Chapter 8
Circulating Tumor Cells and Cancer Stem Cells

Key Topics
- The discovery of circulating tumor cells (CTCs) by Thomas Ashworth
- The metastatic cascade and CTCs
- Site selection by CTCs
- CTC enrichment, isolation, and characterization
- Clinical applications of CTCs

Key Points
- Cancer metastasis involves the detachment and spread of cancer cells in circulation as CTCs, or circulating cancer stem cells (CCSCs). These cells are responsible for the establishment of metastatic deposits and thus a major cause of cancer mortality.
- Because the prognostic relevance of CTCs/CCSCs has been established, there has been a surge in the number of commercial enterprises developing different CTC enrichment and isolation technologies.
- The molecular characterization of CTCs is clinically very informative as well. While CTC analysis does not overcome tumor heterogeneity, the ability to track molecular cancer evolution, especially with acquisition of new drug-resistant mutations, informs therapy decision-making in real time.
8.1 Introduction

In situ carcinomas and benign tumors are potentially curable by surgery and other primary prevention strategies, because these abnormal cells have not breached the basement membrane and have close phenotypic resemblance to their normal counterparts. The word “cancer” indicates the abnormal cell has broken through the basement membrane into the underlying tissue stroma. Thus, by definition, cancer cells are those that have the hallmarks of “detachment,” “deformity,” and “motility.” The “detachment-deformity-motility” phenotype means the cells can actively change their cytoskeletal structures and maneuver in between endothelial cells and into the stroma and eventually the circulation. With the additional hallmarks of proliferation and induction of new blood vessel formation, the cells can also be passively pushed through the porous new blood vessel wall into the circulation. These cells in circulation are referred to as circulating tumor cells (CTCs), and their capture constitutes an aspect of the “liquid biopsy” concept.

Circulating tumor cells tend to home into distant preferential organs where they may remain dormant until they receive the right signals to begin regrowth. These dormant cancer cells, which are found in other organs, but mostly in the bone marrow, are referred to as disseminated tumor cells (DTCs). Though the mechanisms controlling clinical cancer dormancy are largely unknown, both microenvironmental cues and tumor evolution may account for the temporal dormancy, which can last for over a decade. Reversal into active proliferative state accounts in part for disease relapse and associated mortality.

To attain the metastatic phenotype, cancer cells have to undergo partial or complete epithelial-to-mesenchymal transition (EMT), a program or process used by embryonic cells for patterning and establishment of the various parts of the metazoan. Thus, metastatic cancer cells tend to be heterogeneous in regard to the extent of EMT phenotype attained. Conceivably, the process of EMT generates cells expressing epithelial, mesenchymal, and also stem cell markers. The population of cells expressing stem cell markers is referred to as circulating cancer stem cells (CCSCs). These CCSCs are resilient to chemotherapy and radiotherapy and may be the critical cells efficient at establishing metastatic growths. But the rarity of these cells makes it a formidable task to capture and study them.

The hematogenous spread of solid tumors constitutes the leukemic phase of the disease, and this is associated with diverse adverse clinical outcomes. The chances of surgical treatment failure, as well as resistance to other therapies, increase as a function of distance spread of cancer cells, especially with the formation of established metastatic tumors. Disseminated tumor cells, for example, are established prognostic biomarkers predictive of adverse clinical outcome and serve as surrogate markers for the presence of minimal residual disease following curative-intent surgery. Because DTC sampling is invasive, CTC capture and characterization has been explored for similar prognostic and other clinical applications. Several technologies have been developed by both industry and academia for CTC isolation and characterization. The CELLSEARCH® system is the industry
standard, being the first US FDA-approved CTC device for clinical use. However, other commercial devices and technologies are available for CTC capture and analysis.

Admittedly, molecular characterization of CTCs is unlikely to capture entire tumor alterations due to the heterogeneity of cancer cells. Other liquid biopsy approaches such as ctDNA and extracellular vesicular cargo represent better samples for such applications. However, CTC characterization has proven very useful in a number of applications, including study of tumor evolution, mechanisms of drug resistance and relapse, acquisition of metastatic potential, prognostication and detection of minimal residual disease.

### 8.2 Thomas Ashworth and Circulating Tumor Cells

The Australian physician, Thomas R. Ashworth, discovered CTCs in 1869, in his microscopic inquiry of a cancer patient’s blood sample. On examination of blood from a patient who died from metastatic cancer, Ashworth observed cells that closely resembled those of the primary tumor and reasonably noted the implication of this in multiple cancers in the same patient. His observation that “cells identical with those of the cancer itself being seen in the blood may tend to throw some light upon the mode of origin of multiple tumors existing in the same person” is very telling today. Ashworth was able to conjecture based on this observation that these cancer cells must have detached and circulated over long distances to where he sampled the blood. Thus, he noted, “one thing is certain, that if they came from an existing cancer structure, they must have passed through the greater part of the circulatory system to have arrived at the internal saphena vein of the sound leg”. As a deserving tribute, the Ingham Institute in 2014 organized the inaugural Thomas Ashworth CTC Symposium in his honor.

### 8.3 Metastasis and Formation of Circulating Tumor Cells

Circulating tumor cells are metastatic cancer cells that travel long distances in the vascular or lymphatic circulation to colonize distant tissues and organs. Their formation requires the completion of a complex series of activities referred to as the metastatic cascade (Fig. 8.1). These events are controlled by a balance between the activities of a “metastatic effector” and “metastatic suppressor” genetic events. Separate and overlapping molecular genetic cascades regulate tumor formation, progression, and metastasis. It is known, for example, that site-specific metastasis requires different gene expression patterns from primary prognostic expression profile.

The number of tumor cells and emboli in the circulation is a function of tumor size (larger tumors shed more cells than smaller tumors), and this may have
prognostic value that has been explored in CTC clinical applications. In spite of this, the inefficiency of metastatic deposit formation results in just a few cells being capable of forming metastatic tumors. The successful rate of metastatic deposit formation is estimated at 0.01% of CTCs. Circulating tumor cells may extravasate after only 3 days in circulation. However, only ~1:40 extravasated cells will grow at the site and may be able to form micrometastasis, and even much fewer of these (1:100) can actually maintain growth to larger sizes to form tumors. Several reasons account for this inefficiency of metastatic deposit formation:

- Metastasis involves cellular detachment, evasion of anoikis, breach of the basement membrane, stromal interaction, vascular invasion, migration, extravasation, and reestablishment of tumor deposits. These processes require coordinated changes in expression of cell adhesion molecules, proteolytic enzymes, and cytoskeletal organization involving the RHO family members (RHO, RAC, CDC42). Evasion of anoikis, for instance, requires activation of survival pathways such as tropomyosin-related kinase B to inhibit caspase activation [1].
- Many CTCs are destroyed by one of three mechanisms: physical forces due to blood flow, elimination by effective antitumor immunity, and/or elimination by therapeutic agents. Expression of CD47 by colorectal cancer CTCs, for instance, enables escape from recognition by dendritic cells and macrophages [2].
- There is a tight spatiotemporal control of metastatic deposit formation.

