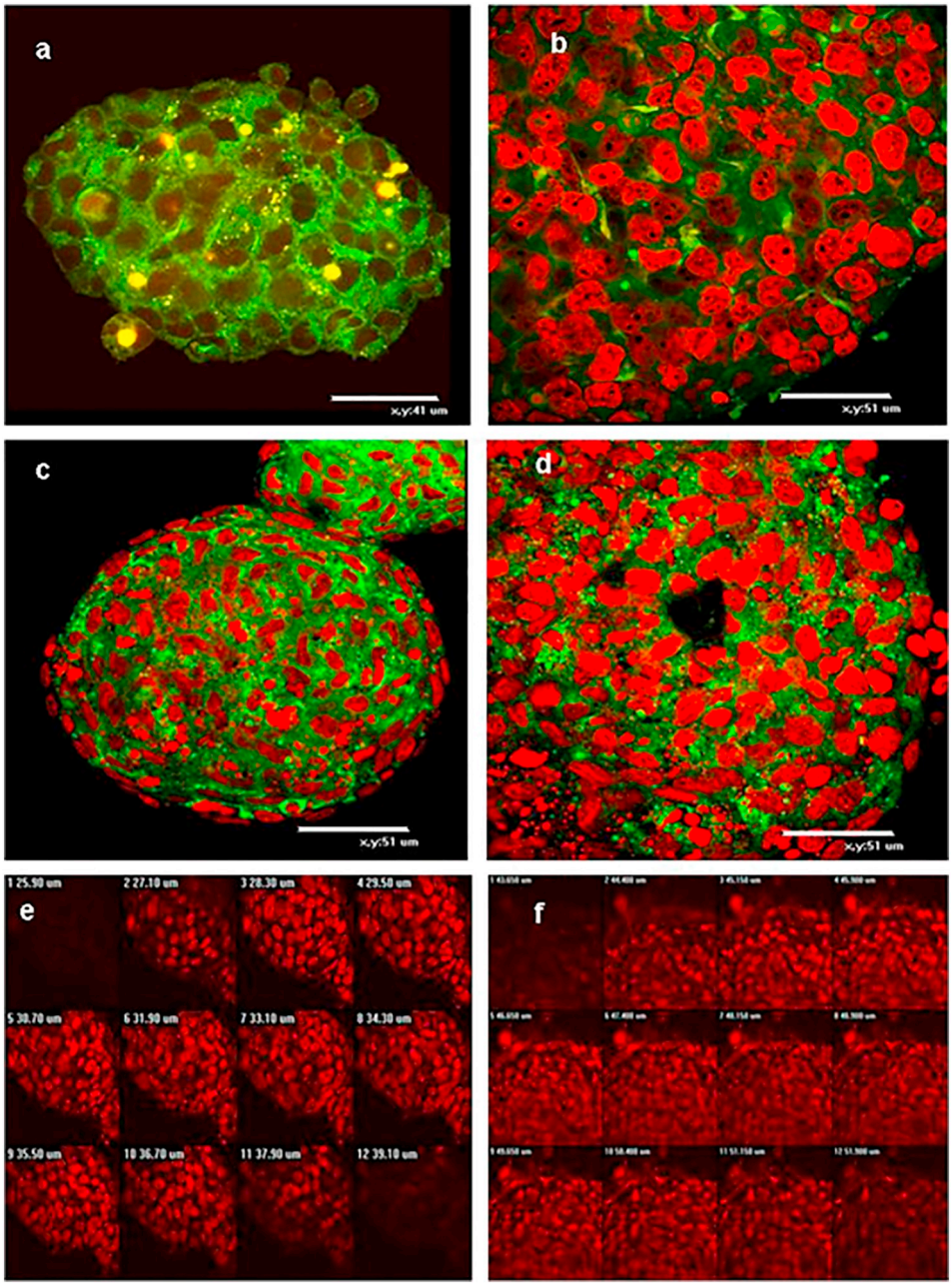

Fig. 7. Penetration of doxorubicin into MCTS. Confocal images of BxPC-3 (a, c, e) or COLO-357 (b, d, f) 48 h MCTS after incubation with 100 ng/mL DOX (red) for 1 h (a, b) or 10 h (c, d). Cells were counterstained with mitochondrial tracker DiOC6 (green). Scale bar corresponds to 50 μm. e-f. Zet-stacks of BxPC-3 (e) or Colo-357 (f) after 10 h incubation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

high level of E-cadherin and desmosomal cadherin DSG3 formed the type I MCTS which were 1–2 order more resistant to antitumor drugs gemcitabine and doxorubicin. Drug resistance of type I 3D cultures was associated with a quiescent state of the cells and a high level of spontaneous apoptosis. Sensitivity to antitumor drugs of the types II and III pancreatic cells was comparable. However, care should be taken when using standard methods such as MTT for the analysis of cell proliferation in MCTS as the metabolism of cells in this condition decreases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.01.034.

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