Disseminating breast cancer stem cells and their molecular characterization

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''I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of "Good Scientific Practice"
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CONTENT

List of abbreviations and definitions

ACOSOG American Colleague of Surgeons Oncology Group

ALDH Aldehydedehydrogenase

ATCC American Type Culture Collection

BM bone marrow

CGH Comparative Genomic Hybridization

CNV copy number variation

CSC Cancer Stem Cells

CTC circulating tumor cells

CK cytokeratin

DNA Desoxyribonucleid Acid

DTC disseminated tumor cells

EMA epithelial membrane antigen

EMT epithelial mesenchymal transition

ESA Epithelial cell surface antigen

FBS Fetal bovine serum

FCS Fetal calf serum

ISET isolation by size of epithelial tumor cells

MEBM Mammary epithelial basal medium

PBS Phosphate buffer serum

PCR Polymerase Chain Reaction

SWOG South West Oncology Group

TNM tumor node metastases

WGA Whole Genome Amplification

Abstract in German

Dem Tumorstammzellmodell liegt die Hypothese zugrunde, dass nur eine kleine Proportion der Tumorzellen die Fähigkeit der Tumorformation, der Selbsterneuerung und der Differenzierung besitzt. In Brustkrebs wurden die Tumorstammzellen mit den Phänotypen $CD44^{+}CD24^{-low}$ und mit Aldehydedehydrogenase Aktivität assoziiert. Während die Präsenz der disseminierenden Tumorzellen und Mikrometastasen die Rationale zum Einsatz adjuvanter Therapieansätze bildet, die Tumorstammzellen dürften eine Erklärung für das Versagen dieser Therapieansätze bieten. In der vorliegenden Dissertation wurde evaluiert, ob sich die derzeitig verwendeten Brustkrebsstammzellmodelle zur Analyse der Heterogenität der Brustkrebszellen mittels umfassender molekularen Charakterisierung eignen und weiters wurden Verfahren zur Evaluierung disseminierender Tumorzellen und deren Zusammenhang mit Tumorstammzellen optimiert.

Die Brustkrebszelllinien MCF7 und SUM159 wurden in adhärenten Bedingungen und als Mammosphären kultiviert. Anschließend wurde Durchflusszytometrisches Sorten anhand von Brustkrebsstammzellmarkern durchgeführt. Als nächster Schritt wurde das DNA Profil mittels Array CGH und das Methylierungsprofil der gesorteten und ungesorteten Populationen, der Mammosphären und der adhärenten Zellkulturen erstellt. Weitere Methoden zur Analyse der disseminierenden Zellen wurden optimiert, inklusive CTC Mikrochip zur Anreicherung der CTC und der Möglichkeit weiterer on chip DNA Analysen.

Mittels Array CGH konnten keine genomischen Unterschiede festgestellt werden und es gibt Hinweise, dass die epigenetische Unterschiede nicht schlüssig sind. Der CTC Mikrochip wurde mit suffizienter Sensitivität reproduziert, und kann nun als Methode zur Evaluierung von peripherem Blut der Brustkrebspatientinnen sowohl auf CTC als auch auf putative Brustkrebsstammzellmarker eingesetzt werden.

Die etablierten Brustkrebsstammzellmodelle in Brustkrebszelllinien dürften nicht geeignet sein, um die molekulare Heterogenität von Brustkrebs zu definieren. Der genaue Prozentsatz der Brustkrebsstammzellen, welche mittels Sphärenbildung und Durchflußzytometrie angereichert werden, bleibt unklar. Diese Arbeit zeigt neuerlich die Notwendigkeit weiterer Ansätze um die intratumorale Heterogenität von Brustkrebs und Disseminierung und deren Zusammenhang mit den Tumorstammzellen aufzudecken.

Abstract in English

The cancer stem cell model hypothesizes the existence of a small proportion of tumor cells capable to sustain tumor formation, self-renewal and differentiation. In breast cancer, these cells were associated with $CD44^{+}CD24^{-low}$ and aldehydedehydrogenase positive phenotype. While the possible presence of early tumor dissemination and micrometastases is the rationale behind the use of systemic adjuvant treatment in patients who have undergone definitive surgery of the primary tumor [1, 2], the presence of cancer stem cells (CSC) may explain the failure of adjuvant chemotherapy in a proportion of early stage breast cancer patients. In this master thesis the suitability of current approaches for breast cancer stem cell analyses to evaluate heterogeneity of breast cancer cells through their extensive molecular characterization was investigated. Furthermore, approaches for evaluation of disseminating tumor cells, and their association with CSC were optimized.

First, the breast cancer cell lines MCF7 and SUM159 were cultured in adherent conditions and as mammospheres. Flow cytometric sorting for breast cancer stem cell markers was performed. Sorted and unsorted populations, mammospheres and adherent cell cultures were subjected to DNA profiling by array-CGH and methylation profiling. Further, methods for analyses of disseminating breast CSC were optimized, including CTC microchip enrichment and analyses of on chip DNA profiling.

Array CGH did not reveal any genomic differences and there is evidence that epigenetic differences cannot in cell lines are indecisve. After CTC microchip optimization sufficient sensitivity was reproduced, and can be further used as method for evaluation of peripheral blood of breast cancer patients for CTC and putative breast cancer stem cell markers.

Established breast cancer stem cell models in breast cancer cell lines may not be suitable to define molecular heterogeneity of breast CSC. The percentage of breast CSC in the enriched fraction remains unknown. This work underscores requirement of additional approaches to reveal intratumoral heterogeneity of breast cancer and disseminating cells and their association with breast CSC.

1 INTRODUCTION

1.1 BREAST CANCER

Breast cancer is the most commonly diagnosed cancer in women, and the leading cause of cancer death in women. Over a million of new cases are diagnosed worldwide each year. Every eight women is diagnosed with breast cancer during her lifetime [3]. The incidence of breast cancer in Austria remains stable since 1997. The risk of disease increases during the lifetime and reaches the maximum between the 5th and 6th decade. Screening methods and better treatment strategies have led to a decrease in mortality in the last decades [4, 5]. However, despite the combined treatment strategies with surgery, radiation and anti-cancer drugs, patients who develop metastatic disease are currently incurable.

More than 90% of breast cancers are adenocarcinomas, either originating from ductal structures (invasive ductal carcinoma) or lobular structures (invasive lobular carcinoma) of breast gland [6]. Both types are supposed to develop from preinvasive forms (carcinoma in situ).

1.2 BREAST CANCER METASTASES

Breast cancer metastasizes early, both through lymphatic vessels and hematogeneously (see figure 1). Lymphatic spread follows along the axillary vein to the axillary lymph nodes and along the thoracic wall to the retrosternal and supraclavicular lymph nodes. Current praxis is to determine the sentinel lymph node, which is regionally the first node. If this lymph node is tumor- free, no additional axillar node dissection is performed [7]. This procedure lowers the morbidity substantially. Hematogeneous metastases of breast cancer are most commonly to the bone (70%), lung (60%), liver (50%) and cerebrum [8].

Tumor node metastases (TNM) staging at the primary diagnosis reflects the clinical and pathological extent of the disease and influences the decision on clinical treatment. Larger primary tumors (T) and involvement of more lymph nodes (N) are associated with

worse prognosis. As already stated above, once distant metastasis is diagnosed, disease becomes incurable.

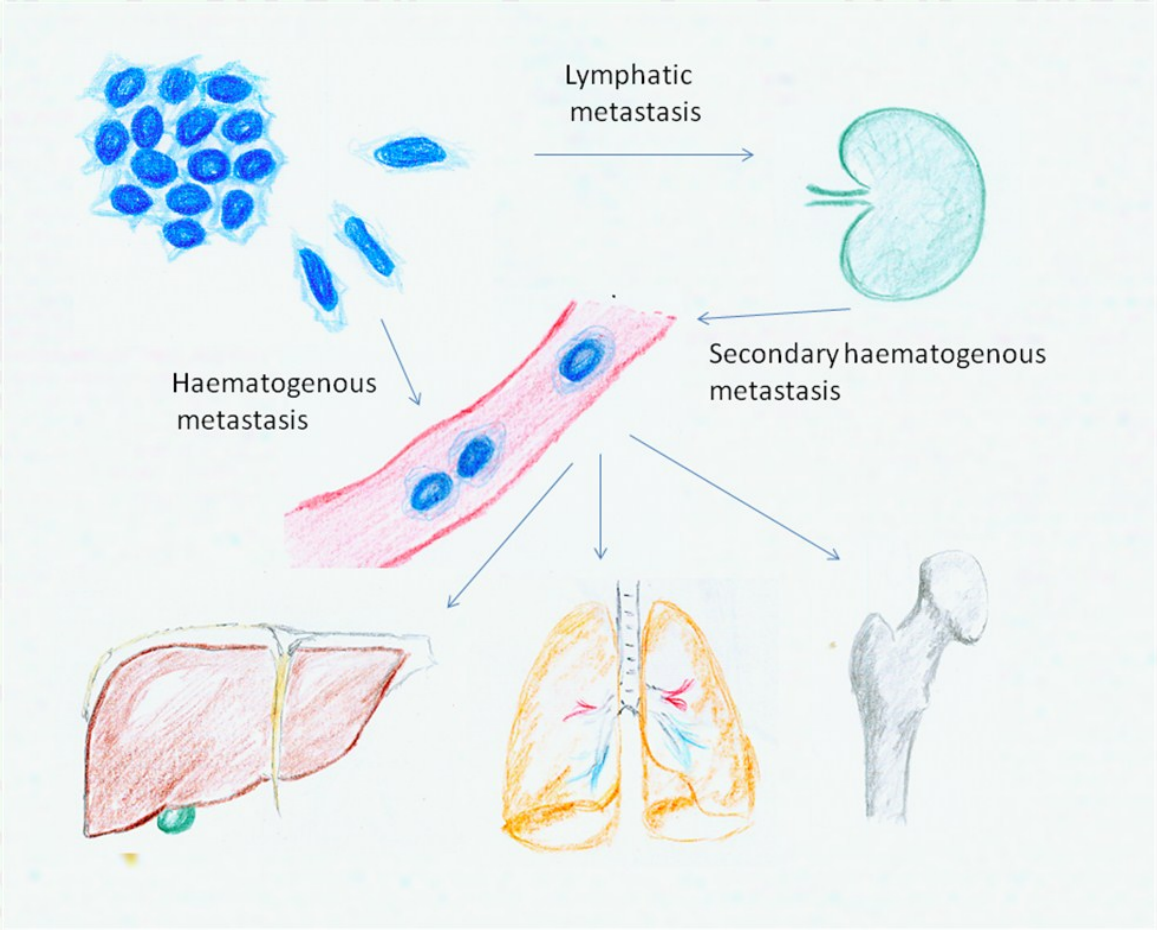

Figure 1 demonstrates schematically the process of metastasis. Single cells or cell groups detach from the primary tumor and invade through the basal membrane into the circulation, either peripheral blood or lymphatic vessels. Eventually, these cells settle down either in distant organs or regional lymph nodes, from where they can secondarily spread through peripheral blood again. At distant sites these cells are believed to interact with the microenvironment and acquire further genetic and epigenetic changes over time, and finally outgrow to metastasis.

1.3 CLINICAL SIGNIFICANCE OF METASTASIS

Major progress has been achieved in the treatment of various cancers, including breast cancer, over the last decade. Nevertheless, metastatic cancer remains incurable. It is gen-

erally believed that distant metastases spread from primary tumor through invasion into circulation and finally distant sites, where they may reinitiate the growth, depending on microenvironment [9]. One of the surrogates of haematological spread are disseminated tumor cells (DTC) detected in bone marrow (BM) aspirations, as demonstrated in one of the first studies of its kind by Redding and colleagues using epithelial membrane antigen (EMA) to identify DTC in BM at the time of primary surgery in women with no overt metastases [10]. Later, several groups demonstrated the prognostic significance of DTC using a combination of epithelial cell surface antigens (ESA) and cytokeratins (CK) to identify DTC in early stage breast carcinoma [11, 12]. Prognostic significance of the presence of DTC in BM aspirates has been established for several cancers, with most evidence related to breast cancer [13]. Because of the invasive procedure of BM aspirations, its limited value of prognostic information and, as recently shown, low number of positive patients [14], aspirations are not routinely performed. Many attempts have been made to optimize the detection of circulating tumor cells (CTC) from peripheral blood. Blood would be the ideal source because of the potential of serial analyses and relative low invasiveness of the procedure. Since a blood draw is frequently performed in patients undergoing treatment, in the majority of cases only additional tubes need to be obtained from patients. Despite the progress in recent years, demonstrating not only the prognostic value, but also the use of CTC in monitoring the response of tumor to systemic therapy in breast and other cancers [15-17], the detection of CTC is far from being widely used in the clinical management of cancer patients.

1.3.1 Differentiation between DTC and CTC

Tumor cells detected in BM of cancer patients are referred to as DTC and those in the blood as CTC. While this is a purely semantic distinction, it is in common usage in the field. Most early studies focused on the detection of DTC. Initially, antibodies to epithelial cell surface antigens like EMA were used to identify DTC by immunostaining of the BM at the time of primary surgery in women diagnosed with breast cancer with no overt metastases [10]. Later on, antibodies to CK were increasingly used for immunohistochemical identification of DTC in early stage breast carcinoma [11, 12]. Most studies demonstrating prognostic significance of the presence of DTC in BM aspirates relate to breast cancer

[13]. Whereas early studies demonstrated the presence of DTC in 30% of breast cancer patients, the incidence of DTC detection is far lower in more contemporary studies, coincident with earlier detection of breast cancer overall due to increased use of screening mammography. Recent report of the results of the American College of Surgeons Oncology Group (ACOSOG) study Z0010, one of the largest prospectively collected clinical trial in stage I and II breast cancer patients, analyzed the prognostic significance of DTC and demonstrated a very low detection rate, with only 3% of BM aspirates containing tumor cells [14]. The presence of DTC was a significant predictor of outcome, although the low incidence of DTC in this highly selected group of patients called in to question the value of its routine use in that setting. Nevertheless, the idea of identifying tumor cells that can be targeted with an adjuvant treatment seems appealing, and many attempts have been made to optimize the detection of tumor cells that circulate in peripheral blood, thus providing minimally invasive access to the early events in metastases. Nevertheless, it remains unclear if CTC are biologically identical to DTC, and thorough characterization of these disseminating tumor cells is needed.

Cancer patients undergoing treatment frequently undergo blood draws for various diagnostic and treatment monitoring reasons. Thus, blood would be an ideal compartment for detection of metastatic tumor cells. Blood draws can easily be performed serially. Recent studies have demonstrated the potential value for a CTC assay to determine prognosis as well as prediction of response to systemic therapy in patients with breast and other cancers [15-17]. Despite a growing body of evidence, the detection and enumeration of CTCs has yet to achieve widespread use in the clinical management of cancer patients, primarily due to the limitations of the currently available technologies in obtaining and analyzing CTCs.

Recognizing the potential value of CTCs as biomarkers for prognosis, prediction of therapeutic response, and as a companion diagnostic in development of novel therapeutics, several new methods have evolved in the last decade for CTC enrichment and evaluation. Various technologies for the enrichment and detection of CTCs have been used [18, 19], and these include density, affinity, and sized based methods. At least to a certain extent these technologies were used also for enrichment of DTC from BM of patients with solid

tumors. As technology in this growing field matures, a substantial impact on clinical practice could follow upon adoption of some of the novel methods. As of today, the only FDA approved technology to clinically monitor CTCs in metastatic cancer patients is CellSearch (Veridex/ J&J).

1.3.2 Review of prognostic and predictive significance of CTCs

The prognostic significance of detection of CTCs in peripheral blood of cancer patients has been demonstrated for several types of cancers. Most of the evidence results from studies based on immunohistochemical detection of CTCs, [15, 16, 20, 21] but several studies have successfully employed a variety of RT- PCR methods to detect epithelial or tumor-derived transcripts as surrogate reporters of CTCs [22, 23]. Similar to DTC, prognostic significance of CTC has mostly been demonstrated in breast cancer, and particularly in metastatic disease.

Seminal studies on CTCs were performed employing the CellSearch method [24]. CellSearch represents an epithelial antigen-affinity-based system for enrichment and detection of CTCs, and has been shown to be reproducible across different independent testing sites [25].

The study performed by Cristofanilli et al represents a landmark study using CellSearch assay. In this study, the authors established the threshold of 5 tumor cells/7.5ml of blood to define the prognostic significance of CTCs in breast cancer patients [15]. This threshold was later repeatedly used in many studies. In this study the predictive significance of changes in CTCs levels during the treatment got recognized. In a follow up study, the authors demonstrated that the changes in CTC levels could predict response to therapy after as early as one cycle of treatment [26].

Several independent studies in metastatic breast cancer patients have confirmed prognostic significance of CTC detected by CellSearch assay [24, 27]. In addition, analogous studies were performed in patients with other cancers, like prostate and colorectal cancer [20, 28]. All these studies concluded that CTC detection was associated with unfavourable outcome. More importantly, serial CTC evaluation was repeatedly demonstrated to be capable of predicting response to treatment early in the course of the therapeutic inter-

vention, and much sooner than serum biomarkers or radiographic examination. These studies led to FDA approval of the CellSearch method as a tool for CTC detection, prognostic evaluation and response monitoring in patients with metastatic breast, prostate, or colon cancer. Therefore, these studies have set an important cornerstone for evaluating the clinical validity of CTCs.

Other methods besides the CellSearch assay have also demonstrated the prognostic significance of CTCs in peripheral blood of cancer patients, and have shown similar association of CTC numbers with shorter overall survival and worse prognosis. These include microbead-based affinity enrichment of CTCs followed by identification by immunohistochemistry [16, 29] , or RT-PCR approaches for detection of CTCs, where multiple epithelial specific and tumor specific transcripts are used as surrogate markers for the presence of CTCs [30, 31].

Establishing CTCs as a biomarker for therapeutic decision-making may improve cancer patient management. As shown by Giuliano et al [32], it may be used to select candidate patients for more aggressive treatment. These patients were the ones with higher CTC levels. Authors suggested a relatively modest treatment plan for patients with lower numbers of CTCs. Breast cancer patients with hormone receptor-positive tumors are commonly observed with progressive disease early on hormonal treatment. In such cases, detection of higher levels of CTC may prove helpful for early identification of patients who would more likely benefit from a chemotherapy regimen.

Aside from their use in determining the prognosis of patients with metastatic cancer, the ability of CTCs to predict the response to an ongoing anticancer treatment at the earliest time point is especially attractive. Several studies have evaluated such predictive potential through monitoring changes in levels of CTCs to determine either response or resistance to a therapy. A significant proportion of patients demonstrating no decrease in the number of CTCs may benefit from intervention in their treatment earlier than is done in the current clinical practice. Two aspects make this important: a potential to decrease costs through an early detection and termination of a failed treatment; and even more importantly, a potential to spare patients the adverse effects of an ineffective treatment. In a recent prospective trial of serial CTC measurements by CellSearch with side-by-side

comparison of several potentially clinically useful serum markers, Bidard et al demonstrated similar performance in terms of prediction of progression-free survival [33]. The question of the clinical utility of CTCs for early decision-making is being evaluated in the prospective collection of blood samples for the Southwest Oncology Group (SWOG) 0500 clinical trial. The trial has stopped recruiting patients and the evaluation of the clinical utility of CTCs may be expected within a year.

