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CIRCULATING TUMOR CELLS AND HISTORIC PERSPECTIVES

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This introduction reviews the history of research on circulating tumor cells (CTCs) with brief reviews of the present opportunities and challenges using CTCs for diagnostic and treatment decisions.

1.1 EARLY STUDIES ON CANCER DORMANCY LED TO THE DEVELOPMENT OF A SENSITIVE ASSAY FOR CTCs (1970–1998)

Prior to my involvement in the development of the first Food and Drug Administration (FDA)-approved capture device for CTCs, our laboratory's research was involved with investigations of the signaling pathways in B lymphocytes. We were studying the mechanisms underlying induction of replication, differentiation, apoptosis, and cell cycle arrest when an important event took place at Stanford University that affected our future plans. In 1980, Slavin and Strober [1] isolated the first murine B-cell lymphoma that spontaneously arose in a BALB/c mouse and allowed us to propagate the tumor. We found that an antibody to the tumor immunoglobulin [anti-idiotype (Id)] injected into the tumor-bearing mice could induce a state of cancer dormancy [2] by its ability to induce cell cycle arrest and apoptosis (antibodies are immunoglobulins; each antibody has a unique antigenicity, so it can stimulate production of an antibody to it, that is, an antibody to an antibody called an anti-idiotypic antibody). Dormancy could last up to 2 years. The population of dormant lymphoma cells in the spleen was

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stable for the 210 days of observation, although a subpopulation of tumor cells were replicating; loss of dormancy occurred at a steady rate during the 2 years of observation; and, about 90% of the lymphoma cells that were now replicating remained Id+ [3–6]. However, the majority of such antibodies had undergone minor changes that made them Id+ variants with decreased or no susceptibility to anti-Id-mediated induction of dormancy. With time, these lymphoma cells regained their full malignant potential; as few as three of these cells transferred progressive tumor growth to syngeneic recipients [6]. Thus, anti-Id suppresses the malignant phenotype, observed in control mice that do not receive anti-Id, by signal transduction mechanisms that override the genetic lesions that cause neoplasia. In searching for potential signaling molecules, alterations in Syk, Lyn, and HS1 were suggested by either the loss of an epitope recognized by a monoclonal antibody or the loss of functional kinase activity [5]. Significant advances in characterizing cancer dormancy followed from studies by Meltzer [7], Stewart [8], and Demichelli [9].

At the same time, the Stevensons reported impressive results of anti-Id therapy of murine B-cell tumors [10, 11], and Levy obtained groundbreaking results in treating patients with B-cell lymphomas with anti-Id serum [12, 13]. Prior conventional treatment could induce long-term remission, but dormant lymphoma cells were not eliminated and virtually all patients eventually died of the disease. Levy treated such patients with anti-Id and the vast majority went into long-term remissions even though tumor cells remained [14]. As with the B-cell tumor lymphoma 1 (BCL1) mouse tumor model, the clinical studies also indicated that the antitumor effect of anti-Id and several other B-cell reactive antibodies related to their ability to act as agonists rather than conventional effector antibodies. Levy's results initiated a burst of research into immunological methods for cancer treatment, which continue to the present day. There are currently over 30 immunologically based drugs in clinical trials to treat patients with various cancers.

These observations in human B-cell lymphoma and in the BCL1 mouse tumor model – that long-lasting tumor dormancy can be induced by antibodies to the tumor immunoglobulin in the face of persisting tumor cells, a portion of which are replicating in the BCL1 tumor – gave us the impetus to study clinical dormancy. We were interested in analyzing those tumor types in which metastases can develop many years after primary tumor removal even though the patient has appeared clinically disease-free. Breast cancer was an excellent example because of the prevalence of the disease and because recurrences occur at a steady rate from 7 to 20 years after mastectomy in patients who appear well [9, 15]. To study the tumor cell dynamics in clinically healthy humans, we needed a relatively noninvasive procedure, namely, a very sensitive blood test to determine whether CTCs were present in a proportion of these patients. However, the methods under development were insensitive, could not be quantified, frequently gave false positives, and were impractical for the challenge.

The history of CTCs began in 1869, when Ashworth [16] described cells in the blood that appeared similar to those observed in the tumor at autopsy. In the mid-twentieth century, there were many claims that CTCs as determined by cytology were commonly seen in cancer patients. However, further studies indicated that hematopoietic cells, particularly megakaryocytes, were responsible for almost all

of these results [17] and such studies were then abandoned. However, there were positive exceptions. Drye *et al*. [18] had convincing evidence that the presence of cancer cells in peripheral blood of 17 patients related to their clinical progress. Engell [19] claimed to have found cancer cells in venous blood draining the tumor as well as in peripheral blood.

Beginning in the 1970s, experimental models were developed to study the events that led to metastases [20, 21]. Metastases had been generally regarded as a late event in the development of epithelial tumors. However, the poor prognosis of patients with clinically localized lung cancer suggested that micrometastases may have taken place before the primary tumor was diagnosed. Pantel *et al*. [22, 23] searched for tumor cells in the bone marrow by immunohistochemistry in patients with breast, gastrointestinal tract, or non-small-cell lung carcinomas with and without evidence of metastases. In the majority of lung cancer patients without metastases, cytokeratin-positive cells were detected at significant concentrations, whereas they were rarely found in controls. The authors concluded that early dissemination of isolated tumor cells is a frequent occurrence in non-small-cell lung carcinomas; in breast and gastrointestinal carcinomas, the majority of these disseminated cells in the bone marrow were in a dormant state. In a series of pioneering experiments, Folkman *et al*. [24, 25] defined a critical role for angiogenesis in the metastatic cascade. Liotta *et al*. [26] studied mechanisms of metastases and focused on the role of angiogenesis. The primary tumor must first develop an adequate vascular supply. This is achieved by balancing angiogenesis-promoting and -inhibiting factors released by tumor cells, inflammatory cells, and extracellular matrices. Although there were problems with the methods for quantification of angiogenesis, the results indicated that increased angiogenesis within the primary tumor resulted in a worse prognosis.

Using a fibrosarcoma model, Liotta also quantified some of the major processes that occur following transplantation of tumor into the leg muscle of a mouse and the subsequent rapid development of pulmonary metastases [27]. He showed that by about Day 4, a vascular network first appeared in the peripheral regions of the tumor and grew throughout the tumor mass by Day 10. Following intratumoral perfusion (using a solution of human hemoglobin, calf serum, Eagle's medium, amino acids, glucose, and insulin), fibrosarcoma tumor cells were detectable in the draining venous blood vessels by Day 5. The number of these cells in the blood stream, both as single cells and tumor clumps (2–30 tumor cells, comprising about 10% of tumor cells), increased rapidly until Day 10–12, after which it diminished. There was a linear relationship between the density of perfused vessels and the concentration of detectable tumor cells in the effluent. Metastases, which were first detectable on Day 10, increased with time and were directly related to the concentration of tumor cell clumps (four or more cells) in the effluent. The aforementioned studies suggested that tumor vascularization and the resultant entry of tumor cell clumps into the circulation were critical events in the initiation of metastases.

These results propagated new types of experiments to look for CTCs [28]. The first procedure was to enrich the small number of tumor cells in the blood. The different techniques that were tried included lysis of RBCs or enrichment of non-RBCs, including positive [29–33] and negative [34] selection by an immunomagnetic procedure.

Antibodies to either an epithelial or a hematopoietic marker were attached to metal beads, mixed with blood, and then removed from the mixture by placement in a magnetic field. Molecular analysis following enrichments was then performed using either immunocytochemical staining or polymerization chain reaction (PCR) or its variants. Immunocytochemical assays, which relied on time-consuming searches for rare cells, were unable to measure tumor burden or to phenotype tumor cells in depth. PCR assays for detecting CTCs involved amplification of specific target DNA sequences and were very sensitive, for example, one tumor cell in 10^6 normal lymphocytes could be detected. Another CTC detection strategy was to amplify tissue-specific mRNA by reverse transcriptase PCR (RT-PCR) for markers characteristic of that tumor's tissue of origin (e.g., to detect circulating prostate or melanoma cells). However, knowledge of that tissue's gene sequence and intron–exon junctions was required for selection of appropriate oligonucleotide primers for the RT-PCR. Fusion genes – due to translocations, interstitial deletions, or chromosomal inversions – may also be detected using RT-PCR, although diagnostic tumor tissue is generally needed to confirm the presence of tumor cells containing the rearranged sequence. However, PCR assays were difficult to quantify and may show false-positive results (due to PCR's high sensitivity, presence of pseudogenes, or introduction of normal tissue-specific cells during biopsy or surgery) and false-negative results (due to low level signals, CTC heterogeneity, or sampling issues). The interpretation and limitations of the assay and its variations are elegantly described by Ghossein *et al*. [28]. Nevertheless, these approaches demonstrated that patients with cancer frequently had evidence of CTCs, even though it was not possible to quantify their number.

1.2 MODERN ERA FOR COUNTING CTCs: 1998–2007

This began with our publication of a very sensitive method to detect, quantify, and immunophenotype CTCs in patients with breast and prostate cancer [35]. Circulating epithelial cells (CECs) were collected by a positive immunomagnetic approach that involved coating metal particles with an antibody to an epithelial cell surface antigen, epithelial cell adhesion molecule (EpCAM), a transmembrane glycoprotein expressed exclusively in epithelia and epithelial-derived neoplasms. (Note that EpCAM is distinct from cytokeratin proteins, which are part of the cytoskeleton and are exclusively intracytoplasmic.) The conjugated particles were mixed with the blood sample and tumor cells were captured in a magnetic field, while the remaining nonadherent blood cells were washed away. The captured epithelial cells were then fluorescently stained with an anti-cytokeratin antibody (CAM 5.2), a nuclear stain, and an anti-CD45 antibody that detects hematopoietic cells. One aliquot of the captured cells was analyzed by flow cytometry and a second by microscopy of the stained slides (in this case, stained with antibodies against cytokeratins 5, 6, 8, 18, Muc-1 glycoprotein, or prostate-specific membrane antigen. The development of this assay was a collaboration between my laboratory and Immunicon Corp. The latter was headed by Liberti [36, 37], an immunologist and an expert in development of magnetic particles; the senior scientist was Terstappen [38], an excellent immunologist

and skilled in flow cytometry. The utility of our method was due to multiple factors: a very effective ferrofluid that maintained its colloidal properties without reacting with blood components and yet could still be magnetically separated; an avid and specific anti-EpCAM antibody; absence of prior enrichment steps, thus eliminating cell loss and preserving cytomorphology; exclusion of circulating cells that stained with a hematopoietic marker; initial analysis using flow cytometry, and, when positive, purification of another simultaneously obtained aliquot of blood for direct staining on slides. Hence, the assay was highly specific and very sensitive. Applying threshold values as a cut-off, excess CECs were detected in 12 of 14 patients with localized breast cancer and in all 3 patients with early prostate cancer, with no false positives in healthy controls.

