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# Three-dimensional Culture System as a Model for Studying Cancer Cell Invasion Capacity and Anticancer Drug Sensitivity

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**Abstract.** *Background: Three-dimensional (3-D) culture systems that simulate the tumor extracellular microenvironment may be appropriate to test cancer cell potential for invasion and tumor cell sensitivity to anticancer drugs. Materials and Methods: Human PC-3 prostate, A549 colon, HT-29 lung and MCF-7 and MDA-MB231 breast cancer cells were embedded and grown in collagen gel surrounded by a fibrin clot. Increasing concentrations of cisplatin, doxorubicin, paclitaxel and 5-fluorouracil were comparatively evaluated for their ability to inhibit tumor cell proliferation and colony formation in vitro. Results: All cells, except MDA, formed colonies in collagen. PC-3, A549 and HT-29 cells massively invaded fibrin forming migratory fronts. Cell colonies were also formed in fibrin (secondary tumor-like structures) apart from migratory fronts; HT-29 cells were the most aggressive in this regard. MDA cells were particularly sensitive to doxorubicin, while MCF-7 cells showed sensitivity to all anticancer regimens tested. A549 cells were the tumor cell type with greatest potential for invasion and were sensitive mostly to cisplatin. PC-3 cells were primarily sensitive to cisplatin and doxorubicin, while HT-29 cells were sensitive to fluorouracil and doxorubicin. Conclusion: 3-D collagen cell culture systems can be used to study cancer cell potential for invasion and their relative sensitivity/resistance to anticancer drugs.*

Monolayer cell culture systems of cancer cell lines are commonly used to evaluate antitumor effects of new anticancer drugs. These standard *in vitro* assays determine the capacity of an anticancer regimen to inhibit cell growth or the potential to induce cancer cell apoptosis (1, 2). However, these assays can neither stratify cancer cells according to their potential for invasion nor evaluate the anticancer drug efficacy with regard to cancer cell invasion and metastasis (3). In addition, most tumor cells are supported by an extracellular matrix microenvironment which plays an important role in the development of anticancer drug refractoriness (4). Thus, the development of *in vitro* assays that simulate the extracellular microenvironment where tumor cells grow can provide important information about tumor cell potential for invasion and the efficiency of anticancer drugs to inhibit both tumor cell growth and the capacity for invasion.

Three-dimensional (3-D) culture systems have allowed the study of the cell-cell interaction implicated in osteoblastic metastasis (5) and tumor-mediated angiogenesis (6). Spheroid cultures using a basement membrane-derived compound, organotypic cultures, soft agar cultures and cells embedded in collagen gel have been developed to investigate the biology of cancer cell lines (7-12). However, cells were limited in further expansion within the 3-D culture matrix support.

Herein we have presented our data on a 3-D cell culture system using cancer cell lines grown in matrices composed of collagen and fibrin, which have enabled us to study tumor cell invasion and cancer cell expansion into collagen matrix. In addition, we assessed and compared the relative capacity of four cytotoxic drugs commonly used in clinical studies to inhibit tumor cell growth and tumor cell capacity for invasion *in vitro*.

## Materials and Methods

Human non-small lung carcinoma cells (A549 cells), colorectal adenocarcinoma cells (HT-29 cells), prostate adenocarcinoma (PC-3 cells) and breast (MCF-7 and MDA-MB-231) cancer cells from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used in our studies. Cells were grown in specific culture media

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such as high glucose-supplemented Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) for A549 cells; McCoy's 5A medium with 10% FBS for HT-29 cells; high glucose-supplemented DMEM with 5% FBS and insulin for MDA-MB-231 and MCF-7 cells with estradiol ($10^{-9}$ M); and DMEM with 5% FBS for PC-3 cells. Glutamine (2 mM) was added to all media. All cells were mycoplasma-free as determined by Hoechst staining.

Cancer cells were embedded in collagen gel at a density of 5 x $10^4$ cells per 200 μl gel in wells of 96-well plates (13). After 2-3h, the gels were removed from the well and sandwiched into a fibrin gel laid down in wells of 24-well plate (6). Thus, the cell-embedded collagen gel was simulating the microenviroment of a primary tumor. For 15 days the fibrin-collagen gels were incubated at 37˚C with 5% $CO_2$ in culture medium. The media were renewed every other day. The ability of cancer cells to migrate from the gel into the fibrin (simulation of tumor invasion) was assessed every other day for 15 days. An antifibrinolytic agent (aprotinin, Trasylol®, Bayer Inc., Etobicoke, ON, Canada) was added at 100U/ml in culture media. Then different concentrations of the chemotherapeutic agents were added at every medium change. After a 30-day period of culture, the collagen-fibrin gels were observed through an inverted microscope (Diaphot, Nikon, Japan) under phase contrast with a long working distance condenser at low magnification (x4 objective). In addition, histological sections were made through the collagen-fibrin gel and stained for hematoxylin, phloxin and saffron.