Serial determination of CTC counts performed in addition to conventional imaging may be highly informative for patient prognosis and therapeutic response. Budd et al performed a comparison of CTC detection and imaging for prediction of outcome in metastatic breast cancer and validated that CTC detection can provide clinically useful data much earlier during the course of treatment [34]. A significant disadvantage of conventional imaging is great inter-reader variability for evaluation of radiographic scans, which may be overcome with automated CTC detection and counting.

However, on the basis of the CTC and DTC studies it seems much more important to understand the biology of these disseminating cells. The biology of the metastatic process is still largely unknown, with each step in the dissemination process representing a significant investigative challenge. The journey of a tumor cell from the primary tumor to a distant site begins with the local invasion of the tumor and intravasation into peripheral circulation. Among the CTC and DTC at least a subpopulation of cells represent the cells driving the progression of disease. One of the crucial aspects of this biological research of disseminating tumor cells (both CTC and DTC) is addressed below.

1.4 TUMOR CELL HETEROGENEITY AND CANCER STEM CELLS

It has long been known that tumors are comprised of heterogeneous cells. There are many indicators of tumor cell heterogeneity, beginning with recognized prognostic and predictive markers of disease. In breast cancer, these markers are estrogen and progesterone receptor, and Her2-neu.

According to the classical stochastic model of disease, tumor cells are heterogeneous, but all these cells have the capability of tumor formation. Opposite to that theory, there is increasing experimental evidence supporting the cancer stem cell model. The cancer stem

cell model favours only a small proportion of cells on the top of the cancer cell hierarchy with the capability of sustaining tumor formation and growth, and self-renewal and differentiation [35]. Figure 2 below depicts the two models next to each other (A- stochastic model, B- cancer stem cell model, modified from Reya et al [36].

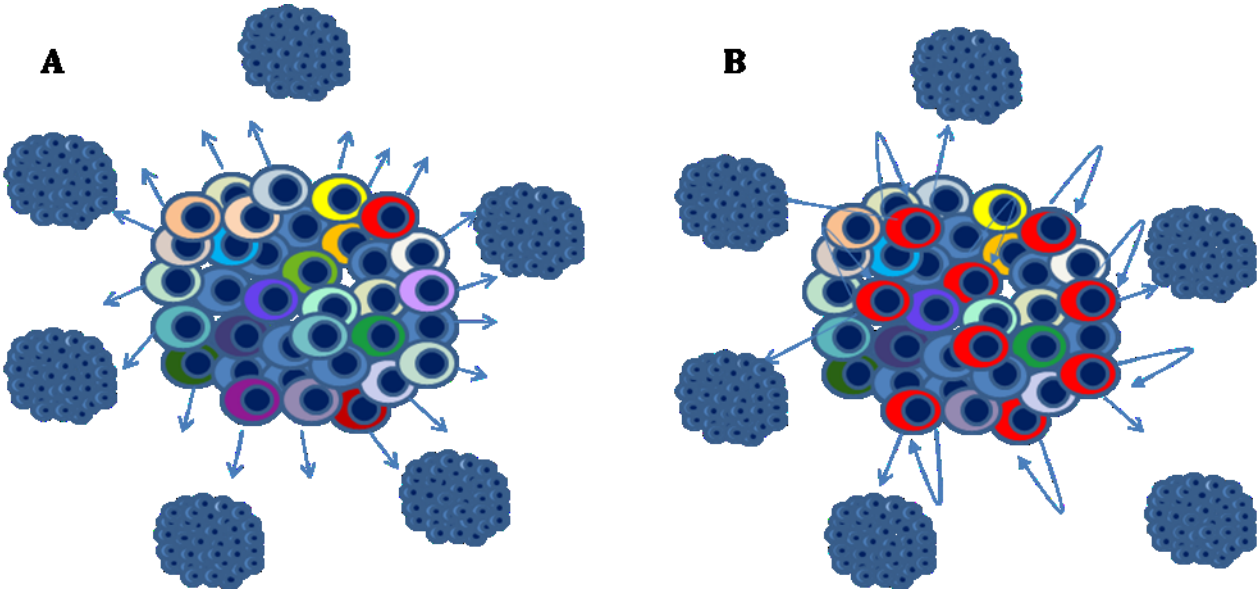

Figure 2 (adapted from Reya et al, Nature, 2001) demonstrates two opposite theories: A. stochastic theory on heterogeneity of tumor cells within a tumor, according to which cells are heterogeneous, and all (most) of the cells have tumorigenic properties and B. stem cell theory on heterogeneity of tumor cells with hierarchical order, with only a small proportion of cells being capable of self renewal, differentiation and tumorigenicity.

Cancer stem cells (CSC) have been identified for hematological malignancies [37], brain tumors [38], breast cancers [39] and several other cancers.

1.1 BREAST CSC

The ability of a tumor cell to undergo epithelial-mesenchymal transition (EMT) has been hypothesized to play a crucial role during the early parts of metastatic process and dissemination. The plasticity of these disseminating tumor cells may also be an attribute of a sub-population of putative CSC within the CTCs. [40]

For breast cancers, CSC, also termed as putative breast CSC, tumorigenic breast cancer cells or tumor initiating breast cancer cells, have been identified to have a $CD44^{+}CD24^{-/low}$ phenotype [39]. Cells with this phenotype have been shown to represent a minor population (10-20%) within primary tumors as well as metastatic tumor formations [39, 41]. Further, $CD44^{+}CD24^{-/low}$ have been shown to be tumorigenic and multipotent, capable of generating cells with different lineages ($CD44^{+}CD24^{-}$, $CD44^{+}CD24^{+}$, $CD44^{-}CD24^{+}$ and $CD44^{-}CD24^{-}$). While the possible presence of early tumor dissemination and micrometastases are the rationale behind the use of systemic adjuvant treatment in patients who have undergone definitive surgery of the primary tumor [1, 2], the presence of CSC may explain the failure of adjuvant chemotherapy in a proportion of early stage breast cancer patients.

The putative breast cancers stem cell phenotype $CD44^{+}CD24^{-/low}$ has been defined in a study performed by Max Wicha and colleagues [39]. The authors minced primary tumors and metastatic lesions and processed them in order to obtain single cells suspension. After flow cytometry sorting they obtained colonies of different populations and implanted them into nude mice. The $CD44^{+}CD24^{-/low}$ population of cells was found to be highly tumorigenic and capable of self-renewal and differentiation.

CD44 is a cell adhesion molecule known to be expressed in most cell types [42, 43] and has been associated with stem cells in normal breast tissue [44]. It has also been suggested to be a homing factor for BM [45]. The role of CD24 is yet to be understood, however, literature suggests that CD24 functions through inhibition of the 'SDF-1 (stromal cell-derived factor-1) - CXCR4' pathway, which normally triggers the migration and recruitment of immune cells. Thus, in breast cancer cell lines enhanced CD24 expression results in suppression of their metastatic potential. In contrast, the metastatic potential of $CD24^{-}$ cells is increased as evidenced by siRNA inhibition [46]. This may explain the high frequency of DTC with $CD44^{+}CD24^{-}$ phenotype in BM of early stage breast cancer patients we have demonstrated [47].

Another marker has recently emerged as a putative stem cell marker for normal tissues and cancer, particularly breast cancer. The expression of aldehydedehydrogenase (ALDH 1, further defined as ALDH) has been used in flow cytometry sorting in Aldefluor assay,

and cells with its expression have been also highly enriched for putative breast CSC. The expression of ALDH in breast cancer tissues has been correlated with worse prognosis [26].

Figure 3 (adapted from Reya et al [36] depicts potential clinical impact of the presence of CSC among tumor cells within the primary tumor and in dissemination.

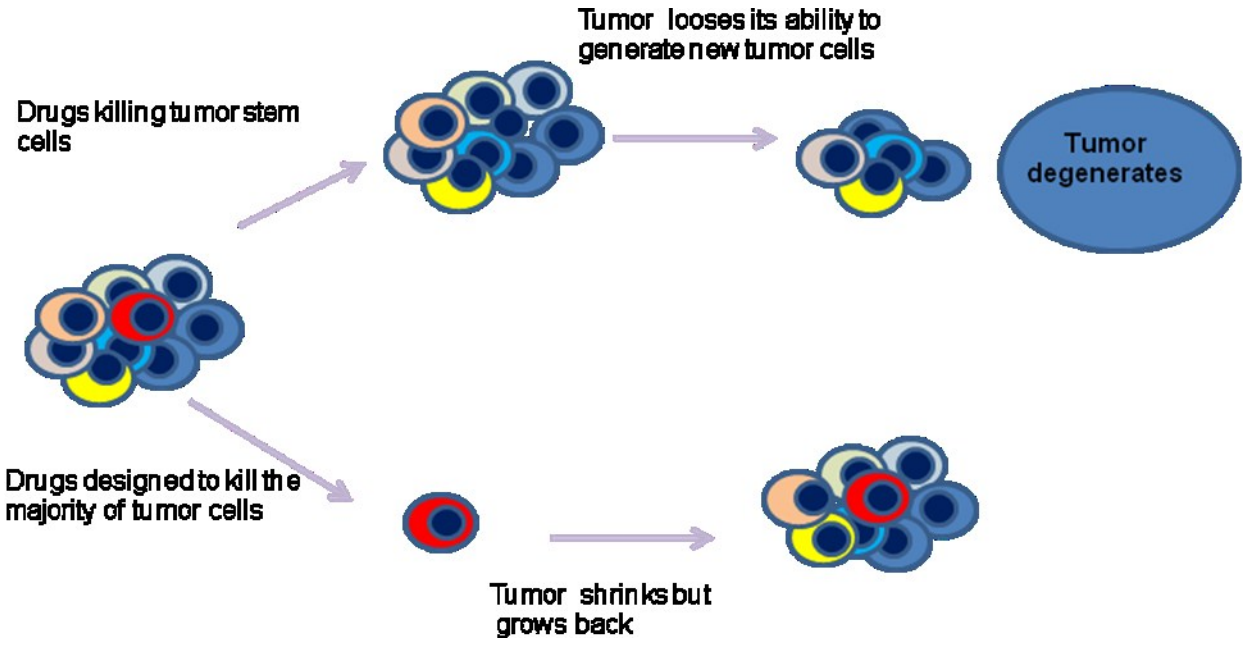

Figure 3 (adapted from Reya et al, 2001), reflecting the clinical implication of cancer stem cell existance. According to the stem cell theory it seems essential to design drugs effective on CSC, otherwise the risk of regrowth remains.

The identification of breast CSC among patients' CTC may represent a promising tool to assess the malignant potential of these cells and help in identification of novel therapeutic targets. The major drawback of this approach is the limited information on the cancer stem cell phenotype, acknowledging further that only a proportion of enriched cells with known markers may be breast CSC. It is still unclear if there are distinct stem cells for intrinsic subtypes of breast cancer, and if so, which markers define them. In a recent paper, Ricardo et al evaluated these established phenotypes and their distribution among the

molecular subtypes [48]. The authors concluded that the methods and biomarkers identifying breast CSC within the distinct molecular subtypes need to be better explored in order to facilitate translation of the cancer stem cell concept to clinical practice.

1.4.1 CSC in dissemination

Breast cancer patient population has a lifetime enhanced risk of death from cancer when compared to matched normal population group [49]. This may be attributable to potential impact of the existence of CSC among the DTC in patients [50]. Pooled analysis [13] of a large number of patients showed that although the presence of DTC was associated with a poor prognosis, a significant proportion of patients still do well even after 10 years. A major reason for this variability could be that a proportion of DTC may be capable of remaining 'dormant', settled in BM and/or distant organs, and these cells are driven towards the recurrent tumorigenesis only after unknown initiation events occur.

These observations and the hypothesis that CSC not only exist within the primary tumor but also exist among cells that metastasize from primary breast cancer to distant locations have led us to perform the analysis of DTC for the described putative breast cancer stem cell phenotype [47]. As expressed by Max Wicha, bringing the field of CSC together with DTC, this study initiated a novel area of research interest [51].

A study analyzing DTCs of patients treated in the ACOSOG Z0010 clinical trial for the putative breast cancer stem cell phenotype was performed, using the $CD44^{+}CD24^{-/low}$ breast cancer stem cell phenotype. This was a novel area of research examining the cancer stem cell phenotype among DTCs and it showed, for the first time, that the large majority of the DTCs exhibited the expression phenotype criteria of putative CSC [47]. Our finding has significantly impacted research on metastasis, as it suggests an enrichment of putative breast cancer cells in metastases. The percentage of such cells within the primary tumor was reported with <10% [52], while our study showed that 72% of DTC showed the putative stem cell phenotype. Further, this result is particularly significant because the analysis was performed in the cohort of stage I and II breast cancer patients, where only 3% of BM samples were shown to be positive for DTC [14]. Also, it contributes significance to earlier findings that putative breast CSC play an important role in pathogenesis of breast

cancer [51] and may offer critical therapeutic targets. In a later study, Reuben et al performed prospective analysis of BM aspirates with flow cytometry sorting, and showed that patients with particularly high risk clinico-pathologic features had high percentages of putative breast CSC in their BMs [53].

Analogous studies on CTCs in metastatic cancer patients have followed. Theodoropoulos et al detected CTCs in 67% of patients, of which 35% of CTCs had the putative stem cell phenotype [54]. Wang et al have used flow cytometry to evaluate peripheral blood of breast cancer patients at different stages and showed that percentage of putative stem cells rose with increasing tumor stage, indicating that these cells can be used to guide the therapy and predict prognosis [55]. Again, these results indicate an emerging role of CSC as therapeutic targets.

1.5 DISSEMINATING TUMOR CELLS, ENRICHMENT AND DETECTION

The greatest challenge in detection of DTC and particularly CTC is their rarity in the blood. Human blood consists of white blood cells ($5–10\times10^6$/ ml), red blood cells ($5–9\times10^9$/ ml), and platelets ($2.5–4\times10^8$/ ml) [25], and very few CTC are usually present, even in patients with known metastatic disease, often less than one CTC per ml of blood [25, 56]. Because of their rarity, CTC have to be enriched prior to detection. A great variety of techniques have been used for enrichment and detection of CTC [57, 58]. Here, only a brief overview is provided.

1.5.1 Enrichment techniques

Techniques for enrichment of disseminating cells and CTC are based on biological and/or physical properties that distinguish CTC from normal cells of the blood. Affinity based enrichment is the most commonly employed strategy to separate CTC from blood cells. Affinity techniques take advantage of distinctive antigens expressed either by CTC but not blood cells (e.g. EpCAM), or by blood cells but not CTC (e.g. CD45). Affinity methods are restricted to CTC that show differential antigen expression and the most common examples are EpCAM expressing epithelial tumor cells. EpCAM based enrichment is an example of enrichment of CTC based on positive selection of tumor cells by targeting the expres-

sion of epithelial markers. Alternatively, negative selection or depletion of non-tumor background cells can be used, where the most typical example is depletion of lymphocytes, which express CD45 [59]. EpCAM is expressed to various degrees by many epithelial cancers and is also known as HEA and BerEp4 [60, 61]. However, many epithelial cancers do not express EpCAM (for example renal cell cancer), and this molecule is heterogeneously expressed by even high expressing tumors (such as breast cancer). EpCAM is also not expressed by non-epithelial cancers, such as melanoma. This represents a major drawback of affinity-based methods. The most common strategy for affinity-based enrichment is immunomagnetic separation using magnetic beads functionalized with antibodies that bind to CTC (positive enrichment) or blood cells (negative enrichment). Immunomagnetic enrichment currently exists in the markets in various formats including columns, beads, and cartridges for automation of the process. More recently, microchip based affinity methods have been developed.

An alternative and older approach is enrichment of cells by their density, a physical property of the cell. An example of such a method is Ficoll Hypaque [62], which separates red blood cells from nucleated cells in the blood or BM, including tumor cells. This method was used for most of studies, which demonstrated the clinical significance of detecting DTC in the BM of early stage breast cancer patients. However, recovery of tumor cells by this method is low, and enrichment is poor.

Another property of tumor cells useful for enrichment is their size [63]. Tumor cells, particularly those derived from solid tumors, are larger than most cells present in blood. While these differences are small, they are consistent. An advantage of this approach is that a broader range of tumors are potentially amenable to size based separation, and heterogeneity of antigen expression is not an obstacle. The idea to use the size for filtering of CTC from peripheral blood is old [64], however, it was not pursued until recently. Several techniques based on size based separation of tumor cells are now either commercially available or under development [65, 66]. I was involved in the development and early evaluation of a size based microfilter for enrichment and detection of CTC [67], which appears to be highly efficient, and much faster, compared to affinity based separa-

tion techniques. This platform can be used for detailed molecular characterisation [68], described more fully below.

In recent meetings on CTC many novel technologies have been presented, with capability of single cell detection, molecular profiling and definition of the biological potential. Many of these systems currently lack clinical data; therefore, their clinical utility is yet to be determined. Most systems rely on affinity separation. After a brief summary of existing CTC detection methods in the next section the most promising novel technologies with clinical evidence will be briefly discussed.

1.5.2 Detection methods

Although enrichment methods for disseminating tumor cells increase the ratio of target (CTC) to background cells, no method results in a pure population of tumor cells. Thus, all separation techniques require a method to distinguish tumor cells from the non-specifically captured cells. This is generally performed by cytomorphological characterisation of cells, immunocytochemical detection of tumor specific antigens or various RT-PCR approaches. Cytomorphological characterisation relies on classification of tumor cells based on their distinct morphological features [66]. Immunocytochemical detection of DTC/ CTC employs antibodies specific for epithelial cells; the most commonly used antibodies are those to a wide range of CK (which are broadly expressed by epithelial tumor cells), including both low- and high molecular weight CKs [69]. Multimarker approaches have been developed, enabling simultaneous visualization of multiple markers on a single cell [70]. Detection of DTC/ CTC by immunocytochemistry (but also other molecular methods) has one major drawback, which is the potential to miss cells not expressing the intended target antigens. As recently shown, this may occur due to epithelial-mesenchymal transition and expression of mesenchymal markers by epithelial CTC [71]. This issue also represents a potential major limitation both for affinity based enrichment and detection of DTC/ CTC.

Several RT- PCR protocols have been developed, striving for quantification and better sensitivity [30, 72-74]. However, it has been demonstrated previously that the detection of DTC/ CTC by RT-PCR based methods has limited specificity, as many of the transcripts

used to identify tumor cells have basal expression levels in non-tumor blood cells [75]. Additionally, the detection of DTC/ CTC by RT-PCR requires the preparation of lysates to recover total RNA, eliminating the ability to perform morphologic analysis on cells of interest. However, one major advantage of molecular methods is their higher potential for standardization based on the superior objectivity.