This prototype is probably the most sensitive detection instrument to the present day. The evidence for calling these CECs, CTCs, was strong. The cells were epithelial, only detected in patients with cancer but not in normal age-matched controls, and there was an excellent correlation in individual patients between their clinical status before and after chemotherapy and the number of CTCs detected.

1.3 PROOF OF MALIGNANCY OF CTCs

The critical nature of this issue made it important to use genetic techniques to support the conclusion that the cells in question were malignant. Therefore, fluorescent *in situ* hybridization (FISH) was used to determine the patterns for chromosomes 1, 8, and 17, which were indicative of malignancy in touch preparations from 74 primary breast cancer patients [39]. Use of the three probes detected aneusomy in 92% of the samples. The genetic abnormalities in the CTCs were compared with those in the primary tumor. CECs from 15 patients with organ-confined breast, kidney, prostate, or colon cancer were analyzed by dual or tricolor FISH using the numerator DNA probes for chromosomes 1, 3, 4, 7, 8, 11, and 17 [40]. In 10 of 13 patients in which touch preparations of the primary tumor tissue were available, the patterns of aneusomy in their CECs matched one or more clones in the primary tumor. We concluded that the CECs were CTCs and that the three negative results were readily explainable by the small number of CECs in several patients (one to two CECs) and the known technical difficulties in counting chromosomes.

1.4 NEW EXPERIMENTS INVOLVING CTCs

There proceeded to be many new experiments extending the immunomagnetic approach for quantifying tumor burden [41–45]. Terstappen [46] led experiments to determine the accuracy and linearity of the CellSearch system in enumerating CTCs in healthy subjects, patients with nonmalignant diseases, and patients with a variety of metastatic carcinomas. The results culminated in the development by Immunicon of the CellSearch instrument (see Chapter 19 for details). It was based on the patent of the 1998 prototype owned by Immunicon and the University of

Texas Southwestern Medical Center. It remains the only FDA-approved instrument for counting CTCs. Johnson and Johnson, Inc. obtained the patent and supported additional clinical trials to refine the ability of CellSearch to help with care of patients with recurrent cancer. The results of clinical trials led by Cristofanilli *et al*. [47] and Hayes *et al*. [48] indicated that the number of CTCs/7.5 ml blood could differentiate between two groups of patients with metastatic breast cancer (MBC) and strikingly different outcomes. Five or more CTCs indicated a very aggressive outcome, whereas less CTCs indicated a better response to treatment with a longer progression-free and overall survival (OS). The prognostic value of CTC enumeration was independent of disease subtype and line of therapy and was superior to or augmented standard anatomical and functional imaging assessment [49]. Similar results have been observed in colorectal, prostate, and lung cancer [50–54]. Also, the longitudinal monitoring of CTCs indicated patients that would fail to benefit from systemic therapies whose disease is associated with extremely rapid progression [47, 48]. These data were confirmed in a recently reported prospective randomized clinical trial conducted by SWOG (formerly Southwest Oncology Group) [55], showing that failure to clear CTCs to below 5/7.5 ml whole blood in patients with MBC after receiving one cycle of first-line chemotherapy was associated with a very poor prognosis. The median OS for this group of patients was 13 months, compared to 23 months for those who presented with elevated CTCs but "cleared" them after one cycle of chemotherapy, and 35 months for those patients who did not have elevated CTC at baseline. However, in this randomized trial, switching from whatever the clinician chose as first-line chemotherapy to an alternative chemotherapeutic regimen did not improve the dismal prognosis for those whose CTCs failed to decline to less than 5/7.5 ml whole blood, most likely because the second-line alternate therapy was no more effective than the first-line therapy. This dampens enthusiasm for only performing enumeration of CTCs, which has previously been the gold standard and major guiding force for prognostication and help with treatment decisions. Additional evidence to be discussed indicates the need for extensive immunophenotypic and genotypic analyses of CTCs and for development of a more sensitive instrument.

1.5 CLINICAL CANCER DORMANCY

The sensitivity of our assay for CTCs allowed us to look for them in patients with breast cancer dormancy. In breast cancer, 15–20% of clinically disease-free patients relapse 7–25 years after mastectomy and, from 10–20 years, the rate of relapse is relatively steady at about 1.5% [9, 15]. Clinical cancer dormancy is also frequently observed in thyroid, renal, and prostate carcinomas and in B-cell lymphoma and melanoma. The current concept is that the persisting cells are nondividing cells. However, in our study involving 36 patients whose mastectomy for cancer occurred 7–22 years previously and who were clinically disease-free, 13 had CTCs [56]. These patients continued to have sustained low levels of CTCs during the next year. Since CTCs develop an apoptotic program after entering the circulation [57–59] and their

half-life is 1–2.4 h in patients whose primary breast cancer was just removed [56], there must coexist a replicating population of tumor cells in these patients with a population that dies at precisely the same rate for many years, perhaps decades. This is one way for keeping a population of persisting cancer cells "dormant." It does not exclude that there is another tumor cell population in these patients that is not replicating. Also, it appears that some patients with early-stage breast cancer and disseminated tumor cells (DTCs) in their bone marrow may not relapse for long periods of time [60–62]. Their tumor cells may represent a dormant population or the same replicating population described earlier or both.

Several possible mechanisms underlying the dormant state have been extensively investigated. These include an antitumor immune response, as first proposed by Thomas [63] and Burnett [64], and lack of vascularization, as proposed by Folkman and colleagues [24, 65]. Aguirre-Ghiso [66] has accumulated considerable evidence for a role for stress signaling in inducing dormancy in models of human cancer.

We hypothesize that the size of the persisting cancer cell population may be stabilized by some of the same mechanisms that control the size of organs and subsets of cells [67]. Our attraction to this hypothesis is that the organ-control mechanisms are precise as is the balance between replication and cell death of the persisting tumor cells in breast cancer dormancy. This does not exclude contributions from other mechanisms less likely to be so precise. Evolution has given mammals and other species the regulatory equipment to keep organs at a precise size and to restore that size if the organ is altered in size [68, 69]. Thus, each cell type in the blood is kept at a relatively constant level barring disease. The same is true for solid organs. For example, if half the liver is removed, liver cells that are quiescent suddenly begin to divide and stop dividing only when the liver has reached normal size [70]. The stem cells that give rise to the differentiated cells in each organ control the size of that organ. Organisms from Drosophila to mammals have evolved complex mechanisms to coordinate cell proliferation with cell death to prevent inappropriate proliferation of somatic cells. Asymmetric replication in which one cell differentiates and dies and the other is a renewal cell is one strategy.

Many molecules and signaling pathways have been implicated in organ size control in Drosophila, and there is considerable conservation of the signaling molecules between Drosophila and humans [71]. These include, but are not limited to, IGF-1 and IGF-2 [72], IRS1-4 [73], TSC1, and TSC2 [74].

Another important example is the Hippo signaling pathway. It plays a major role in controlling eye and wing growth in Drosophila and size control of some organs in mice [75]. Again, there is significant conservation in the components of this pathway between humans and Drosophila and there is mounting evidence that this pathway can act as a tumor suppressor as well as controlling organ size in humans [75, 76]. Very recent studies have revealed some of its downstream transcriptional targets and their potential role as a suppressor of cancer. In breast cancer cells, the mevalonate metabolic pathway, or its inhibitor simvastatin, exerts regulation upon the Hippo signaling pathway and appears to be involved in tumor suppression [77]. Statins, which block the mevalonate pathway, are reported to have multiple anticancer effects, for example, antiproliferation and anti-invasive properties against mammary

carcinoma [78, 79] The mevalonate pathway also regulates the transcription of *RHAMM* (receptor for hyaluronan-mediated motility or CD168), a breast cancer susceptibility gene [80, 81]. Its hyperexpression is associated with tumor development and progression to metastases [82], and its expression is upregulated in a variety of human cancers, including breast [83]. These results emphasize the possibility that size-controlling pathways may affect the persisting tumor cell population in clinical breast cancer dormancy.

It is possible that all "survivors" of breast cancer have a small number of tumor cells somewhere in their body, the growth of which is controlled usually for the lifetime of the host. If the mechanisms underlying clinical tumor dormancy and relapse were understood, it is possible that appropriate targeting drugs could be developed, which could eliminate or control these persistent tumor cells and prevent their transformation into growing metastases. Thus, cancers with late recurrences could be treated after removal of the primary tumor as chronic diseases to be controlled by relatively nontoxic therapy.

1.6 HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR 2 (HER2) GENE AMPLIFICATION CAN BE ACQUIRED AS BREAST CANCER PROGRESSES

Use of trastuzumab (Herceptin), a humanized monoclonal antibody that targets HER2 on breast cancer cells, to treat patients with HER2-overexpressing breast cancers provided impressive clinical results in both the metastatic and adjuvant settings [84–86]. However, the accepted dogma that HER-negative tumors could never become HER2 amplified with recurrence was puzzling. Patients whose primary tumors were HER2-negative and who developed breast cancer recurrence were ineligible for treatment with Herceptin under the assumption that they would not have tumor cells that would be blocked by this antibody. However, the plausibility of this assumption is under question, considering the ability of HER2 amplification to confer aggressiveness on the tumor and the known evolution of neoplastic cells to constantly mutate to more aggressive phenotypes. Studies of HER2 status of primary and discontinuous recurrences of breast cancer tumors indicated that there could be discordance among them, with HER2-positive tumors developing HER2-negative metastases and HER2-negative tumors developing HER2-positive metastases [87, 88]. Wülfing *et al*. [89] demonstrated that HER2-positive CTCs were associated with a poor prognosis and identified HER2-positive CTCs in patients with primary HER2-negative breast cancer, thus further challenging this concept.

We, therefore, embarked on a study to determine the relationship between the HER2 status of CTCs and the primary tumor tissue and to determine the HER2 status of CTCs in recurrent breast cancer [90]. CTCs were captured and evaluated for HER2 gene status by fluorescence *in situ* hybridization. In 31 patients with primary breast cancer who had not received Herceptin, there was 97% concordance in the HER2 status of the primary tumor and corresponding CTCs, with no false positives. Amplification of HER2 in CTCs was shown to be a reliable surrogate marker

for amplification in the primary tumor, and HER2 protein expression in CTCs was sufficient to make a definitive diagnosis of HER2 gene status. However, of 24 patients with recurrent breast cancer whose primary tumor was HER2-negative, nine (38%) acquired HER2 gene amplification in their CTCs during cancer progression. Four of these were treated with therapy that included Herceptin. One patient who was terminal from liver and kidney failure had a complete response and lived for another 2 ½ years, and two had partial responses.