Taxol or paclitaxel (Bristol-Myers-Squibb Pharmaceutical Group, Montreal, QC, Canada), 5-fluorouracil (Adrucil, Pharmacia & Upjohn Inc., Mississauga, ON, Canada), doxorubicin (Novopharm, Toronto, ON, Canada) and cisplatin (Faulding Canada, Inc., Montreal, QC, Canada) were tested at an increasing concentrations (0.1 nM to 10 μM). Anticancer drug assessments were compared to control 3-D cultures.

## Results

In general, the cancer cells formed: (i) cell aggregates in collagen gels (*i.e.*, primary tumor), except for MDA cells, (ii) cell expansions from collagen gel to fibrin showing clear migratory fronts (*i.e.*, invasion), and (iii) cell aggregates in fibrin clot, which were apart from the migratory fronts in the sense of forming secondary tumor-like structures. The development of cell aggregates in collagen gels was clearly observed on histological sections after 30 days of culture. The two formations in fibrin were evident under phase contrast analyses at different time periods, but their development depended on the cell types as described below.

Control 3-D culture systems containing PC-3 cells have shown the ability to form small cancer cell aggregates in the collagen gel as primary tumor and cell migration in a spark-shape manner from the collagen gel boundaries into fibrin (Figure 1). The fibrin gel was also invaded by secondary tumor-like structures which contained cell aggregates of stellar-like or spheroid-like shape, however, these formations were clearly distinct from the cells migrating in the invasion-like fronts. Cisplatin and doxorubicin did not alter the formation of migratory fronts until reaching a concentration greater than 10 μM, whereas secondary tumor-like structures were observed at a concentration of 1 μM of these two drugs. Paclitaxel and fluorouracil did not alter the formation of secondary tumor-like structures until reaching concentrations greater than 10 μM. However, cell aggregates in collagen gels were effectively decreased after fluorouracil treatment at a concentration of 1 μM.

Control 3-D culture systems containing HT-29 cells showed a large number of secondary tumor-like structures with a stellar shape that became more spherical as they detached and migrated from the migratory fronts (Figure 2). The latter had a limited outgrowth. Cell aggregates in collagen gel were present in the control cultures, but limited in size and cell content compared to those formed by PC-3, A549 and MCF-7 cells in the collagen gel. Numerous secondary tumor-like structures were observed in the presence of cisplatin up to a concentration greater than 1 μM and only a few spheroid secondary tumor-like structures were seen at 10 μM. Low doses (10-100 nM) of doxorubicin diminished the expansion of migratory fronts and the development of secondary tumor-like structures. Higher concentrations (1 and 10 μM) of doxorubicin eliminated the migratory front, but still very few spheroid secondary tumor-like structures were present at those concentrations. The paclitaxel regimen resulted in few spheroid secondary tumor-like structures with no migratory front until reaching concentrations greater than 1 μM. Fluorouracil reduced the size of the migratory fronts and secondary tumor-like structures at low concentration (10 nM), but some small spherical tumors were still present at 10 μM. A decrease in aggregates and cell content in collagen gels was seen only when a 10 μM fluorouracil regimen was reached. The other three drugs tested did not diminish the development of primary tumors in collagen gel.

Control 3-D culture systems containing A549 cells showed the ability to form numerous and dense cell aggregates in the collagen gel and large migratory fronts in the fibrin gel which were heterogenous (aggregated structures), but the development of separate secondary tumor-like structures was limited compared to the other cell types investigated (Figure 3). Numerous cell aggregates in the collagen gel were observed even at 10 μM of any drug tested. Cisplatin reduced the migratory fronts up to concentrations greater than 10 nM, accompanied by very few secondary tumor-like structures. Doxorubicin and paclitaxel reduced the size of the migratory fronts only at 10 μM concentration, but secondary tumor-like structures had disappeared at lower doses (0.1-1 μM) of these two drugs. Fluorouracil did not induce a regression of the migratory fronts and secondary tumor-like structures at any doses.