All these methods are time consuming and cost intensive. Recent progress in technology focuses either on lowering the costs of analyses or facilitating molecular characterization of DTC/ CTC or ideally both.

The CellSearch System by Veridex/ Johnson and Johnson is still the only FDA approved method for detection of CTC in breast, colon and prostate cancer. None of the other methods has yet undergone such careful preclinical and clinical validation. The CellSearch method has been shown in multiple studies to identify patients with metastatic cancer who will have a shorter survival, and more importantly, which can identify patients that are responding, or not responding, to systemic therapy, often after the first treatment, based on the number of tumor cells identified in 7.5 ml of blood. The FDA approval for CellSearch is for prognostic evaluation and therapeutic response monitoring in patients with metastatic breast, prostate, or colon cancer [15, 20, 21, 26, 28, 76]; CellSearch is a fully automated system for detection of CTC, and has been shown to be reproducible across different independent testing sites. Briefly, the immunomagnetic enrichment is based on positive selection using EpCAM labelled iron oxide nanoparticles, and subsequent detection of CK positive CTC cells. CellSearch adds an additional step to identify cells that express CD45; cells that express CKs, but lack the expression of CD45, and that have the cytomorphologic characteristics of tumor cells (appropriate size, presence of a nucleus, and appropriate nuclear to cytoplasmic ratio), are counted as tumor cells. After enrichment and labelling, positive events are sorted, and are reviewed by trained personnel for the above characteristics. A 7.5 ml sample of blood is thus evaluated for the presence, and number, of CTC. The system possesses the capability of additional detection channels for Her2 neu, however, the ability to interrogate the CTC is limited. In a study comparing different enrichment methods, we demonstrated that CellSearch outperformed three other methods, which included another immunomagnetic positive enrich-

ment method, depletion of CD45 positive cells (negative enrichment) and density based enrichment by OncoQuick [77]. Recently at the international meeting on CTC technologies data were presented by Michail Ignatiadis (Ass. Professor, Jules Bordet Institute, Brussels, Belgium, Advances in Circulating Tumor Cells, Athens, 26th-29th September 2012) regarding the reproducibility of CellSearch across the experienced laboratories indicating 40% discordance rates, which is of concern.

1.5.3 Size based microchips

The size of tumor cells has been increasingly used as a property of tumor cells for their enrichment. Several platforms using size as the capture method have been described [63]. At University of Southern California developed simple size-based filter for isolation of CTC has the potential for integrated downstream RNA, DNA and multimarker protein characterization. The ability to fabricate high-density pores enabled us to enhance the enrichment factor and recovery rate. In initial studies recovery rates were >90% for capturing cultured cancer cells spiked in peripheral blood. The filtration of a 7.5 ml blood sample required less than 3 min for processing. Possible downstream analyses include the possibility of molecular analysis of captured cells [68]. Recently, a report on a comparison of **isolation by size** of epithelial tumor cells (ISET) with CellSearch was published [78]. Again, discordant results were obtained by both methods, indicating the limitations of EpCAM based enrichment. More recently, the ScreenCell device (ScreenCell, Paris, France) has been developed, employing similar principles as the ISET platform, where CTC are enriched in a size-based fashion. Although commercially available, no significant clinical data has yet been reported using this technology. A major difference between the CTC filter developed by Richard Cote's group as compared with virtually all other sized based separation platforms is the density and regularity of the pores. Most filters are track-etched, and produced by ionizing radiation. This results in an irregular pore distribution, with a low density of pores, and significant overlap of pores resulting in holes large enough to allow CTC to pass through. In contrast, the CTC microfilter developed by Cote uses advanced lithography techniques, resulting in a highly regular and dense pore pattern, and the ability to precisely vary the size and shape of the pores. An alternative design of these filters allows for the capture of live cells [68]. Live cell capture may become

increasingly important, as understanding the functional status of CTC becomes critical in discovery and management of cancer.

1.6 ENRICHMENT OF PUTATIVE BREAST CSC

To enrich breast CSC, currently either flow cytometry sorting for defined breast cancer stem cell phenotypes ($CD44^+CD24^-lineage^-$ or $ALDH^+$) or enrichment by cell culture in non-adherent conditions is typically used. Defined phenotypes of breast CSC were associated with cancer stem cell properties, and it remains unknown what percentage of cells are breast CSC. Similarly, growth of cells in non-adherent conditions and formation of spheres is attributed to CSC. However, also for the spheroid cultures the real percentage of CSC is unknown. Research on biology of CSC for breast cancer and other cancers is restricted to these enrichment approaches, along with animal models to demonstrate tumorigenicity of certain cell type and to evaluate further biological properties.

2 HYPOTHESIS AND SPECIFIC AIMS

Breast cancer is still an incurable disease and this possibly at least to a part due to the presence of putative breast CSC that seem to play an important role in the local and distant progression of the disease. These cells are increasingly known as being resistant to conventional therapies. The overall hypothesis of this project was that definition of intratumoral heterogeneity and its association with putative breast CSC and dissemination may provide a powerful tool to assess the malignant potential of putative breast CSC. Enrichment and characterization of disseminating breast CSC may help to identify biomarkers of breast cancer disease and further identify potential therapeutic targets. The ability to address the role of CSC in heterogeneity and dissemination in clinical samples requires reliable technical approaches. In my thesis existing technologies have been validated in breast cancer cell lines, and heterogeneity of the breast CSC models evaluated in these cell lines. Further, multimarker immunofluorescence protocols for putative breast CSC markers were adapted and CTC microfilter optimized. These technologicl approaches were optimized to facilitate future studies in clinical samples.

3 METHODS AND RESULTS

3.1 CELL CULTURE AND SPHERE FORMATION

3.1.1 Cell lines

3.1.1.1 MCF7:

MCF7 cell line originates from the pleural effusion of a 69-years-old Caucasian woman with metastatic adenocarcinoma of the breast. This cell line has several characteristics of differentiated mammary epithelium, including the ability to process estradiol via cytoplasmic estrogen receptors. MCF7 cells were obtained from American Type Culture Collection ATCC (Manassas, VA, USA) and grown according to the instructions in DMEM (PAA Laboratories GmbH, NJ, USA) containing 10% fetal bovine serum (FBS) gold (Gibco, Invitrogen corp.NY, USA), 1% Sodium Pyruvate (PAA Laboratories GmbH) and 1% Penicillin-Streptomycin (Lonza, Basel, Switzerland).

3.1.1.2 SUM159:

SUM159 cells were purchased from Dr. Stephen Ethier from Asterand (Detroit, MI, USA). SUM159 were developed from primary, inflammatory invasive ductal breast cancer. Cells are estrogen and progesterone receptor negative. SUM159 cells were grown in Ham's F-12 medium (PAA Laboratories GmbH) containing 0.5% Insulin (Peprotech, NY, USA), 1% HEPES (PAA Laboratories GmbH), 0.1% Hydrocortison (Lonza) and 5% FBS (Gibco, Invitrogen). The culture media were changed three times a week.

3.1.1.3 SK-BR3:

These epithelial cells were also obtained from ATCC and grown in RPMI 1640 (Gibco) with 10% FBS and 1% Penicillin-Streptomycin. This cell line is also developed from pleural effusion of a metastatic breast adenocarcinoma.

3.1.1.4 MDA- MB 231

MDA-MB 231 cell lines were purchased from ATCC and cultured in MEM (PAA laboratories GmbH) containing 10% FBS and 1% Penicillin-Streptomycin. These epithelial cells are originated from pleural effusion of women with metastatic breast adenocarcinoma.

3.1.2 Cell Culture

All cell lines were cultured in a humidified incubator at 37°C and 5% CO_2. Cell lines were harvested at about 90% confluency. Thereafter, cells were washed with 2-4 ml phosphate buffer saline (PBS) (Gibco) depending on the size of the culture flask ($25cm^2$-$162cm^2$) (Corning, New York, USA) to remove serum containing trypsin inhibitor. Trypsin-EDTA (Gibco) was then added to the cells and incubated for 4 minutes at 37°C to detach them from the flask. The detachment was observed under the inverted microscope Olympus CKX41. The trypsin was inhibited with the growth medium and the cells aspirated. The cell suspension was transferred to a 50 ml centrifuge tube and centrifuged at 800 rpm for 4 minutes in Haereus Megafuge 1.0R. After centrifugation, the supernatant was discarded and the cell pellet resuspended in fresh growth medium. Appropriate aliquots of the cell suspension were added to new culture vessels.

For most of the experiments the two breast cancer cell lines MCF7 and SUM159 were used. Table 1 below describes the cell lines according to the recognized prognostic and predictive biomarkers of breast cancer.

Table 1: List of breast cancer cell lines used for experiments in this work and their molecular characterization according to the recognized biomarkers.

Breast cancer cell lines	Triple receptor status	ER	PR	Her2 neu
SUM159	Triple negative			
MDA-MB231	Triple negative			
SK-BR3	HR − Her2 neu +			
MCF7	HR + Her2 neu −			

Negative
intermediately positive
highly positive

(ER estrogen receptor, PR progesteron receptor, HR hormone receptors)

3.1.3 Mammospheres

The mammosphere cultures were generated either from viable single floating cells or after flow-cytometry sorting of the cells. Cells were collected from the supernatant of confluent MCF7 or SUM159 cells by centrifugation at 125g for 4 minutes. After washing with PBS cells were resuspended with 5ml medium. For cultivation of mammospheres serum-free Mammary Epithelial Basal Medium (MEBM; Lonza) was used with 10ng/ml fibroblast growth factor (FGF), 20ng/ml epithelial growth factor (EGF) (both from Peprotech, New York, USA) diluted in 0.1% Bovine Serum Albumin (BSA)-PBS, 20ng/ml B27 supplement and heparin (1000 IE/ml), (all obtained from Peprotech). The cultures were performed without antibiotics. The cell number was determined using the CASY cell counter system and $5x10^4$ cells were seeded in a $25cm^2$ flask. For the cultivation of floating cells ultra low attachment culture flasks (Corning) were used. The mammospheres were cultured for one week/ten days at 37°C and 5% CO_2 before splitting or medium change. For these purposes the medium was transferred into a 50ml centrifugal tube and centrifuged as mentioned above to collect the cells. After the first passage the spheroids were filtered on a 40 µm cell strainer (BD Bioscience, Scwechat, Austria) and singularized with TrypLE (Gibco). TrypLE was added to the pellet and incubated in a water bath at 37°C for 4 minutes. Then the cells were resuspended in the double volume of PBS and centrifuged to inactivate the enzyme. Finally the cellss were dissolved in MEBM plus supplements and seeded, separated into two flasks for expansion or transferred into a $75cm^2$ low attachement culture flask to form spheres. For the mammosphere culture of pleural effusion protocol was slightly adapted and is described below.

3.1.4 Results

After testing different approaches for all the experiments, mammospheres were generated from floating cells. On average, SUM159 would form spheres within 5 days, and MCF7 after 10 days. After the first passage, growing spheres were filtered with the 40µm cell strainer (BD) and dissociated as described above. These secondary spheres were evaluated presently. Standardized dilution assay to determine the sphere formation efficacy was not performed. What could be observed in the way the culture was performed was, that

SUM159 not only needed half of the time to form spheres, but also the number of spheres generated from the same number of cells was higher. With respect to the mammosphere forming capacity, there was no significant difference if floating cells or cells in the putative stem cell sorted fraction were cultured in non-adherent conditions. Thus, for most of experiments with mammospheres generated from cell lines floating cells were used.

As stated, MCF7 and SUM159 were both able to generate mammospheres in non-adherent conditions. For generation of mammospheres in first passage, MCF7 remained in non-adherent conditions for 10 days. After filtration and dissociation of spheres, another 10 days were needed for generation of secondary spheres. SUM159 cells were able to build spheres after 5 days. In analogy to the MCF7 mammosphere culture, after filtration and dissociation secondary spheres were generated. Also for secondary SUM159 mammospheres 5 days were needed. Parental cultures and corresponding representative spheres are depicted in figure 4. As indicated in the figure and suggested previously, SUM159 were capable of forming significantly larger spheres within half of the time. Single spheres and pools of spheres were then manually picked and subjected to further profiling analyses. The cell culture was repeatedly reproducible.

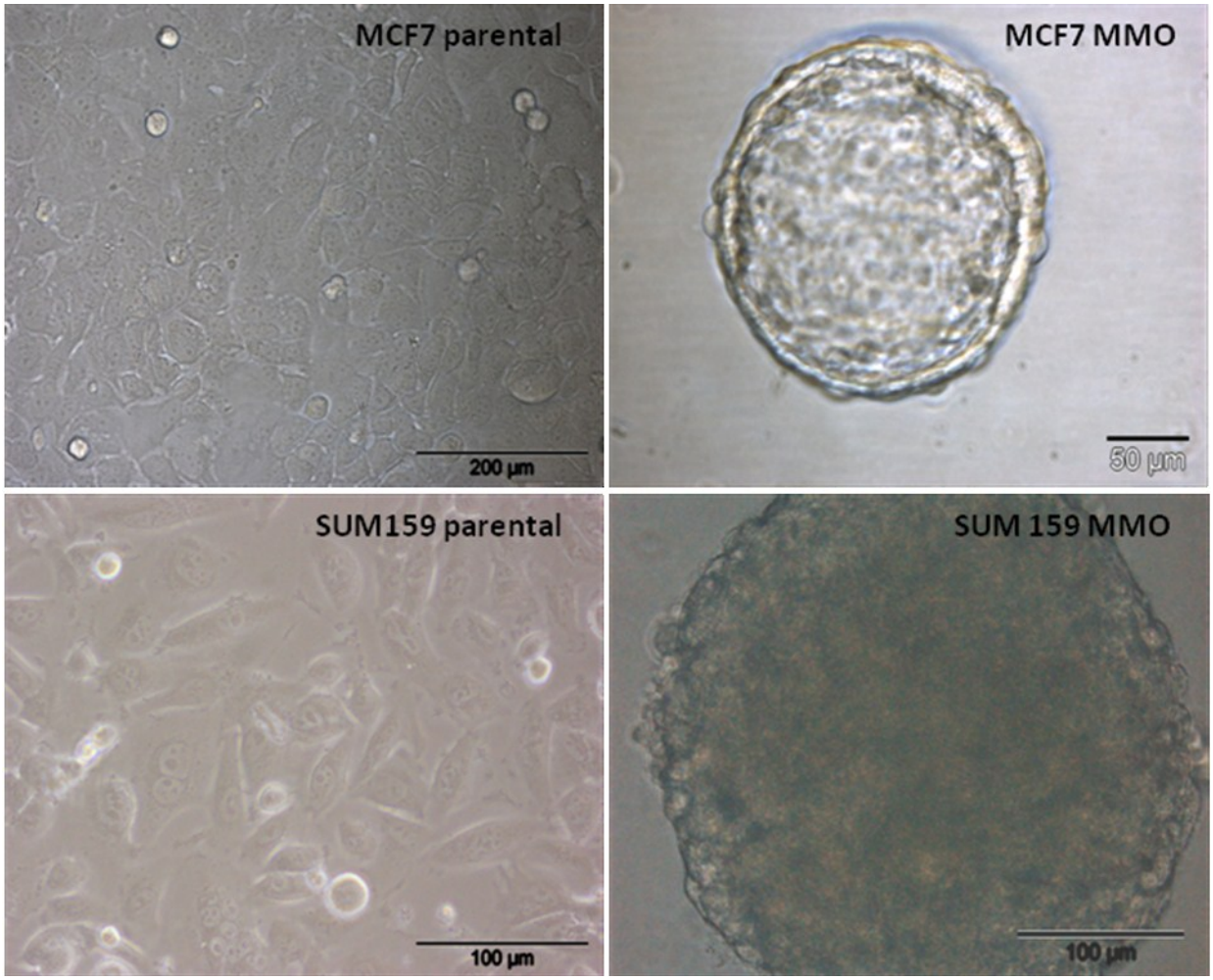

Figure 4: Representative images from parental MCF7 and SUM159 cells and corresponding mammospheres (MMO). MCF7 and SUM159 breast cancer cells were grown in standard medium as recommended by the supplier. For the sphere formation assay, viable floating single cells were harvested from the supernatant of confluent breast cancer cell lines and seeded in ultra-low attachment flasks.

3.2 FLOW CYTOMETRY SORTING

3.2.1 Methods

Antibodies used to sort cultured breast cancer cells from cell lines were anti-CD44 APC and anti-CD24PE (BD Bioscience). The protocol was adapted from Al-Hajj et al [39]. Further, the Aldefluor assay Kit (STEMCELL Technologies, Grenoble, France) was used for the detection of ALDH. This protocol was adapted from Ginestier et al [26] and performed as previously published [81].

Cells without staining were integrated as a negative control in all experiments. Isotype IgG_1, purchased from BD Bioscience was included as a negative control. All antibodies were pretitered on breast cancer cell lines to determine their optimal dilutions before use. Dead cells were eliminated by using the viability dye 7 Actinomycin D (7-AAD, Invitrogen, Leek, the Netherlands).

3.2.1.1 Staining of the cells

For all sorting experiments cells were detached with Accutase (PAA Laboratories GmbH) for 5 min at 37°C.

When using the Aldefluor protocol dissociated cells were suspended in Aldefluor assay buffer to a concentration of 10^6cells/ml. When using anti-CD44 and anti-CD24, cells were suspended in PBS/2% fetal calf serum (FCS) to a concentration of 10^6 per 100 µl.

In preparation for flow cytometry sorting, staining of the cells was performed in following different ways:

(1) After 20 min incubation on ice with blocking buffer (consisting of horse serum diluted 1:20 in 6% bovine serum albumin/ PBS solution), aliquots of antibodies (CD44 APC and CD24 FITC), each at a dilution of 1:40 in a final concentration of 0.08 µg/ml and 5 µg/ml, respectively, were added and staining performed as previously published [39]. Briefly, after centrifugation the pellet was dissolved in 100 µl PBS/ FCS and 2 µl of the antibodies (CD24-PE, CD44-APC or ESA-FITC) were mixed with the cells which where incubated for 20 minutes on ice. For triple staining the antibodies were diluted and 2,5 µl of each antibody was added to the cells. Then the cells were washed with 500 µl PBS/ FCS and centrifuged

again. Before sorting, the cells were resuspended in 100 µl PBS/ FCS, filtered and rinsed with 100 µl PBS/ FCS.

(2) To measure and isolate cells with high ALDH activity, the Aldefluor assay was performed according to the manufacture's guidelines. To activate the Aldefluor reagent, 25µl DMSO was added to the vial with the dry substrate, mixed and let to stand for 1 minute. After adding 25µl 2N HCl it was mixed and incubated for 15 minutes at room temperature. Afterwards, 360µl of the Assay Buffer was added.