Subsequently, Fehm *et al*. [91] showed that between 32% and 49% of patients with HER2-negative breast cancers (78 patients) had HER2-positive CTCs after recurrence depending upon the CTC test used. In a study by Riethdorf *et al*. [92], examining blood samples from patients with large operable or locally advanced nonmetastatic breast cancer eligible to undergo neoadjuvant chemotherapy, among 26 patients with primary tumors classified as HER2-negative, approximately 20% had CTCs with strong HER2 expression. However, some of the patients had only one CTC. Others also confirmed discordance between HER2-negative primary tumors and HER2-positive CTCs and DTCs in breast and gastrointestinal cancers [93–95]. Paik *et al*. [96] followed 1787 patients in a study comparing standard adjuvant chemotherapy with and without trastuzumab and concluded that some patients, who were later classified as having HER2-negative primary tumors by immunohistochemistry and FISH testing, appeared to benefit from trastuzumab treatment. It is important to develop more sensitive CTC capture techniques to increase the number of CTCs that can be examined in nonmetastatic patients and to understand the biology of cells surviving trastuzumab treatment that are likely responsible for metastatic spread. Further, amplification may not be the only mechanism of HER2 overexpression in micrometastases. For example, Ithimakin *et al*. [97] have shown that MCF-7 breast cancer cells, which normally only express low levels of HER2, upregulate HER2 expression without amplification when placed into estrogen-free murine bone marrow. Moreover, bone metastases in 12 of 14 (87%) patients with hormone-receptor-positive HER2-negative breast cancer showed significantly higher HER2 protein expression than present in matched primary tumors. These data suggest that HER2 regulation may be plastic in some types of breast cancer cells (specifically, the luminal subtype) and highlight the importance of using the proper assay to measure protein levels. A current clinical trial, National Surgical Adjuvant Breast and Bowel Project B-47, is evaluating whether the addition of trastuzumab to chemotherapy improves invasive disease-free survival in patients with low HER2 expression.

1.7 uPAR AND HER2 CO-AMPLIFICATION

Activation of the urokinase plasminogen activator (UPA) system is associated with a poor prognosis in breast cancer, with the greatest amount of evidence for UPA overexpression. However, it is well documented that UPA receptor (uPAR) overexpression is also associated with increased tumor aggressiveness and worse disease-free survival and OS in breast and other cancers [95–97]. The uPAR gene on chromosome 19, encodes a 35 kDa protease, with a glycosyl phosphatidyl anchor linking it to the

outer cell membrane. Interaction of uPAR and UPA causes cleavage of plasminogen and activation of plasmin (a serine protease), which degrades several extracellular matrix (ECM) components and also activates matrix metalloproteinases (MMPs). The result is proteolytic degradation of ECM, thereby allowing tissue penetration, thereby facilitating metastasis. Additional signaling pathways are activated resulting in cellular replication, motility, and remodeling of the ECM. Therefore, overexpression of uPAR in breast and other cancers is associated with a poor prognosis [98–100].

Since overexpression of HER2 also predicts a poor prognosis, we thought it is important to analyze both HER2 and uPAR protein and gene expression in CTCs and touch preps of primary breast carcinomas and CTCs of patients with advanced breast carcinomas. Our results showed amplification of the uPAR gene in 20–25% of breast cancer patients and a marked bias for amplification of both oncogenes in the same tumor cell [101]. The amplification was responsible for overexpression of both genes. Coamplification suggests cross talk and cooperation between the HER2 and uPAR signaling pathways. This represents an intriguing finding for further studies that could lead to additional antitumor therapy.

Recent studies of Meng and coworkers [102] showed that tumor cell growth could be suppressed and apoptosis induced by using RNA interference (RNAi) to deplete either HER2 or uPAR or both in cell lines. With depletion of both genes, MAPK signaling pathways were suppressed and there was decreased ERK activity and a high P38/ERK activity ratio. Growth suppression and induction of apoptosis were further augmented when uPAR downregulation was used in conjunction with trastuzumab. It was concluded that targeting HER2 and uPAR has a synergistic inhibitory effect on breast cancer cells.

LeBeau *et al*. [103] investigated a possible role for recombinant human anti-uPAR antibodies in treating breast cancer. *In vitro*, these antibodies exhibited strong binding to the surface of cancer cells expressing uPAR. Using the antibodies for *in vivo* imaging, they detected uPAR expression in triple-negative breast cancer (TNBC) tumor xenografts and micrometastases. When the antibodies were used for monotherapy and radioimmunoassay studies, there was significant decrease in tumor growth and tumor burden in the TNBC xenograft model. These results suggest use of such uPAR targeting for therapy of highly aggressive breast cancers. Moreover, the simultaneous targeting of both uPAR and HER2 might also be considered.

1.8 EPITHELIAL–MESENCHYMAL TRANSITION (EMT)

EMT is a conversion of an epithelial to mesenchymal phenotype that occurs during embryonic development in all vertebrates. It was first recognized as a distinct process in 1982 by Greenburg and Hay [104] and currently has been well reviewed by Bednarz-Knoll *et al.* [105] and others [106, 107]. Epithelial markers include cytokeratins 8, 18, and 19, E-cadherins, Mucin-1, occludins, and others; mesenchymal markers include vimentin, N-cadherin, F-actin, nuclear beta-catenin, and others. The mesenchymal morphology causes increased motility and thereby facilitates migration

into the circulation. Conversely, circulating mesenchymal cells can participate in the formation of epithelial organs through mesenchymal–epithelial transition (MET). This process is not well understood, for example, whether it may start in the blood or only after extravasation into the site of the future organ.

EMT may be stimulated by growth factors such as EGF or TGF-beta or transcription factors such as Twist, Snail, or Slug. Thiery [108] implicated EMT in tumor metastasis based on studies with human cancer cell lines and mouse models. This idea was extended in the stem cell hypothesis that states that the EMT-transformed stem cells are the tumor cells responsible for the development and propagation of cancer [109]. This has profound implications for the diagnosis and treatment of cancer. CTCs with stem cell markers such as CD44+, CD24-/low and ALDH1+ are frequently seen in epithelial cancers [110]. We will not discuss this important hypothesis, but there are extensive reviews evaluating the evidence [111–115].

EMT is usually demonstrated by downregulation or relocalization of E-cadherin and appearance of mesenchymal markers such as vimentin [105–107]. CTCs are detected when the size of the vimentin-positive areas within the primary tumor has increased [116]. Decreased E-cadherin expression in primary tumors also correlates with appearance of CTCs [117]. In several tumor types, the majority of CTCs were negative for E-cadherin expression and stained positive or overexpressed vimentin [117–119]. Notably, when analyzed as single cells or clusters, CTCs show heterogeneous expression of EMT-associated markers, including TGF-beta, FOXC1, ZEB1, ZEB2, Fibronectin, Twist, and others [118–122].

There is accumulating evidence that CTCs showing EMT or a cancer stem cell phenotype may have enhanced capacity for metastatic growth [123, 124]. Therefore, it is particularly important to both count and analyze such CTCs in depth. There are still several issues with counting CTCs with a mesenchymal phenotype. Some of the EMT-related markers are present on nonneoplastic cells – for example, vimentin is expressed in monocytes and activated macrophages [125]. Hence, using an epithelial marker for CTC capture is necessary, but it eliminates the possibility of detection of a CTC that may have lost some of its epithelial phenotype and displays only mesenchymal markers [126, 127]. Because there are a variety of CTCs expressing different proportions of epithelial versus mesenchymal markers [121], more studies are needed on the different combinations of mesenchymal and epithelial markers to determine the contribution of each type to the development of metastases.

This is an extremely important field with a great deal of “catching-up-to-do.” There is every reason to assume that adding mesenchymal markers to the various capture assays for CTCs under development will improve sensitivity. However, there is ample evidence that patients with TNBC, which lack expression of estrogen receptor, progesterone receptor, and HER2 and which comprise the majority of the basal-like molecular subtype, are equally likely to have elevated CTCs when measured by CellSearch as do patients with other intrinsic subtypes [128, 129]. Moreover, CTCs, when enumerated by CellSearch, are highly prognostic, suggesting that cells with metastatic potential are being counted. Nonetheless, there is sufficient evidence for redoing many of the clinical trials that used CellSearch

when an instrument employing both antiepithelial and antimesenchymal antibodies is developed that can also meet the criteria for performing multicenter trials. Indeed, the studies of Chalmers and coworkers [130] using negative selection raise new questions concerning which biomarkers of CTCs are important for identification of those that can cause metastases.

1.9 NEW INSTRUMENTS TO CAPTURE CTCs

In 2007, another major advance took place when a microfluidic platform (the CTC chip) capable of capturing CTCs was described by the Toner and Haber group [131]. In contrast to immunomagnetic purification, CTCs were captured by EpCAM-coated microposts under controlled laminar flow conditions without any preprocessing of samples. Prior theoretical analyses determined the geometric arrangement, distance between microposts, and volumetric flow rate to maximize cell attachment. The resultant CTC chip contained 78,000 microposts within a 9.7 cm^2 surface area. The advantages of this instrument included flow velocity that optimized duration of cell–micropost interaction; low shear stress to minimize damage to the CTCs; the ability to do on-chip immunostain counting and characterization of live cells; and its very small size. The instrument was able to capture CTCs in patients with different kinds of metastatic disease. Although there were major concerns about specificity that affected interest in this particular instrument, it stimulated a major effort to develop microfluidic-based instruments to capture CTCs. Improvements were then made, and a new high-throughput microfluidic mixing device, the herringbone chip (HB-Chip), providing greater specificity and sensitivity was introduced by the same group [132]. The new design used microvortices to maximize the number of interactions between CTCs and the antibody-coated chip surface. CTCs were detected in 14 of 15 (93%) patients with metastatic disease. Captured CTCs could be visualized using both immunofluorescence conjugated antibodies or standard immunocytochemical stains. More recently, this group has developed another microfluidic cell sorting device, the CTC-iChip, which uses size-based lateral displacement, inertial focusing, and magnetophoresis to achieve both antigen-dependent and antigen-independent CTC capture from whole blood. It is capable of isolating EpCAM-positive (with the ^{pos}CTC-iChip) and EpCAM-negative (with the ^{neg}CTC-iChip) CTCs [133].

Many new instruments, as well as multiple filter devices, have been produced based on differences between cancer and hematopoietic cells. The pros and cons of these are elegantly discussed by Alix-Panabières and Pantel [134] and in the following chapters of this book. It is not yet clear which approach will provide the best capture, which also will depend on specific application needs.

