In this issue: LAB-ON-A-CHIP TECHNOLOGY

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

eJIFCC

Future prospects in breast cancer research – cancer stem cells

Floor Wolbers[1], Henk R. Franke[2], Joost M. Klaase[3], Mariël Brinkhuis[4], Albert van den Berg[1], István Vermes[1]
[1]BIOS, Lab on a Chip Group, MESA+ Institute for Nanotechnology, University of Twente, Enschede, The Netherlands
[2]Department of Obstetrics and Gynaecology
[3]Department of Surgery, Medisch Spectrum Twente, Hospital Group, Enschede
[4]Laboratory for Pathology East Netherlands, Enschede

Corresponding Author:

dr. Floor Wolbers
BIOS, Lab on a Chip group
MESA+ Institute for Nanotechnology
University of Twente
PO Box 217, 7500 AE Enschede
Tel.:+31-53-489-3944/F: +31-53-489-3595
e-mail: f.wolbers@utwente.nl

Abstract

Breast cancer is one of the leading causes of cancer deaths among women. Although significant advances in the prevention, diagnosis and management are made, still every year half a million women die of breast cancer. Personalised treatment has the potential to increase treatment efficacy, and hence decrease mortality rates. Moreover, understanding cancer biology and translating this knowledge to the clinic, will improve the breast cancer therapy regime tremendously. Recently, it has been proposed that cancer stem cells (CSC) play an important role in tumour biology. CSC have the ability for self-renewal and are pivotal in setting the heterogeneous character of a tumour. Additionally, CSC possess several characteristics that make them resistant and more aggressive to the conventional chemo- and radiotherapy. Nowadays, breast cancer therapy is focused on killing the differentiated tumour cells, leaving the CSC unharmed, potentially causing recurrence of the disease and metastasis. Specific targeting of the CSC will improve the disease-free survival of breast cancer patients. In this article, two methods are described, aiming at specifically attacking the differentiated tumour cells ('Apoptosis chip') and the cancer stem cell. For this, microfluidics is used.

Keywords: apoptosis, breast cancer, cancer stem cell, drug screening, mammospheres, microfluidics

Introduction

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 (5,6), with three recent papers in Nature and Science (7-9). 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 are clinically validated (10) Nowadays, the use of microtechnologies for cell biology applications (11), and specifically for cancer, receives rapidly growing attention (12). Lab-on-a-Chip technology is a promising platform for personalised oncology to predict response or resistance to therapy, such 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, first research 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 1ab). The 'Apoptosis chip' is made of the polymer poly(dimethylsiloxane) (PDMS), and combines cell culture, drug screening and apoptosis detection in one single device. The advantages of using this 'Apoptosis chip' for such drug sensitivity assays in comparison to the existing conventional assays are multiple (13). 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, microfluidics facilitate high-throughput dose-response analysis with limited number of cells. Hence, cell passaging to increase cell concentration, is 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 (14). Morphological different responses were analysed 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 (15). 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 on chip for up to 7 days (16). For drug screening, the 'Apoptosis chip' was coupled to a flow system (Figure 1c). We analysed the effect of 3 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