3.2.1.2 Flow cytometry sorting and analysis

The cells were first washed with Aldefluor Assay Buffer, detached with Accutase and resuspended in Assay Buffer again. Then the cells were centrifuged at 800rpm for 4 minutes. The cell pellet was then resuspended in 4 ml Assay Buffer. Per $1x10^6$ cells one ml of Assay Buffer was used.

For each sample to be tested one "test" and one "control" tube was prepared. 1 ml of the cell suspension was added to the test tube. 5 µl of the DEAB solution was added to the control tube and closed to prevent evaporation of the solution containing 95% ethanol. 5µl of the thawed activated Aldefluor substrate per ml sample was added to the sample. After adding 500µl of the mixture the enzymatic reaction starts immediately. Both tubes were incubated in a water bath at 37°C for 45 minutes.

Finally, cells were suspended in different buffer systems and Flow cytometry sorting performed on the fluorescence activation cell sorter (FACS) Aria (Beckton Dickinson, San Jose, CA). Live cells were investigated on the basis of forward and side scatter and gates were determined by analyzing unstained cells, isotype stains and single stains. Side scatter and forward scatter profiles were used to eliminate cell doublets. The isolated cell populations were not assessed for purity due to the low number of cells expected. The acquired data were analyzed using the Diva software (BD Bioscience).

3.2.2 Results

3.2.2.1 Phenotypic charachterization by flow- cytometry analyses for CD44 and CD24

First, cell lines were characterized according to the putative breast cancer stem cell phenotype determined by CD44 and CD24. In the figure 5 representative data are shown for SUM159 and MCF7. Flow cytometry figure shows the percentage of cells enriched in the $CD44^{+}CD24^{-}$ fraction, and bars demonstrate the percentages next to each other. It can be clearly seen that SUM159 had a high population of $CD44^{+}CD24^{-}$ cells, whereas in MCF7 cells, this population represented <2%. These experiments were repeated at least three times, and the results were comparable.

3.2.2.2 Assessment of Aldehydedehydrogenase positivity

Further, the $ALDH^{+}$ population of the cells was determined. The Aldefluor assay was performed to evaluate ALDH positivity of the cells. Representative data of the Aldefluor assay on SUM159 and MCF7 cells are shown in figure 6. The results are demonstrated in the same manner as for CD44 and CD24. The Aldeflluor assay was performed three times for SUM 159 and MCF7 cells, respectively.

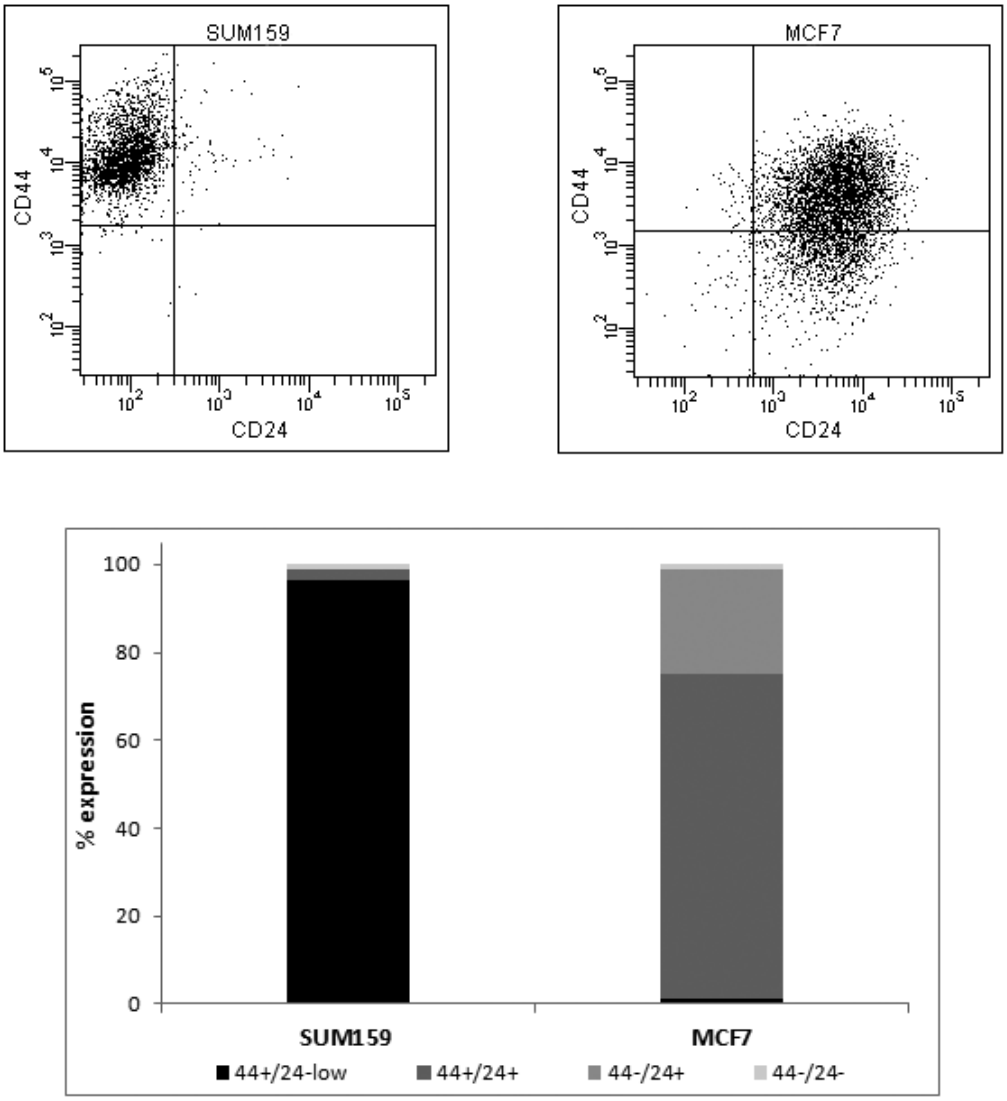

Figure 5: Estimation of the CD44^{+}CD24^{-} CSC phenotype in breast cancer cell lines: the flow cytometry figure shows percentage of cells enriched in the CD44^{+}CD24^{-} fraction, and bars demonstrate the percentages next to each other.

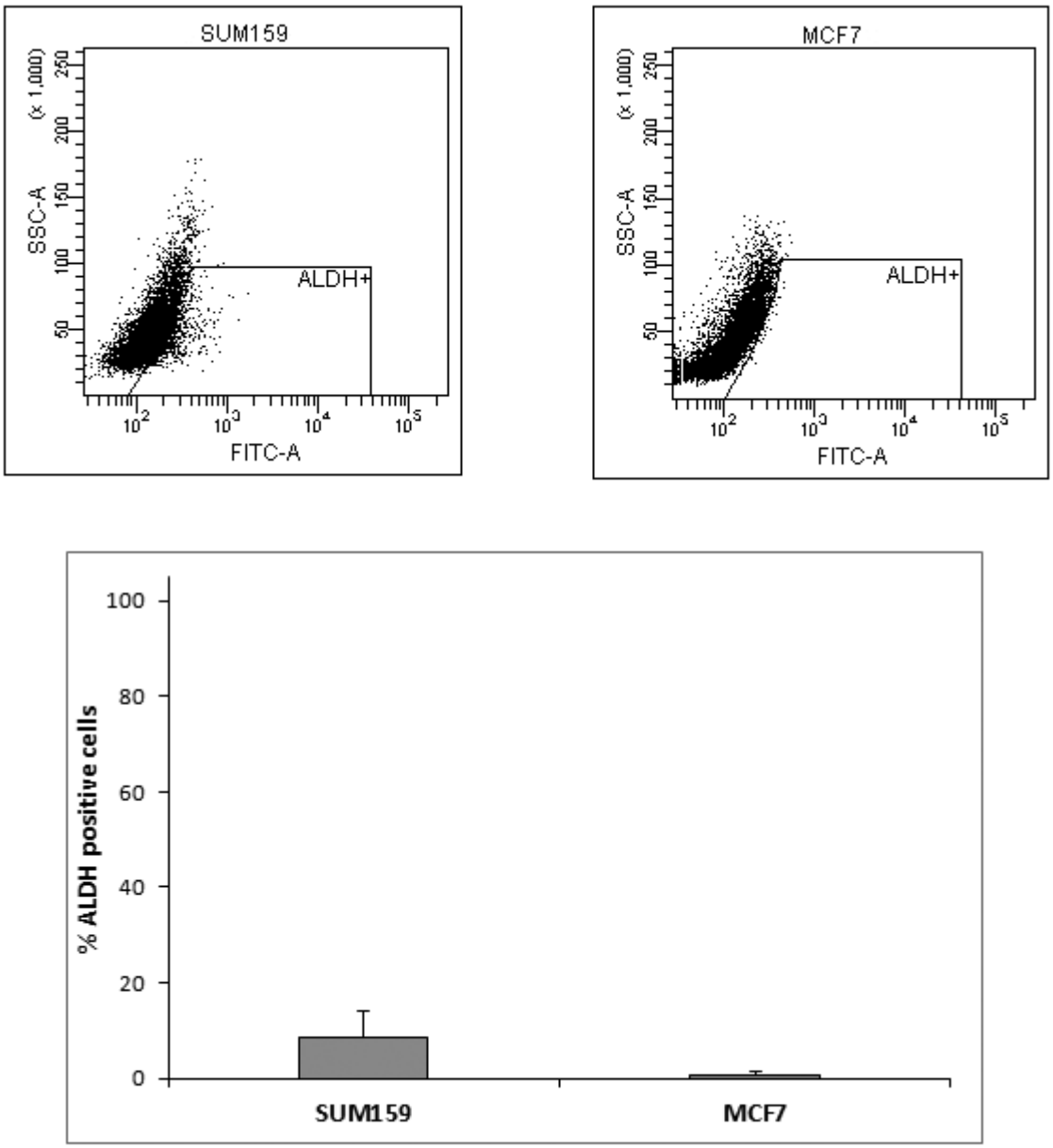

Figure 6: ALDH$^+$ positivity in breast cancer cell lines: the flow cytometry figure shows percentage of cells enriched in the ALDH$^+$ fraction, and bars demonstrate the percentages next to each other.

Table 2 is a list of flow cytometry sorting results in all analyzed cell lines

Table 2: List of cell lines and the percentage of putative breast cancer stem cell phentoypes

Cell line	$CD44^+CD24^-$	$ALDH^+$	Classification
MCF7	1.1±0.21	1,9±1.30	luminal
SUM159	94.3±6.69	8.8±4.89	basal
SKBR3	n.d.	13.3±10.68	luminal
MDA 231	81.8±6.30	n.d.	basal

3.3 EVALUATION OF GENOMIC PROFILES

3.3.1 Methods

3.3.1.1 DNA Extraction

Genomic DNA from cultured cells was extracted using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA was dissolved in a final volume of 100 µL buffer and quantified spectrophotometrically using a BioPhotometer (Eppendorf, Hamburg, Germany).

3.3.1.2 Evaluation of genomic profiles

Evaluation of genomic profiles was performed in collaboration with the Institute of Human Genetics. Whole genome amplification (WGA), and array comparative genomic hybridization (CGH) analyses were performed in collaboration with Martina Auer, Dr. Elen Heitzer and Ao. Prof. Jochen Geigl as described below.

3.3.1.3 Whole Genome Amplification (WGA)

From SUM159 and MCF7 pool of mammospheres, and ten single mammospheres were collected from the same passage, respectively and analyzed in comparison with genomic DNA. These experiments were repeated, and were reproducible.

DNA extracted from different cell populations from the sorted SUM159 cell line ($ALDH^+$, $ALDH^-$, $CD44^+CD24^-$, $CD44^-CD24^+$) and manually picked cells from mammosphere cultures of SUM159 and MCF7 cells were amplified using the WGA-4 single cell amplification kit (Sigma-Aldrich, St.Louis, MO, USA) following the instructions of the manufacturer. To prevent any loss of genomic material, cells were sorted/ picked directly into tubes where cell lysis and WGA were performed.

Briefly, the volume of the sorted and manually picked samples was adjusted with water to 9µl. After addition of lysis/ fragmentation buffer and proteinase K the suspensions were incubated at 50°C for one hour and subsequently heated to 99°C for four minutes. Next the Omniplex libraries were prepared by ligation of universal priming sites. Therefore, the appropriate buffers were added and the samples were denatured at 95°C for two minutes. The enzyme mix was added and the samples were incubated as follows: 16°C for 20 minutes, 24°C for 20 minutes, 37°C for 20 minutes, 75°C for 5 minutes, 4°C hold. These products were used as templates in the WGA reaction. PCR reaction was set up and amplification was performed in a thermal cycler (95°C for 3min, 25 cycles of 94°C for 30 seconds and 65°C for 5 minutes, hold 4°C) by adding 7.5 µL of 10 × Amplification Master Mix, 48.5 µL of nuclease-free water and 5 µL WGA DNA polymerase. Amplified samples were purified using GenElute PCR Clean-up Kit (Sigma-Aldrich) and quantified by measuring absorbance on a NanoDropTM Spectrophotometer (Thermo Scientific). For quality control a multiplex PCR was performed using 1 µl of amplified DNA as a template, as previousl published [79]. Multiplex PCR reaction makes use of four primer sets producing products with 100, 200, 300 and 400 bp fragments from nonoverlapping sites in the GAPDH gene. The final volume was 30 µl. Following eight primers were used: 100F gttccaatatgattccaccc; 100R ctcctggaagatggtgatgg; 200F aggtggagcgaggctagc; 200R ttttgcggtggaaatgtcct; 300F aggtgagacattcttgctgg; 300R tccactaaccagtcagcgtc; 400F acagtccatgccatcactgc and 400R

gcttgacaaagtggtcgttg. PCR reaction was carried out with 10mM Tris-HCl pH 8,8, 1.5 mM MgCl, 75 mM KCl, 0.2 mM dNTPs, 1U Taq DNA polymerase for 4 min at 94°C, 35 cycles each of 1 min 94°C, 1 min 56°C and 3 min 72°C, followed by 7 min 72°C and ending at 15°C. After addition of 6 μl loading dye, 10 μl of each sample was analysed on a 1.5% TBE agarose ethidium bromide-stained gel. Samples with significant lower concentrations after WGA than the expected average concentration of 250ng/µl, or samples that showed only one band in van Beers PCR were excluded from further analyses.

3.3.1.4 Array Comparative Genomic Hybridization (CGH)

Array CGH was carried out using a genome-wide oligonucleotide microarray platform (Human genome CGH 60K microarray kit, Agilent Techologies, Santa Clara, CA, USA), following the instructions of the manufacturer (protocol version 6.0). Commercially available male reference DNA (Promega, Madison, WI, USA) was used.

DNA from SUM159 and MCF7, whole genome amplified DNA of cultured mammospheres of both cell lines and whole genome amplified DNA of sorted SUM159 was digested with resitriction endonucleases arthrobacter luteus I (ALUI) and RSAI (Promega) at 37°C for 120 minutes, followed by an enzyme inactivating step at 65°C for 20 minutes. A smear between 2000 and 100 bp on a 1% agaorse gel indicated successful digestion.

Samples were then labeled with the Bioprime array CGH genomic labeling system (Invitrogen) according to the manufacturer's instructions. In brief, 500 ng test DNA and reference DNA were differentially labeled with dCTP-Cy5 or dCTP-Cy3 (GE Healthcare, Milwaukee, WI, USA). Unincorporated nucleotides were removed using a QIAquick PCR purification kit (Qiagen). Before hybridization the probes were measured by NanoDrop 1000 (Thermo Scientific) by determining the absorbance at 260 nm (DNA), 550 nm (cyanine 3), and 650 nm (cyanine 5), and were denatured by incubation at 95 °C for 2 min followed by cooling to room temperature. Array hybridization was carried out at 65 °C with about 200 ng probes/array in Agilent HI-RPM hybridization buffer. Slides were scanned using a microarray scanner (Agilent Technologies, Santa Clara, CA, USA), and images were preprocessed using Feature Extraction and DNA Workbench 5.0.14 (Agilent Technologies). In addition, data normalization and calculation of ratio values was performed using the Fea-

ture Extraction software 9.1 from Agilent Technologies. Evaluation of data was done based on the previously published algorithm in R (www.r-project.org) [80]. The algorithm calculates ratio values with different window sizes that differ significantly from the ratio profile's mean and are therefore considered as over- or under-represented graphically indicated in a single green or red bar for gained or lost regions, respectively. Furthermore, the algorithm generates a table with all localizations of significant calls which allows detailed mapping of each copy number variation (CNV).

3.3.2 Results

3.3.2.1 Profiles of adherent parental cell lines and putative stem cells grown in mammospheres

To evaluate whether copy number status is changing during mammosphere formation array CGH on 10 manually picked spheres from sphere culture of SUM159 and MCF7 were evaluated, respectively. These results were compared to genomic DNA, and to a pool of spheres.

From both SUM159 and MCF7 two spheres were excluded from further analyses due to low DNA concentrations after WGA or insufficient amplification in the multiplex PCR. Figure 7 below demonstrates multiplex PCR from DNA extracted from MCF7 single spheres (1-10), with the highest quality of DNA samples where all four products are shown in the gel. In figure 8 corresponding data is shown for SUM159.

Mean DNA concentrations after WGA were 249.7ng/µl and 216.8ng/µl for SUM159 and MCF7, respectively. For MCF7 no significant copy number differences between genomic DNA of the parental cell line and the corresponding mammospheres could be observed. All samples showed the same genomic aberrations including high-level gains at 8q, 15q, 17q and 20q, and losses at 1p, 8p, 11q, 11q, 13q, 18q and 22q. In addition, most regions of chromosome 4 were underrepresented. These changes are in line with previously published copy number profiles for MCF7 [17, 81]. Figure 9 depicts the described profiles.

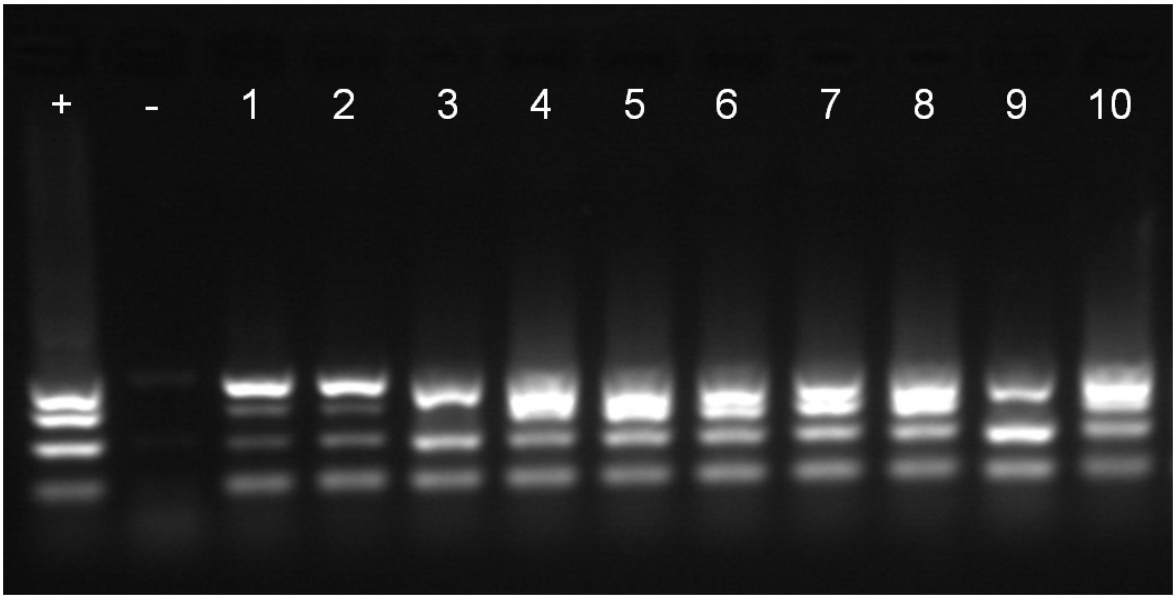

Figure 7: Multiplex PCR from DNA extracted and amplified from ten single MCF7 mammospheres.