Another major issue is that current methods for CTC detection result in capture of tumor cells of which the majority are apoptotic [57–59]. It is important to have an alternative procedure of isolating viable CTCs that are responsible for metastases and characterizing this population. Yao *et al.* [135] placed individual CTCs in

nanowells and measured short-term viability, invasiveness, and secretory profiles. Only a rare subset showed anoikis resistance or invasive capacity; most viable cells were nonproliferative (quiescent). Paris *et al*. [136] used a novel functional cell separation method to isolate viable CTCs from patients with prostate cancer. This method relies on the ability of CTCs to invade a collagen adhesion matrix (CAM) and, hence, collects viable cells for subsequent cellular and genomic analyses. Another functional approach for counting viable CTCs is the epithelial immunospot (EPISPOT) assay, which detects target proteins (e.g., cytokeratin-19) secreted by live cells remaining in leukocyte-depleted blood [137]. In this prospective multicenter study of OS in 254 patients with MBC, OS correlated with live CTC status as determined by the EPISPOT assay; when EPISPOT and CellSearch system results were combined, there was better prediction of OS than either method alone.

Also, of particular interest is the development of a new immunomagnetic cell separator, the MagSweeper, which gently enriches live target cells from whole blood and is highly effective at eliminating any nonspecifically bound cells without significantly affecting cell viability or gene expression [138]. The MagSweeper thus enables capture of viable CTCs or DTCs whose RNA or DNA can be profiled at the single cell level [120, 139–141] or propagated in culture [139, 142]. There are multiple other recent microfluidic approaches that isolate live unlabeled CTCs, including those developed by Lim and coworkers [143], Gascoyne and coworkers [144], and Di Carlo and coworkers [145]. Also, under evaluation is an internally based EpCAM-functionalized capture wire that is inserted into a peripheral arm vein for 30 min, exposing the capture wire to 1.5–3 l of blood. Of note is that median numbers of CTCs captured from patients with breast cancer and those with nonsmall-cell lung cancer were 5.5 and 16, respectively [146].

1.10 GENOTYPIC ANALYSES

An important future goal is the development of personalized therapy for patients with primary malignant tumors or recurrent cancer. As previously emphasized, it is not known which cells from the primary tumor will cause metastases, if and when they are in the circulation at sufficient concentrations for this to occur, nor the appropriate treatment for each patient to either prevent metastases or treat metastatic disease. A critical step to answer these questions will be the genotypic, transcriptional, and/or protein signatures of the particular cells in question. CTCs are the obvious population to be so analyzed, for at the single cell level, they can show both genomic and transcriptional heterogeneity and changes over time. However, results of CTC analysis can be discordant from those in the primary tumor and distant metastases [139]. Hence, analysis of both CTCs and DTCs may provide independent clinical information relevant to treatment decisions [94, 139, 147, 148]. For example, persistence of bone marrow metastases after diagnosis of breast cancer was associated with increased risk for subsequent relapses and death [60–62]. Also, isolated DTCs can remain viable and some can be propagated in culture while maintaining the original

mutational status [139, 149, 150]. They may serve as a resource for investigating new drug therapies. Recently, improved techniques for propagating CTCs in culture represent a major step forward for facilitating both *in vitro* and *in vivo* personalized drug testing [151–153].

There are already several examples of genotypic analyses that could eventually impact drug selection on individual patients. In colorectal cancer, *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* exon 20 (but not *PIK3CA* exon 9) mutations have been established as negative predictors for treatment with EGFR inhibitors [154–156]. The first comprehensive genomic profiling of CTCs using array comparative genomic hybridization (arrayCGH) and next-generation sequencing was performed by Speicher's group [157] in primary tumors, metastases, and single CTCs from stage IV colorectal carcinoma patients; the sequencing was performed using a panel of 68 colorectal cancer-associated genes. In individual patients, similar copy number changes or driver gene mutations in the primary tumor and metastases were also identified in corresponding CTCs. However, additional mutations were detected exclusively in some CTCs (including "private mutations" defined as being found in only one CTC). Ultradeep sequencing of the primary tumor and metastases was then performed, which identified most of the private mutations as present at the subclonal level in the tumors from these same patients. These findings emphasize the importance of CTC analysis and monitoring tumor genomes that are likely to change during progression, treatment, and relapse.

A single-cell arrayCGH assay from Klein was optimized to enable reliable detection of structural copy number changes as small as 0.1 Mb in single CTCs, allowing identification of CTC heterogeneity and observation of chromosomal changes that occur over the course of treatment; in particular, this method identified the emergence of aberrant clones likely selected by therapy that may contribute to chemotherapy resistance [158].

Whole-exome sequencing of single CTCs from patients with metastatic castration-resistant prostate cancer has been recently performed using a census-based approach in a proof of principal study by Love's group that showed impressively high fidelity [141]: 70% of CTC mutations were present in multiple cores of matched tissue, and 90% of "early trunk mutations" identified in tumor tissue were detected in the CTC exomes.

Lianidou's group measured multiplex gene expression of enriched CTCs using a scalable liquid bead array assay capable of handling minute samples [159]. Another study by Sieuwerts *et al.* [160] measured the expression of highly expressed genes in pooled CTCs and demonstrated that transcriptional profiling of low numbers of CTCs within a high background of leukocytes was feasible.

High-dimensional single-cell transcriptional profiling was first performed by Jeffrey's group on individual CTCs from patients with breast cancer [120] and later by Huang and coworkers [118] in prostate cancer, both studies demonstrating CTC heterogeneity within even a single blood draw, a finding that would have been missed in pooled samples of CTCs, and leading to speculation regarding whether different populations of CTCs may require different therapies (similar to the discussion about

mixed populations of HER2-positive and -negative CTCs). Moreover, marked gene expression differences between CTCs and single cells from proliferating cell lines brought into question the "fit for purpose" of standard cancer cell lines in new drug discovery [120].

The employment of leukapheresis enabled CTC screening from large volumes of blood and identified larger numbers of CTCs, a technique that could be potentially helpful in molecularly evaluating CTCs in early disease and in defining which cells contribute to the metastatic process for determining treatment options [161].

CTCs can also be interrogated for metastasis suppressors that inhibit metastasis without blocking primary tumor growth. This is accomplished by regulating signaling pathways that inhibit proliferation, cell migration, and growth at the secondary site. One example is breast cancer metastasis suppressor-1 (*BRMS1*) [162], which differentially regulates the expression of multiple genes leading to suppression of metastases without blocking orthotropic tumor growth. Chimonidou *et al.* [163] showed that the *BRMS1* promoter is methylated in CTCs isolated from 1/3 of patients with early breast cancer and in 44% of those with metastatic disease, compared to 8.7% in healthy individuals. *BRMS1* promoter methylation results in the transcriptional repression of this metastasis suppressor gene and, when found in the primary tumor, predicts poorer disease-free survival [164]. These results indicate the potential clinical relevance of identifying this methylation event in CTCs from patients with operable breast cancer.

Another recent step forward is the identification and characterization of breast cancer CTCs that can cause brain metastasis by Zhang *et al.* [151]. By successfully culturing CTCs from patients with MBC, they developed CTC cell lines including three from patients who had brain metastases. Selecting markers HER2+/EGFR+/HPSC+/Notch1+, a high proportion metastasized to the brain and lungs after injection into immunodeficient animals, whereas parental CTCs metastasized only to the lung. Hence, there may be therapeutic interventions that can prevent CTCs from colonizing distant organs such as the brain.

Is there a role for DNA analysis in addition to the aforementioned cellular analyses? Cell-free DNA (cfDNA) or circulating tumor DNA (ctDNA) may derive from lysed tumor cells, from secretion by macrophages that have ingested necrotic cells, or potentially from CTCs themselves. Mutations associated with therapeutic resistance can be detected in ctDNA up to 10 months prior to detection by imaging; this may thereby allow clinicians to change therapy sooner [165–167]. ctDNA has also been used for early detection of cancer [168–170]. Such information is likely to supplement that obtained from CTC analysis.

However, there are issues for the future. Is ctDNA derived from dying tumor cells sensitive to a given therapy and/or derived from resistant tumor cells that persist? Another concern is determining those patient- and/or tumor-specific mutations or epigenetic alterations that should be analyzed. Finally, prior to acceptance as a technique for clinical biomarker detection, both ctDNA and CTCs will require proof of analytical validation with standardized, reproducible assays and proven clinical utility in particular patient populations, as aptly discussed by Danila *et al.* [171].

1.11 CONCLUSIONS

The potential importance of CTCs is generally accepted. The buzzword "liquid biopsy" confirms the relatively noninvasive nature of this means for monitoring or investigating the biology of both primary malignant tumors and recurrent disease. However, perhaps even more importantly, CTCs represent a critical tumor cell population for understanding metastatic potential and, eventually, determining choice of treatment for individual patients. It is likely that they would also aid in the development of novel therapies. However, the degree to which this helps the oncologist treat his or her patient at present has come into question. CTC enumeration is moderately prognostic in early-stage breast cancer but probably does not outweigh what is already known from tumor size/characteristics and evaluation of lymph node involvement. It can monitor patients for recurrence, but so can tumor markers such as CA-15.3 [172, 173] although such markers may show tumor subtype dependence [174]. There is ample evidence for the relation between CTC count and prognosis in metastatic breast, prostate, and colon cancer, with prediction of disease progression long before symptoms or imaging changes. But what is the evidence that it changes treatment or that earlier treatment is helpful? Early change in therapy based on persistently elevated CTC counts after starting a drug has not yet changed patient outcome [55]. Of course, this could be caused by ineffective treatment. Regardless, measurement of biomarkers and genotype will be needed in addition to CTC enumeration. Also, the increasing evidence that a portion of CTCs that have undergone EMT may have escaped detection by the only FDA-approved instrument for counting CTCs is a major issue. This concern is heightened by the probability that CTCs with a mesenchymal phenotype may play a major role in development of metastases. Thus, mesenchymal as well as epithelial markers must now be used to capture CTCs, which must in turn be genotypically characterized in clinical trials. Indeed, negative selection methodology for CTCs represents another important pathway for determining the biomarkers and immunophenotype of those CTCs responsible for metastatic disease.

However, even the most extensive characterization of CTCs does not necessarily solve the problem of the complexity of cancer. When will a particular cancer metastasize? The primary tumor cell population and the CTCs are extremely heterogeneous and constantly change over time. This is true also for metastatic seeding, which can be dormant for the lifetime of the patient; there may be dormant cells that then regrow, or such cells may develop metastases from the onset. Even when a treatment strategy has been developed for a particular cancer population, it may not apply for a specific patient's tumor. Jeffrey [139, 165] has stressed that additional information, such as analyses of DTCs from the bone marrow and/or ctDNA, may be used to augment in-depth genotypic analyses of CTCs to maximize the patient's specific information needed for monitoring and optimizing individual therapy in real time.