Control 3-D culture systems containing MCF-7 cells did not show any migratory front, but rather cell aggregates mainly at the boundaries of the collagen gels which expanded slightly into the fibrin gel (Figure 4). In addition, few spherical-shaped

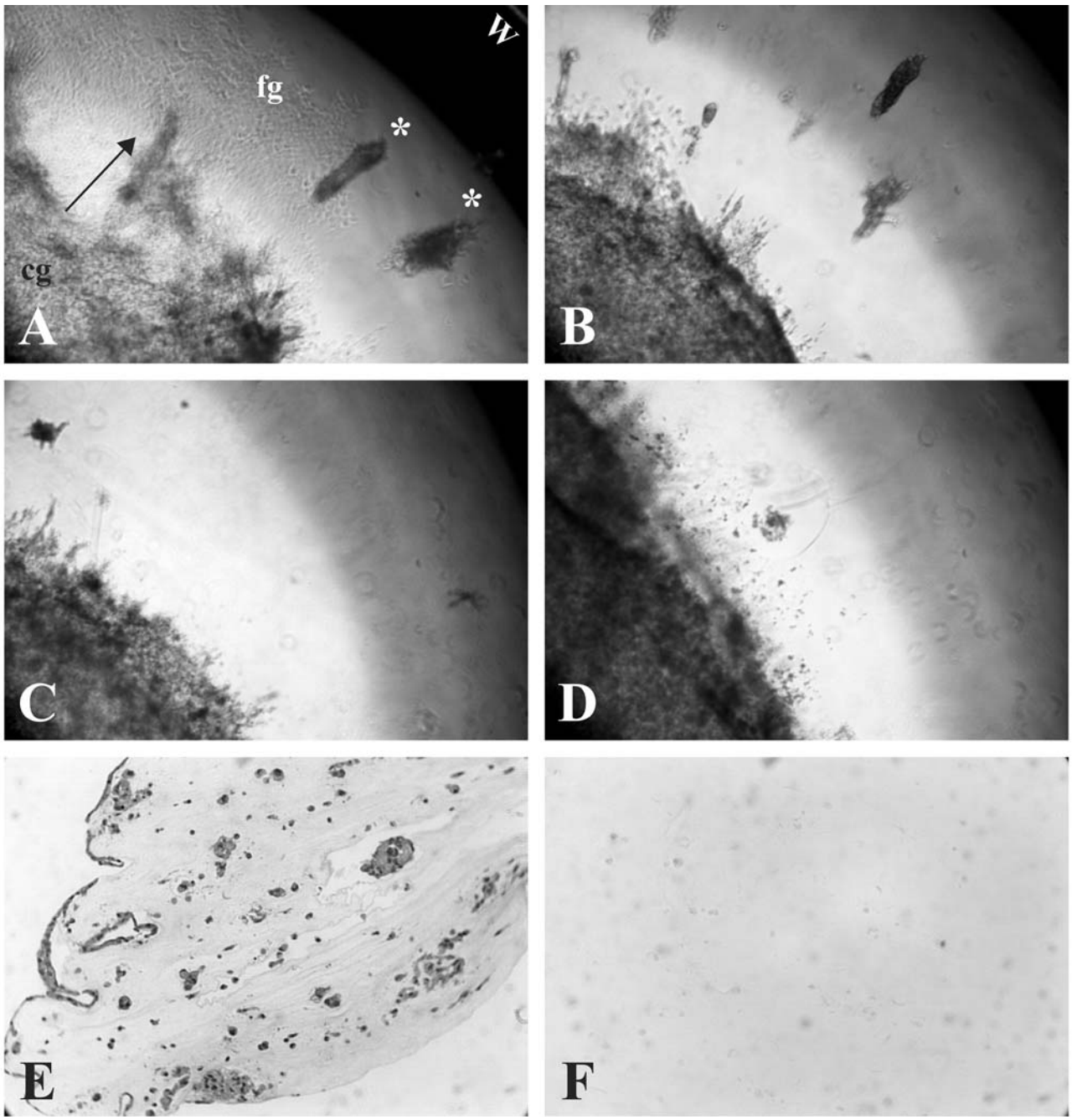


Figure 1. *Observation of PC-3 prostate cancer cells in collagen gel (cg) and their expansion into fibrin gel (fg); w represents the well wall. In control cell culture (A), PC-3 cells exhibited a migratory front (arrow) and secondary tumor-like structures (asterisks) as observed by phase contrast. These formations were slightly decreased with low drug concentrations such as 100nM doxorubicin (B), but significantly with 10 μM paclitaxel (C) and cisplatin (D). Histological sections of collagen gels show numerous tumor cells in the control cultures (E) and almost none with 10 μM cisplatin (F). (A-D: x25; E & F: x180).*

tumors were seen in the fibrin gel as distinct entities. Cisplatin diminished the density of cell aggregates in the collagen gel and the size of secondary tumor-like structures in fibrin gel up to concentrations greater than 1nM. Secondary tumor-like structures were not present at 1 and 10 μM cisplatin. Doxorubicin and paclitaxel induced similar effects to cisplatin, except that no secondary tumor-like structure was seen at 10nM and above. Fluorouracil reduced cell agglomerates in the collagen gel and secondary tumor-like structures in fibrin up to concentrations greater than 1nM.