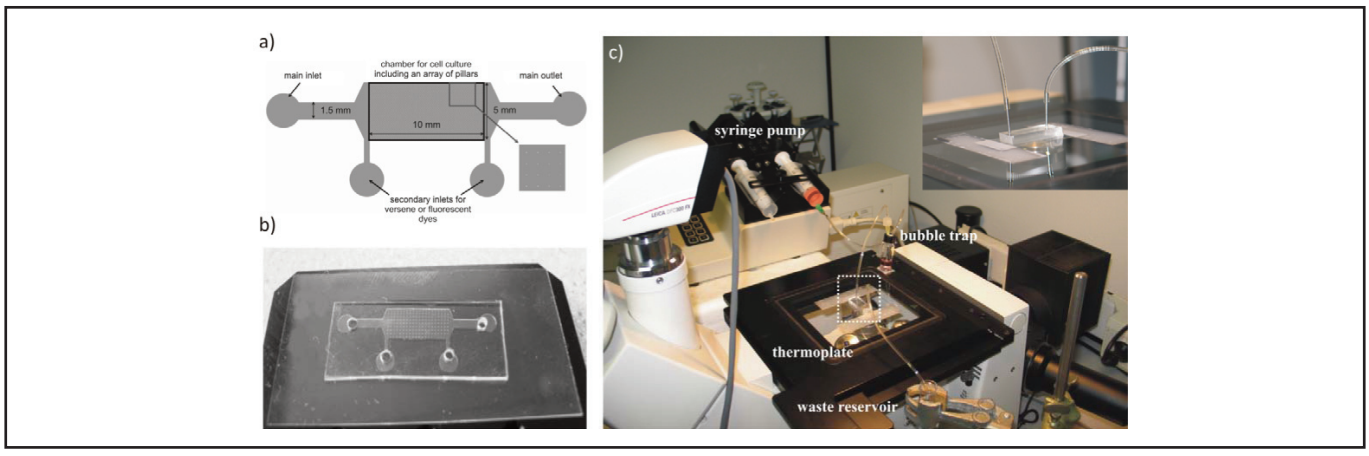

Figure 1
a) Schematic drawing of the microfluidic 'Apoptosis chip' and b) picture of the final chip for drug screening. The chip is made of PDMS and sealed onto a microscope slide. Total volume is approximately 4.4 µl. c) Picture of the flow system. Large picture presents an overview, with an insert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

time and the occurrence of round cells (Figure 2). It is hypothesized that over 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 over 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 (17). Any tumour cell can contribute 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 finite capacity to replicate (due to shortening of the telomeres) and hence 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 self-renewal pathways, leading to tumour formation (5,6). Hence, only a subset of cells can initiate tumourigenesis. These so-called cancer stem cells (CSC) have the ability for self-renewal and 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 (5,18). 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) (18). Currently, breast cancer therapy is focused on killing the differentiated tumour cells, leaving the CSC