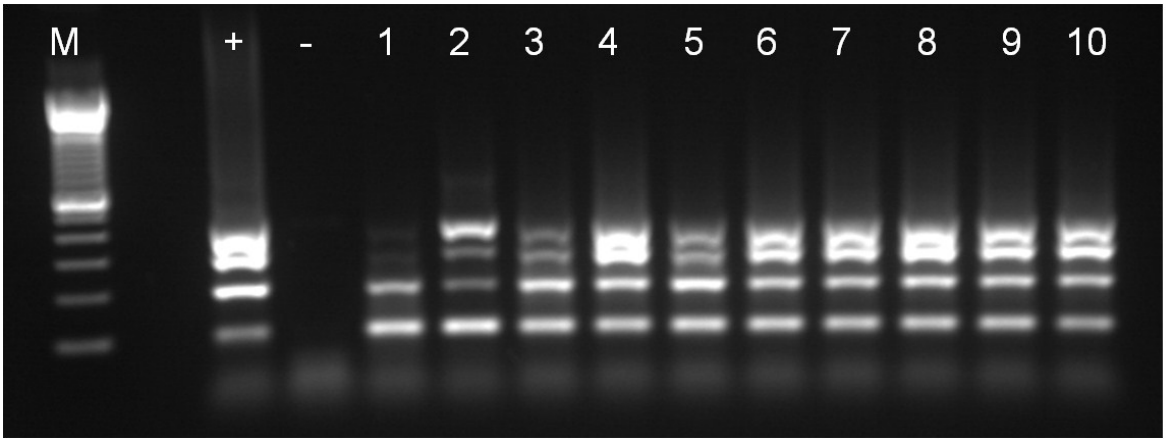

Figure 8: Multiplex PCR from DNA extacted and amplified from ten single SUM159 mammospheres

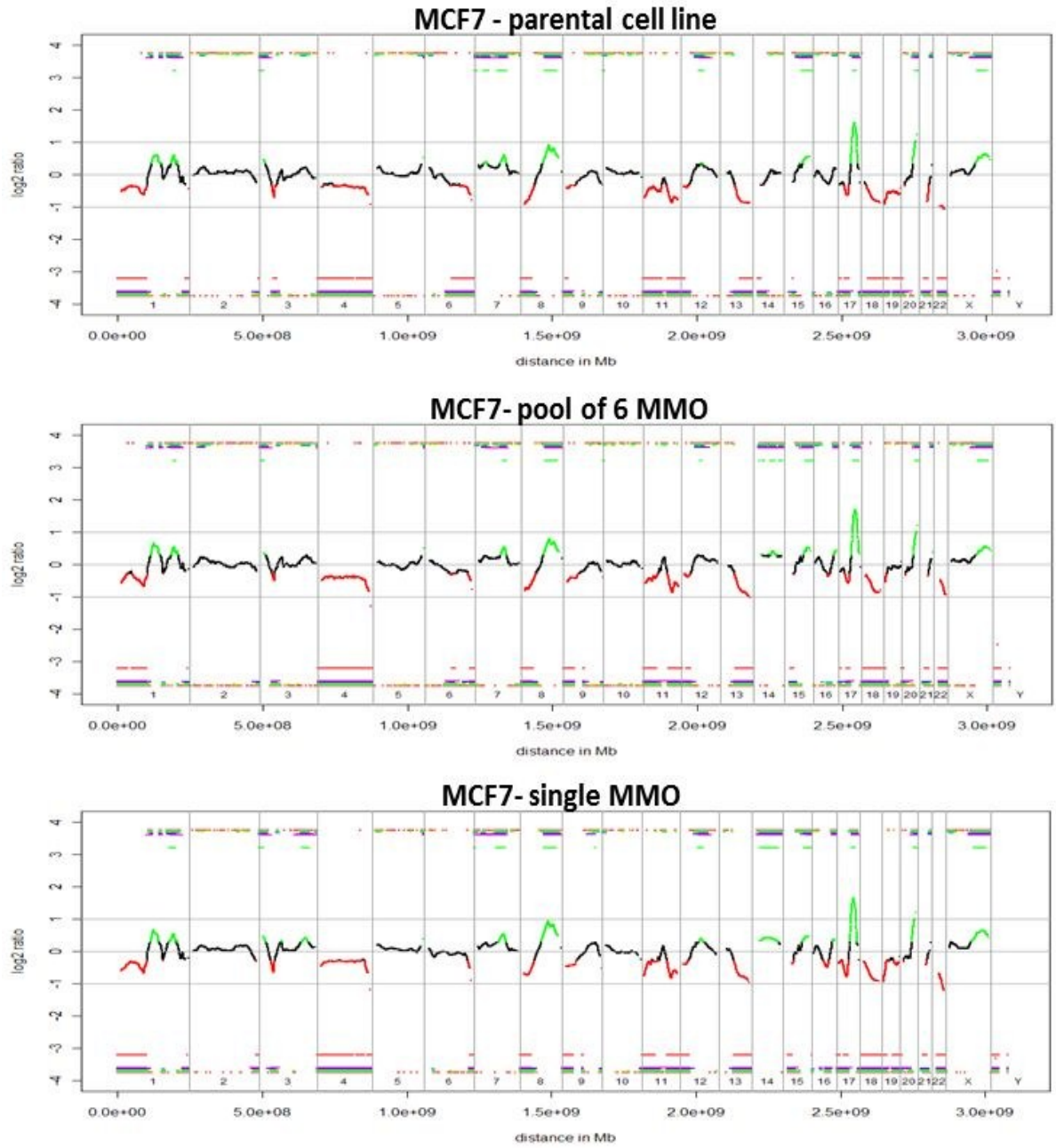

Figure 9: Array CGH profiles obtained by analyses of MCF7 genomic DNA (upper figure), pool of mammospheres (middle figure) and a single mammosphere (lower figure).

MMO- mammosphere

For SUM159 similar results were obtained. Multiplex PCR products from DNA extracted from SUM159 single spheres (1-10) are shown in the figure 10.

High-level amplifications of 3q and 5p, losses at 17p and 21q, and a complete loss of chromosomes 4, 19 and 22 in all samples, the parental cell lines and in all single mammospheres were observed.

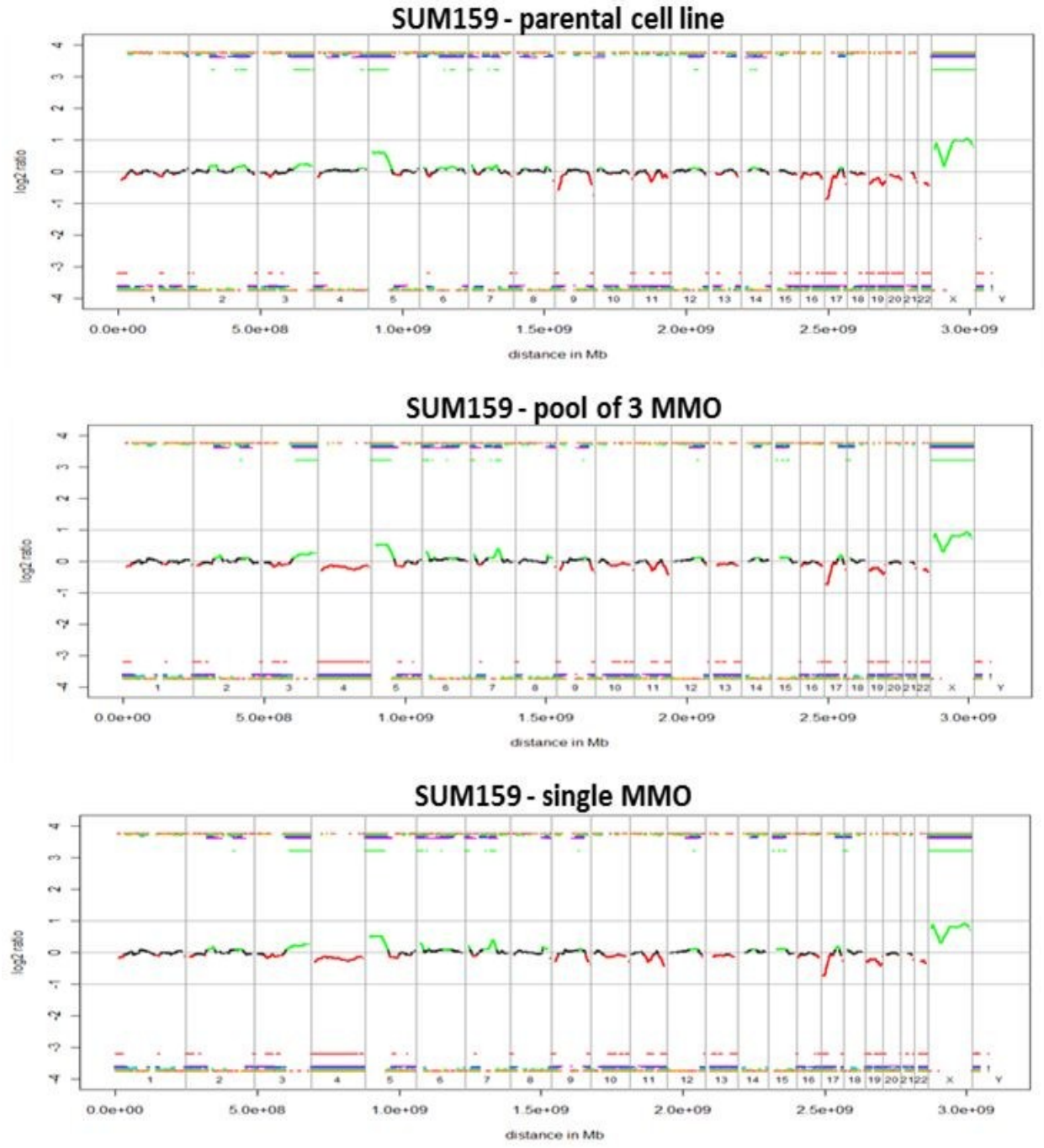

Figure 10: Array CGH profiles obtained by analyses of SUM159 genomic DNA (upper figure), pool of mammospheres (middle figure) and a single mammosphere (lower figure)

MMO- mammosphere

3.3.2.2 *Profiles of putative breast cancer stem cell subpopulations*

Since mammospheres displayed no significant copy number difference compared to the parental cell lines, further evaluation was performed to determine whether different cell subpopulations of SUM159 exhibit distinct differences in their genomic aberration profiles. Therefore, four isolated cell populations after sorting ($ALDH^+$, $ALDH^-$, $CD44^+CD24^-$, $CD44^-CD24^+$) were subjected to WGA followed by copy number analysis using array CGH. However, even after separation of cell populations no differences in the copy number profile were detectable ($ALDH^+$ and $ALDH^-$ cells are shown in figure 11 and $CD44^+CD24^-$ cells vs. $CD44^-CD24^+$ in figure 12).

Minor differences in CNVs that mainly include single, non-adjacent oligonucleotides most likely represent artifacts introduced during the amplification process. Nevertheless, amplification artifacts with the single cell amplification, which may result in under- (e.g. allele drop out) or over-representations (e.g. preferential amplifications) are proven to be rare according to the previously reported protocol [80].

Finally, the profiles are summarized in the heat maps. In figure 13 the heat maps of aray CGH-profiles from SUM159 parental cell line compared to sorted subpopulations and to single mammospheres are depicted, and in figure 14 heat maps of analzed MCF7 profiles are shown.

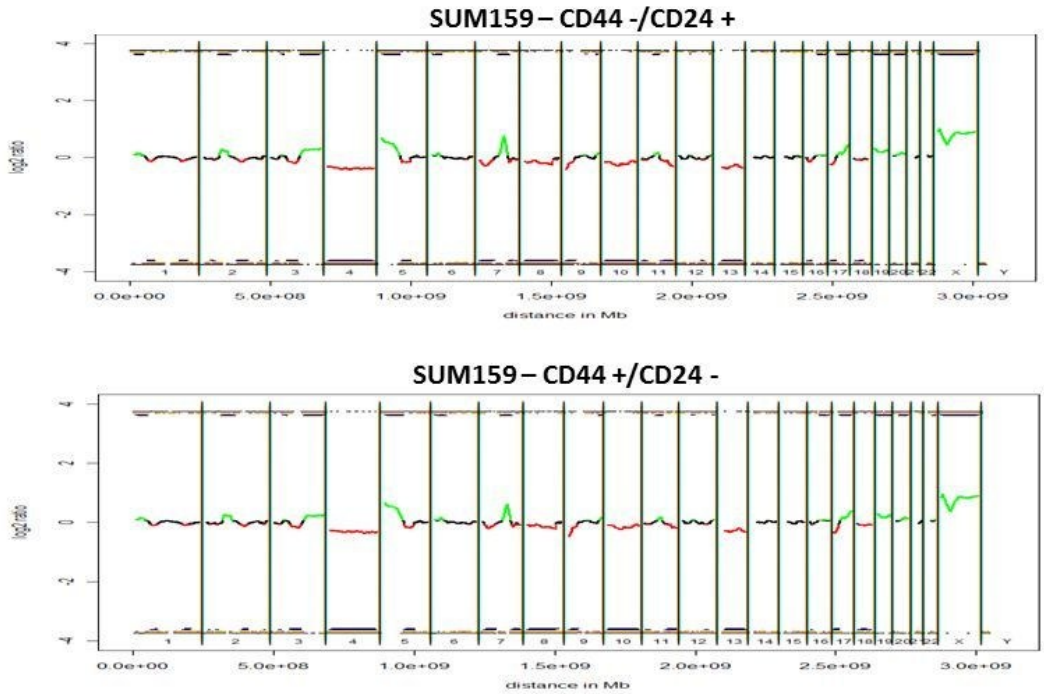

Figure 11: Array CGH profiles obtained by analyses of SUM159 $CD44^{+}CD24^{-}$ population of cells (putative breast cancer stem cell phenotype, upper figure) and representative population of other cell profiles (lower figure);

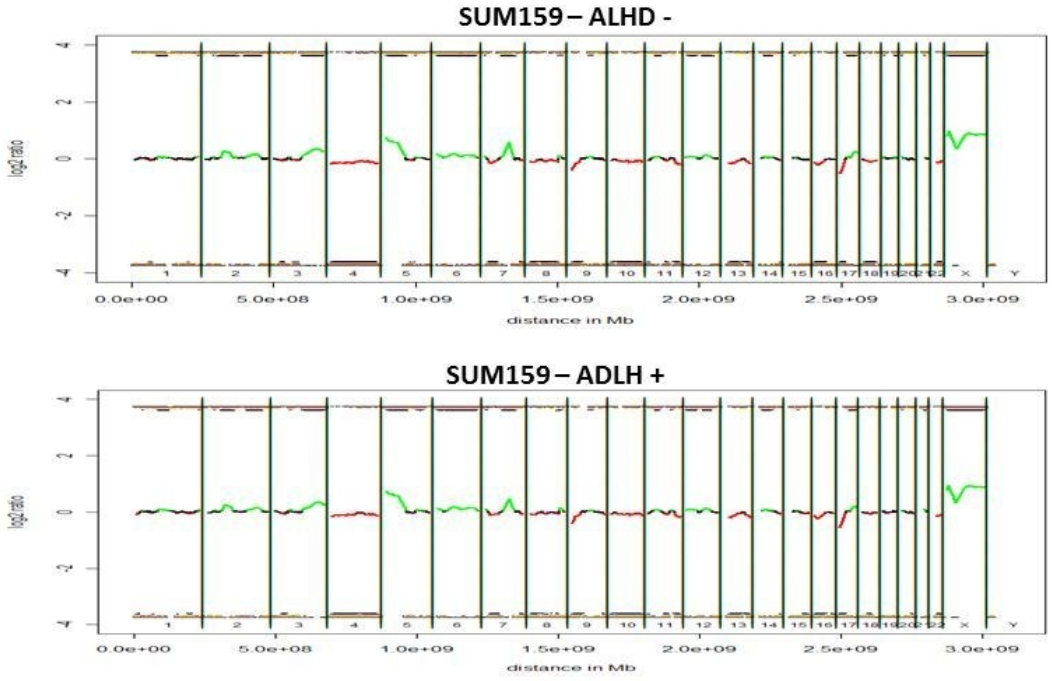

Figure 12: Array CGH profiles obtained by analyses of SUM159 $ALDH^{+}$ population of cells (putative breast cancer stem cell population, upper figure) and $ALDH^{-}$ population of cells (lower figure)