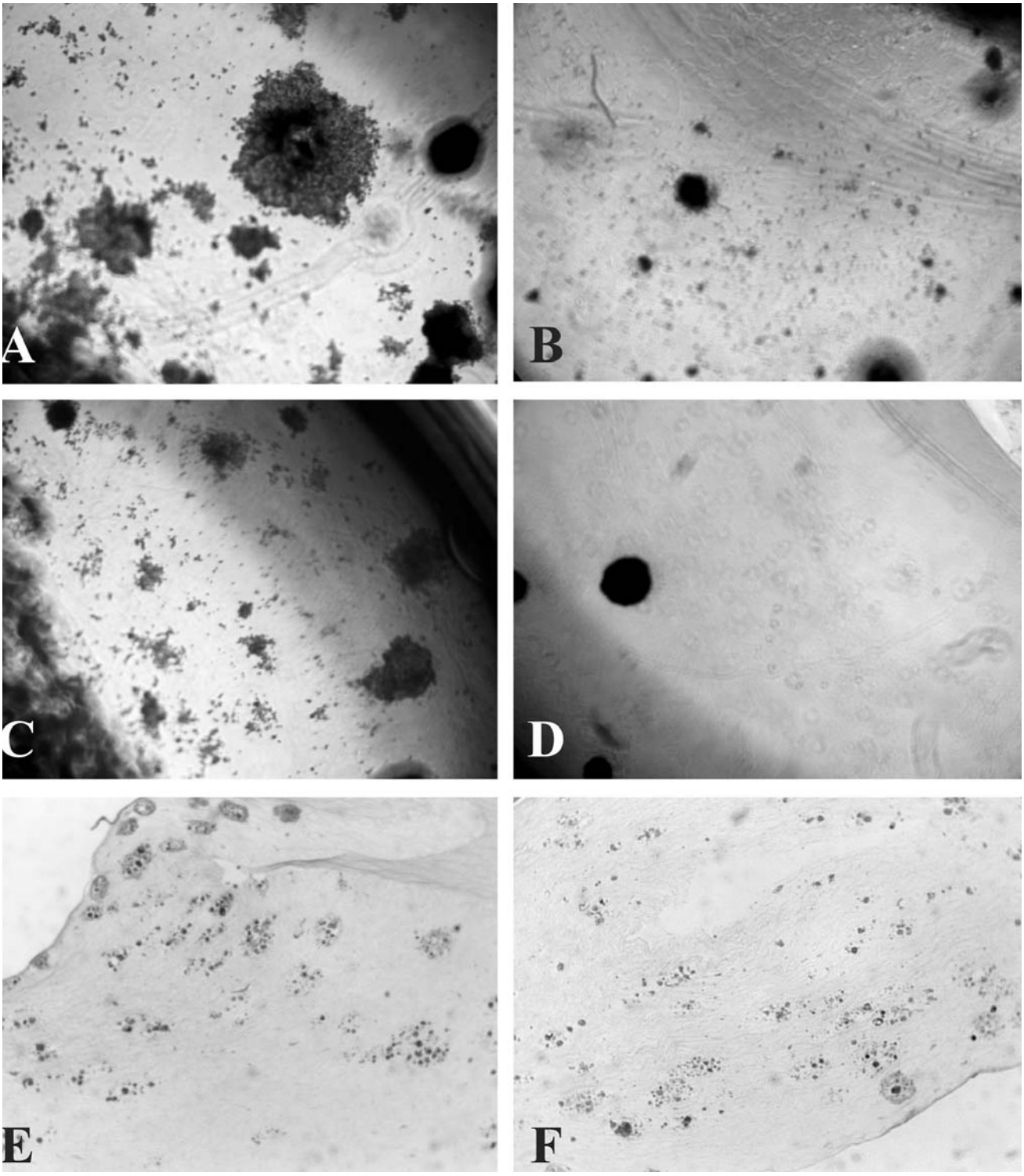


Figure 2. *HT-29 cells extensively migrate into the fibrin gel. Migratory fronts and secondary tumor-like structure formation were present in control cultures and in the presence of 1nM fluorouracil (A). Increasing concentrations of fluorouracil decreased cancer cell invasion (10nM: B). Expansion was still present in the presence of paclitaxel (100nM: C). Few secondary tumor-like structures were seen at 1 μM fluorouracil (D). Histological sections show cell clumps in collagen gels (control: E) which slightly diminished in the presence of 1 μM cisplatin (F). (A-D: x25; E & F: x180).*