What is the potential role for a CTC assay to become a general blood test to detect cancers at an earlier stage? Progress in treating epithelial cancers over the last 60 years has been relatively modest. Patients with clinical recurrences still succumb to disease, although their lifespan can be prolonged with chemotherapy. There are a large number of new immunologically based therapies in clinical trials,

but it is too early to make efficacy predictions. It seems reasonable, therefore, to develop a diagnostic blood test as a high priority. This is not a generally accepted view for several reasons: it is argued that earlier diagnosis might not be beneficial; there can be false positives from mucosal inflammation and other factors; and localizing the organ source might be a problem. These are valid concerns but not of sufficient weight to stop the effort. Earlier diagnosis will certainly save some lives and perhaps a significant proportion; the other issues have possible solutions. The most important concern and the one less articulated is the presence of CTCs in a patient who should not be treated. The finding in breast cancer dormancy that a substantial proportion of patients many years after mastectomy who are disease-free, most of whom will live a normal lifespan, have CTCs and the evidence that the presence of breast cancer cells in the bone marrow does not necessarily lead to growing metastases over a period as long as one decade both raise this issue [56, 60–62]. Of course, the dormancy patients have had their primary tumor removed and patients with bone marrow metastases have received treatment. Nevertheless, there may be a significant proportion of patients, particularly in the elderly age group, who develop CTCs and would be best left untreated. However, this is an issue for the future when a diagnostic blood test has been developed.

The prospects are favorable for developing such a blood test. There is a myriad of new CTC assays under development. However, a recurrent problem reflected in the literature is the unstated assumption that a cell from a tumor line that has the same approximate phenotype as a CTC can be used as an accurate guide to the level of sensitivity of a particular capture procedure. CTCs are far more fragile. Many of the current instruments are overrated with regard to sensitivity. That is solely determined by CTC capture in very early primary cancers. It is too early to make a choice on a particular approach, for example, immunomagnetic, microfluidic, or the many other approaches. In the next 5 years or so, there may be sufficient clinical data to move ahead with a government-sponsored multicenter trial using an optimal instrument. In this regard, it is critical to also make full use of an optimal immunological "cocktail" [175]. The capacity to immunologically engineer antibodies with low picomolar (pM) affinity constants makes it possible to use univalent Fv antibody fragments of molecular weight 35,000. Because the affinity of divalent antibodies can be 1000–10,000-fold higher than conventional secondary antibodies from immunized animals, such univalent fragments still have strong avidity. This is a critical point because they allow combinations of antibodies to different epitopes to be used without the problems of steric hindrance that would develop if native antibodies of 150,000 molecular weight were employed. Hence, specificities to more than one epithelial, mesenchymal, and organ-specific marker can be tried on the same specimen in various combinations and in different time sequences. Deng *et al.* [176] have shown that antibody to intracellular cytokeratin (CK) together with anti-EpCAM antibodies increases assay sensitivity. It is also possible to attach a short polypeptide to the polypeptide holding the Fv light (L)- and heavy (H)-chain antigen-combining regions

together, thereby increasing the Fv's potential range of attachment to the corresponding epitopes on the CTCs [175]. Such an arrangement could be likened to individual arms of an "immunological octopus."

Nonimmunoglobulin molecules with a high degree of specificity and picomolar affinities can be engineered from either polypeptides (protein scaffolds) [177] or nucleic acids (aptamers) [178]. The latter can be as small as 19 nucleotides with 55 pM affinity to its specific antigen [179]. Protein scaffolds with pM affinities have been produced to EpCAM [180], EGFR2/HER2 [181], and HER2, the latter being a 6 kDa Affibody [182]. Combinations between antibodies and scaffolds have also been produced, for example, a llama H chain variable domain fragment and a non-Ig scaffold protein resulted in a protein with an affinity of 0.54 pM to its specific antigen [183]. The aforementioned changes to the immunological portion of the assays should help to increase their sensitivity.

This is a particularly exciting time for scientists studying CTCs. There are opportunities for obtaining large amounts of new data concerning the genomes and potential behavior of these cells and for utilizing that information to better understand the metastatic cascade. At the same time, it is necessary to redefine the criteria and instrumentation for capturing and enumerating CTCs and to determine how this new knowledge should be funneled into particular clinical trials designed to improve diagnosis and therapy. The latter will include a host of recently developed immunologic- and inflammatory-specific cancer drugs that together with earlier diagnosis could result in a major advance in treatment.

REFERENCES

1. Slavin, S. and Strober, S. (1978) Spontaneous murine B-cell leukaemia. *Nature*, **272**, 624–626.
2. Krolick, K.A., Isakson, P.C., Uhr, J.W., and Vitetta, E.S. (1979) BCL1, a murine model for chronic lymphocytic leukemia: use of the surface immunoglobulin idiotype for the detection and treatment of tumor. *Immunological Reviews*, **48**, 81–106.
3. Knapp, M.R., Jones, P.P., Black, S.J. *et al.* (1979) Characterization of a spontaneous murine B cell leukemia (BCL1). I. Cell surface expression of IgM, IgD, Ia, and FcR. *Journal of Immunology*, **123**, 992–999.
4. Uhr, J.W., Tucker, T., May, R.D. *et al.* (1991) Cancer dormancy: studies of the murine BCL1 lymphoma. *Cancer Research*, **51**, 5045s–5053s.
5. Vitetta, E.S., Tucker, T.F., Racila, E. *et al.* (1997) Tumor dormancy and cell signaling. V. Regrowth of BCL1 tumor after dormancy is established. *Blood*, **89**, 4425–4436.
6. Scheuermann, R.H., Racila, E., Tucker, T. *et al.* (1994) Lyn tyrosine kinase signals cell cycle arrest but not apoptosis in B-lineage lymphoma cells. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 4048–4052.
7. Meltzer, A. (1990) Dormancy and breast cancer. *Journal of Surgical Oncology*, **43**, 181–188.
8. Stewart, T.H., Hollinshead, A.C., and Raman, S. (1991) Tumour dormancy: initiation, maintenance and termination in animals and humans. *Canadian Journal of Surgery*, **34**, 321–325.

9. Demichelli, R., Abbattista, A., Miceli, R. *et al.* (1996) Time distribution of the recurrence risk for breast cancer patients undergoing mastectomy: further support about the concept of tumor dormancy. *Breast Cancer Research and Treatment*, **41**, 177–185.
10. George, A.J. and Stevenson, F.K. (1989) Prospects for the treatment of B cell tumors using idiotypic vaccination. *International Reviews of Immunology*, **4**, 271–310.
11. Stevenson, F.K., George, A.J., and Glennie, M.J. (1990) Anti-idiotypic therapy of leukemias and lymphomas. *Chemical Immunology*, **48**, 126–166.
12. Meeker, T., Lowder, J., Cleary, M.L. *et al.* (1985) Emergence of idiotype variants during treatment of B-cell lymphoma with anti-idiotype antibodies. *New England Journal of Medicine*, **312**, 1658–1665.
13. Levy, R. and Miller, R.A. (1990) Therapy of lymphoma directed at idiotypes. *Journal of the National Cancer Institute Monographs*, **10**, 61–68.
14. Miller, R.A., Maloney, D.G., Warnke, R., and Levy, R. (1982) Treatment of B-cell lymphoma with monoclonal anti-idiotype antibody. *New England Journal of Medicine*, **306**, 517–522.
15. Karrison, T.G., Ferguson, D.J., and Meier, P. (1999) Dormancy of mammary carcinoma after mastectomy. *Journal of the National Cancer Institute*, **91**, 80–85.
16. Ashworth, T. (1869) A case of cancer in which cells similar to those in the tumours were seen in the blood after death. *Australian Medical Journal*, **14**, 146–147.
17. Christopherson, W.M. (1965) Cancer cells in the peripheral blood: a second look. *Acta Cytologica*, **9**, 169–174.
18. Drye, J.C., Rumage, W.T. Jr., and Anderson, D. (1962) Prognostic import of circulating cancer cells after curative surgery: a long time follow up study. *Annals of Surgery*, **155**, 733–740.
19. Engell, H.C. (1955) Cancer cells in the circulating blood; a clinical study on the occurrence of cancer cells in the peripheral blood and in venous blood draining the tumour area at operation. *Acta Chirurgica Scandinavica. Supplementum*, **201**, 1–70.
20. Fidler, I.J. (1973) The relationship of embolic homogeneity, number, size and viability to the incidence of experimental metastasis. *European Journal of Cancer*, **9**, 223–227.
21. Fidler, I.J. (1978) Tumor heterogeneity and the biology of cancer invasion and metastasis. *Cancer Research*, **38**, 2651–2660.
22. Pantel, K., Schlimok, G., Braun, S. *et al.* (1993) Differential expression of proliferation-associated molecules in individual micrometastatic carcinoma cells. *Journal of the National Cancer Institute*, **85**, 1419–1424.
23. Pantel, K., Izbicki, J., Passlick, B. *et al.* (1996) Frequency and prognostic significance of isolated tumour cells in bone marrow of patients with non-small-cell lung cancer without overt metastases. *Lancet*, **347**, 649–653.
24. Folkman, J. (1971) Tumor angiogenesis: therapeutic implications. *New England Journal of Medicine*, **285**, 1182–1186.
25. Folkman, J., Watson, K., Ingber, D., and Hanahan, D. (1989) Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature*, **339**, 58–61.
26. Liotta, L.A., Steeg, P.S., and Stetler-Stevenson, W.G. (1991) Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell*, **64**, 327–336.
27. Liotta, L.A., Kleinerman, J., and Saidel, G.M. (1974) Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. *Cancer Research*, **34**, 997–1004.