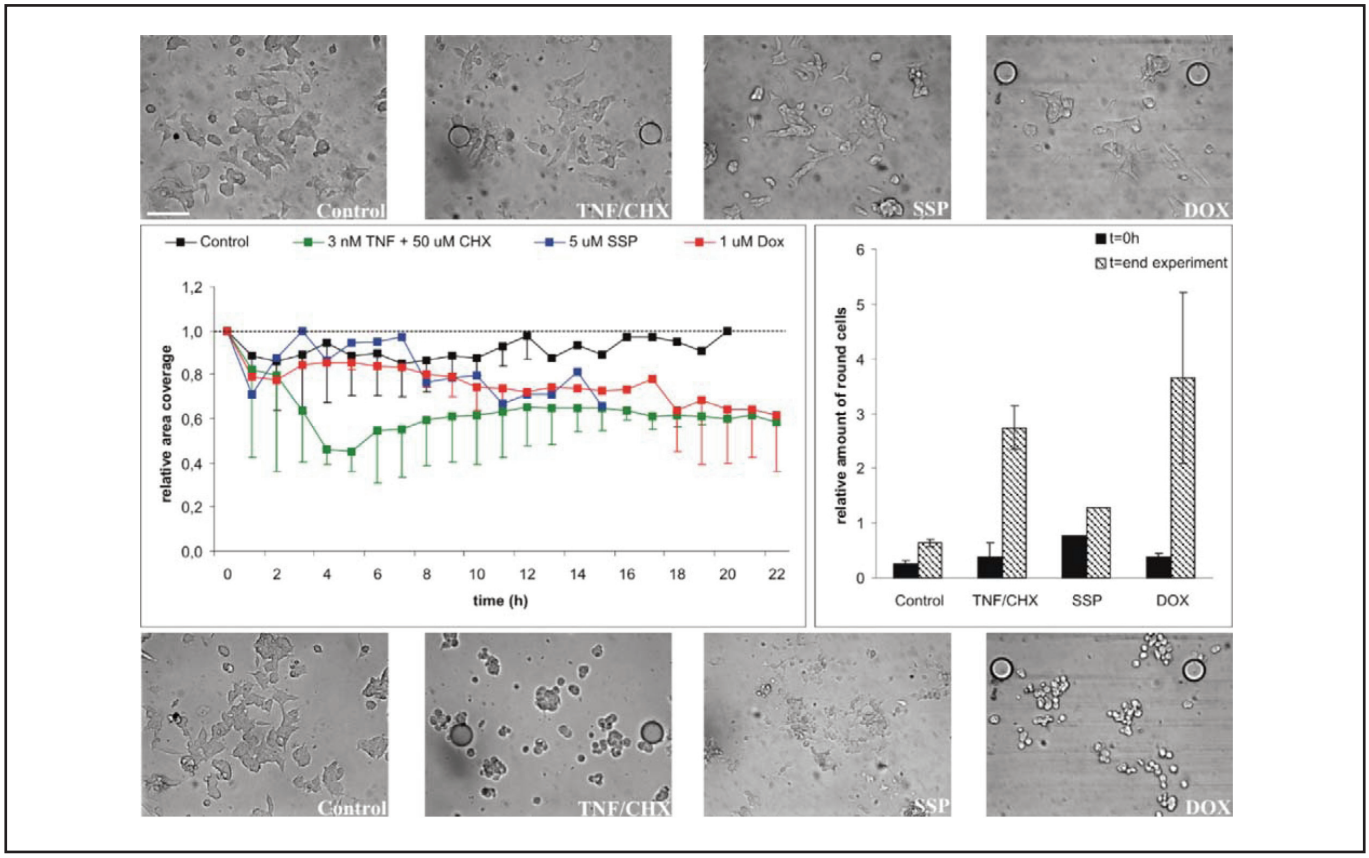

Figure 2
Drug screening in the microfluidic 'Apoptosis chip'. MCF-7 cells were incubated with various drugs (3 nM TNF-α in combination with 50 µM CHX; 5 µM SSP; 1 µM DOX) and the morphological responses were analysed over time. Upper row of pictures shows MCF-7 cells at the start of the flow experiment and the lower row of pictures after incubation with the corresponding drug under a continuous flow of 1 µl/min. Scale bar is 50 µm and is representative for all pictures. In the middle graphs, the relative area coverage over 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 over time.

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, with detailed set indicators, will improve the disease-free-survival of cancer patients tremendously (5,6,18).

