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Breast cancer research on chip

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Recently, significant advances in the prevention, diagnosis and management of breast cancer have been made. Nevertheless, worldwide, every year, 460,000 women die of breast cancer (1). The conventional approach to cancer therapy is to provide treatment according to the organ or tissue in which the cancer originates. Currently, the selection of which breast cancer therapy is based on a broad scale of factors, including a patient's age and tumour characteristics, such as nodal stage, the presence of oestrogen receptors and the Her-2/neu status (2). However, the various protocols that exist for chemo- and hormone therapy have different and limited rates of success. Often, this approach to cancer treatment is referred to as 'trial and error' or 'one-size-fits-all' (3). This practice is inefficient and frequently results in inappropriate therapy and treatment-related toxicity. In contrast, personalised treatment has the potential to increase efficacy and decrease toxicity. Nowadays, it is known that cancer develops as a result of multiple genetic defects and that individuals with the same type of cancer often have dissimilar genetic defects in their tumours (4). This finding explains why patients who seem to have similar cancers respond in a heterogeneous manner to antitumour agents and show clearly the huge obstacle to providing effective treatments for cancer. The hypothesis that stem cells play an important role in tumour biology receives a lot of attention (5). These so-called cancer stem cells (CSC) have the

ability for self-renewal and are pivotal in setting the heterogeneous character of a tumour. Besides influencing the origin and growth of tumours, these CSC play an important role in developing metastasis. For personalised medicine, individual treatment regimes have to be set to define the best treatment possible for every patient. Currently, personalised treatment is most advanced for breast cancer. To achieve personalised treatment for cancer, (bio)markers for determining prognosis, predicting response to therapy, and predicting severe toxicity related to treatment are needed (3). DNA/RNA-microarrays for breast cancer prognosis, but also prediction, are very promising and at present clinical validation is ongoing (6). Recently, the use of microtechnologies for cell biology applications, and specifically for cancer, has received rapidly growing attention (7). Lab-on-a-Chip technology is a promising platform for personalised oncology to predict response or resistance to therapy, so that the individual patient receives the right drug. Even though results from *in vitro* assays can't be directly and uniformly translated to the *in vivo* situation, the *in vitro* approach to determining drug sensitivity and resistance continues to have great potential to spare patients the morbidity of ineffective treatment. Here, the development of a microfluidic chip ('Apoptosis chip') to screen the effect of well-known antitumour drugs on human breast cancer cells is described. Moreover, preliminary results on cancer stem cells are shown.

Apoptosis chip

The microfluidic 'Apoptosis chip' consists of a main channel which broadens into a chamber for cell culture (figure 1a). The 'Apoptosis chip' is made of the polymer poly(dimethylsiloxane) (PDMS), and combines cell culture, drug screening and apoptosis detection in one single device.

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The advantages of using this ‘Apoptosis chip’ for such drug sensitivity assays in comparison to the existing conventional assays are multiple (8). Predominantly, the fluidic component enables the continuous flow of nutrients and drug over the cells and exposes the cells to mechanical forces (shear stress). Moreover, fluidics facilitate high-throughput dose-response analysis with limited number of cells. Hence, various passages of cell culture are not necessary, preventing cellular modifications. Our focus is on studying the process of apoptosis, as the goal of breast cancer therapy is to induce cell death. Suppression of apoptosis is known to cause or contribute to cancer (9). Morphological different responses were analyzed in real-time at an individual cell level, advisory as apoptosis is a process that only takes a few hours and does not occur simultaneously in all the cells of a population (10). Hence, performing these experiments on chip will provide us with new insights in the apoptotic cascade (is the specific drug ‘hitting the target’, *i.e.* kills the tumour cells?) which will promote drug development and thus cancer diagnostics. Experimental work in our group demonstrated that oestrogen receptor (ER) positive invasive lobular carcinoma cells (MCF-7) could be stationary cultured in the ‘Apoptosis chip’ for up to 7 days (11). For drug screening, the ‘Apoptosis chip’ was coupled to a flow system (Figure 1c). We analysed the effect of 3 different drugs on MCF-7 cells: tumour necrosis factor (TNF)- α in combination with the protein-synthesis inhibitor cycloheximide (CHX), the protein kinase inhibitor staurosporine (SSP), and the well-known chemotherapeuticum doxorubicin (DOX). Although MCF-7 cells lack caspase-3, the main mediator in the apoptotic cascade, these cells remain responsive to many apoptotic stimuli. In the presence of TNF- α /CHX and DOX, specific apoptotic characteristics were demonstrated optically in detail, at a single-cell level and in real-time: cells obtained a round, shrunken morphology, cells moved actively