28. Ghossein, R.A., Bhattacharya, S., and Rosai, J. (1999) Molecular detection of micrometastases and circulating tumor cells in solid tumors. *Clinical Cancer Research*, **5**, 1950–1960.
29. Hardingham, J.E., Kotasek, D., Farmer, B. *et al.* (1993) Immunobead-PCR: a technique for the detection of circulating tumor cells using immunomagnetic beads and the polymerase chain reaction. *Cancer Research*, **53**, 3455–3458.
30. Hardingham, J.E., Kotasek, D., Sage, R.E. *et al.* (1995) Detection of circulating tumor cells in colorectal cancer by immunobead-PCR is a sensitive prognostic marker for relapse of disease. *Molecular Medicine*, **1**, 789–794.
31. Wong, L.S., Bateman, W.J., Morris, A.G., and Fraser, I.A. (1995) Detection of circulating tumor cells with the magnetic activated cell sorter. *British Journal of Surgery*, **82**, 1333–1337.
32. Makarovskiy, A.N., Ackerley, W. 3rd, Wojcik, L. *et al.* (1997) Application of immunomagnetic beads in combination with RT-PCR for the detection of circulating prostate cancer cells. *Journal of Clinical Laboratory Analysis*, **11**, 346–350.
33. Martin, V.M., Siewert, C., Scharl, A. *et al.* (1998) Immunomagnetic enrichment of disseminated epithelial tumor cells from peripheral blood by MACS. *Experimental Hematology*, **26**, 252–264.
34. Shibata, K., Mori, M., Kitano, S., and Akiyoshi, T. (1998) Detection of ras gene mutations in peripheral blood of carcinoma patients using CD45 immunomagnetic separation and nested mutant allele specific amplification. *International Journal of Oncology*, **12**, 1333–1338.
35. Racila, E., Euhus, D., Weiss, A.J. *et al.* (1998) Detection and characterization of carcinoma cells in the blood. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 4589–4594.
36. Liberti, P.A. and Feeley, B. (1991) Analytical- and process-scale cell separation with bioreceptor ferrofluids and high-gradient magnetic separation, in *Cell Separation Science and Technology* (eds D.S. Kompala and P. Todd), American Chemical Society, Washington, DC, pp. 268–288.
37. Kemshead, J.T., Hancock, J.P., and Liberti, P.A. (1994) A model system for the enrichment of tumor cells from peripheral blood and bone marrow using immunomagnetic ferrofluids. *Progress in Clinical and Biological Research*, **389**, 593–600.
38. Terstappen, L.W., Meiners, H., and Loken, M.R. (1989) A rapid sample preparation technique for flow cytometric analysis of immunofluorescence allowing absolute enumeration of cell subpopulations. *Journal of Immunological Methods*, **123**, 103–112.
39. Fehm, T., Morrison, L., Saboorian, H. *et al.* (2002) Patterns of aneusomy for three chromosomes in individual cells from breast cancer. *Breast Cancer Research and Treatment*, **75**, 227–239.
40. Fehm, T., Sagalowsky, A., Clifford, E. *et al.* (2002) Cytogenetic evidence that circulating epithelial cells in patients with carcinoma are malignant. *Clinical Cancer Research*, **8**, 2073–2084.
41. Witzig, T.E., Bossy, B., Kimlinger, T. *et al.* (2002) Detection of circulating cytokeratin-positive cells in the blood of breast cancer patients using immunomagnetic enrichment and digital microscopy. *Clinical Cancer Research*, **8**, 1085–1091.
42. Ulmer, A., Schmidt-Kittler, O., Fischer, J. *et al.* (2004) Immunomagnetic enrichment, genomic characterization, and prognostic impact of circulating melanoma cells. *Clinical Cancer Research*, **10**, 531–537.

43. Mangan, K.F., Leonardo, J., Mullaney, M.T. *et al.* (1999) A rapid two-step method for elimination of bcl-2/IgH positive non-Hodgkin's lymphoma cells from human blood or marrow stem cells, employing immunomagnetic purging with streptavidin-coated ferrofluids. *Cytotherapy*, **1**, 287–293.
44. Liberti, P.A., Rao, C.G., and Terstappen, L.W.M.M. (2001) Optimization of ferrofluids and protocols for the enrichment of breast tumor cells in blood. *J. Magnetism Magnetic Materials*, **225**, 301–307.
45. Tibbe, A.G., de Grooth, B.G., Greve, J. *et al.* (2001) Cell analysis system based on immunomagnetic cell selection and alignment followed by immunofluorescent analysis using compact disk technologies. *Cytometry*, **43**, 31–37.
46. Allard, W.J., Matera, J., Miller, M.C. *et al.* (2004) Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clinical Cancer Research*, **10**, 6897–6904.
47. Cristofanilli, M., Hayes, D.F., Budd, G.T. *et al.* (2005) Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. *Journal of Clinical Oncology*, **23**, 1420–1430.
48. Hayes, D.F., Cristofanilli, M., Budd, G.T. *et al.* (2006) Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. *Clinical Cancer Research*, **12**, 4218–4224.
49. Budd, G.T., Cristofanilli, M., Ellis, M.J. *et al.* (2006) Circulating tumor cells versus imaging – predicting overall survival in metastatic breast cancer. *Clinical Cancer Research*, **12**, 6403–6409.
50. Cohen, S.J., Punt, C.J., Iannotti, N. *et al.* (2009) Prognostic significance of circulating tumor cells in patients with metastatic colorectal cancer. *Annals of Oncology*, **20**, 1223–1229.
51. de Bono, J.S., Scher, H.I., Montgomery, R.B. *et al.* (2008) Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. *Clinical Cancer Research*, **14**, 6302–6309.
52. Hodgkinson, C.L., Morrow, C.J., Li, Y. *et al.* (2014) Tumorigenicity and genetic profiling of circulating tumor cells in small-cell lung cancer. *Nature Medicine*, **20**, 897–903.
53. Hou, J.M., Krebs, M.G., Lancashire, L. *et al.* (2012) Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer. *Journal of Clinical Oncology*, **30**, 525–532.
54. Krebs, M.G., Sloane, R., Priest, L. *et al.* (2011) Evaluation and prognostic significance of circulating tumor cells in patients with non-small-cell lung cancer. *Journal of Clinical Oncology*, **29**, 1556–1563.
55. Smerage, J.B., Barlow, W.E., Hortobagyi, G.N. *et al.* (2014) Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. *Journal of Clinical Oncology*, **32**, 3483–3489.
56. Meng, S., Tripathy, D., Frenkel, E.P. *et al.* (2004) Circulating tumor cells in patients with breast cancer dormancy. *Clinical Cancer Research*, **10**, 8152–8162.
57. Méhes, G., Witt, A., Kubista, E., and Ambrose, P.F. (2001) Circulating breast cancer cells are frequently apoptotic. *American Journal of Pathology*, **159**, 17–20.
58. Smerage, J.B., Budd, G.T., Doyle, G.V. *et al.* (2013) Monitoring apoptosis and Bcl-2 on circulating tumor cells in patients with metastatic breast cancer. *Molecular Oncology*, **7**, 680–692.

59. Kallergi, G., Konstantinidis, G., Markomanolaki, H. *et al.* (2013) Apoptotic circulating tumor cells in early and metastatic breast cancer patients. *Molecular Cancer Therapeutics*, **12**, 1886–1895.

60. Pantel, K., Müller, V., Auer, M. *et al.* (2003) Detection and clinical implications of early systemic tumor cell dissemination in breast cancer. *Clinical Cancer Research*, **9**, 6326–6334.

61. Wiedswang, G., Borgen, E., Kåresen, R. *et al.* (2004) Isolated tumor cells in bone marrow three years after diagnosis in disease-free breast cancer patients predict unfavorable clinical outcome. *Clinical Cancer Research*, **10**, 5342–5348.

62. Klein, C.A. (2009) Parallel progression of primary tumors and metastases. *Nature Reviews Cancer*, **9**, 302–312.

63. Thomas, L. (1959) Reactions to homologous tissue antigens in relations to hypersensitivity, in *Cellular and Humoral Aspects of the Hypersensitive States* (ed H.S. Lawrence), Hoeber-Harper, New York, p. 529.

64. Burnet, F.M. (1970) The concept of immune surveillance. *Progress in Experimental Tumor Research*, **13**, 1–27.

65. Gimbrone, M.A., Leapman, S.B., Cotran, R.S., and Folkman, J. (1972) Tumor dormancy *in vivo* by prevention of neovascularization. *Journal of Experimental Medicine*, **136**, 261–276.

66. Aguirre-Ghiso, J.A. (2007) Models, mechanisms and clinical evidence for cancer dormancy. *Nature Reviews Cancer*, **7**, 834–846.

67. Uhr, J.W. and Pantel, K. (2011) Controversies in clinical cancer dormancy. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 12396–12400.

68. Conlon, I. and Raff, M. (1999) Size control in animal development. *Cell*, **96**, 235–244.

69. Day, S.J. and Lawrence, P.A. (2000) Measuring dimensions: the regulation of size and shape. *Development*, **127**, 2977–2987.

70. Michalopoulous, G.K. and DeFrances, M.C. (1997) Liver regeneration. *Science*, **276**, 60–66.

71. Verdu, J., Buratovich, M.A., Wilder, E.L., and Birnbaum, M.J. (1999) Cell-autonomous regulation of cell and organ growth in Drosophila by Akt/PKB. *Nature Cell Biology*, **1**, 500–506.

72. Zaina, S. and Squire, S. (1998) The soluble type 2 insulin-like growth factor (IGF-II) receptor reduces organ size by IFG-II-mediated and IGF-II-independent mechanisms. *Journal of Biological Chemistry*, **273**, 28610–28616.

73. Böhni, R., Riesgo-Escovar, J., Oldham, S. *et al.* (1999) Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1-4. *Cell*, **97**, 865–875.

74. Gao, X., Zhang, Y., Arrazola, P. *et al.* (2002) Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. *Nature Cell Biology*, **4**, 699–704.

75. Pan, D. (2007) Hippo signaling in organ size control. *Genes and Development*, **21**, 886–897.

76. Harvey, K. and Tapon, N. (2007) The Salvador-Warts-Hippo pathway - an emerging tumour-suppressor network. *Nature Reviews Cancer*, **7**, 182–191.

77. Wang, Z., Wu, Y., Wang, H. *et al.* (2014) Interplay of mevalonate and Hippo pathways regulate RHAMM transcription via YAP to modulate breast cancer cell motility. *Proceedings of the National Academy of Sciences of the United States of America*, **111**, E89–98.

78. Demierre, M.F., Higgins, P.D., Gruber, S.B. *et al.* (2005) Statins and cancer prevention. *Nature Reviews Cancer*, **5**, 930–942.

79. Clendening, J.W. and Penn, L.Z. (2012) Targeting tumor cell metabolism with statins. *Oncogene*, **31**, 4967–4978.

80. Pujana, M.A., Han, J.D., Starita, L.M. *et al.* (2007) Network modeling links breast cancer susceptibility and centrosome dysfunction. *Nature Genetics*, **39**, 1338–1349.

81. Maxwell, C.A., Benítez, J., Gómez-Baldó, L. *et al.* (2011) Interplay between BRCA1 and RHAMM regulates epithelial apicobasal polarization and may influence risk of breast cancer. *PLoS Biology*, **9**, e1001199.

82. Wang, C., Thor, A.D., Moore, D.H. 2nd *et al.* (1998) The overexpression of RHAMM, a hyaluronan-binding protein that regulates ras signaling, correlates with overexpression of mitogen-activated protein kinase and is a significant parameter in breast cancer progression. *Clinical Cancer Research*, **4**, 567–576.

83. Velseh, M. and Turley, E.A. (2011) Hyaluronan metabolism in remodeling extracellular matrix: probes for imaging and therapy of breast cancer. *Integrative Biology*, **3**, 304–315.