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 two separate methods, i.e., conventional flow cytometry and mammosphere culture. CSC are defined as cells which express the CD326 and CD44 receptor and lack the CD24 receptor (19) The number of CSC in human breast tumour tissue, as measured with flow cytometry, was < 0.5% in human breast tumour tissue and < 5% in the MCF-7 cell line. CSC were cultured as mammospheres to enable drug screening experiments. Culturing MCF-7 cells as mammospheres in specific stem cell medium (DMEM/F12 medium supplemented with penicillin, streptomycin, insulin, hydrocortisone, epidermal growth factor and fibroblast growth factor) under non-adhesive conditions, resulted in a mammosphere formation efficiency of 0.035%, which means that 3.5 mammospheres developed out of the 1000 single cells plated. The addition of an extra growth stimulant B27 increased the efficiency with a factor 4.7, to 0.164%. B27 is a mixture of amino acids, vitamins, hormones and anti-oxidants and is a custom supplement for neural cell culture20, but is now widely used in other cell cultures and experiments. Mammospheres could be cultured up to passage 6, however, the self-renewal capacity was maximal at passage 2. In our experiments, only passage 1 mammospheres were used, with the addition of B27 to the stem cell medium. To perform drug screening experiments to specifically target CSC, the developed mammospheres must be filtered out of the batch of single (dead) cells. Conventional centrifugation is not applicable (e.g., centrifugation destroys mammospheres), and therefore a Lab-on-a-Chip device is developed to trap mammospheres and subsequently perform drug screening experiments (Figure 3). The microfluidic device is based on the work of Tan and Takeuchi21 and used for cell-based assays (22). The device consists of a meandering channel (R2) and a straight channel (R1), with a total of 28 traps (Figure 3ab). The channels are designed such that when a trap is empty, the straight channel has a lower flow resistance than the meandering channel, which results in a main flow along the straight channel (Figure 3c left). Hence, a mammosphere carried by this flow, will be trapped (Figure 3c middle). Entrapment of a mammosphere increases the resistance along the straight channel, and redirects the main fluid flow to the meandering channel towards the next trap (Figure 3c right). Mammosphere loading was accomplished by manually placing the cell solution in the inlet and drawing the mammospheres through the device, using a syringe pump. The trapping efficiency reached 85% (Figure 4). Trapping did not negatively influence cell viability (positive Calcein-AM staining after trapping). Moreover, a broad range of mammospheres sizes could be trapped. After trapping, the diameter of the trapped mammospheres was measured (Figure 5, diameter control t=0/doxo t=0). Doxorubicin (1 µM) was added via a syringe pump, to remove the non-trapped mammospheres and to ensure that all mammospheres had been in direct contact with the drug. In the control chip, solely medium was flown. The device was placed in the incubator for static overnight incubation. After 24h, the diameter of the mammospheres was measured for a second time. Figure 5 shows that in the presence of doxorubicin, the diameter of the