over the surface (‘filopodia’) and cells and cell fragments (apoptotic bodies) were pinched off. Moreover, the heterogeneity of the apoptotic process clearly emerges. Conventional flow cytometry lacks these advantages. To quantify apoptosis, we set two new parameters: the area coverage in time and the occurrence of round cells (figure 2). It is expected that in time in the presence of the apoptotic stimulus, the area coverage will decrease and the amount of round cells increase. Measuring the area coverage and amount of round cells in time turned out to be specific for apoptosis, demonstrating a 2-fold decrease in area coverage and a 4-6 times increase in the number of round cells in the presence of TNF- α /CHX and DOX. SSP, which initiated necrosis, showed no stable changes in these parameters. Implementation of electrodes will enable impedance measurements to monitor changes in cellular behaviour, such as cell growth, migration, detachment and cell death in real-time and fully automated.

Cancer stem cell research

In the traditional model of tumourigenesis, tumours arise from a series of sequential and random mutations (12). Any tumour cell can participate in tumour growth, however due to genetic drift and natural selection for the fittest, the most aggressive cells drive tumour progression. A major argument against this model is the prolonged period required to develop the first mutation that subsequently leads to malignant tumour formation. In many tissues in which tumours arise, differentiated cells have a short lifespan and a limited opportunity to accumulate the multiple mutations required for tumour development. Therefore, recently, a new model has been proposed, which considers that tissue stem cells or progenitor cells undergo mutations that deregulate normal self-renewal pathways, leading to tumour formation (5). Hence, only a subset of cells can initiate tumourigenesis. These so-called cancer stem cells (CSC) have the ability for self-renewal and