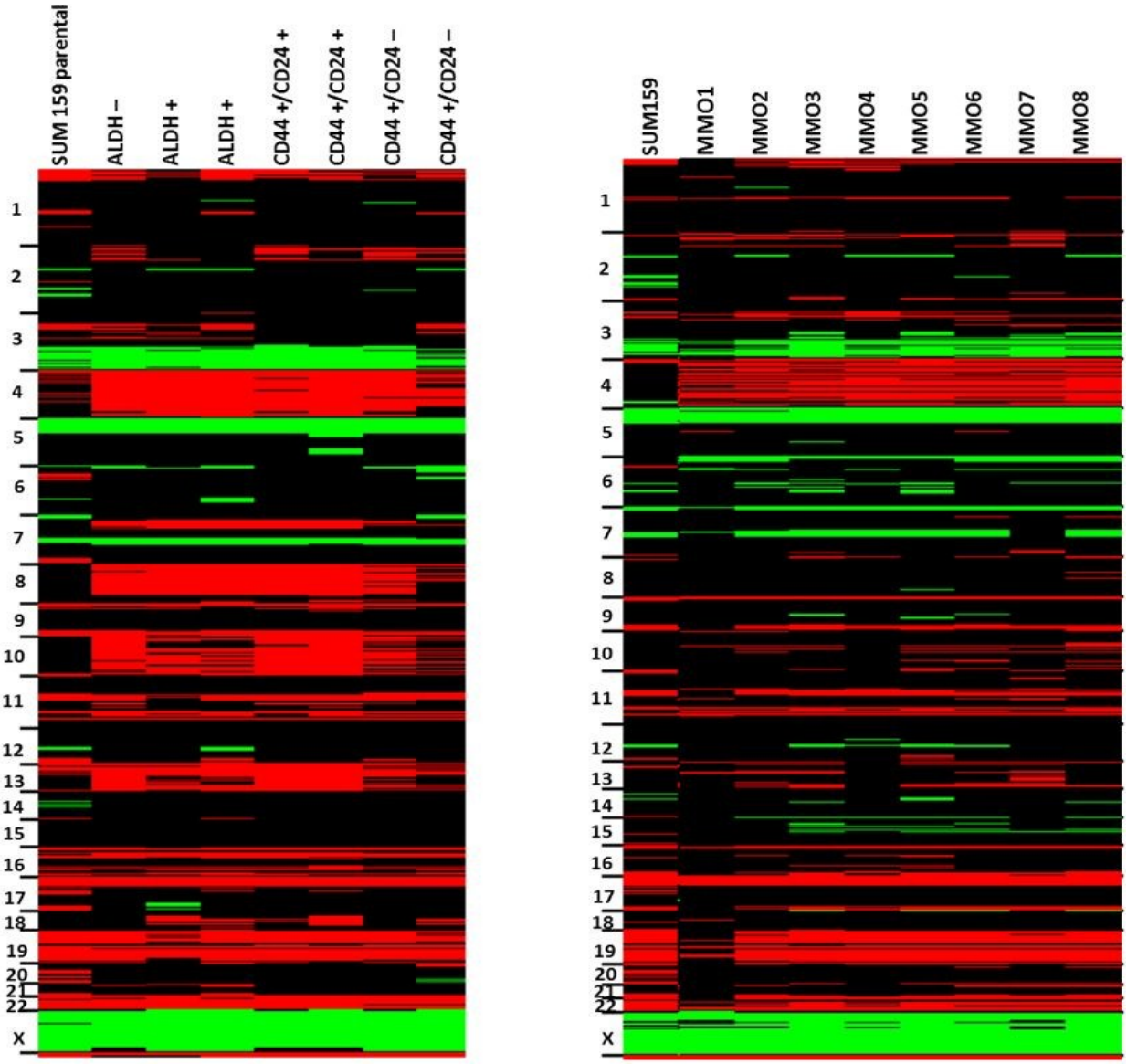

Figure 13: Heat maps of CGH-profiles from SUM159 parental cell line compared to sorted subpopulations and to single mammosphere. Male reference DNA was used in all experiments and therefore female plasma DNA samples have a relative over-representation of the X chromosome and an under- representation of the Y-chromosome (black: balanced; red: under-represented; green: over-represented)

MMO- mammosphere

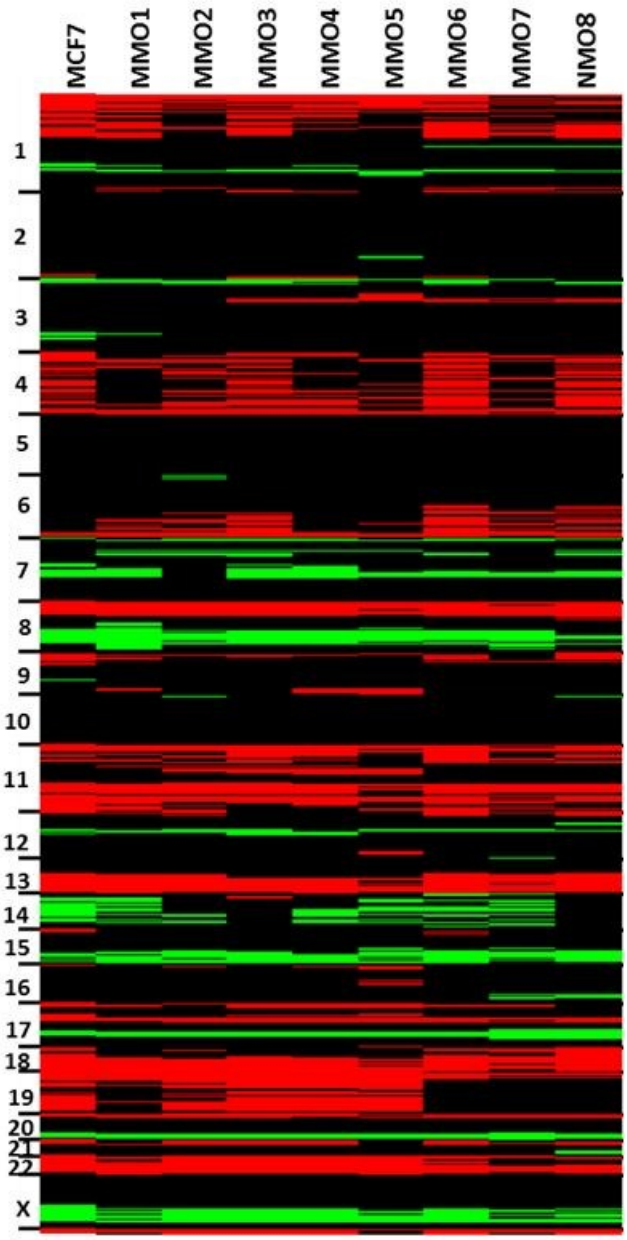

Figure 14: Heat maps of CGH-profiles from MCF7parental cell line compared to single mammosphere. Male reference DNA was again used in all experiment, and thus, female plasma DNA samples have a relative over-representation of the X chromosome and an under- representation of the Y-chromosome (black: balanced; red: under-represented; green: over-represented)

MMO- mammosphere

3.4 EVALUATION OF PLEURAL EFFUSIONS

Pleural effusions were obtained from metastatic breast cancer patients where pleural punction obtained by thoracentesis was performed as a treatment intervention in patients with dyspnea caused by malignant pleural effusion. After preparing samples for diagnostic procedure the remaining fluid is typically disposed. Breast cancer is the second most common cause of malignant pleural effusions [82] . Malignant effusions including pleural effusions may be regarded as a representative source of dissociated disseminating cells. Remarkably, most of the breast cancer cell lines were generated from malignant pleural effusions.

The analysis of pleural effusions of breast cancer patients was approved by the local ethics committee of the Medical University of Graz. After obtaining written informed consent from patients and the treatment procedure pleural fluid was collected and further processed in the research laboratory of Division of Oncology at the Department of Internal Medicine. Patients included in the study were seen at the outpatient care unit at the Department of Pneumonology, where paracentesis was performed and peural effusions collected. At present, a total of 14 pleural effusions from metastatic breast cancer patients were processed.

After pleural effusions were obtained, samples were picked up in the outpatient care unit at the Department of Pneumonology and processing started shortly after. The fluid was transferred into autoclaved falcon tubes (BD Biosciences), and centrifuged at 1300 rpm for 10 minutes at room temperature. After removing supernatant, cells were pooled (depending on the total cell count). Red blood cell lyses buffer was added and cells incubated in the centrifuge for 5 minutes and thereafter centrifuged at 1300 rpm for another 10 minutes. Supernatant was removed, and if cell pellet was still bloody, the lysis was repeated in the same manner. Thereafter, 70um filtration was performed, the filter with captured large cells removed, and the flow- through further processed. After another centrifugation step supernatant was removed, and cell resuspended in PBS with 2% Penicillin-Streptomycin (Lonza). For the cell counting a 1:2 dilution with trypan blue was prepared and cells were incubated for 5 minutes. 20µl of cells were pipetted into the Fuch-Rosenthal chamber and counted. The percentage of viable cells and the total cell count

were determined. Thereafter, cells were seeded in culture flasks ($6x10^6$ cells/ 162 cm^2 flask or $2x10^7$ per 500 cm^2 flask). The added medium contained Dulbecco's Modified Eagle's Medium (DMEM), low glucose + 10% FCS and 2% Penicillin-Streptomycin solution.

3.4.1 GenomiPhi whole genome amplification

GenomiPhi (GE Health Care, Biociences, Pittsburg, PA; USA) is a highly efficient and representative whole-genome amplification kit. It is a kit enabling for multiple displacement amplification for single and or few cells. For array CGH profiling, DNA extracted from unsorted cells, and $ALDH^+$ and $ALDH^-$ fraction was WGA amplified by GenomiPhi. After amplification, on average 40 ng/µl DNA was obtained.

3.4.2 Putative breast cancer stem cell subpopulations and mammospheres

14 pleural effusions were collected and analysed so far. In table 3 the number of cells from aspirates is listed and also the percentages of the putative stem cell fraction according to the phenotype $CD44^+CD24^-$ and $ALDH^+$ cells are demonstrated. The average percentage of the $CD44^+CD24^{-/low}$ phenotype was 7.7% and of ALDH+ cells 6.6%. Further, in the column spheres it is indicated with a. (available) when spheres from the according fraction were generated. Representative spheres generated from pleural effusions are depicted below (figure 15). N.a. indicates that no spheres could be generated.

At present, no trend towards one of the sorted fractions regarding the mammosphere forming capacity could be identified. For such analyses, the number of patients is too small. Also, the main focus of my thesis was to evaluate if such approach was feasible.

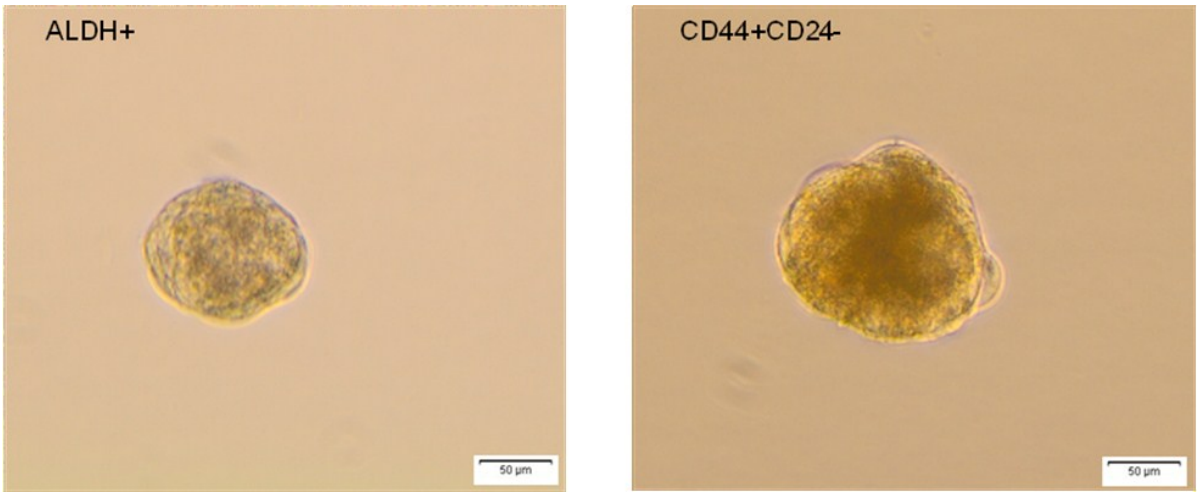

Figure 15: representative mammospheres generated from ALDH^{+} population and CD44^{+}CD24^{-} $^{-}$population of cells sorted from pleural effusions of breast cancer patients. The mammosphere depicted on the right represents a mammosphere from CD44^{+}CD24^{-} fraction and the mammosphere depicted on the left represents a sphere from ALDH^{+} fraction from the patient number 11.

Table 3: Characteristics of analysed pleural effusions

Patient number	Total cell number	$CD44^+CD24^-$ (%)	Spheres	$ALDH^+$ (%)	Spheres
1	$4x10^8$	-	n.a.	-	n.a.
2	$8x10^6$	8,00	n.a.	1,00	n.a.
3	$4,3x10^6/5,2x10^8$	12,00	n.a.	1,00	n.a.
4	$2,4x10^8$	14,00	n.a.	4,00	n.a.
5	$4,3x10^8$	15,00	a.	13,00	a.
6	$7,1x10^6$	-	n.a.	1,00	n.a.
7	$3,2x10^8$	3,00	n.a.	3,00	n.a.
8	$5,5x10^8/2,6x10^8$	9,00	a.	12,00	a.
9	$9,01x10^8$	2,00	n.a.	8,00	n.a.
10	$1,1x10^8$	4,00	n.a.	9,00	n.a.
11	$2,3x10^9$	6,00	a.	6,00	a.
12	$3x10^9$	2,00	n.a.	2,00	n.a.
13	$4,7x10^7$	-	n.a.	12,00	n.a.
14	$1x10^8$	10,00	n.a.	14,00	n.a.

3.4.3 Genomic profiles of pleural effusions

So far the first pleural effusion was analyzed and sorted fractions according to $ALDH^+$ and $ALDH^-$ fraction were compated. The amount of DNA after extraction from sorted fractions

was low, therefore DNA whole genome amplification had to be performed. Results are demonstrated in the figure 16.

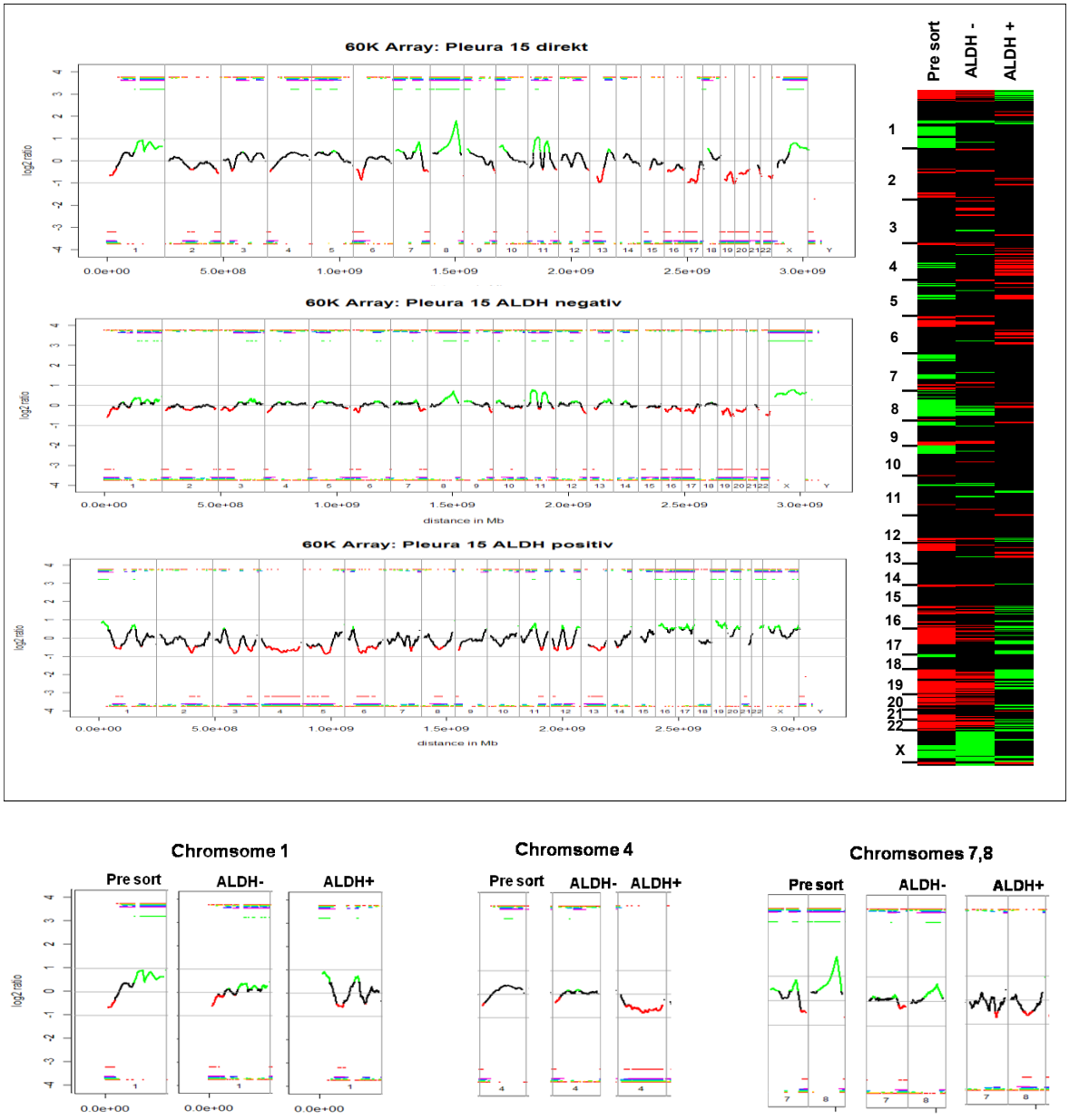

Figure 16: 60k array performed from one pleural effusion. Comparison of profiles from DNA extracted from unsorted cells, ALDH$^-$ and ALDH$^+$ population. The lower part of the figure demonstrates chromosomes where differences were detected in the putative breast cancer stem cell population (ALDH$^+$) compared to the unsorted cells and ALDH$^-$ population.

It has to be noted that it is only one sample where array CGH profile was successfully generated so far, and further analyses and material collections are ongoing, in order to follow up on this question. The first analyses revealed different profile of $ALDH^+$ population on three chromosomes, when compared to genomic DNA and $ALDH^-$ population, as shown in the lower part of the figure 16.

3.5 EVALUATION OF SIZE BASED MICROCHIP FOR CTC/ DTC ENRICHMENT

3.5.1 CTC Microfilter design and enrichment procedure

CTC microfilter developed originally by Professor Richard Cote in collaboration with Caltech is a membrane filter designed to handle larger sample volume. Micromachined membrane filters, which have precise geometrical and thickness control, can have better performance. The particle membrane filters ($8 \times 8\ mm^2$) have circular through holes. Based on the precise geometrical design with pores of 8 µm and regular distance between the two holes, the CTC microfilter represents a method for size based separation of particles, with a great potential for processing of up to 10ml of peripheral blood in few minutes. The design of the filter is shown in the figure 17. Figure 18 and 19 demonstrate the setup of the filter and the set up of sample filtration.

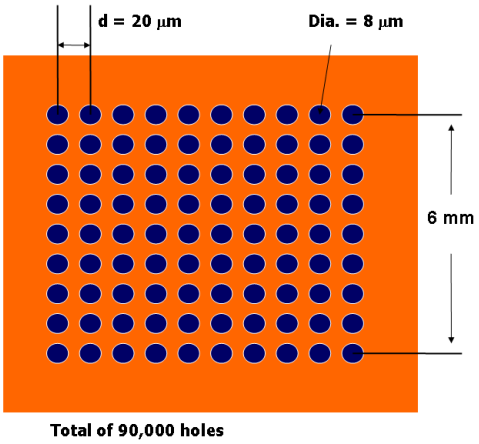

Figure 17. Design of the microfilter: Since the design is based on the computer program the density and the form of the pores is variable. For the blood and BM analysis the presented design was used.

At the time my thesis was performed, the CTC microfilter was cut to a 10x10 mm^2 piece and introduced into a PDMS chamber designed for repeated use. In figure 18, the set up of the device into the predesigned chamber is demonstrated.