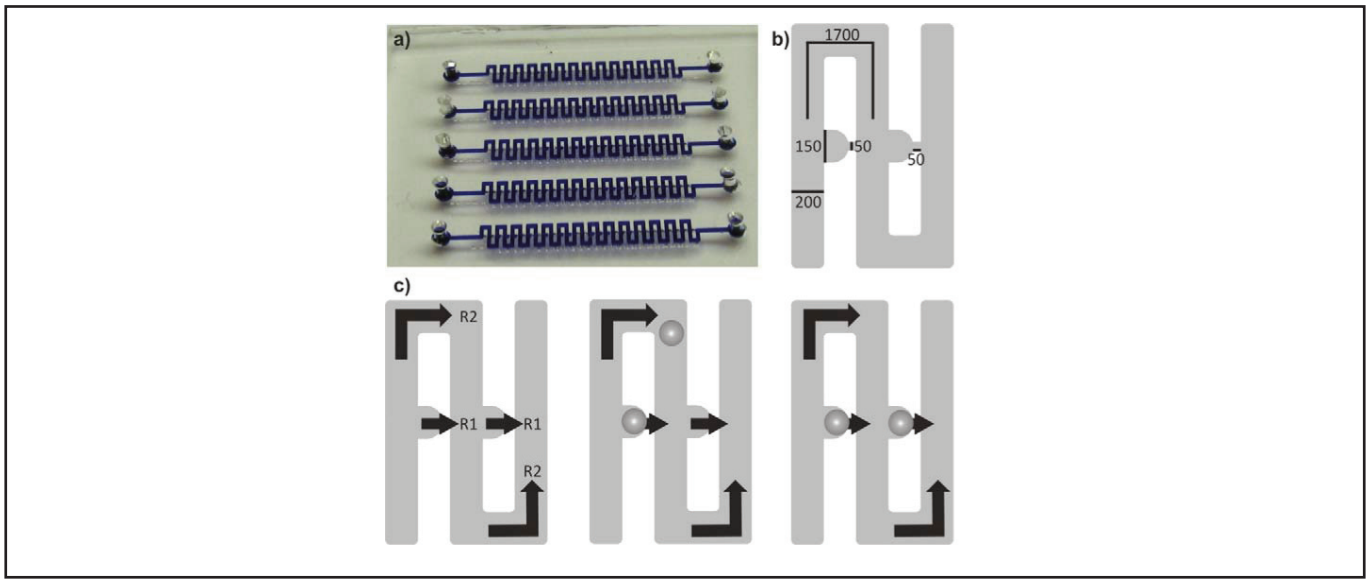

Figure 3

a) Picture of the microfluidic device for trapping mammospheres. A total of 5 trapping devices are shown, with 28 trapping sites each. b) Dimensions of the trapping structure are in µm. Depth is 150 µm. c) Trapping mechanism. When a trap is empty (left), the straight channel (R1) has a lower flow resistance than the meandering channel (R2), R1>R2, which results in a main flow along the straight channel and mammospheres carried by this flow, will be trapped (middle). Entrapments causes R2>R1, resulting in redirection of the flow towards the meandering channel towards the next trap (right). Grey circle depicts a mammosphere.

Figure 4
Mammosphere trapping. Mammospheres of passage 1 were stained with 2 µM Calcein-AM. Inlet is positioned at the left, the outlet at the right. Scale bar is 400 µm.

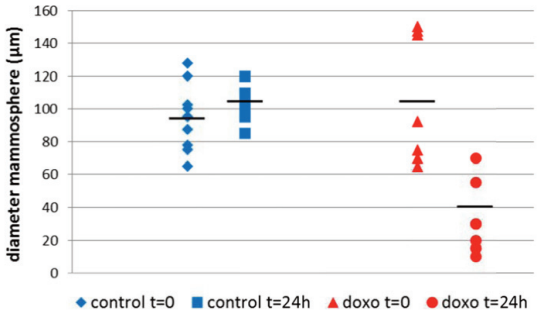

Figure 5
Effect of doxorubicin on mammosphere diameter. The diameter of individual trapped mammospheres is plotted. Black horizontal line represents the mean. In the control chip, 10 mammospheres were trapped and cultured for 24h, in the doxorubicin chip 7 mammospheres were trapped and incubated with doxorubicin for 24h.

mammospheres decreased (doxo t=24h) from 106 µm (±39 µm) to 41 µm (±21 µm). The diameter of control mammospheres remained unchanged. Moreover, fluorescent staining demonstrates that cellular viability was impaired in the presence of doxorubicin (Figure 6). Hence, doxorubicin has an effect on differentiated tumour cells, but to analyse if doxorubicin also has an effect on the cancer stem cells, the self-renewal potency had to be determined. For this, the mammospheres from the control chip and doxorubicin-chip were removed from the chip, disintegrated and plated as single cells in stem cell specific medium. After 7 days, the number of mammospheres was counted. In the control, 15 mammospheres formed, and after doxorubicin treatment only 1 mammosphere developed. The sphere formation efficiency at T1 was 0.9%, and hence increased to 1.35% for the control and decreased to 0.13% after doxorubicin treatment (Figure 7).
The experiments with doxorubicin were repeated (n=4) and relative results are plotted in Figure 8. The mammosphere diameter before adding doxorubicin (set at 1.0; black column) was compared with the mammosphere diameter after 24h doxorubicin treatment (middle-grey column), and as explained previously, a clear decrease in diameter is demonstrated. Furthermore, the mammospheres which arose in the second passage (T2) after doxorubicin treatment (light-grey column) were smaller than the mammospheres of T1 (set at 1.0, black column). Moreover, the self-renewal potency was decreased with a factor 4 (white column; self-renewal potency at T1 was set at 1.0 = dark-grey column).
Thus, doxorubicin affects cancer stem cell viability, however, in all 4 experiments at least one cancer stem cell survived treatment. Ideally, breast cancer treatment is successful if no cells (i.e., differentiated tumour cells and CSC) endure treatment. Therefore, further experiments are focused on analysing (combination of) drugs in varying doses, sequential steps and incubation times to specifically target CSC and obtain a self-renewal potency of zero. First steps have been made. Mammospheres were characterized with immunocytochemistry to identify possible targets. Mammospheres express the oestrogen- (ER; 40%), progesterone- (PR; 20%) and vitamin D- (VDR; range 30%-90%) receptor. Horwitz et al (23= demonstrated that in the T47D cell line (ER^+/PR^+ ductal carcinoma), CSC ($CD44^+$ cells) were negative for ER and PR, and that their progeny acquired steroid receptors. Fillmore et al (24) showed that in MCF-7 cells, 20-25% of the cancer stem cell population ($CD44^+/CD24^-/CD326^+$) expressed ER. In human breast carcinomas, $CD44^+$ cells showed low abundance of ER.25 Based on these studies, breast CSC are ER-/low and PR-, and it is therefore likely that CSC are not responsive to these steroid hormones. Normal breast stem cells are (typically) ER and PR negative (26). If CSC are positive for the VDR still needs to be unravelled. However, it is an interesting phenomenon that vitamin D might be a promising target to act on the CSC, but also on the differentiated tumour cells, because vitamin D deficiency is associated with increased breast cancer risk and decreased breast cancer survival (27-28).