Fig. 8.1 The metastatic cascade. Loss of E-cadherin-dependent intercellular adhesions is an early event in metastasis of epithelial cancers. EMT follows this process, whereby cells acquire migratory phenotypes. Various molecules are then deployed for basement membrane disruption and eventual invasion of the surrounding stromal matrix. Intravasation then occurs, which could be augmented by tumor angiogenesis. Intravasation could also occur via lymphatics with eventual emptying into the bloodstream. Cells in circulation must overcome all the hazardous environmental cues including immunosurveillance and chemotherapeutic agents. Some cells migrate as microemboli, which may help prevent anoikis. Extravasation and homing occur at the target organ with eventual regrowth and formation of metastatic deposits. Extravasated tumor cells may also remain in a dormant state for possibly several years before regrowth.
Microenvironmental conduciveness at the site of metastasis is important for successful tumor cell proliferation and regrowth.

Many cells shed into the circulation may be executing apoptosis or other cell death processes and hence unable to establish tumor foci at distant sites.

Molecular and gene expressional changes that control cell-cell communication, as well as growth factor receptor signaling, play important roles in successful colonization of the metastatic site.

Metastasis requires reactivation of embryonic morphogenetic pathways needed to complete epithelial-to-mesenchymal transition (EMT) and vice versa (MET). EMT is dedifferentiation of epithelial cells into fibroblastoid cells that are migratory with altered mesenchymal gene expression. This process involves loss of E-cadherin functions and increased vimentin expression and is coordinated by several signaling pathways including the RAF/MEK/MAPK, PI3K/AKT, NF-kB, TGFβ, WNT, Notch, Hedgehog, and even hypoxia.

8.3.1 Basement Membrane Disruption

Normal epithelial cells usually require signals from the extracellular matrix (ECM) including the basement membrane to maintain their polarity. Cells can control the assembly and disassembly of the ECM. For cancer cells, the anchorage-independent growth frees them from normal epithelial-ECM control.

The beginning of locoregional or distant spread of the neoplastic cell is basement membrane derangement. This feature is a hallmark of cancer and differentiates benign tumors and premalignant lesions from invasive cancers. Once the basement membrane is breached, the number of cancer cells that have escaped cannot be estimated, or can it be ascertained that the tumor is organ confined, even if no gross or histopathologic evidence of local invasion is visible. Benign tumors can be large with highly disorganized epithelial structures, yet they consistently maintain intact basement membranes around them. Similarly, although in situ neoplasias have cellular atypia, they maintain intact basement membranes, and it is only when this membrane is disrupted that cancer can be diagnosed and warranting aggressive treatment. Indeed, what determines whether or not an in situ neoplastic cell breaches the basement membrane is unknown, hence the disagreement of establishing them as precursor lesions to the associated cancer. Compromised basement membrane is the definite initial process of tumor spread.

Migratory cancer cells require alterations in cell-cell and cell-ECM adhesions. This involves several cell surface receptors, but the type employed for such events depends on the type of tumor. Almost all invasive cancers express some receptors for the major adhesion molecules including integrins, cadherin, immunoglobulin superfamily members, and CD44. Decrease cell and matrix adhesion is required for escape, and contrary, at arrest and extravasation, increased cell and matrix adhesion is needed.
8.3.2 Invasiveness

Invasive tumor cells need to acquire special properties and complete an ordered cascade of events to be successful in establishing distant metastatic deposits. These properties and events include:

- Detachment from other cells without executing anoikis.
- Acquiring the ability to move and migrate (EMT).
- Breaching of the basement membrane.
- Acquiring the ability to intravasate (move between endothelial cells) into existing blood vessels or new blood vessels. This process involves cytoskeletal dynamics.
- Escaping from cell death signals, immune surveillance, and therapeutic agents.
- Ability to circulate as single cells or as cell clusters (microemboli or collective migration).
- Recognition and responding to distant site signals.
- Acquiring the ability to extravasate at a distant site.
- Encountering a microenvironment conducive for implantation, proliferation, and regrowth.

These actions require epigenetic and genetic changes that enable transition from epithelial-to-mesenchymal phenotype at initiation of invasion and the reverse from mesenchymal-to-epithelial phenotype at distant destination.

8.3.3 Molecular Genetic Control of Metastasis

Metastatic competence and behavior of cancer cells involve a complex array of events, orchestrated by several molecular signaling pathways. Molecules such as RHO GTPases, HGF, MMPs, IGF2, VEGFs, and autotaxin, among many others, play different roles in cancer cell metastasis. However, some established markers include changes in cell adhesion molecules (CAMs – cadherin, integrin, selectin, IgSF), CD44, FAK, BMP7, MMPs, and epithelial protein lost in neoplasia (EPLIN).

The loss of cell adhesion is an initial requirement for the cell to begin its egress. In the normal epithelium, cell junctions, namely, desmosomes and tight junctions, cohesively hold cells together. The formation and maintenance of these junctions are mediated by calcium-dependent interactions controlled by biomolecules such as cadherins. The cadherin (or calcium-dependent adhesion molecules) gene family of adhesion molecules is single-pass membrane glycoproteins with cytosolic, membranous, and extracellular segments (Fig. 8.2). The over 100 family members are subcategorized into five subfamilies, namely, classic types I and II cadherins, desmosomal cadherins, protocadherins, and cadherin-related proteins. Homotypic epithelial cell adhesions are regulated by epithelial cell classic cadherins or E-cadherin. The cytoplasmic or cytosolic component of E-cadherin physically
interacts with the actin cytoskeleton through catenins. The extracellular segment of cadherin has five domains that mediate the epithelial cell-to-cell homotypic adhesions.

The catenin family of proteins forms complexes with cadherins. This family includes α, β, γ, and δ catenins. In addition to its cell adhesion functions, β-catenin is also a member of the canonical WNT signaling pathway. Under physiologic conditions, β-catenin is sequestered in an adhesion complex formed by α-catenin, p120 CAS, and the intracellular domain of E-cadherin (Fig. 8.2). This macromolecular complex is what tatters E-cadherin to the cytosolic actin cytoskeleton, thus maintaining normal cell-cell adhesion. The loss of E-cadherin-catenin complex, and hence loss of epithelial cell adhesion, results in the release of β-catenin from the complex. This loss of cell adhesion obviously frees in situ cancer cells to begin their migration. The released β-catenin also augments the neoplastic phenotype and may enhance migratory activity of the cancer cell. The released β-catenin accumulates in the cytosol and is usually kept in check by ubiquitin-mediated proteasomal degradation. The free β-catenin binds to APC and is phosphorylated by GSK3β for degradation. The absence or loss of APC functions, as occurs through mutations (e.g., in CRC), or loss of GSK3β functions possibly via WNT proto-oncogene
signaling, renders intracellular buildup of β-catenin. Intracellular entrance of cytosolic β-catenin activates TCF/LEF1 family of transcription factors that induce expression of various oncogenes including cMYC and CCND1.

Changes in expression or function of E-cadherin or β-catenin through mutations, loss of heterozygosity, promoter hypermethylation, or proteolytic modifications can lead to loss of function and cell adhesion. Proteolytic modification of E-cadherin is demonstrated in mammary epithelial cells. Stromelysin-1, a member of the MMP gene family, can degrade the extracellular domain of E-cadherin resulting in loss of epithelial cell adhesion. Indeed, constitutive expression of stromelysin-1 in mammary epithelial cells causes EMT phenotype characterized by downregulation of cytokeratin expression, increased vimentin and MMP9 expression, and loss of catenin from adhesion complexes.