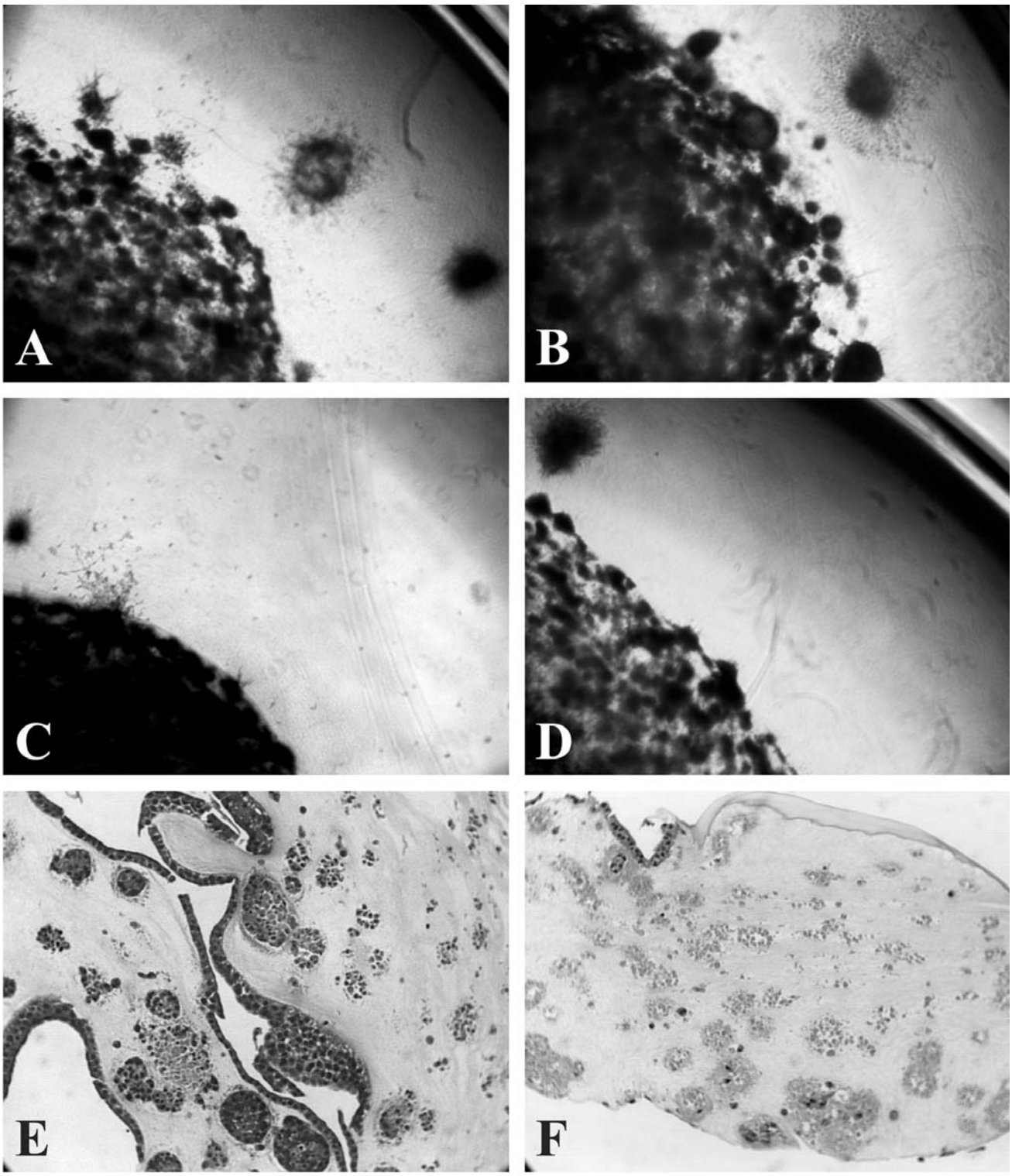


Figure 3. *A549 cells exhibited an intense expansion from the collagen gel as observed in control cultures (A) and in the presence of 1 μM doxorubicin (B). At high concentrations (10 μM) of cisplatin (C) and fluorouracil (D) less invasive structures were seen. Histological sections show the invasive characteristics of these cells (control: E) and that cell density decreased with 1 μM paclitaxel. (A-D: x25; E & F: x180).*