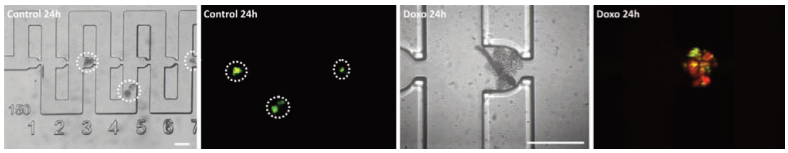

Figure 6
Fluorescent staining of mammospheres under control conditions and after doxorubicin incubation. Mammospheres were stained with Calcein-AM (green) and propidium iodide (nucleus, red) to check viability. Scale bar is 200 µm

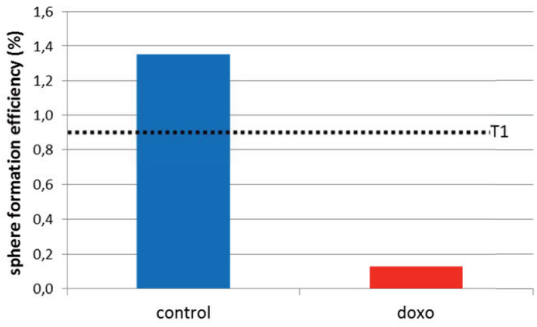

Figure 7
The sphere formation efficiency at T2. Mammospheres were cultured under control conditions or in the presence of 1 µM doxorubicin (doxo) for 24h. Thereafter, mammospheres were removed from the chip, disintegrated and plated as single cells in stem cell specific medium. The number of newly formed mammospheres was calculated after 7 days and the self-renewal potency was determined.

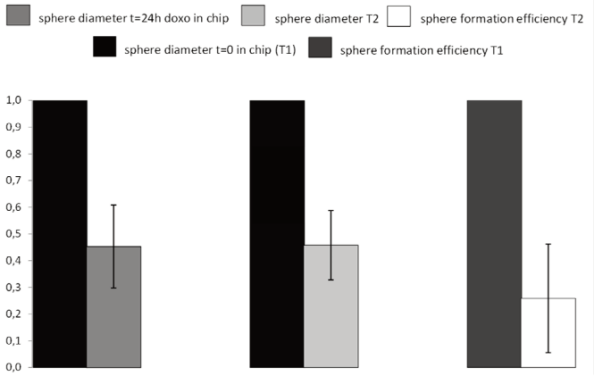

Figure 8
Relative results of doxorubicin treated mammospheres. Mammospheres were trapped and incubated with doxorubicin for 24h. The diameter of the trapped mammospheres at t=0 (=T1, set at 1.0; black column) was compared with the diameter after doxorubicin treatment (middle-grey column), and compared with the diameter of the mammospheres which arose in T2 (light-grey column). Moreover, the self-renewal potency of T1 (set at 1.0; dark-grey column) was compared with the self-renewal potency of T2 (white column). Data represents results of 4 individual experiments.