In addition to E-cadherin, the other family members also play roles in EMT and cancer invasiveness. The cadherin “switch” phenomenon underlies EMT. The downregulation of E-cadherin in cancer cells is often associated with increased CDH2/N-cadherin or neuronal cadherin. CDH2 binds to and activates the FGFR signaling pathway and also enhances β-catenin nuclear induction of EMT genes. Moreover, CDH1 expression, when coincident with expression of CDH3, causes CDH3-mediated IGFR signaling and p120 phosphorylation to mediate cancer cell migration and invasiveness. Vascular endothelial cadherin or CDH5/VE-cadherin interacts with CDH2 to promote tumor growth, vasculogenesis, and intravasation. Truncated cadherin or CDH13/T-cadherin expression leads to EGFR signaling pathway activation. Thus, a multitude of cadherin actions, which may be due to tumor-specific induction (e.g., CDH17/liver-intestine or LI-cadherin and gastrointestinal cancer metastasis), underlie cancer metastasis and formation of CTCs.

Another molecule with established roles in cancer metastasis is CD44. CD44 is a transmembrane glycoprotein receptor for a ubiquitous component of the ECM, hyaluronan or hyaluronic acid (HA). The CD44, located on chromosome 11p13, has 20 exons that undergo alternative splicing to produce several isoforms referred to as variants (v). CD44 interacts with many extracellular components including collagens, osteopontin, and MMPs, but it is a major receptor for hyaluronic acid. Hyaluronic acid is upregulated during angiogenesis and is intrinsically an endothelial cell mitogen. Similarly, CD44 is overexpressed by many neoplastic cells and promotes cellular growth and invasion. The interaction of CD44 and HA facilitates binding and adhesion of cancer cells to the ECM, which promotes cancer invasion and metastasis. CD44 is also a cancer stem cell (CSC) marker and is involved in CSC invasive abilities. In colorectal cancer, CD44v6 expression in CSCs is involved in mediating the metastasis of these cells. CD44+ CD24−/low Lin− breast cancer stem cells can intravasate into the circulation and metastasize. Additionally, CD44+ cells can efficiently establish tumors, indicating their important role in metastasis and establishment of new metastatic deposits.

A role for the immunoglobulin superfamily (IgSF) in cancer metastasis is well known. The IgSF is a very diverse group of proteins that include PECAM-1, ICAM-1, MCAM, NCAM, L1CAM, ALCAM, TCR, viral receptors, and MHC molecules. Molecularly, they are related by the shared immunoglobulin homology unit, which
is comprised of 70–110 amino acids that are organized into seven to nine β-pleated sheets. Structurally, many members of the group have N-terminal extracellular, a single transmembrane, and C-terminal cytoplasmic domains. The extracellular domain interacts with other members of the group (homophilic interactions) or with integrins or other carbohydrate moieties (heterophilic interactions), while the cytoplasmic unit interacts with the cytoskeleton and adaptor proteins that could mediate intracellular signaling. Functionally, the IgSF members are involved in cell-cell interactions. Cancer cells overexpress many members of the IgSF, which are implicated in cancer progression and metastasis. For instance, melanoma cell adhesion molecule (MCAM) mediates progression of melanoma and breast and prostate cancer cells. Similarly, the progression of several solid tumors is associated with altered expression of IgSF members.

### 8.3.4 Epithelial-to-Mesenchymal Transition

Epithelial-to-mesenchymal transition (EMT) mediates invasive behavior of most solid tumors. The majority of solid tumors are of epithelial origin, i.e., they are carcinomas. Epithelial cells are polarized in nature with apical and basal ends. The basal side interacts with the basement membrane through hemidesmosomes. In the epithelium, the cells are connected together by tight junctions toward their epical regions. While the epithelial cells can move within the epithelium, they never breach the basement membrane and enter into the ECM.

Greenburg and Hay [3] first observed the transition of epithelial cells into mesenchymal migratory phenotype and hence named this process EMT. Cultured adult anterior lens and embryonic cells in 3D collagen gels revealed changing morphology from polar epithelial to elongated mesenchymal cells that detached and migrated through the 3D gel as single cells. This process requires loss of cell-cell adhesion and acquisition of motile migratory ability in association with tissue reorganization, all of which recapitulate embryonic tissue patterning processes.

There are three types of EMT. Type I EMT operates during patterning and development of multicellular organisms. In the formation of some epithelial tissues, type I EMT cells revert to their epithelial phenotypes (epithelial plasticity). Type II EMT is described for the migration of cells during wound healing. Here the cells at the leading edges extend lamellipodia and migrate to close the wound. Thus, the leading keratinocytes (which have undergone partial EMT) drag the adjacent cells with them. The EMT-like process that occurs in cancer is referred to as type III EMT.

A functional hallmark of EMT is the change from stationary to mobile and migratory cell that can invade the ECM. However, other defining characteristics include altered morphology and differentiation marker expression. Morphological changes from apicobasal polarity to dispersed spindle-shaped cells with changes in cell-cell adhesion markers and cytokeratin intermediate filament to vimentin filament and fibronectin underlie EMT.
The defined molecular genetic control of EMT includes activation of several developmental signaling pathways and hypoxia that induce specific transcription factors to orchestrate the transition. An early event is loss of E-cadherin/CDH1 expression. The expression of this gene is lost early during carcinogenesis, and germline mutations in this gene underlie familial gastric cancers. In addition to mutations and epigenetic gene silencing, signaling pathways also modulate E-cadherin functions. Tyrosine kinase receptor signaling, for example, negatively controls E-cadherin cell-cell adhesion functions. This pathway can phosphorylate β-catenin and the cytoplasmic domain of E-cadherin. Phosphorylated E-cadherin binds to Hakai E3 ligase that is internalized in endosomes and recycled to the cell surface or destroyed in lysosomes. Phosphorylated β-catenin is destroyed in proteasomes. These events initiate invasive behavior as cells lose adhesion and begin to migrate and spread.

Established transcription factors that mediate EMT include FOXC2, TWIST1, SNAI1, SLUG, GOOSECOID, SIP1 (ZEB2), and δEF1 (ZEB1). These markers are expressed by invasive tumors and facilitate invasive behaviors. In addition to other functions, SNAI1, SLUG, and SIP1 can repress E-cadherin expression. Upstream embryonic signaling pathways and hypoxia control the expression of these transcription factors as well. These pathways include TGFβ, WNT, and NOTCH signaling. For example, GSK3β phosphorylates SNAI1 for degradation. Hence, WNT signaling that inactivates GSK3β stabilizes SNAI1. Similarly, SNAI1 is activated by hypoxia, and TGFβ can induce expression of SIP1, SLUG, and SNAI1.