84. Slamon, D.J., Clark, G.M., Wong, S.G. *et al.* (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*, **235**, 177–182.

85. Romond, E.H., Perez, E.A., Bryant, J. *et al.* (2005) Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *New England Journal of Medicine*, **353**, 1673–1684.

86. Piccart-Gebhart, M.J., Procter, M., Leyland-Jones, B. *et al.* (2005) Herceptin Adjuvant (HERA) Trial Study Team. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *New England Journal of Medicine*, **353** (16), 1659–1672.

87. Sekido, Y., Umemura, S., Takekoshi, S. *et al.* (2003) Heterogeneous gene alterations in primary breast cancer contribute to discordance between primary and asynchronous metastatic/recurrent sites: HER2 gene amplification and p53 mutation. *International Journal of Oncology*, **22**, 1225–1232.

88. Houssami, N., Macaskill, P., Balleine, R.L. *et al.* (2011) HER2 discordance between primary breast cancer and its paired metastasis: tumor biology or test artefact? Insights through meta-analysis. *Breast Cancer Research and Treatment*, **129**, 659–674.

89. Wülfing, P., Borchard, J., Buerger, H. *et al.* (2006) HER2-positive circulating tumor cells indicate poor clinical outcome in stage I to III breast cancer patients. *Clinical Cancer Research*, **12**, 1715–1720.

90. Meng, S., Tripathy, D., Shete, S. *et al.* (2004) HER-2 gene amplification can be acquired as breast cancer progresses. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 9393–9398.

91. Fehm, T., Müller, V., Aktas, B. *et al.* (2010) HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective multicenter trial. *Breast Cancer Research and Treatment*, **124**, 403–412.

92. Riethdorf, S., Müller, V., Zhang, L. *et al.* (2010) Detection and HER2 expression of circulating tumor cells: prospective monitoring in breast cancer patients treated in the neoadjuvant GeparQuattro trial. *Clinical Cancer Research*, **16**, 2634–2645.

93. Pestrin, M., Bessi, S., Galardi, F. *et al.* (2009) Correlation of HER2 status between primary tumors and corresponding circulating tumor cells in advanced breast cancer patients. *Breast Cancer Research and Treatment*, **118**, 523–530.

94. Krishnamurthy, S., Bischoff, F., Ann Mayer, J. *et al.* (2013) Discordance in HER2 gene amplification in circulating and disseminated tumor cells in patients with operable breast cancer. *Cancer Medicine*, **2**, 226–233.
95. Iwatsuki, M., Toyoshima, K., Watanabe, M. *et al.* (2013) Frequency of HER2 expression of circulating tumour cells in patients with metastatic or recurrent gastrointestinal cancer. *British Journal of Cancer*, **109**, 2829–2832.
96. Paik, S., Kim, C., and Wolmark, N. (2008) Her2 status and benefit from adjuvant trastuzumab in breast cancer. *New England Journal of Medicine*, **358**, 1409–1411.
97. Ithimakin, S., Day, K.C., Malik, F. *et al.* (2013) HER2 drives luminal breast cancer stem cells in the absence of HER2 amplification: implications for efficacy of adjuvant trastuzumab. *Cancer Research*, **73**, 1635–1646.
98. Sidenius, N. and Blasi, F. (2003) The urokinase plasminogen activator system in cancer: recent advances and implication for prognosis and therapy. *Cancer Metastasis Reviews*, **22**, 205–222.
99. Hemsen, A., Riethdorf, L., Brünner, N. *et al.* (2003) Comparative evaluation of urokinase-type plasminogen activator receptor expression in primary breast carcinomas and on metastatic tumor cells. *International Journal of Cancer*, **107**, 903–909.
100. Riisbro, R., Christensen, I.J., Piironen, T. *et al.* (2002) Prognostic significance of soluble urokinase plasminogen activator receptor in serum and cytosol of tumor tissue from patients with primary breast cancer. *Clinical Cancer Research*, **5**, 1132–1141.
101. Meng, S., Tripathy, D., Shete, S. *et al.* (2006) uPar and HER-2 gene status in individual breast cancer cells from blood and tissues. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 17361–17365.
102. Li, C., Cao, S., Liu, Z. *et al.* (2010) RNAi-mediated downregulation of uPAR synergizes with targeting of HER2 through the ERK pathway in breast cancer cells. *International Journal of Cancer*, **127**, 1507–1516.
103. LeBeau, A.M., Duriseti, S., Murphy, S.T. *et al.* (2013) Targeting uPAR with antagonistic recombinant human antibodies in aggressive breast cancer. *Cancer Research*, **73**, 2070–2081.
104. Greenburg, G. and Hay, E.D. (1982) Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. *Journal of Cell Biology*, **95**, 333–339.
105. Bednarz-Knoll, N., Alix-Panabières, C., and Pantel, K. (2012) Plasticity of disseminating cancer cells in patients with epithelial malignancies. *Cancer and Metastasis Reviews*, **31**, 673–687.
106. Thompson, E.W., Newgreen, D.F., and Tarin, D. (2005) Carcinoma invasion and metastasis: a role for epithelial-mesenchymal transition? *Cancer Research*, **65**, 5991–5995.
107. Polyak, K. and Weinberg, R.A. (2009) Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nature Reviews Cancer*, **9**, 265–273.
108. Thiery, J.P. (2002) Epithelial-mesenchymal transitions in tumour progression. *Nature Reviews Cancer*, **2**, 442–454.
109. Wicha, M.S., Liu, S., and Dontu, G. (2006) Cancer stem cells: an old idea – a paradigm shift. *Cancer Research*, **66** (4), 1883–1890.
110. Kasimir-Bauer, S., Hoffmann, O., Wallwiener, D. *et al.* (2012) Expression of stem cell and epithelial-mesenchymal transition markers in primary breast cancer patients with circulating tumor cells. *Breast Cancer Research*, **14**, R15.

111. Krawczyk, N., Meier-Stiegen, F., Banys, M. *et al.* (2014) Expression of stem cell and epithelial-mesenchymal transition markers in circulating tumor cells of breast cancer patients. *Biomed Research International*, **2014**, 415721.
112. Gupta, P.B., Chaffer, C.L., and Weinberg, R.A. (2009) Cancer stem cells: mirage or reality? *Nature Medicine*, **15**, 1010–1012.
113. Farnie, G. and Clarke, R.B. (2007) Mammary stem cells and breast cancer – role of Notch signalling. *Stem Cell Reviews*, **3**, 169–175.
114. Lathia, J.D., Venere, M., Rao, M.S., and Rich, J.N. (2011) Seeing is believing: are cancer stem cells the Loch Ness monster of tumor biology? *Stem Cell Reviews*, **7**, 227–237.
115. Patel, P. and Chen, E.l. (2012) Cancer stem cells, tumor dormancy, and metastasis. *Front Endocrinology*, **3**, 125.
116. Bonnomet, A., Syne, L., Brysse, A. *et al.* (2012) A dynamic *in vivo* model of epithelial-to-mesenchymal transitions in circulating tumor cells and metastases of breast cancer. *Oncogene*, **31**, 3741–3753.
117. Loric, S., Paradis, V., Gala, J.L. *et al.* (2001) Abnormal E-cadherin expression and prostate cell blood dissemination as markers of biological recurrences in cancer. *European Journal of Cancer*, **37**, 1475–1481.
118. Chen, C.L., Mahalingam, D., Osmulski, P. *et al.* (2013) Single-cell analysis of circulating tumor cells identifies cumulative expression patterns of EMT-related genes in metastatic prostate cancer. *Prostate*, **73**, 813–826.
119. Hou, J.M., Krebs, M., Ward, T. *et al.* (2011) Circulating tumor cells as a window on metastasis biology in lung cancer. *American Journal of Pathology*, **178**, 989–996.
120. Powell, A.A., Talasaz, A.H., Zhang, H. *et al.* (2012) Single cell profiling of circulating tumor cells: transcriptional heterogeneity and diversity from breast cancer cell lines. *PloS One*, **7**, e33788.
121. Yu, M., Bardia, A., Wittner, B.S. *et al.* (2013) Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science*, **339**, 580–584.
122. Kallergi, G., Papadaki, M.A., Politaki, E. *et al.* (2011) Epithelial to mesenchymal transition markers expressed in circulating tumour cells of early and metastatic breast cancer patients. *Breast Cancer Research*, **13**, R59.
123. Raimondi, C., Gradilone, A., Naso, G. *et al.* (2011) Epithelial-mesenchymal transition and stemness features in circulating tumor cells from breast cancer patients. *Breast Cancer Research and Treatment*, **130**, 449–455.
124. Gradilone, A., Naso, G., Raimondi, C. *et al.* (2011) Circulating tumor cells (CTCs) in metastatic breast cancer (MBC): prognosis, drug resistance and phenotypic characterization. *Annals of Oncology*, **22**, 86–92.
125. Mor-Vaknin, N., Punturieri, A., Sitwala, K., and Markovitz, D.M. (2003) Vimentin is secreted by activated macrophages. *Nature Cell Biology*, **5**, 59–63.
126. Königsberg, R., Obermayr, E., Bises, G. *et al.* (2011) Detection of EpCAM positive and negative circulating tumor cells in metastatic breast cancer patients. *Acta Oncologica*, **50**, 700–710.
127. Gorges, T.M., Tinhofer, I., Drosch, M. *et al.* (2012) Circulating tumour cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. *BMC Cancer*, **12**, 178.
128. Peeters, D.J., van Dam, P.J., Van den Eynden, G.G. *et al.* (2014) Detection and prognostic significance of circulating tumour cells in patients with metastatic breast cancer according to immunohistochemical subtypes. *British Journal of Cancer*, **110**, 375–383.

129. Wallwiener, M., Hartkopf, A.D., Baccelli, I. *et al.* (2013) The prognostic impact of circulating tumor cells in subtypes of metastatic breast cancer. *Breast Cancer Research and Treatment*, **137**, 503–510.

130. Yang, L., Lang, J.C., Balasubramanian, P. *et al.* (2009) Optimization of an enrichment process for circulating tumor cells from the blood of head and neck cancer patients through depletion of normal cells. *Biotechnology and Bioengineering*, **102**, 521–534.

131. Nagrath, S., Sequist, L.V., Maheswaran, S. *et al.* (2007) Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature*, **450**, 1235–1239.

132. Stott, S.L., Hsu, C.H., Tsukrov, D.I. *et al.* (2010) Isolation of circulating tumor cells using a microvortex-generating herringbone-chip. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 18392–18397.

133. Karabacak, N.M., Spuhler, P.S., Fachin, F. *et al.* (2014) Microfluidic, marker-free isolation of circulating tumor cells from blood samples. *Nature Protocols*, **9**, 694–710.