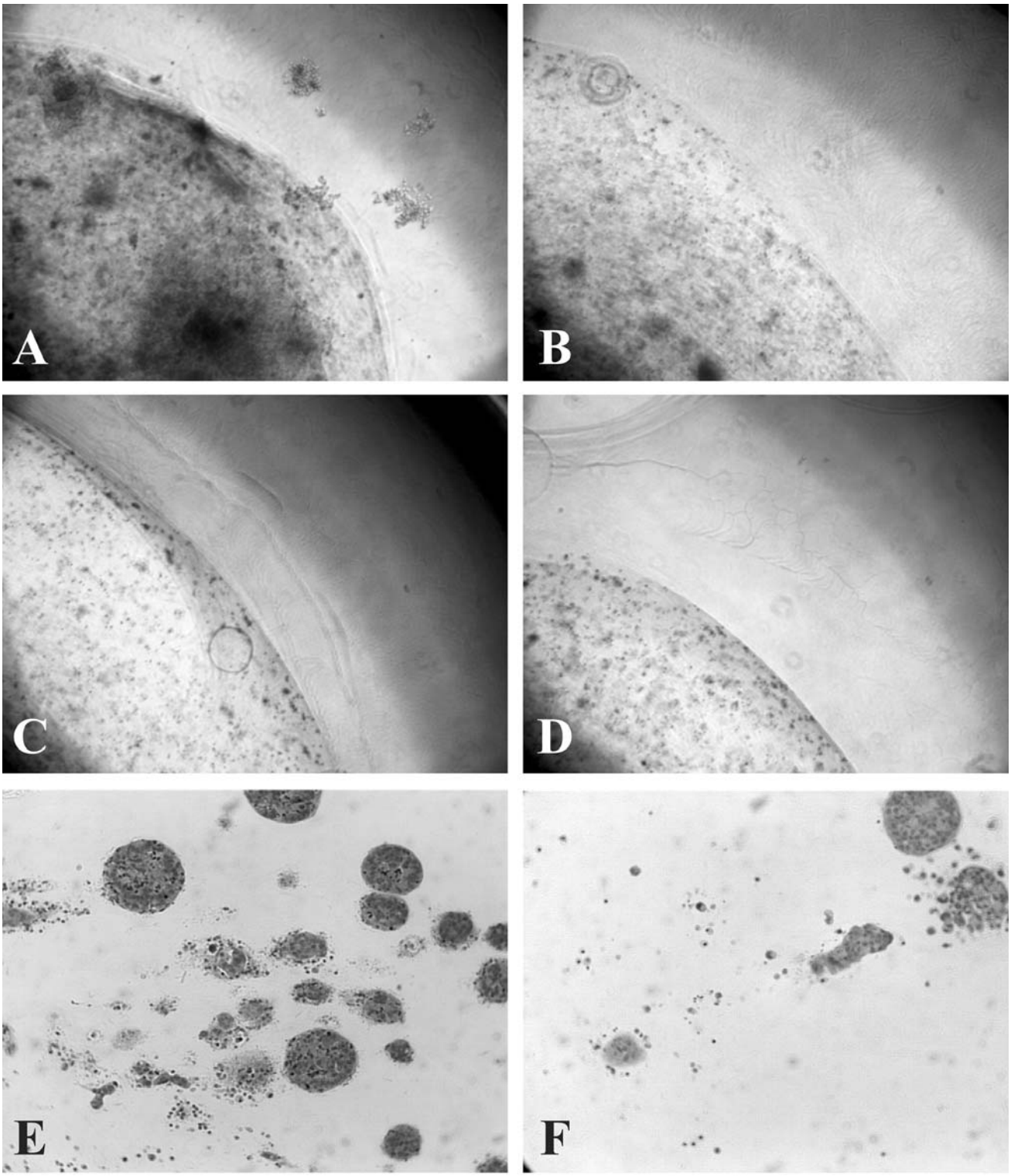


Figure 4. *MCF-7 cells appear less invasive with dense cell clumps within collagen gels as observed by phase contrast and on histological sections of control cultures (A & E). In the presence of 100nM paclitaxel (B) and high concentrations (10 μM) of cisplatin (C) and paclitaxel (D & F), the cell density in collagen gels diminished and secondary tumor-like structure was not present in fibrin. (A-D: x25; E & F: x180).*