In the majority of invasive cancers, EMT is not a stable binary condition whereby cells are strictly epithelial or mesenchymal with reference to morphology and/or gene expressional changes (Table 8.1). It may be very transient and unnoticeable, and a complete mesenchymal phenotype may not be necessary for invasion. Additionally, some cancer cells may invade without undergoing EMT. Typical EMT is demonstrated in just a few cancers, especially diffuse-type gastric and invasive lobular breast cancers. The majority of these cancers lose E-cadherin expression and overexpress the EMT transcription factor TWIST1. Additionally, nuclear β-catenin accumulation in colorectal cancer cells with APC or β-catenin mutations is associated with increased expression of fibronectin and loss of E-cadherin expression. Novel approaches are required for the isolation of CTCs from these tumors, as the epithelial marker technology will miss them. Notwithstanding, the majority of CTCs, even invasive breast cancer, maintain epithelial morphology and express epithelial cell markers (EpCAM, CK) that have been successfully used for their isolation.

Distant metastatic deposits are epithelial in nature, often resembling the primary tumor of origin. Thus, EMT must be a reversible process. Even if tumors initially invade as mesenchymal cells, they must reverse at some point back to epithelial phenotypes before implantation and growth at distant sites [4].
8.3.5 Circulating Tumor Cell Formation Independent of EMT

Cancer cells can also enter the circulation through other mechanisms including passive spread, as summarized:

- The work by Aceto et al. [5] suggests that CTCs can be released as 2–50 cell clusters held together by plakoglobin-dependent intercellular adhesions.
- The microtubule-organizing center (the centrosome) is amplified in some tumors, and those cancer cells are thought to enter the circulation in a non-EMT mechanism.
- Reduced intercellular adhesion is achieved via RAC1 signaling that induces Arp2/3-dependent actin cytoskeletal polymerization [6].
- Additionally, tumor angiogenesis generates leaky blood vessels. With tumor growth and expansion, cells or cluster of cells can be passively pushed or dislodged into these vessels and be carried away as CTCs.

These EMT-independent mechanisms should generate CTCs very suitable for epithelial marker-based assays. Similarly, migrating cell clusters should overcome anoikis.

8.4 Distant Site Selection: The Anatomic vs. Seed and Soil Hypotheses

Anatomic organization of tissues, their vasculature, and lymphatic supply provides facile explanation for target organ invasion by tumors. In this scenario, the organ initially encountered by the primary tumor cells in circulation serves as the target of choice. Thus, lymphatic and vascular drainage from the primary tumor site to regional organs or tissues accounts for such spread. A classical example is the drainage of the colon and colorectal cancer (CRC) hepatic spread. The hepatic capillaries are the first encountered by CRC cells in the blood (hepatic portal circulation), and hence the liver remains the most frequent initial site of CRC metastases. Such locoregional spread occurs in many cancers. However, such simplistic explanation cannot account for all cancer metastasis, especially to long
distant organs. Tumors and site molecular characterization appear to offer some explanation for such target organ choices.

Stephen Paget conceived the “seed and soil” hypothesis in 1889. These early cancer researchers observed the predilection for some cancers to metastasize to the same organ. In the work of Paget, breast cancer spread to the liver, which is a frequent occurrence could not be accounted for by the easy accessibility of the liver by mammary blood supply, because other organs bathed by such circulating blood rarely developed metastases. Thus, Paget concluded that certain cancer cells (the seeds) could only successfully establish metastatic deposits in selective organs (the soil) that have suitable growth microenvironments (Table 8.2). Three important concepts characterize the “seed and soil” mechanism of cancer spread:

- Metastatic cells evolve as those that are fit to succeed in all phases of the entire metastatic process. Thus, a successful metastatic cell must complete the metastatic cascade.
- Primary tumors and their metastases consist of genetically diverse cells.

### Table 8.2 Examples of “seeds” and “soils” of some cancers

<table>
<thead>
<tr>
<th>Primary cancer (seed)</th>
<th>Distant metastatic sites (soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma</td>
<td>Lymph node, lung, liver, brain, gastrointestinal tract, bones</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Bone marrow, lung, liver, lymph nodes, brain</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>Liver, bone, bone marrow, adrenals, brain, lymph nodes, contralateral lung, subcutaneous tissues, pancreas</td>
</tr>
<tr>
<td>Pleural mesothelioma</td>
<td>Retroperitoneal lymph nodes, contralateral lung and pleura, brain, spine, thyroid, prostate</td>
</tr>
<tr>
<td>Head and neck cancer</td>
<td>Lung, bone, liver</td>
</tr>
<tr>
<td>Salivary gland cancer</td>
<td>Lung</td>
</tr>
<tr>
<td>Thyroid cancer</td>
<td>Lung, bone</td>
</tr>
<tr>
<td>Esophageal cancer</td>
<td>Lymph nodes, bone</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>Liver, lungs, brain</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>Liver, peritoneal cavity, lungs</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>Liver, lung</td>
</tr>
<tr>
<td>Hepatobiliary cancer</td>
<td>Liver, peritoneal cavity, lungs, pleura, brain, bone</td>
</tr>
<tr>
<td>Renal cell carcinoma</td>
<td>Bone, liver, lung, brain, distant lymph nodes</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>Retroperitoneal lymph nodes, lung, bone, liver</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>Bone, lung liver</td>
</tr>
<tr>
<td>Testicular cancer</td>
<td>Lung, liver, bone, other visceral sites</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>Liver, lung, bone, supraclavicular and axillary lymph nodes</td>
</tr>
<tr>
<td>Endometrial cancer</td>
<td>Vagina, lung, intra-abdominal</td>
</tr>
<tr>
<td>Cervical</td>
<td>Mediastinum and supraclavicular lymph nodes, lung, peritoneal cavity, bone</td>
</tr>
<tr>
<td>Vaginal cancer</td>
<td>Aortic lymph nodes, lung, bone</td>
</tr>
<tr>
<td>Vulvar cancer</td>
<td>Iliac lymph nodes</td>
</tr>
<tr>
<td>Gestational trophoblastic tumors</td>
<td>Lung, kidney, GIT, spleen, liver, brain</td>
</tr>
</tbody>
</table>
Metastases generally develop in a site-specific manner. Because the microenvironments (the soil) of target organs are genetically and histologically different, specific cancer cells are only capable of colonizing one specific organ [7].

Two important complementary events are required for successful seed and soil interaction and establishment of metastases. The tumor cells must produce signaling factors responsive to the stroma and tumor microenvironment, but similarly the microenvironment must respond appropriately to these signals to permit habitation and growth of the new arrivals. Successful metastasis to an organ is precluded if this interaction fails, explaining why not all organs, even if the tumor cells encounter them, will be successful homes or soils for the seeds. Regulated activation of specific chemokines, cytokines, and proteases may guide metastasis to a specific site or organ. For example, lung metastasis may be predicted by the expression of inhibitor of differentiation-1 (ID-1), VCAM-1, MMP-1, and CXCL-12. Thus, breast cancer cells that express CXCR4 can selectively home into lungs because of the high expression of its only ligand CXCL12 [8].

Molecular characterization of tumors is providing insights into how “seeds” chose their “soils.” Gene expression analyses of metastatic tumors have identified specific genetic signatures of some tumors for specific target organs. For instance, genes that mediate the metastasis of breast cancer to bone are different from those that mediate metastasis to the lung. Thus, different sets of genes create the right environment for tumor cells to specifically interact with the stromal cells of specific target organs [9].