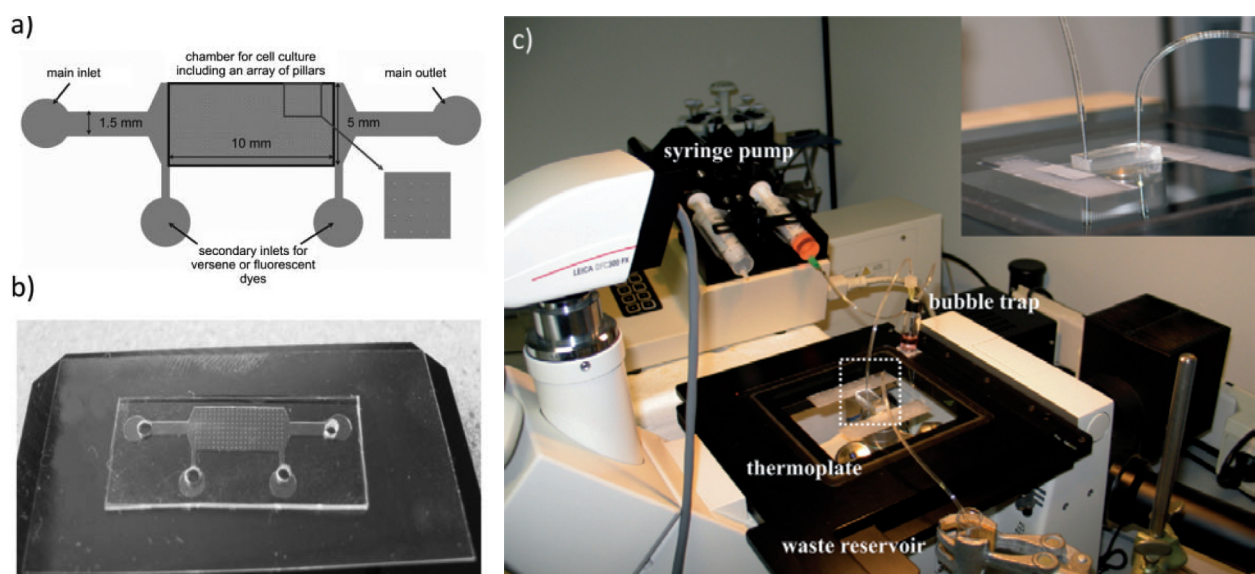


Figure 1. a) Schematic drawing of the microfluidic ‘Apoptosis chip’ and b) picture of the final chip for drug screening. The chip is made of PDMS and sealed onto a microscope slide. Total volume is approximately 4.4 μ l. c) Picture of the flow system. Large picture presents an overview, with an insert of a close-up (dashed square), showing the connection of the flow system to the microfluidic ‘Apoptosis chip’.

are pivotal in setting the heterogeneous character of a tumour. The existence of CSC was first documented in acute myelogenous leukemia, but nowadays CSC are demonstrated in many solid tumours, such as breast cancer (5, 13). The CSC hypothesis has important clinical implications for treatment of breast cancer, because CSC possess several characteristics that make them resistant, and more aggressive, to conventional chemo- and radiotherapy (*e.g.*, quiescent state, DNA-repair during self-renewal, multidrug resistance transporters, expression of high levels of anti-apoptotic proteins and the enzyme aldehyde dehydrogenase 1) (13). Currently, breast cancer therapy is focused on killing the differentiated tumour cells, leaving the CSC unharmed. These survived CSC can form a new tumour (recurrence of disease) and metastasize to other parts in the body. Hence, specific targeting of these CSC will improve the disease-free-survival of cancer patients tremendously (5, 13).

To develop specific targets against CSC, first the CSC have to be identified and subsequently isolated from the total breast tumour population. For this, we used conventional flow cytometry. CSC are defined as cells which express the CD326 and CD44 receptor and lack the CD24 receptor (14). The number of CSC in

human breast tumour tissue was $< 0.5\%$ and $< 5\%$ in the MCF-7 cell line. CSC were cultured as mammospheres to enable drug screening experiments. Mammospheres could be cultured up to passage 6, however, the self-renewal capacity was maximal at passage 2. Mammospheres were characterized with immunocytochemistry to identify possible targets. Mammospheres express the oestrogen- (40%), progesteron- (20%) and vitamin D- (range 30%-90%) receptor. Vitamin D plays an important role in bone metabolism and osteoporosis, as well as in malignant disorders (15). Vitamin D deficiency is associated with increased breast cancer risk and decreased breast cancer survival. The probable anticarcinogenic properties of vitamin D may constitute a promising targeted therapy. *In vitro* results demonstrated that the biologically active hormone $1,25(\text{OH})_2\text{D}$ induced an increased apoptosis/proliferation ratio in MCF-7 cells (15). In addition, $1,25(\text{OH})_2\text{D}$ showed to be involved in the induction of stem cell differentiation and the decrease in self-renewal. Therefore, $1,25(\text{OH})_2\text{D}$ is a promising target to act on the CSC, but also on the differentiated tumour cells. Next experiments are focused on culture mammospheres on chip and analyze the effect of vitamin D on initiating apoptosis in differentiated tumour cells and affecting the self-renewal capacity of CSC.