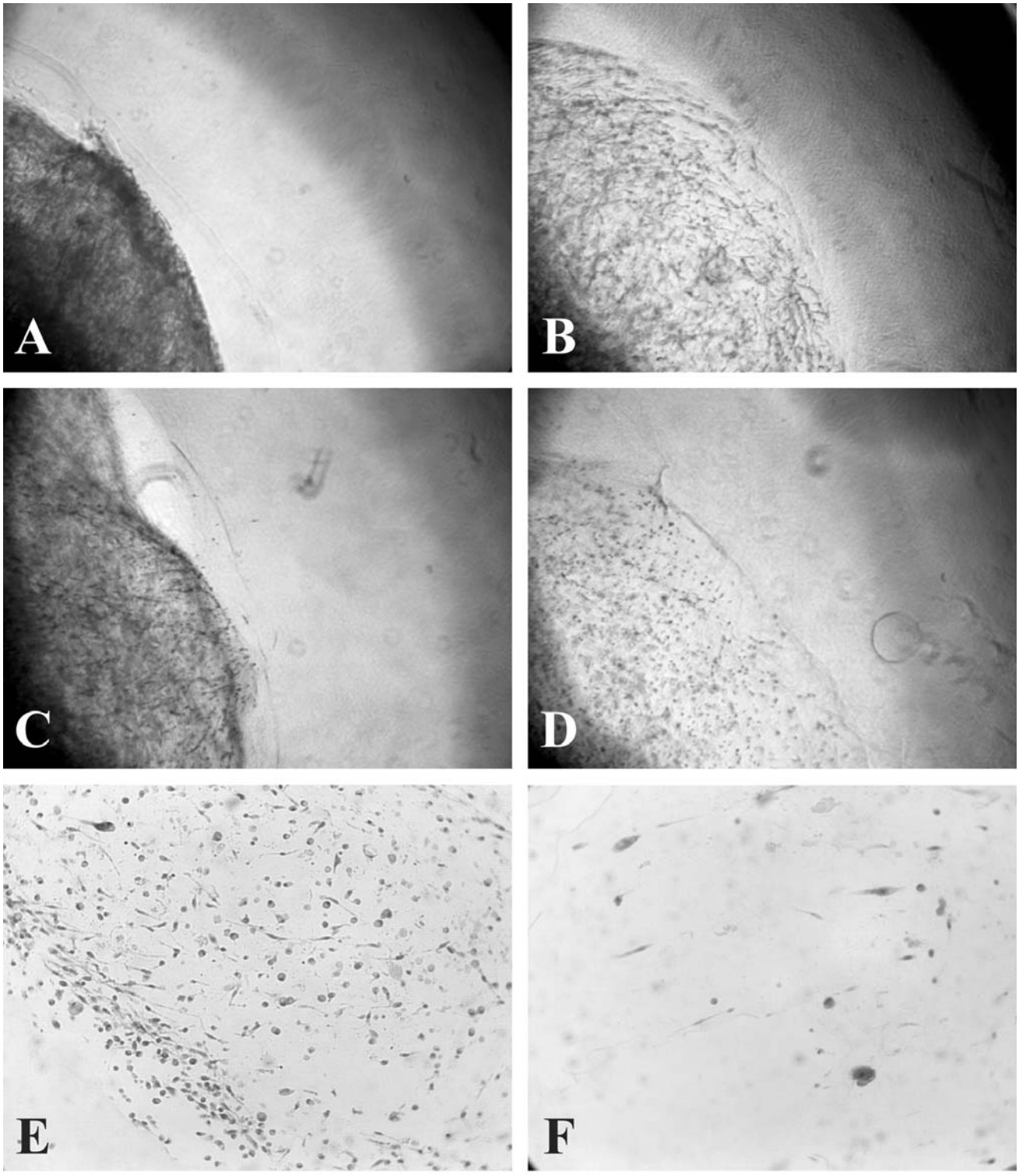


Figure 5. *MDA-MB-231 cells did not invade the fibrin gel. Cells remained dense in the collagen gels of control cultures (A). The density decreased with any anticancer agents as observed with 100nM doxorubicin (B), 1 μM (C) and 10 μM (D) fluorouracil. Histological sections show a dense cell population in the control cultures (E) and few cells with 1 μM paclitaxel (F). (A-D: x25; E & F: x180).*

Control 3-D culture systems containing MDA-MB-231 cells showed that cells were sparsely distributed in the collagen gel and appeared dense, particularly at the collagen gel boundary (Figure 5). However, it was difficult by phase contrast to observe clear migratory fronts. In addition, there was no secondary tumor-like structure in fibrin. The cell density in collagen gels decreased as concentrations of the investigated drugs increased and, more specifically, until reaching concentrations greater than 100nM doxorubicin, 1 and 10 μM cisplatin and 10 μM paclitaxel and fluorouracil. Histological sections of gels confirmed the effects of drug concentrations as described above on the decreasing cell density within collagen.