8.5 Enrichment and Isolation of Circulating Tumor Cells

Circulating tumor cells have been enriched, isolated, and characterized using different methods and techniques (Fig. 8.3). About 80% of all solid tumors are of epithelial origin. Hence, many CTCs are from epithelial cancers. In view of this, and the differences between epithelial cells and blood cells in regard to size and other physical properties, many earlier CTC separation techniques have involved exploitation of such physical differences. These physical property-based separation approaches include gradient centrifugation, filtration, and use of microelectrochemical systems. Because of the differences in molecular marker expression between epithelial cells and blood formed elements, newer methods have utilized tumor cell membrane unique epithelial surface markers such as EpCAM, for CTC isolation. These methods include immunomagnetic bead-based approaches and variations of this including the use of microfluidic platforms and nanotechnology approaches. There are currently >40 methods and techniques developed for CTC enrichment and isolation, and many more are being developed and optimized.
8.5.1 The Filtration Principle

This procedure enables direct enrichment of CTCs based on size. Many of the normal cellular elements (RBCs, WBCs, and platelets) of blood are 8–10 μm in diameter, with epithelial cells being much larger with mean diameter of ~10 μm. In general, this separation method incorporates a polycarbonate membrane filter with pores of ~8 μm to separate most peripheral blood cells from epithelial cell components, which are mostly CTCs in advanced cancer patients. The method has some advantages over immunologic-based cytometry such as CELLSEARCH® and other CTC-chips. Because heterogeneity and EMT can result in loss of some tumor marker expression, this non-immunologic approach captures additional CTCs not amenable to antigen-antibody interaction technologies. Putatively, it thus captures more CTCs. Also, size-based methods and the herringbone (HB) chip are among the reliable methods for capturing circulating tumor microemboli (CTM). Described below are commercially developed CTC isolation techniques and devices based of the filtration principle.

Fig. 8.3 Circulating tumor cell (CTC) enrichment, isolation, and characterization. CTCs can be analyzed using the various indicated methods of enrichment and characterization.
8.5.1.1 ISET®

Prof. Patrizia Paterlini-Brechot, the founder of Rarecells, developed the isolation by size of epithelial tumor (ISET) technology for CTC isolation. The technology is based on CTC isolation base on the sizes of epithelial tumor cells. The procedure involves first the dilution of blood in RBC lysis buffer before being applied to the filtration membrane on the ISET device. The membrane has about ten wells with pore sizes of 8 μm. Following filtration, the membranes are washed and removed for staining and other analyses. The sensitivity threshold of this system is one CTC per 10 ml of blood treated within 4 h of collection.

8.5.1.2 ScreenCell®

Originally described by Vona et al. [10] and developed by ScreenCell, the ScreenCell® device enables CTC isolation by size using microporous membrane filters. Different filters are used to separate fixed cells for cytometry (ScreenCell® Cyto) and live cells for molecular biologic applications (ScreenCell® MB) or cell culture (ScreenCell® CC). The 18 μm-thick filter has a smooth hydrophilic surface with pore sizes of 7.5 ± 0.36 μm for isolating fixed cells and 6.5 ± 0.33 μm for capturing live cells. Similar to ISET, red blood cell lysis precedes filtration and the membranes are washed in PBS prior to use in downstream applications.

8.5.1.3 CellSieve™

Creatv MicroTech has developed an identical isolation method called CellSieve™. This uses biocompatible polymer filters of 13 mm diameters containing 7 μm pores for CTC capture. Pore density can be as high as 160,000 per 9 mm diameter.

8.5.1.4 Parsortix

The Parsortix system, developed by Angle plc (UK), uses a simple patented microfluidic technology to isolate CTCs based on size and compressibility (CTCs are less deformable than blood cells). A disposable cassette, which is of the size of a microscope slide, is placed in a clamp, and as blood flows through it, the Parsortix system automatically separates CTCs, which can then be stained and enumerated or harvested for other applications.
8.5.1.5 JETTA

DeNovo Sciences developed the JETTA technology for CTC isolation. It uses microfluidic slides with 56,320 capture chambers to isolate CTCs based on size. There are customized reagents and cartridges for breast, prostate, and colorectal cancers. This device has >85% cell capture efficiency. Downstream applications of captured cells include immunohistochemistry, FISH, mRNA analysis, and PCR. Slides from JETTA can be transferred to the Vanguard Imaging System that prepares them for evaluation. The JETTA100 is a manual system; however, the new JETTA400 is a fully automated system that handles samples from input to result delivery.

8.5.1.6 ClearCell®

Clearbridge BioMedics has developed the ClearCell® system for CTC isolation. This label-free automated CTC enrichment system is based on a patented microfluidic biochip. This microfluidic biochip (CTChip®FR1) isolates CTCs based on size and deformity using Dean Flow Fractionation (DFF). Cells are focused within the microfluidic channels using the DFF process such that larger cells flow along the inner wall and smaller cells away from it. The entire process is complete in under an hour, and isolated viable cells can be used for multiple downstream applications.

8.5.1.7 ApoStream®

Developed by ApoCell, the ApoStream® uses differences in the dielectric properties of CTCs and blood cells to isolate CTCs. Dielectric properties of a cell depend on their size, volume, membrane area, density, and conductivity, which differ between cells. By using dielectrophoresis (DEP) field assist, cells are attracted to or repelled from a charged electrode. DEP forces pull CTCs toward electrodes on the chamber floor, repelling blood cells away into the eluent. The attracted tumor cells are then collected at the floor of the chamber through a port. The other blood cells are carried away in the eluent.

8.5.2 Centrifugation Principle

Density gradient centrifugation isolates both CTCs and mononuclear cells from blood. It involves centrifugation in density gradient (e.g., Ficoll) to create layers of cells based on their sizes and densities in relationship to the density of the suspension medium. The layers generated after the spin are from top to bottom, plasma,
CTCs and mononuclear cells (densities \(< 1.077 \text{ g/mL}\)), density gradient media (e.g., Ficoll), and then PMN leukocytes and RBCs (density \(> 1.077 \text{ g/mL}\)). Available kits for such cell isolation include Lymphoprep™ (Axis-Shield) and OncoQuick (Greiner Bio-One GmbH).

### 8.5.2.1 OncoQuick®

OncoQuick® (Greiner Bio-One GmbH) is a research use only product for the enrichment of CTCs from 15 to 30 mls of uncoagulated whole blood based on density gradient centrifugation. The 50 mL OncoQuick® centrifugation tube has two compartments separated by a porous barrier. The lower compartment has the blue-colored separation medium, while the upper chamber is where whole blood is loaded for separation. The sample is span at 1600 g at 4°C for 20 min, resulting in column stratification of cells based on buoyant densities. The denser RBCs and PMN cells migrate through the barrier and displace the separation medium into the upper compartment. The less dense CTCs and other mononuclear cells are trapped between the separation medium and plasma. Targeted CTCs are harvested, washed, and used for downstream applications.