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 also be 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.

ACKNOWLEDGEMENTS

Financial support from the Dutch technology association STW and technical assistance of J.G. Bomer, P.M. ter Braak, S. Le Gac, A.D. van der Meer, L.I. Segerink, L.M. Griep, M. Ymker, M. Engels, J. Swennenhuis, M. Kristen and S. Heijink are gratefully acknowledged.

This article is modified from Wolbers F., et al., Ned Tijdschr Klin Chem Labgeneesk 2012; 37: 88-91, with new research results on the cancer stem cell.

References

1. J. Ferlay, H. Shin, F. Bray, et al. Int. J. Cancer 127 (2010) 2893-2917
2. G.K. Malhotra, X. Zhao, H. Band et al. Cancer Biol Ther 10 (2010) 955-960
3. M.J. Duffy, J. Crown. Clin. Chem. 54 (2008) 1770-1779
4. L.J. van 't Veer, R. Bernards. Nature 452 (2008) 564-570
5. T. Reya, S.J. Morrison, M.F. Clarke, et al. Nature 414 (2001) 105-111
6. H. Clevers. Nat Med 17 (2011) 313-319
7. J. Chen, Y. Li, T. Yu, et al. Nature (2012) doi: 10.1038/nature11287
8. G. Driessens, B. Beck, A. Caauwe, et al. Nature (2012) doi: 10.1038/nature11344
9. A.G. Schepers, H.J. Snippert, D.E. Stange, et al. Science (2012) doi: 10.1126/science.1224676
10. M. Knauer, S. Mook, E.J. Rutgers, et al. Breast Cancer Res Treat 120 (2010) 655-661
11. A.D. van der Meer, A. van den Berg. Integr Biol (Camb) 4 (2012) 461-70
12. D. Wlodkowic, J.M. Cooper. Curr Opin Chem Biol 14 (2010) 556-567
13. F. Wolbers, C. Haanen, H. Andersson, et al. Lab-on-Chips for Cellomics, Kluwer Academic Publishers, Dordrecht (2004) 197-224
14. I. Vermes, C. Haanen. Adv Clin Chem 31 (1994) 177-246
15. F. Wolbers, P. Buijtenhuijs, C. Haanen, et al. Apoptosis 9 (2004) 385-392
16. J. Komen, F. Wolbers, H.R. Franke, et al. Biomed Microdev 10 (2008) 727-737
17. M. Kakarala, M.S. Wicha. J Clin Oncol 26 (2008) 2813-2820
18. N.Y. Frank, T. Schatton, M.H. Frank. J Clin Invest 120 (2010) 41-50
19. S.P. McDermott, M.S. Wicha. Mol Oncol 4 (2010) 404-419
20. P.J. Price, G.J. Brewer. Protocols for Neural Cell Culture, Humana Press Inc., New York (1994) 255-264
21. W. Tan, S. Takeuchi. PNAS 104 (2007) 1146-1151
22. J. Frimat, M. Becker, Y. Chaing, et al. Lab Chip 11 (2011) 231–237
23. K.B. Horwitz, W.W. Dye, J.C. Harrell, et al. PNAS 105 (2008) 5774-5779
24. C.M. Fillmore, P.B. Gupta, J.A. Rudnick, et al. PNAS 107 (2010) 21737–21742
25. M. Shipitsin, L.L. Cambell, P. Argani, et al. Cancer Cell 11(2007) 259-273
26. J.A. Eden. Menopause 17 (2010) 801-810
27. C.F. Garland, E.D. Gorham, S.B. Mohr, et al. Ann Epidemiol 7 (2009) 468-483
28. S. Veldhuis, F. Wolbers, O. Brouckaert, et al. Menopause 18 (2011) 319-322