Peripheral blood from healthy controls and/ or cancer patients is drawn into CellSave tubes (CellSearch, Veridex, Johnson and Johnson, South Raritan, NJ, USA). According to analyses of peripheral blood from cancer patients, analyses of healthy controls with spiked tumor cells were performed at Medical University Graz. The recovery rate was deterimed in a controlled manner. Defined number of tumor cells was spiked into 7.5ml of peripheral blood. The number of tumor cells ranged from 50- 200. The experiments were optimized untill consistent recovery rates of >90% were obtained for three times. After these optimizations steps, the CTC microfilter became available for the first time in Europe.

Prior to filtration, samples were diluted with PBS and 3.7% formalin/ PBS solution in a ratio 50%:40%:10%. Total volume was 15ml and was filtered through one CTC microfilter. After the filtration of the samples through the microfilter, the microfilters were transferred onto glass slide and the staining performed on the filter. The staining performed after filtration was either pancytokeratin staining as described below, or combinations of antibodies for determination of putative breast cancer stem cell markers (see below).

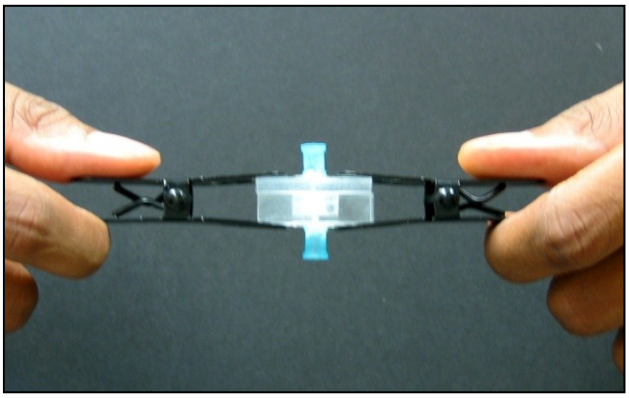

Figure 18: Device set up, CTC microfilter is used in the equipment shown in the figure. It is important to set up the device in an airtight manner. In the future, ideally a ready to use set up for a single sample filtration will be available.

In early studies, filtration was performed manually, but in recent studies the filtration of the samples is performed by a controlled pump. This step allowed for controlled filtration

rate resulting in better morphology of captured cells. The set up for filtration with the pump is depicted in figure 19. Filtration is performed at a flow rate 75ml/h.

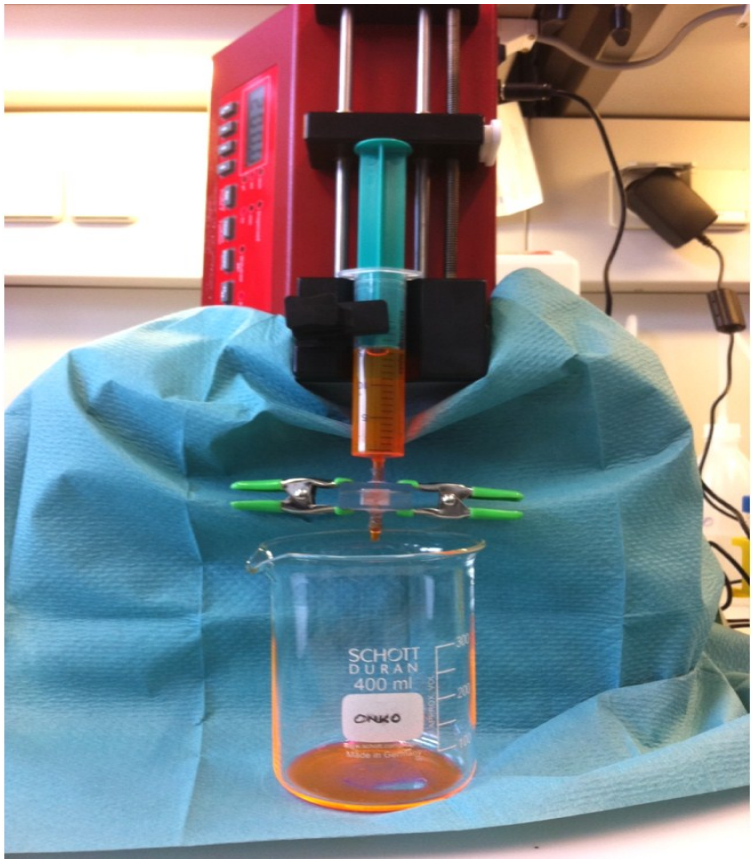

Figure 19: Filtration set up. As opposite to the previous manual filtration, the filtration of samples is performed by a controlled pump. The simple set up of the pump and filtration process is demonstrated. It can be clearly seenfrom the figure that also the flow through can easily be collected and analysed.

3.5.2 CTC Microfilter evaluation

The CTC Microfilter was tested in a model system using multiple cancer cell lines and a high recovery rate (>90%) was consistently obtained. Further, in a comparison with CellSearch CTC could be detected in 51 of 57 patients with metastatic prostate, colon, breast or bladder cancer using the microdevice, compared with only 26 patients with the CellSearch method. On average, 5.5 times more CTC were recovered by the microfilter device than by CellSearch [67]. Superior recovery rate and consistently higher number of patients with detected CTC and higher average number of CTC detected per patients could be demonstrated, and therefore, potential of clinical utility of the microdevice.

The transfer of technology to the Medical University of Graz was successfull. After careful optimization, and consistent recovery rate reaching 90% the confidency to analyze cancer patients became sufficient.

In an independent project, the utility of CTC microchip as enrichment platform for BM aspirates and detection of DTC was demonstrated. Since the frequency of DTC has repeatedly been reported to be much higher in BM aspirates in comparison to PB, it was essential to characterize the performance of the CTC microfilter in DTC detection from BM aspirates. Due to presence of larger cells and higher viscosity and cell density of BM aspirates, such analysis could be more challenging than analysis of blood.

BM aspirates from 8 patients with hematological diseases were successfully processed utilizing a model system where 10 - 100000 cultured breast cancer cells (MCF7) were spiked in BM aspirates. The same dilution was used as for peripheral blood. At least one DTC was detected when 10 cells were spiked into 5ml of BM. Up to 6 ml of BM successfully passed through a filter (6x6mm square area) with 8 µm pores. The captured cells were subjected to analysis by immunofluorescence, with direct evaluation on the membrane. In table 4 the origin of BM aspirates is listed.

Table 4: Origin of BM aspirates analyzed in the present study

Pt Nr	Disease	Actual BM aspiration result
1	Mb Hodgkin	Without infiltration, normocellular
2	Multiple Myeloma	Low number of BM infiltrating cells
3	Multiple Myeloma IgG	15% BM infiltration
4	MGUS IgM	< 10% infiltration with plasma cells
5	Acute myeloic leukemia	Without infiltration, normocellular
6	Policytaemia rubra vera	Middle hypercellularity
7	Ess. Thromocytemia	Middle enhancement of megakaryopoiesis
8	Multiple Myeloma IgA κ	30% BM infiltration
9	Folicular Lymphoma Grad2	10% BM infiltration

3.5.3 Array CGH of MCF7 Single Cell

In order to evaluate the feasibility of DNA analyses of cells microdissected from CTC microfilter and subsequently optimize the array CGH technology of the cells, we have performed series of analyses. We used the breast cancer cell line MCF7. After the capture of the cells on membrane, cells were either washed with H_20 and directly microdissected or the immunofluorescence for CK was performed. Single cells were isolated by laser microdissection using precise and contact-free laser microdissection system (PALM, Zeiss). Importantly, the CTC microfilter was dissectible, with relatively low amount of energy, thus preserving the quality of DNA (figure 20). After microdissection, whole genome amplifica-

tion has been performed using whole genome amplification kit (Sigma Aldrich). The DNA quality was tested previously by multiplex PCR, as described above, and in the majority of the cases substantial DNA quality was obtained. The multiplex PCR was performed according to the previously published protocol for evaluation of the DNA quality prior to CGH analysis [79]. After performing whole genome amplification whole genome 44K Agilent array was performed.

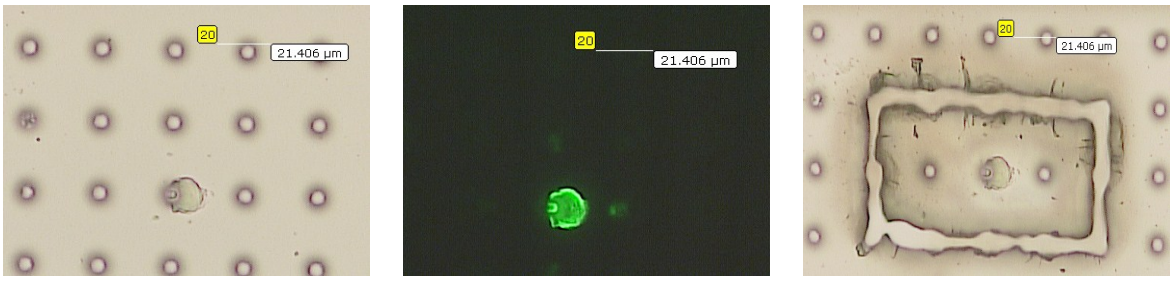

Figure 20: shows the captured MCF7 breast cancer cell (a), immunofluorescence image of the cell stained for panCK (b) and microdissected filter with the cell on the microdissected part (c). The cell is then catapulted and collected in a tube.

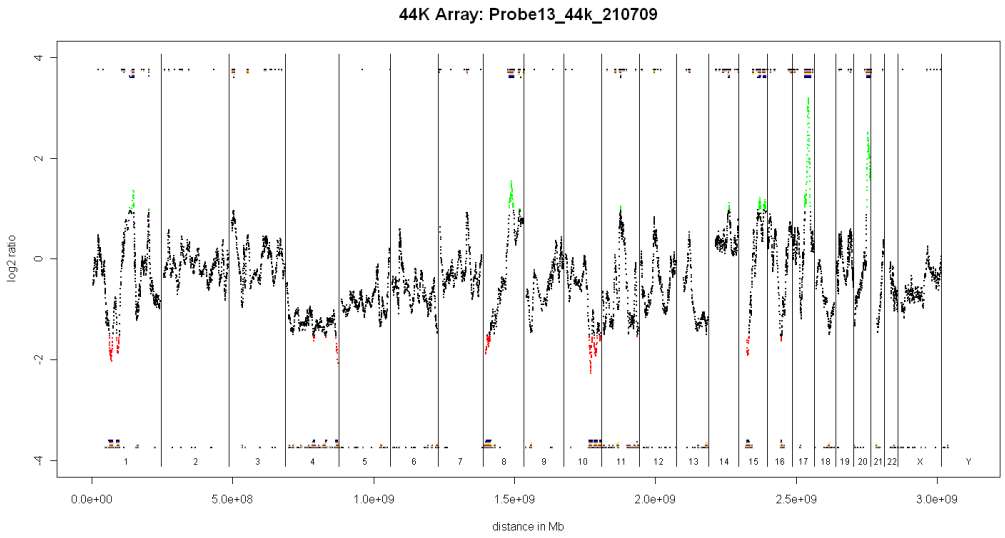

Figure 21: Representative whole genome 44K Agilent array of a MCF7 single cell. DNA gains are illustrated in green above the X-axis and the losses are shown in red below the X-axis. This is row data before computer based refinement was made.

The feasibility of arrayCGH profiling of single tumor cells from the CTC microchip as shown in the figure 21 will add to the potential to analyse heterogeneity associated with CSC within primary tumors, CTC/DTC and metastases and to define their role in metastatic process.

3.6 IMMUNOFLUROESCENCE FOR PUTATIVE BREAST CANCER STEM CELLS

3.6.1 Mammosphere embedding

For optimization and evaluation of feasibility of immunofluorescent staining for putative breast cancer stem cell markers, mammospheres were paraffin embedded. To collect the spheres, 100 µm cell strainer was placed over 50ml falcon tubes (BD Biosciences), and mammospheres transferred form the ultralow attachment flask and filtered. After filtration, mammospheres were washed with 3ml PBS. Cell strainer was turned over, and mammospheres relieved from the surface into falcon tubes with 2ml of the cell culture medium. Spheres were transferred into 2ml Eppendorf tubes and centrigued at 800 rpm for 5 minutes. Spheres were washed twice with PBS and centrifuged shortly. Thereafter, supernatant was removed. 100 µl of citrated plasma (Sigma- Aldrich) and 200 µl of Thromborel S (OUHP29, Siemens) were added to the spheres. The tubes were left for 20 minutes to harden and transferred to cassettes for paraffine embedding. The samples were fixed for 15 minutes with 3.7% formalin, then washed with PBS. After dewatering with high concentrated ethanol, paraffine preheated overnight at 65°C was poured over the samples.

3.6.2 Assessment for breast cancer stem cell markers

Multimarker immunofluorescence for four markers individually, panCK (AE1/ AE3, LabVision/Thermo Fisher Scientific), CD44 (clone 156-3C11, LabVision), CD24 (clone SN3b, LabVision) and ALDH (ALDH1, clone 44, BD Biosciences), were optimized. The first multimarker immunofluorescence protocol was established with three primary mouse antibodies

directly conjugated to the secondary antibody coupled with a dye. Only anti CD24 could not be conjugated, since it was IgM isotype. The conjugation of the primary anitbodies was performed with the DyLight Microscale Antibody Labeling Kit (Thermo Scientific) according to the manufacturer's protocol [81].

With the current LSM equipment (Zeiss LSM, Zeiss, Oberkochen, Germany) at Center for Medical Research, Medical University of Graz, triple immunofluorescence with panCK, CD44 and 24 with DAPI counterstain, and double immunofluorescence with panCK and ALDH with DAPI counterstain for visualisation of the nuclei can be reliably performed. Representative data is shown in figure 22. Cytospins from Aldefluor sorted MDA-MB-231 were prepared and fixed with 3.7% paraformaldehyde solution for 30 minutes at 37°C. (100 ml of paraformaldehyde solution consisted of 10ml PBS, 33.4 ml of 11.1% formaldehyde, 90µl Triton-X 100 and 56 ml of distilled water). Aldefluor sorted cells were used to obtain higher fraction of ALDH positive cells. Imaging analyses was performed with a Zeiss LSM 510 META confocal laser scanning microscope equeipped with four lasers. DyLight 405 was detected with detector LP 420, DyLight 633 with META 646.7-689.5 nm, DyLight 549 with META 561.1-614.6 nm and DyLight 488 with META 507.6-550.4. These protocols were further adapted. The images shown below (figure 22, 23 and 24) were obtained by sequential scanning of each channel and a merged picture was automatically generated by the software. The nuclear staining was detected by simultaneous use of transmitted light channel (ChD9) and detector for DyLight 488. The analysis was performed with the LSM 510 software for generation of cross talk free multifluorescence images (including transmittion light image).

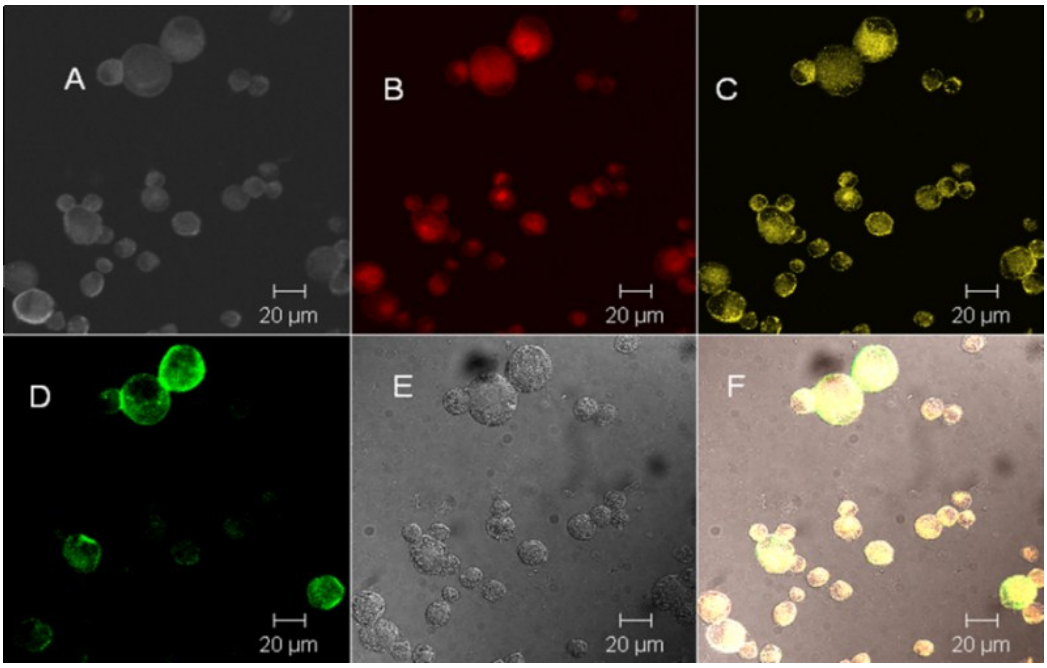

Figure 22: Quadruple immune-fluorescence on a cytospin from ALDH sorted MDA-MB-231. A. CD24 (blue, secondary labeled with DyLight 405), B. ALDH (red, primary conjugated with DyLight 633), C. panCK (yellow, primary conjugated with DyLight 549) and D. CD44 (green, primary conjugated with DyLight 488). The panel E depicts cells in transmission light and F. demonstrates the merged figure.

To simplify the protocols, combination of fewer markers were used. CK antibody was chosen from a different species, to omit crossreactions and the need for conjugation of the primary antibody. Only CD44 was conjugated repeatedly and the results were always stable with a bright immunofluorescence.

3.6.2.1 Multimarker immunofluorescence for paraffine embedded spheres

Paraffine microsections were prepared over night at 60°C. Deparaffinization was performed in xylol 100% for 20 minutes, and then ethanol 100%, 90%, 70% and 50% for 2 minutes, respectively. Thereafter, sections were washed with PBS and antigen retrieval was performed. panCK rabbit (Z0622, Dako) was used at a dilution 1:300, CD24 antibody (see above) was used at a dilution 1:50, CD44 was used at 1:150 and ALDH at a dilution 1:100. As secondary antibodies, Alexa Fluor 488 goat anti rabbit (A11029, Liffe technolo-

gies), Alexa Fluor 550 rabbit anti mouse (35557, Pierce), ADyLight 650 rabbit anti mouse (84545, Pierce) were used. In tables 5 and 6 the summary of results for positivity of the markers in analyzed mammospheres is listed. In table 5 results from the combination of CK and ALDH staining are shown, and in table 6 results from the combination CK and CD44. Please note that the ALDH staining with the used antibody was negative, although particularly SUM159 cells contained a positive fraction in the Aldefluor sorting. This finding was reproducible and implicates that sensitivity of immunofluorescence for the ALDH is lower than with the Aldefluor assay. Similar findings were shown by Ginestier et al [26]. For this reason in the multicolour immunofluorescence the Aldefluor sorted cells were used for staining to ensure the positivity of ALDH. The sensitivity of the ALDH antibody seems low, regardeless of the fixation used (data not shown).