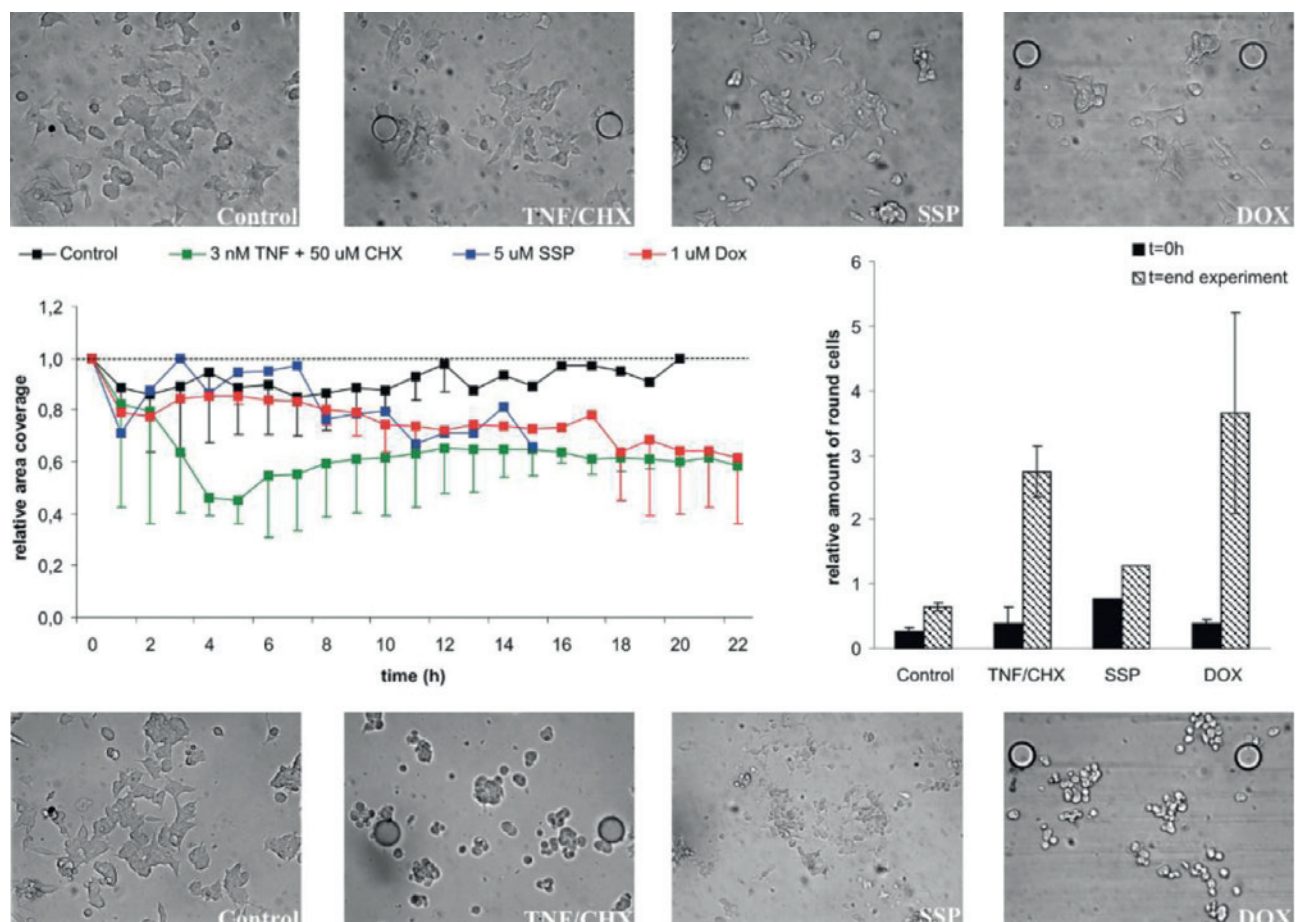


Figure 2. Drug screening in the microfluidic 'Apoptosis chip'. MCF-7 cells were incubated with various drugs (3 nM TNF- α in combination with 50 μM CHX; 5 μM SSP; 1 μM DOX) and the morphological responses were analysed in time. Upper row of pictures shows MCF-7 cells at the start of the flow experiment and the lower row of pictures after incubation with the corresponding drug under a continuous flow of 1 $\mu\text{l}/\text{min}$. Magnification is 20x. In the middle graphs, the relative area coverage in time and the relative amount of round cells are plotted. The mean relative values with SD are shown. Dashed line refers to no change in area coverage in time.

Outlook

Nowadays, much research effort is put forward on understanding cancer biology and translating this knowledge towards the clinic. Our work fits perfectly within this scope. Eventually, we aim that our microfluidic chip will be implemented in the current breast cancer therapy regime. In this way, patients will be also treated by focusing on specific oncogenic pathways that are activated in their particular tumour, rather than only on the tumour's location or histologic features. Moreover, specific targeting of the CSC might prevent metastasis and recurrence of the cancer. Hence, microtechnology has great potential for the clinic to individualise treatment towards personalised medicine.

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