### 8.5.2.2 Lymphoprep™

Lymphoprep™ (Axis-Shield) is a ready-to-use media composed of reagents with composition conducive for the isolation of mononuclear cells in blood (monocytes and lymphocytes). Reported in 1968 by Boyum, this method relies on density disparity between mononuclear cells and other circulating cells [11]. Mononuclear cells have a much lower buoyant density than RBCs and PMN cells. Most mononuclear cell densities are \(< 1.077 \text{ g/mL}\). Hence, centrifugation of blood in iso-osmotic medium of density 1.077 g/mL enables sedimentation of RBCs and PMNs, while entrapping mononuclear cells at the interface between the media and plasma. Optimal isolation requires mixing blood with saline at a 1:1 dilution and spinning at 600 g for 20 min at 20 °C. While this kit is not specified for CTC isolation, the identical buoyant densities enable the isolation of both cell types.

### 8.5.3 Immunomagnetic Bead Principle

This is a common method for CTC enrichment. It can be applied to whole blood or to cellular components obtained by centrifugation or filtration principles. Isolated cells can be analyzed by various molecular methods (RT-PCR) or enumerated after staining. Also FISH analysis can be performed on cell isolates.

The principle of cell isolation is simple. This could include only positive selection or both positive and negative selections (Fig. 8.3). Positive selection of
CTCs involves the use of antibodies attached to magnetic beads. These antibodies target epithelial or tumor-associated cell surface antigens such as human epithelial antigens, cytokeratins, epithelial cell adhesion molecules, oncofetal antigens (e.g., CEA), PSA, HER2, and several other markers. The inclusion of antibodies that target leukocytes (CD45, CD61 expressing cells) enables negative selection that can then be followed by positive selection. The method enables isolation and detection of living cells, quantification, and/or direct visualization of CTCs. These epithelial marker immunomagnetic enrichment methods include the CELLSEARCH®, AdnaTest, and various microfluidic devices.

8.5.3.1 CELLSEARCH®

CELLSEARCH® (Veridex) is an automated CTC isolation and enumeration assay approved by the FDA for management of metastatic cancer. It has been useful for enumerating CTCs for the prediction of overall survival and progression-free survival of metastatic breast, colorectal, and prostate cancer patients, but it is also being applied to the study of other epithelial tumors as well. The CELLSEARCH® CTC kit is used for selection, identification, and enumeration of epithelial CTCs in blood (requires 5–15 mls of blood). It uses an electromagnetic bead approach whereby ferrofluids loaded with EpCAM antibodies capture CTCs that are then visualized or detected with a cocktail of antibodies against positive (CTCs) and negative (other cells) cells.

The assay includes the CellSave preservative tubes that contain stabilizers for CTCs for up to 96 h to enable shipment at room temperature from distant locations. Additionally, optimal performance is monitored by inclusion of a control kit that contains low and high concentrations of fixed breast cancer cell line. The entire process from whole blood intake, aspiration of anti-EpCAM ferrofluid and buffer, magnetic incubation, cell separation, staining cartridge analysis, and presentation of fluorescent images for classification of CTCs is highly automated.

8.5.3.2 AdnaTest

AdnaTest (AdnaGen) is an epithelial cell marker-based CTC isolation process. It uses a proprietary blood collection kit (AdnaCollect blood) to obtain samples from patients for CTC isolation based on epithelial (EpCAM) marker expression. This procedure involves two main processes. The first is the AdnaTest Cancer Select that positively enriches CTCs using cancer-specific Select Beads applied to 5 mls of blood. The isolated CTCs are then subjected to the AdnaTest Cancer Detect analysis, which is an RT-PCR analysis of the sample. Cancer gene-specific primer pairs are employed for CTC detection. For example, the AdnaTest Breast Cancer Detect kit uses primers that target MUC-1, HER2, and GA733-2, with ACTB as the housekeeper control gene.
8.5.3.3 Microfluidic Technologies

The application of microfluidic technology has enhanced CTC isolation from whole unprocessed blood. The prototype, developed at Harvard by Mehmet Toner’s group for CTC isolation, enables more cells to be captured per milliliter of blood than the industry standard, CELLSEARCH® [12–14]. This novel approach was able to capture ~50 CTCs per milliliter of blood compared to CELLSEARCH® sensitivity of 1 CTC/mL. Additionally, this microfluidic system enhances the purity of CTCs for subsequent enumeration and downstream molecular analysis. The version 1 chip, dubbed the CTC-chip, comprised of a silicon chamber with 78,000 EpCAM-coated microposts used for epithelial cell capture. Controlled laminar flow of 2–4 mls of unprocessed whole blood through the chamber facilitates CTC capture while reducing shear stress to cells. Viable captured CTCs can be fixed, permeabilized, and stained with DAPI, pan-cytokeratin, and CD45 FITC antibodies for imaging and enumeration. Alternatively, molecular analysis can be performed on biomolecules obtained from lysed CTCs on the chip.

While this proof of principle had enhanced CTC isolation, it had its own shortcomings. First, laminar flow of blood in a uniaxial streamline precludes some cells from making contacts with the microposts; hence, they escape from being captured. Second, the complex geometric design of microposts precludes high-throughput manufacturing that is necessary for large-scale clinical applications. To overcome these limitations, an improved version, called the herringbone chip (HB-Chip named after its configuration that resembles bones of a herring), was developed [15]. The HB design includes microvortex mixing of blood that disrupts the streamline flow and thus increases the interaction of CTCs with EpCAM-coated walls. It uses calibrated microfluidic flow patterns to drive cells into contact with the surface ridges. This simple geometry (without microposts) enables scale-up manufacturing for clinical evaluation. Additional advantage of the HB-chip is that the chamber is made of transparent materials that facilitate image resolution including possible use of electron microscopy in CTC analysis. In a proof of principle study of metastatic prostate cancer patients, the HB-chip demonstrated a median CTC capture of 63 per milliliter, which is a slight improvement over the CTC-chip.

8.5.3.4 On-Q-ity C5 Microfluidic Technology

On-Q-ity has developed proprietary technologies combining biomarkers and CTC analyses for personalized medicine including cancer treatment decisions and monitoring. The On-Q-ity C5 microfluidic chip uses dual capture technology to increase the sensitivity of CTC isolation from blood. This novel approach uses a combination of two microfluidic technologies into one, namely, separation based on antibody affinity method and size filtration technology. The blood flows in a streamline fashion through gradient gap sizes between posts that decrease from 40 μm to
12 μm. Additionally, the posts are coated with EpCAM antibodies. Because not all CTCs express EpCAM, this dual method enables CTC capture based on EpCAM expression and also enables non-EpCAM-expressing cells to be captured based on size. This dual capture method is superior to either technology used alone. Indeed, it can isolate between 1 and 30 CTCs in just 3 mls of blood, which is an improved sensitivity over other methods. Captured CTCs are detected in a similar fashion to CELLSEARCH®, which is staining with DAPI, CK, and CD45 antibodies.

8.5.3.5 CTC-iChip

The CTC-iChip is a novel CTC isolation device. In this process, whole blood treated with anti-CD45 and anti-CD66b magnetic beads (to capture WBCs) is applied to the CTC-iChip microfluidic device. The chip houses two separate microfluidic devices, but with three distinct microfluidic components that serve different purposes. The first part eliminates RBCs and platelets by separating nucleated cells (WBCs and CTCs) by size-based deflection referred to as deterministic lateral displacement. The second microfluidic component uses inertial focusing to line up the nucleated cells for subsequent separation. Finally, magnetic separation and magnetophoresis enable the separation of unlabeled CTCs from bead-coated WBCs [16].