134. Alix-Panabières, C. and Pantel, K. (2014) Technologies for detection of circulating tumor cells: facts and vision. *Lab on a Chip*, **14**, 57–62.

135. Yao, X., Choudhury, A.D., Yamanaka, Y.J. *et al.* (2014) Functional analysis of single cells identifies a rare subset of circulating tumor cells with malignant traits. *Integrative Biology*, **6**, 388–398.

136. Paris, P.L., Kobayashi, Y., Zhao, Q. *et al.* (2009) Functional phenotyping and genotyping of circulating tumor cells from patients with castration resistant prostate cancer. *Cancer Letters*, **277**, 164–173.

137. Ramirez, J.M., Fehm, T., Orsini, M. *et al.* (2014) Prognostic relevance of viable circulating tumor cells detected by EPISPOT in metastatic breast cancer patients. *Clinical Chemistry*, **60**, 214–221.

138. Talasaz, A.H., Powell, A.A., Huber, D.E. *et al.* (2009) Isolating highly enriched populations of circulating epithelial cells and other rare cells from blood using a magnetic sweeper device. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 3970–3975.

139. Deng, G., Krishnakumar, S., Powell, A.A. *et al.* (2014) Single cell mutational analysis of PIK3CA in circulating tumor cells and metastases in breast cancer reveals heterogeneity, discordance, and mutation persistence in cultured disseminated tumor cells from bone marrow. *BMC Cancer*, **14**, 456.

140. Cann, G.M., Gulzar, Z.G., Cooper, S. *et al.* (2012) mRNA-Seq of single prostate cancer circulating tumor cells reveals recapitulation of gene expression and pathways found in prostate cancer. *PloS One*, **7**, e49144.

141. Lohr, J.G., Adalsteinsson, V.A., Cibulskis, K. *et al.* (2014) Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer. *Nature Biotechnology*, **32**, 479–484.

142. Ameri, K., Luong, R., Zhang, H. *et al.* (2010) Circulating tumour cells demonstrate an altered response to hypoxia and an aggressive phenotype. *British Journal of Cancer*, **102**, 561–569.

143. Khoo, B.L., Warkiani, M.E., Tan, D.S. *et al.* (2014) Clinical validation of an ultra high-throughput spiral microfluidics for the detection and enrichment of viable circulating tumor cells. *PloS One*, **9**, e99409.

144. Shim, S., Stemke-Hale, K., Tsimberidou, A.M. *et al.* (2013) Antibody-independent isolation of circulating tumor cells by continuous-flow dielectrophoresis. *Biomicrofluidics*, **7**, 11807.

145. Sollier, E., Go, D.E., Che, J. *et al.* (2014) Size-selective collection of circulating tumor cells using Vortex technology. *Lab on a Chip*, **14**, 63–77.

146. Saucedo-Zeni, N., Mewes, S., Niestroj, R. *et al.* (2012) A novel method for the in vivo isolation of circulating tumor cells from peripheral blood of cancer patients using a functionalized and structured medical wire. *International Journal of Oncology*, **41**, 1241–1250.

147. Wiedswang, G., Borgen, E., Schirmer, C. *et al.* (2006) Comparison of the clinical significance of occult tumor cells in blood and bone marrow in breast cancer. *International Journal of Cancer*, **118**, 2013–2019.

148. Fehm, T., Hoffmann, O., Aktas, B. *et al.* (2009) Detection and characterization of circulating tumor cells in blood of primary breast cancer patients by RT-PCR and comparison to status of bone marrow disseminated cells. *Breast Cancer Research*, **11**, R59.

149. Ross, A.A., Cooper, B.W., Lazarus, H.M. *et al.* (1993) Detection and viability of tumor cells in peripheral blood stem cell collections from breast cancer patients using immunocytochemical and clonogenic assay techniques. *Blood*, **82**, 2605–2610.

150. Solakoglu, O., Maierhofer, C., Lahr, G. *et al.* (2002) Heterogeneous proliferative potential of occult metastatic cells in bone marrow of patients with solid epithelial tumors. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 2246–2251.

151. Zhang, L., Ridgway, L.D., Wetzel, M.D. *et al.* (2013) The identification and characterization of breast cancer CTCs competent for brain metastasis. *Science Translational Medicine*, **5**, 180ra48.

152. Pizon, M., Zimon, D., Carl, S. *et al.* (2013) Heterogeneity of circulating epithelial tumour cells from individual patients with respect to expression profiles and clonal growth (sphere formation) in breast cancer. *Ecancermedicalscience*, **7**, 343.

153. Yu, M., Bardia, A., Aceto, N. *et al.* (2014) Cancer therapy. Ex vivo culture of circulating breast tumor cells for individualized testing of drug susceptibility. *Science*, **345**, 216–220.

154. Misale, S., Yaeger, R., Hobor, S. *et al.* (2012) Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature*, **486**, 532–536.

155. Diaz, L.A. Jr., Williams, R.T., Wu, J. *et al.* (2012) The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature*, **486**, 537–540.

156. De Roock, W., Claes, B., Bernasconi, D. *et al. et al.* (2010) Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncology*, **11**, 753–762.

157. Heitzer, E., Auer, M., Gasch, C. *et al.* (2013) Complex tumor genomes inferred from single circulating tumor cells by array-CGH and next-generation sequencing. *Cancer Research*, **73**, 2965–2975.

158. Czyż, Z.T., Hoffmann, M., Schlimok, G. *et al.* (2014) Reliable single cell array CGH for clinical samples. *PloS One*, **9**, e85907.

159. Markou, A., Strati, A., Malamos, N. *et al.* (2011) Molecular characterization of circulating tumor cells in breast cancer by a liquid bead array hybridization assay. *Clinical Chemistry*, **57**, 421–430.

160. Sieuwerts, A.M., Mostert, B., Bolt-de Vries, J. *et al.* (2011) mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients. *Clinical Cancer Research*, **17**, 3600–3618.

161. Fischer, J.C., Niederacher, D., Topp, S.A. *et al.* (2013) Diagnostic leukapheresis enables reliable detection of circulating tumor cells of nonmetastatic cancer patients. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 16580–16585.

162. Seraj, M.J., Samant, R.S., Verderame, M.F., and Welch, D.R. (2000) Functional evidence for a novel human breast carcinoma metastasis suppressor, BRMS1, encoded at chromosome 11q13. *Cancer Research*, **60**, 2764–2769.

163. Chimonidou, M., Strati, A., Tzitzira, A. *et al.* (2011) DNA methylation of tumor suppressor and metastasis suppressor genes in circulating tumor cells. *Clinical Chemistry*, **57**, 1169–1177.

164. Chimonidou, M., Kallergi, G., Georgoulias, V. *et al.* (2013) Breast cancer metastasis suppressor-1 promoter methylation in primary breast tumors and corresponding circulating tumor cells. *Molecular Cancer Research*, **11**, 1248–1257.

165. Kidess, E. and Jeffrey, S.S. (2013) Circulating tumor cells versus tumor-derived cell-free DNA: rivals or partners in cancer care in the era of single-cell analysis? *Genome Medicine*, **5**, 70.

166. Murtaza, M., Dawson, S.J., Tsui, D.W. *et al.* (2013) Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature*, **497**, 108–112.

167. Dawson, S.J., Tsui, D.W., Murtaza, M. *et al.* (2013) Analysis of circulating tumor DNA to monitor metastatic breast cancer. *New England Journal of Medicine*, **368**, 1199–1209.

168. Schwarzenbach, H., Hoon, D.S., and Pantel, K. (2011) Cell-free nucleic acids as biomarkers in cancer patients. *Nature Reviews Cancer*, **11**, 426–437.

169. Bettegowda, C., Sausen, M., Leary, R.J. *et al.* (2014) Detection of circulating tumor DNA in early- and late-stage human malignancies. *Science Translational Medicine*, **6**, 224ra24.

170. Newman, A.M., Bratman, S.V., To, J. *et al.* (2014) An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. *Nature Medicine*, **20**, 548–554.

171. Danila, D.C., Pantel, K., Fleisher, M., and Scher, H.I. (2011) Circulating tumors cells as biomarkers: progress toward biomarker qualification. *Cancer Journal*, **17**, 438–450.

172. Barak, V., Carlin, D., Sulkes, A. *et al.* (1988) CA15-3 serum levels in breast cancer and other malignancies--correlation with clinical course. *Israel Journal of Medical Sciences*, **24**, 623–627.

173. Hashim, Z.M. (2014) The significance of CA15-3 in breast cancer patients and its relationship to HER-2 receptor status. *International Journal of Immunopathology and Pharmacology*, **27**, 45–51.

174. Yerushalmi, R., Tyldesley, S., Kennecke, H. *et al.* (2012) Tumor markers in metastatic breast cancer subtypes: frequency of elevation and correlation with outcome. *Annals of Oncology*, **23**, 338–345.

175. Uhr, J.W. (2013) The clinical potential of circulating tumor cells; the need to incorporate a modern "immunological cocktail" in the assay. *Cancers*, **5**, 1739–1747.

176. Deng, G., Herrier, M., Burgess, D. *et al.* (2008) Enrichment with anti-cytokeratin alone or combined with anti-EpCAM antibodies significantly increases the sensitivity for circulating tumor cell detection in metastatic breast cancer patients. *Breast Cancer Research*, **10**, R69.

177. Skerra, A. (2000) Engineered protein scaffolds for molecular recognition. *Journal of Molecular Recognition*, **13**, 167–187.

178. McKeague, M. and Derosa, M.C. (2012) Challenges and opportunities for small molecule aptamer development. *Journal of Nucleic Acids*, **2012**, 748913.

179. Shigdar, S., Lin, J., Yu, Y. *et al.* (2011) RNA aptamer against a cancer stem cell marker epithelial cell adhesion molecule. *Cancer Science*, **102**, 991–998.

180. Stefan, N., Martin-Killias, P., Wyss-Stoeckle, S. *et al.* (2011) DARPins recognizing the tumor-associated antigen EpCAM selected by phage and ribosome display and engineered for multivalency. *Journal of Molecular Biology*, **413**, 826–843.

181. Theurillat, J.P., Dreier, B., Nagy-Davidescu, G. *et al.* (2010) Designed ankyrin repeat proteins: a novel tool for testing epidermal growth factor receptor 2 expression in breast cancer. *Modern Pathology*, **23**, 1289–1297.

182. Orlova, A., Magnusson, M., Eriksson, T.L. *et al.* (2006) Tumor imaging using a picomolar affinity HER2 binding affibody molecule. *Cancer Research*, **66**, 4339–4348.

183. Järviluoma, A., Strandin, T., Lülf, S. *et al.* (2012) High-affinity target binding engineered via fusion of a single-domain antibody fragment with a ligand-tailored SH3 domain. *PloS One*, **7**, e40331.