## Discussion

Monolayer cell culture systems allow the study of cell biology with regards to cell differentiation, growth and function, however, they do not provide evidence for the cell-to-cell and cell-to-extracellular matrix interactions that modulate key processes implicated in the architecture of normal and cancer tissues (14-16). One *in vitro* approach to the analysis of the cell interactions in a system that retains the architecture of tissue is the "organ culture". However, long-term organ culture is compromised by gas and nutrient exchanges to maintain the cell viability throughout the tissue mass (17). A second *in vitro* approach is to grow disaggregated cells to high density within a 3-D reconstituted extracellular matrix that closely simulates the geometric microenvironment of tissues in the body (5).

The composition of our 3-D system was designed based on their predictive role in cancer cell biology. Thus, collagen is the most represented extracellular matrix component of malignant tumor where metalloproteinases play an important role in producing tumor invasion (15, 16, 18). Fibrin is a major extravasated protein that facilitates cancer cell invasion through protease activation (*e.g.*, plasmin), as previously shown *in vivo* (19-21). This setting was established to reflect the local stage of tumor invasion. From a pathological point of view, we report that our 3-D system allowed the study of three distinct events of tumor cell biology: (i) colony formation in collagen may represent intra-tumoral cell expansion (primary tumor), (ii) the formation of migratory fronts, which were initiated from tumor cells grown in collagen gel towards fibrin compartment (tumor cell invasion), and (iii) the formation of cell colonies in fibrin, anatomically separated from the invasion fronts and primary tumor, representing new growths of migrating cells (secondary tumor-like structures).

*In vitro* formation of a cancer cell colony or spheroids has been previously observed in soft agar cultures and after release of cells from plastic support (8, 10, 12). Such cell colonies resemble those observed in our collagen and fibrin gels. Cancer cells embedded in a single extracellular matrix are limited in their expansion, whereas our 3-D system facilitates further expansion of tumor cells. Similarly this advantage is also observed with organotypic cultures of tumors (7, 9). However, the latter have shown that non cancerous cells, such as capillary endothelial cells, preferentially overgrow from the tumor fragments (22).

Our 3-D system enabled the stratification of an individual tumor cell line's capacity to produce the above mentioned events, thereby stratifying four tumor cell lines according to their potential for local growth, invasion and metastasis *in vitro*. Apparently, the A549 cells were the most aggressive tumor cell type in this regard, followed by PC-3 and HT-29 cells, whereas MCF-7 and MDA cells were the least invasive cell types tested. Furthermore, our 3-D system proved efficient in stratifying various cytotoxic drugs *vis-à-vis* their potential to inhibit tumor cell growth, tumor cell capacity for invasion and tumor cell capacity for metastasis in a dose-dependent manner.

Analysis of drug efficiency in our 3-D system showed that it depends on the dose and on the cell type, as expected, but it also depends on the events that occurred in the collagen, or in fibrin. For example, we documented that higher doses were necessary to target the colonies of PC-3, A549 and HT-29 cells in collagen gel, whereas the formation of migratory fronts and separate colonies in the fibrin compartment were more sensitive to low doses of the drugs tested.

Drug resistance is likely to occur in our 3-D system. Resistance to doxorubicin has been previously reported for PC-3 cells embedded in collagen gel (11). This resistance was observed only for the cell colonies within the collagen gel of our 3-D system. Conversely, doxorubicin resistance was not observed for the migratory and re-growth characteristics of PC-3 cells in fibrin. It has been reported that the 3-D microenvironment facilitates drug resistance generation in which integrin signaling plays an important role (4, 23). Integrin signaling may also vary according to the extracellular matrix (24). Furthermore, HT-29 cells, the most invasive cells tested, are particularly resistant to paclitaxel. A549 cells were resistant to the drugs tested. A549 cell-derived tumors are known to have a poor prognosis with frequently intrinsic resistant phenotype (25).

Breast cancer cells, such as MDA-231 and MCF-7 cells, have limited invasion capacity in fibrin. It is unlikely that fibrin is inappropriate since these cells are initially derived from the pleural cavity with fibrin extravasation. In addition, a plasmin inhibitor was added to our system to avoid fibrinolysis that would have occurred in a few days of culture. Conversely, in invasive tumors, structural changes in the extracellular matrix are necessary for cell migration during neoplastic invasion in which proteases play an important role (26, 27). Despite the addition of a plasmin inhibitor, invasive cancer cells such as A549, HT-29 and PC-3 cells, the latter originating from bone metastases, still had the capacity to migrate and expand.

In conclusion, our 3-D system offers a new approach for *in vitro* screening of anticancer drugs. In addition, the formation of cell colonies in the fibrin compartment is an interesting feature for further studying cell invasiveness and metastatic growth. Quantification of those events will implement the predicted efficacy of anticancer drugs in a 3-D composite extracellular environment.

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