8.5.3.6 IsoFlux™ System

Fluxion biosciences developed and commercialized a fully automated microfluidic technology for isolating viable CTCs and other rare cancer and immune cells for downstream applications. This proprietary microfluidic technology platform uses immunomagnetic bead capture procedure and is flexible in its application because cocktails of antibodies can be used to target different cell types such as CTCs, cancer stem cells, cells undergoing EMT, or immune cells. In principle, 7–10 mls of whole blood is first devoid of RBS through Ficoll gradient centrifugation, and the remaining cells are coupled with IsoFlux beads carrying one or more antibodies (e.g., EpCAM, EGFR, HER2, N-cadherin, etc). The sample is then loaded onto the IsoFlux cartridge that contains microfluidic flow channels at the bottom. At the cell isolation zone of the flow channels, cells coated with beads are pulled up to the top surface of the cartridge by neodymium magnet, while uncoated WBCs run into a waste well. The isolated cells can be enumerated or detected using immunofluorescence, and fluorescence in situ hybridization, or used for molecular analysis. The rate of CTC detection (>5 CTCs) ranges from ~60 % for lung cancer to ~100 % for breast cancer.
8.5.3.7 GILUPI CellCollector™

The GILUPI CellCollector™ (GILUPI GmbH) method is the first in vivo CTC capture microfluidic device. It consists of an EpCAM-coated wire device that is placed through a cannula into a vein of the patient. It is then left in place for 30 min, during which time an estimated 1.5 L of blood passes over the device. This process increases the chance and number of captured CTCs. In clinical validation studies, the CellCollector™ was able to isolate significantly high numbers of CTCs even in early-stage cancer patients. This has potential for monitoring early micrometastasis and minimal residual disease. Its detection rate of CTCs in patients with various solid tumors is up to 70%.

8.5.3.8 CytoTrack

CytoTrack CT11™ technology is a rare cell (such as CTCs) scanning and isolation technology on solid surface. This approach to CTC detection marries conventional CD/DVD and flow cytometry technologies. RBCs in the collected sample are lysed, and the sample then stained, evenly spread, and immobilized on a CytoTrack disk. The disk has a high capacity that enables even up to 30 mls of blood to be evenly spread on. FITC-stained CK and other markers enable fluorescent scanning of positive cells. The disk with the sample is then placed in the CytoTrack FM3 scanner. The disk rotates at a very high speed, while scanning at high resolution of up to 100 million cells per minute takes place. The CytoTrack data management system processes and displays the results. The exact position of each target CTC detected is captured on the disk. High-quality images of CTCs are produced and displayed in gallery format for review. Each target is revisited, reanalyzed, and verified. CTCs are often seen in clusters, but single-cell genotypic and phenotypic analysis is possible. After CTC enumeration, the CytoPicker™ can lift off cells from the glass surface into Eppendorf tubes for other uses.

8.5.4 Other CTC Separation Methods

Vitatex technologies developed the cell adhesion matrix (CAM)-based CTC isolation technique. The CAM-based iCTC platform exploits the invasive properties of cancer cells to isolate metastatic-initiating circulating tumor cells (iCTCs). When patient blood samples are applied to the CAM-coated tubes (Vita-Cap™) or culture plates (Vita-Assay™), iCTCs preferentially adhere to CAM. The captured iCTCs are labeled due to their ingestion of FITC- or TRITC-labeled CAM. Isolated iCTCs can be used for cytometric or molecular analysis.
8.5.5 Detection and Characterization of CTCs

Following CTC enrichment, isolation, and capture, the cells have to be identified and characterized. Various cytometric and molecular biologic techniques enable bona fide CTC detection. Other downstream characterizations include single-cell genomics and next-generation sequencing.

Circulating tumor cells have been identified and characterized using PCR assays targeting tumor-associated transcripts. For example, in gastrointestinal cancer CTC analysis of blood samples from patients with esophageal cancer, density gradient enrichment of CTCs followed by RT-PCR for several mRNAs, including CEA, BIRC5, and ERCC1, has proved to be important in cancer management. Patients with persistently elevated CTCs as demonstrated by CEA mRNA levels are more likely to have a recurrent tumor. Similarly, survivin mRNA levels significantly fall following complete surgical tumor resection. Additionally, a significant decrease in survivin-expressing CTCs is found in patients with adenocarcinoma on neoadjuvant chemoradiation. Patients who respond poorly to neoadjuvant radiotherapy have significantly elevated CTC levels as assayed by ERCC1 mRNA. Carcinoembryonic antigen RT-PCR after density gradient centrifugation of samples from patients with gastric cancer revealed absence in healthy controls and patients with benign conditions, but positive in 36.8% of cancer patients. Carcinoembryonic antigen positivity correlated with depth of tumor invasion, and elevated levels were associated with the likelihood of systemic spread [17]. While qPCR has increased sensitivity for CTC detection, it has limitations such as primer specificity, heterogeneous gene expression by CTCs, and possible tumor marker gene expression by normal blood cells.

Circulating tumor cell enumeration and hence their absolute numbers have prognostic value in patients with different types of solid tumors. Various technologies use fluorescence immunocytochemistry to label, enumerate, and characterize CTCs following enrichment and/or isolation. Additionally, genetic analysis is also possible through fluorescence in situ hybridization to look for genetic and chromosomal alterations. For example, the CELLSEARCH® system stains for cell nuclear using DAPI, cytokeratins (8, 18, and/or 19) with anti-CK-PE antibodies, and leukocytes with anti-CD45-APC following enrichment. Cells that have dual positive and negative staining (CD45+/CK-PE+ or CD45−/CK-PE−) are excluded from enumeration. Thus, only EpCAM+, CK-PE+, and CD45− cells are scored as CTCs and used for patient management. Additional fluorescence channel is available for user-defined marker identification. For instance, this channel can be used for markers such as vimentin, E-cadherin, androgen receptor, PSA, HER2, or EGFR.

An additional armamentarium to CTC enumeration and characterization is the development of objective automated image analysis systems. The Ariol cellular imaging system is a fully automated method used to scan slides to identify CTCs using three fluorescent channels (for cytokeratins, CD45, and nuclear staining). Additionally, it enables bright-field imaging to delineate cell morphology. An
advantage of the system is the ability to quantify DNA ploidy, enabling genetic and chromosomal analysis of CTCs.

Viable CTCs have been detected and characterized using the EPISPOT (epithelial immunospot) assay. This assay enables real-time viable CTC detection based on secretion of specific cancer cell proteins. The enriched cells are cultured on membranes coated with specific antibodies that capture the secreted proteins by the CTCs. These proteins are then detected with fluorochrome-labeled secondary antibodies. This assay detected CK19-negative and MUC1-secreting circulating breast cancer cells even in patients with early-stage localized disease (54 % stage M0) [18].

8.6 Circulating Cancer Stem Cells

In many solid tumors, epithelial, mesenchymal, and CSC markers have been demonstrated in CTCs enriched from the same patient. In some patients negative for CTCs by standard definition, markers of EMT have been detected, indicating that these patients will be deemed CTC negative, and possibly classified as having favorable disease outcome, when indeed they harbor exclusively CTCs with mesenchymal phenotypes. In some instances, a single CTC can co-express both epithelial and mesenchymal markers. High expression of mesenchymal markers is associated with treatment resistance and worse prognosis. Thus, the development of technologies to capture these EMT cells and circulating CSCs such as the AdnaTest EMT-1/stem cell (with TWIST, AKT2, PI3K, and ALDH1) is commendable.