Table 5: Double immunofluorescence for CK and ALDH in mammospheres generated from MCF7 and SUM 159

	CK- AF488 sek.	**ALDH- DL550sek.**
MS- MCF-7	+++ ~ 10% Hintergrund	neg.
MS- SUM159	+ ~ 30 -50%	neg.

Table 6: Double immunofluorescence for CK and CD44 in mammospheres generated from MCF7 and SUM159

	CK- AF488 sek.	**CD-44 DL650 sekundär**
MS- MCF-7	+++ ~ 10% Hintergrund	+++ 80%
MS- SUM159	+ ~ 30 -50%	++++ 100%

A representative figure of MCF7 mammosphere immunostained for CK and CD44 is shown in figure 23.

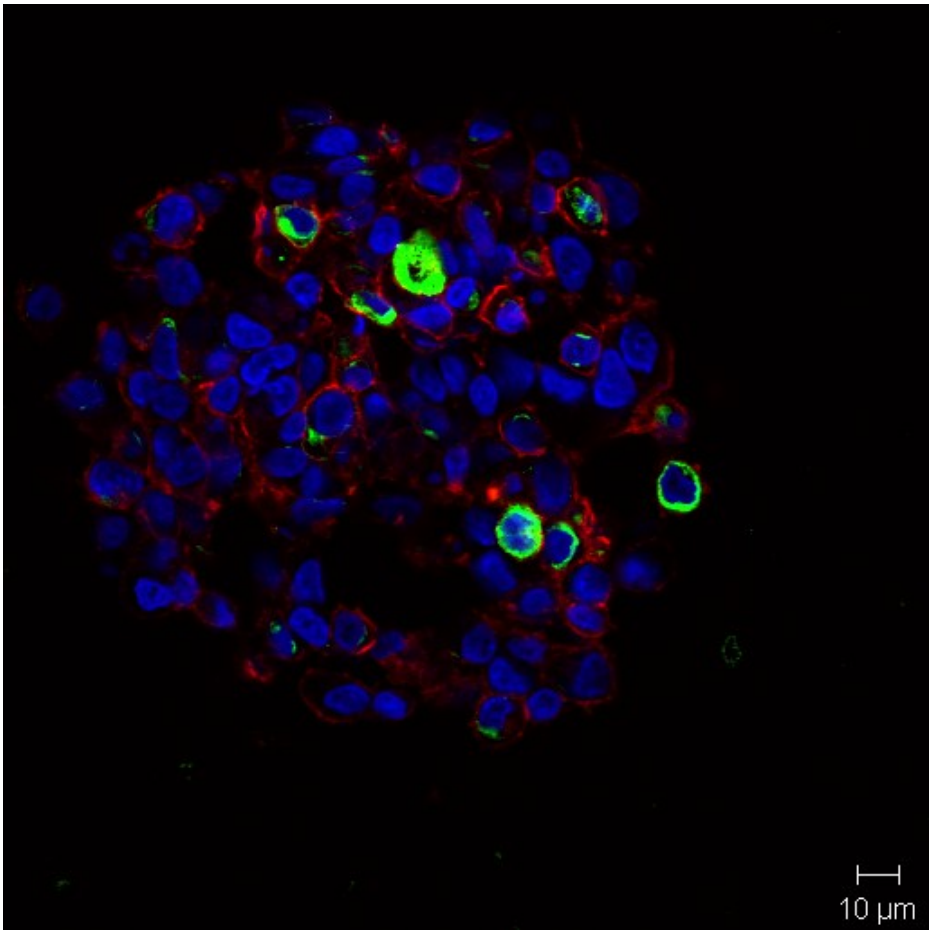

Figure 23 represents an immunostained section of paraffine embedded MCF7 mammosphere, stained with CK/ AF 488 (green), CD44/ DL650 (red) and DAPI (blue).

3.6.2.2 Multimarker immunofluorescence on CTC microfilter

To evaluate the feasibilty of the staining for putative breast cancer stem cell markers on CTC microfilter, cells were admixed with PBS and filtered. After filtration, filters with captured cells were fixed with 3.7% formaldehyde and triton X 100 for 30 minutes. Then, filters were washed three times with PBS for 5 minutes, and blocking buffer was added. The panel of primary antibodies was added for 60 min at the room temperature, and then panels of secondary antibodies with Dyes were added, according to the labeling described

above. This procedure was repeated few times, and results were stable. A representative figure of cells captured on filter, and stained for CK and CD44 is shown below in figure 24.

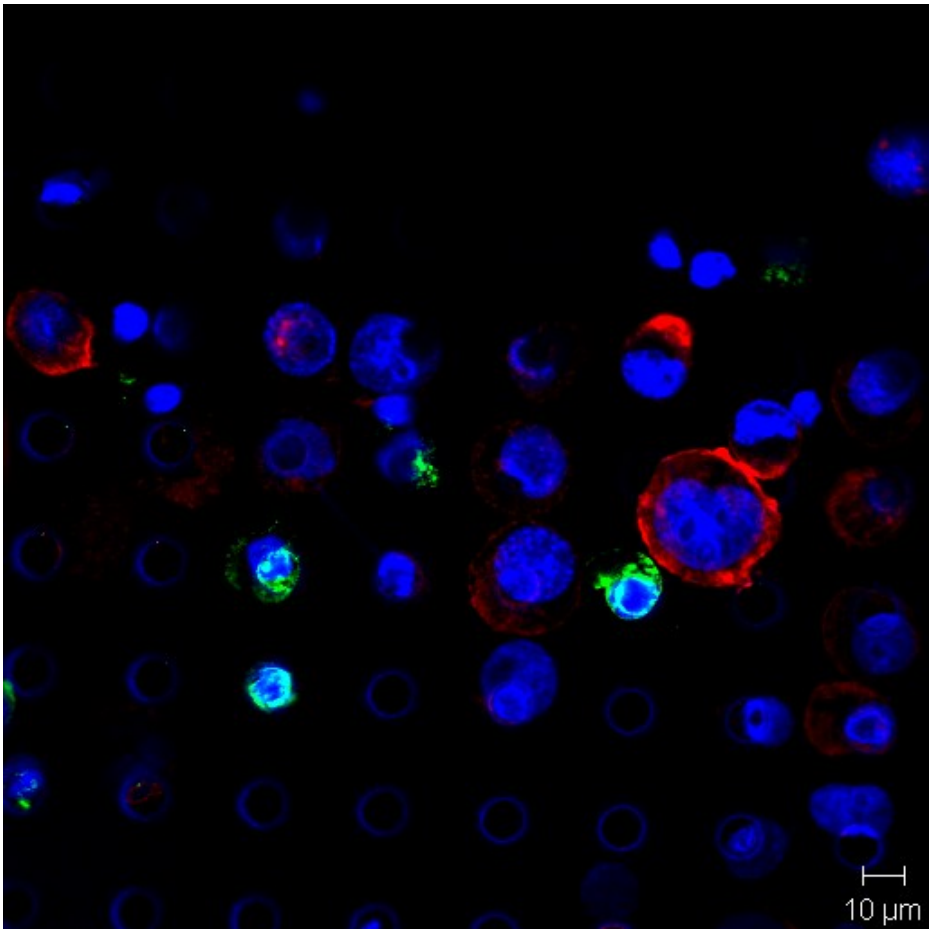

Figure 24 is a representative figure for cells captured on CTC microfilter and stained directly on the filter. On filter mmunostaining for CK/ AF 488 (green), CD44/DL650 (red) and DAPI (blue) is demonstrated.

4 DISCUSSION

The molecular intertumor heterogeneity of breast cancers has been increasingly studied for almost two decades [83]. In parallel, the intratumor heterogeneity has gained a great interest, in part due to development of novel targeted agents for treatment of cancer [84]. At least to some extent, this intratumor heterogeneity may be caused by existence of CSC, and it became increasingly important to study CSC and their role in tumorigenesis, progression and metastasis [51].

Despite the increasing interest, definition and analysis of CSC remains a challenge. Among the approaches to enrich and study the cancer stem cell biology are *in vitro* cultures and flow cytometry sorting based on defined putative phenotypes of CSC for different diseases [26, 38, 39, 85], along with the gold standard xenograft transplantation models. In order to evaluate the role of CSC in dissemination, their unique properties have to be established. This represents a challenge for detection, for understanding of metastatic processes, and ultimately for better treatment strategies. With defined markers and properties of CSC all these components would be facilitated. In the present thesis, my focus was on evaluation and optimization of technologies for studies of intratumoral heterogeneity and for enrichment and analyses of CSC from breast cancer cell lines, pleural effusions, and cells in dissemination.

Genetic profiles of breast cancer cell lines were analyzed in the attempt to associate breast cancer heterogeneity with the presence of putative CSC. Therefore, parental breast cancer cell lines and mammospheres originating from the parental cell line and distinct populations defined by putative breast cancer stem cell phenotypes were compared. In most of these analyses two biologically distinct breast cancer cell lines were used: MCF7 as a representative luminal breast cancer cell line and SUM159 as a representative triple negative cell line. The phenotypes used to enrich for breast CSC were established early by Al Hajj [39] and Ginestier [26], and are commonly used to study breast

CSC. The ability of these two cell lines to form mammospheres was demonstrated [81, 86, 87]. The percentages of putative breast CSC populations obtained in both cell lines were comparable with published results [87-89]. Basal like cell lines contain higher percentages of $CD44^{+}CD24^{-low}$ cells (SUM159 and MDA231).

The analyses of array CGH profiles represent the first comparison of array CGH profiles between parental cells and mammospheres for both cell lines, indicating that there is no difference in genomic aberrations between adherent cell cultures and putatively enriched breast CSC population from these cultures. Although sphere formation assay and flow cytometry sorting are commonly used and widely accepted method to enrich for CSC, not all cells within the sphere population or populations phenotypically defined as putative breast CSC represent cells with stem-cell properties. In fact, CSC population probably forms a minor fraction. As a result, the extent of dilution of the CSC signature, both genetically and epigenetically, is unknown. Therefore, it is of particular importance that array profiles across the analyzed samples (parental cells, mammospheres, $CD44^{+}CD24^{-/low}$ population of cells vs. others, and $ALDH^{+}$ cells vs. $ALDH^{-}$) were equal. Putative CSC populations defined by stem cell phenotypes were analyzed only for SUM159 cells. This was mainly due to the fact that in the breast cancer cell line MCF7 a very low percentage of $CD44^{+}CD24^{-}$ and $ALDH^{+}$ cells, respectively, were detected. The genomic profile of MCF7 cell line was comparable with results published for adherent cell cultures [17, 81]. Matching data for SUM159 could not be found. As expected based on their very distinct biology, MCF7 and SUM159 had substantially different genomic profiles.

Analogue analyses of pleural effusions were feasible to a certain extent. Pleural effusions have the advantage over primary tumor tissue or metastasis tissue, since cells are capable of surviving in fluid, do not have to be disassociated and cell count is larger than in DTC and CTC, theoretically facilitating further analyses. In fact, pleural effusions may be enriched for breast CSC and were the source for most established breast cancer cell lines. However, large amount of material has to be processed in order to obtain cells of interest. Fourteen pleural effusions were processed so far. The average percentage of the $CD44^{+}CD24^{-/low}$ phenotype in the fourteen pleural effusions was 7,7% and the average percentage of $ALDH^{+}$ cells was 6,6%. These results are in line with the published results

[26, 39] and confirm that cells with the putative breast CSC phenotype represent the minority of populations within the tumor cells. No conclusions on population with a higher potential for mammosphere formation from cells obtained from pleural effusions can be drawn from these analyses, since from both fractions ($ALDH^+$ and $CD44^+CD24^{-/low}$) in three cases mammospheres were generated.

One successful genomic profile from the pleural effusion of a breast cancer patient could be generated. The process was laborious and many optimizations steps were needed in order to proceed to the evaluation of the genomic profile. The cell counts and the amount of DNA extracted directly from the samples and sorted populations were mostly not sufficient to perform the whole comparative analyses. Nevertheless, in this one sample CNV in three chromosomes could be observed. In further few samples the DNA quality was lower. In the comparison of $ALDH^+$ sorted population of cells regions on chromosome 1, 4 and 7, 8 with significant differences of $ALDH^+$ fraction in comparison with the $ALDH^-$ cells and unsorted cells could be identified. This first result already indicates, that the analyses of breast cancer cell lines does not allow to draw conclusions for breast cancer tissues, and thourough analyses of patients tissues will have to be performed, in order to understand the role of CSC in heterogeneity of breast cancer. For this purpose, additional collection of primary tumors was initiated in order to enhance the success rate of generating substantial amount of DNA for definitive analyses.

As already indicated above, the alternative approach to collect and analyze primary breast cancers and establish primary breast cancer cell lines and mammospheres and characterization of these cell lines may be of greater promise. Collaboration was initiated with Department of Gynecology, and currently the primary procedure for tissue processing and primary cell cultures is being optimized and is therefore not described in more detail here. This approach is not equal to analyses of pleural effusions and may provide different information, particularly since patients undergoing primary surgery are patients at the early stage of disease, but, nevertheless, it holds a great promise for further understanding of the role of breast CSC.

Proposed plasticity and dynamic property of CSC has been frequently associated with the ability of cells to undergo epithelial to mesenchymal transition and reverse. Further, the

likelihood of switching stem cell markers off and back on as another aspect of plasticity of CSC suggest an underlying epigenetic mechanism [90, 91]. These properties may determine metastatic potential of tumors [92] and be the reason why most of the cells detected in dissemination have the putative breast CSC phenotype, as shown previously [47]. Epigenetic regulation of common CSC genes was recently demonstrated in triple negative breast cancer [93]. Therefore, in the master thesis by Daniela Scwarzenbacher an extended analyses of heterogeneity of CSC focused on methylation profiles of putative breast CSC. Selected pathways were chosen according to the potentially important pathways involved in stem cell biology, including stem cell transcription factors, homeobox genes, wnt signaling and epithelial to mesenchymal transition.

For reasons mentioned previously, methylation patterns unique to CSC may be masked by the methylation profile of other cells (e.g. progenitor cells). Indeed, as Daniela Schwarzenbacher showed in her master thesis, bisulfite pyrosequencing revealed only subtle differences in methylation levels between parental and mammosphere cells [94]. Replicate analyses with bisulfate pyrosequencing showed consistent results. Different methylation levels between sorted populations and mammosphere cells were identified. This may reflect heterogeneity in CSC but also different enrichment of CSC. As already stated, enrichment factor for CSC is unknown for both approaches. There were no methylation differences detected between sorted subpopulations analysed in the SUM 159 cell line. In this cell line the majority of cells were associated with $CD44^{+}CD24^{-}$ CSC phenotype. SUM159 cells are more aggressive, basal like, with mesenchymal characteristics, and with the majority of cells being similar, detection of differentially expressed markers was less likely.

Several groups have used similar approach in attempt to associate intratumor heterogeneity with the presence of CSC. Clearly, the main goal of such approach is to understand the biologic mechanism and finally identify biomarkers of CSC to design better treatment strategies. One of the first studies analyzing epigenetic regulation of putative CSC was published by Hernandez-Vargas et al, revealing that JAK-STAT pathway was activated in putative breast CSC defined as $CD44^{+}CD24^{-}$ MCF7 cells. Hypomethylation of the gene components of the JAK- STAT pathway was demonstrated further in mammospheres [95].

In this study, authors demonstrated not only a potential definition of biomarkers associated with CSC, but provided also evidence of underlying epigenetic regulation of CSC properties. In a similar analysis in hepatocellular carcinoma, authors identified epigenetic regulation of wnt signaling as a potential biomarker of early detection and therapeutic targeting [96]. Epigenetic mechanisms and the role of miRNA regulation of CSC was also demonstrated [97, 98] providing a new insight into biology and potential definition of novel therapeutic targets. These analyses are promising and require further evaluation.

In master thesis by Daniela Schwarzenbacher also mRNA expression analyses has been performed [94]. These analyses suggested that also mechanisms than DNA promoter methylation may be responsible for regulating gene expression. DNA promoter hypermethylation is known to downregulate mRNA expression and subsequently protein expression [99].Alternative studies have demonstrated further mechanisms involved in epigenetic silencing of proteins, including histone modifications, long noncoding RNAs and miRNAs [100]. These mechanisms are not thoroughly understood and are emerging study topics. All together, results on heterogeneity presented here along with the epigenetic analyses performed in Schwarzenbacher master thesis [94] suggest that heterogeneity patterns have to be evaluated further to provide more definitive results. Moreover, these results clearly underpin the hypothesis that epigenetic mechanisms seem to play a major role in the regulation of CSC. Epigenetic heterogeneity may reveal more promising results. Also these analyses should be extended to patient samples, in order to validate results, and possibly find higher heterogeneity then present in cell lines.

Along with the analyses of heterogeneity of breast cancer cell lines and mammospheres derived from cell lines, further technological approaches were established and optimized, including the sphere formation, sphere paraffine embedding, multimarker immunofluorescence both in paraffine embedded spheres and on CTC microfilter. CTC microfilter technology was optimized, both in terms of sensitivity and post-capture analyses. For future analyses of clinical samples it was critical to optimize the filtration and immunostaining on CTC microfilter. CTC microfilter was evaluated for the feasibility of bone marrow enrichment, and cells could be successfully recovered. Furthermore, also single cells could be captured and array CGH performed from single cells or few cells microdis-

sected from the CTC microfilter. These technologies are essential in order to address the heterogeneity in clinical samples, and compare primary tumors, CTC, DTC, and metastatic sites for molecular heterogeneity and its association with putative CSC. The CTC microfilter technology was adapted both for CTC capture with sufficient sensitivity but also for further characterisation of clinical samples for putative stem cell markers and genomic analyses. In the multimarker immunofluorescence, the lower sensitivity of the ALDH immunostaining remains constant. The ALDH antibody (ALDH1, clone 44, BD Biosciences) was used in the current analyses and by Ginestier et al. The data are conform in suggestion that a lower percentage of the cells with ALDH expression is detected when compared to Aldefluor. This has to be taken into account when further analyses of clinical samples are performed. Taken all these technologies together, including also the flow cytometry sorting based on CD44/CD24 and ALDH, it is obvious, that protocols are now established to perform comprehensive comparison studies of primary tumor tissues, cells in dissemination, and solid metastasis, in order to understand the role of breast cancer stem cells in progression, metastases, and therapy reseistance.

In conclusion, in this thesis important technological approaches were established, to facilitate further studies of the role of breast CSC in molecular heterogeneity of breast cancer and dissemination and breast cancer progression. Future studies based on presented technologies may add to this understanding and will be followed further. DTC and CTC will be addressed in the context of systemic disease, in comparison with primary tumors and metastases. Stem cell hypothesis has to be further challenged, new markers and more efficient approaches and models are needed in order to better define the biology of stem cells and identify biomarkers for dissemination, early diagnosis and treatment with higher precision.

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