The process of cancer metastasis produces cells that acquire various phenotypes conducive to their functions. This process generates cells that express stem cell markers. These cells with self-renewal abilities are identified as cancer stem cells (CSCs) or cancer stemlike cells. In addition to their stemness, these cells express mesenchymal markers and are mobile and can intravasate into the circulation and become CCSCs. Circulating CSCs have been detected with epithelial markers, suggestive of transition from mesenchymal to epithelial cells in preparation for invasion and colonization of distant sites.

However, the accurate characterization of CCSC requires the use of just a few reliable markers. Markers with strong association to CCSCs include aldehyde dehydrogenase 1 (ALDH1), a putative breast cancer prognostic biomarker, CD44 cell surface glycoprotein involved in cell invasion and metastasis, as well as gangliosides 2 and 3 (GD2 and GD3). Another ganglioside, GD1a, is however a putative marker of CCSCs. The xenobiotic extrusion pump protein, ADCG2, is highly expressed by CSCs and may be involved in resistance mechanisms to chemotherapy. Thus, its expression in CTCs may indicate some components of stemness.

Conceivably, only a minute percentage (0.01 %) of all CTCs possess the ability to form metastatic deposits, and these are the rare cells that harbor stem cell-like features. Indeed, in breast cancer patients, many DTCs in bone marrow are CD44+/

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CD24$^{low}$, and this finding is associated with aggressive tumor behavior and successful establishment of metastatic tumors [19]. Circulating CSCs may be responsible for therapy resistance and hence treatment failures in patients with advanced-stage disease. Targeting these cells should be of utmost importance in cancer biology and pharmaceutical developments. To this end, numerous agents are in development and at various stages of clinical trials to target CCSCs and the mechanisms by which they are generated. Inhibitors of the Hedgehog, WNT, Notch, PI3K/AKT/mTOR, and other signaling pathways are developed and have shown promise at destroying CSCs. The Hedgehog pathway inhibitor, vismodegib, for instance, inhibits the growth of tamoxifen-resistant breast cancer cells. RKF118-310, an inhibitor of the WNT pathway, eradicates breast cancer stem cells, and γ-secretase inhibitors (GSIs) that block the Notch pathway are able to kill CSCs. The GSI, MK-0752 (RO4929097) has shown promise in phase I and II clinical trials in patients with metastatic breast cancer. The PI3K inhibitor, everolimus (RAD001), blocks CSC growth and has shown promise for treating breast cancer patients resistant to conventional therapy. This drug may be useful in trastuzumab-resistant patients and has undergone phase III clinical trials for this purpose (NCT00876395). The preliminary finding is that progression-free survival was insignificant between groups; however, a 7.2-month prolongation was observed with addition of everolimus in the hormone receptor-negative, HER2-positive patients [20].

8.7 Clinical Applications of CTCs

Traditionally, bone marrow aspiration for DTC detection has been used for prognostication of many solid tumors. Tumor micrometastasis preferentially homes into the bone marrow to establish dormancy from where they recirculate to preferred “soils” to establish metastatic deposits. The preferred site could be, or include, the primary site or organ of the tumor. Thus, the presence of DTCs indicates the possible future formation of both distant metastasis and local relapse.

Circulating tumor cell detection is not useful for early cancer detection, although it has an ancillary role in establishing the diagnosis of primary tumors. In clinically localized cancer (M0), CTC evaluation helps determine the presence of minimal residual disease. However, CTCs are established as prognostic biomarkers for several solid tumors. In some cases, CTC characterization has even proven superior to conventional imaging and other clinical metrics of treatment response prediction. In early-stage disease without clinical evidence of metastasis, CTC presence, albeit low, is of significant value in detecting minimal residual disease, and this correlates with prognostic factors such as disease-free survival, disease-specific survival, and overall survival. Expectedly, CTC detection rates are much higher in patients with metastatic disease than those with localized cancer. In patients with advanced-stage disease, baseline and posttreatment CTC numbers or presence predict worse outcome.
Molecular characterization of CTCs is clinically very informative in identifying therapeutic targets as well as uncovering the mechanisms of tumor evolution relevant to treatment resistance. The transition of the cancer cell from its initiation to metastatic phenotype involves acquiring additional specific hallmarks of cancer. The biologic mechanisms evoked to achieve the propensities to invade surrounding tissues and metastasize require overcoming selective pressures. To circumvent these pressures, some clones evolve demonstrating altered genomic expression and mutational status. In the advent of personalized oncology, targeted therapies are available for treating cancers with specific genetic signatures. Traditionally though, primary tumor tissue is interrogated for these changes to inform clinical decision-making. However, such therapies are targeted mostly at minimal residual disease and metastatic cells in circulation. It is well established that CTCs and CCSCs can have different genetic makeups from that of the primary tumor. For example, breast cancer patients with HER2⁺ and/or ER⁺ primary tumors are found to have HER2⁻ and/or ER⁻ CTCs and vice versa. Similarly, BRAF and KRAS mutation status may differ between primary tumors and CTCs from the same tumor. The presence of KRAS mutant CTCs may explain treatment failures with anti-EGFR agents in CRC patients whose primary tumors have wild-type KRAS genotype. Thus, longitudinal sampling for CTCs informs appropriate clinical decision-making and also enables the study of various therapy-resistant mechanisms.

Finally, CTC detection and characterization can be useful in monitoring or determining the risk of disease recurrence after surgery or radiotherapy, as well as the origin of the tumor, if the primary is in doubt. Circulating tumor cells can also serve as surrogate endpoint biomarkers in clinical trials and in the development of companion diagnostics.

8.8 Summary

- Cancer invasion leads to the release of cancer cells and stem cells into the circulation as CTCs and CCSCs. These cells may circulate as single cells or as tumor microemboli.
- CTC formation involves specific gene expressional changes leading to phenotypic changes in the cell. An initial change from epithelial-to-mesenchymal phenotype helps their mobility and exits into the circulation. At the metastatic site, they reverse this phenotypic change and become epithelial to establish a metastatic deposit.
- CTCs may remain dormant in the bone marrow or in other tissues for years, and when given the right cues, they may reenter the circulation and subsequently form metastatic disease.
- Tumor metastasis and treatment failure are major contributing factors to cancer mortality. A reason for the failure in cancer treatment is the possible lack of response by a rare cell type, the CCSC, to treatment interventions.
Another problem with cancer therapy is that targeted therapy decisions are often made based on the genotype of the primary tumor. Not only will tumor heterogeneity preclude a complete capture of the genetic alterations, but also metastasis is associated with tumor genetic evolution. Thus, effective cancer treatment should target CTCs and CCSCs in real time.

CTCs can be enriched, isolated, and characterized using various technologies including centrifugation, filtration, immunologic capture, and molecular approaches.

CTCs have prognostic value in cancer management. Not surprising, therefore, following the FDA clearance of CELLSEARCH® for clinical applications, a plethora of companies have emerged with novel technologies for CTC enrichment, isolation, and characterization.

References