Chapter 10
Understanding Cancer Invasion and Metastasis

Cancer is a deadly disease in large part because, if not stopped, will generally evolve to the metastatic stage, i.e., cancer cells spread from the primary site to new locations (generally different organs) through blood circulation or the lymphatic system. For largely unknown reasons, metastatic cancers tend to exhibit distinct growth patterns from its primary cancer counterpart, growing substantially faster and metastasizing more easily. Recent statistics show that metastatic cancer is responsible for approximately 90 % of all cancer-related mortalities. While it is known to be the deadliest stage of a cancer, the current understanding of the biology of metastatic cancer is rather limited. Some of the very basic questions such as: *what drives a primary cancer to metastasize*; *why some cancers tend to metastasize more easily than the other cancers, e.g., melanoma versus basal cell carcinoma*; and *why metastatic cancers tend to grow much faster than the corresponding primary cancer*, still have no clear answers. This may be the result of: (1) the true challenging nature of these questions, and (2) the lack of adequate investment and hence efforts into metastatic cancer research. This unfortunate reality is probably due to the general belief in the field that little can be done once a cancer has metastasized.

In this and the following chapter, we present the current knowledge about the potential drivers of metastasis, the key mechanisms in executing cancer metastasis and our recent understanding about the biology of metastatic cancers in their new microenvironment. As in the previous chapters, cancer evolution is viewed as a process for the diseased cells to escape from the deadly pressures imposed on them from their microenvironment. As part of their adaption to the challenges, the altered metabolism of the cells may be responsible to a significant degree for their increasingly more challenging microenvironment, this following the initial pressure caused by the accumulation of glucose metabolites due to chronic hypoxia and/or ROS accumulation (see Chap. 5).

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10.1 Local Invasion by Cancer Cells

The first step in cancer metastasis is tumor invasion, i.e., cancer cells breach their basement membrane (a type of ECM) and enter the stromal compartment, where stromal cells (fibroblasts and pericytes), immune cells and blood capillaries reside, as introduced in Chap. 1 and depicted in Fig. 10.1. To understand the process of tumor invasion, one needs to first understand how epithelial cells, from which most of the solid tumors evolve, are organized to facilitate their division and inhibition of division when needed.

10.1.1 Tumor Invasion and the Roles Played by Hyaluronic Acid

Epithelial cells are arranged adjacent to each other, much like a sheet, on top of the basement membrane, which is a knitted network consisting of collagen and hyaluronic acid fibrils and multiple types of linker proteins such as fibronectins, elastins and laminins (Hay 1981), as discussed in Chap. 1 and a few other chapters. It is known that cell-cell contacts inhibit cell division, a phenomenon referred to as *contact inhibition* under physiological conditions, and their anchorage to the basement membrane is generally required before they can divide. Structurally, cell-cell adhesion is provided by *adherens junctions*, one of the three types of intercellular junctions connecting two neighboring cells while the other two types, *tight* and

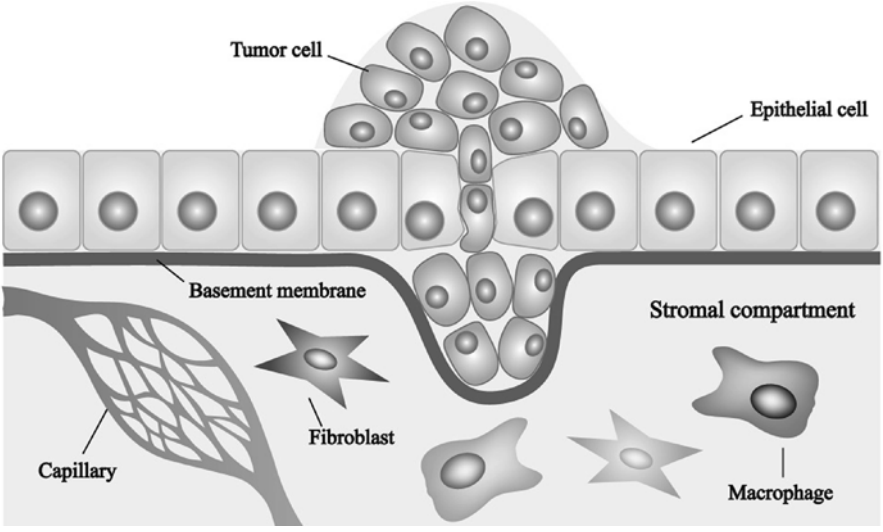

Fig. 10.1 A schematic of epithelial cells located above a basement membrane and associated stromal compartment, along with developing neoplastic cells

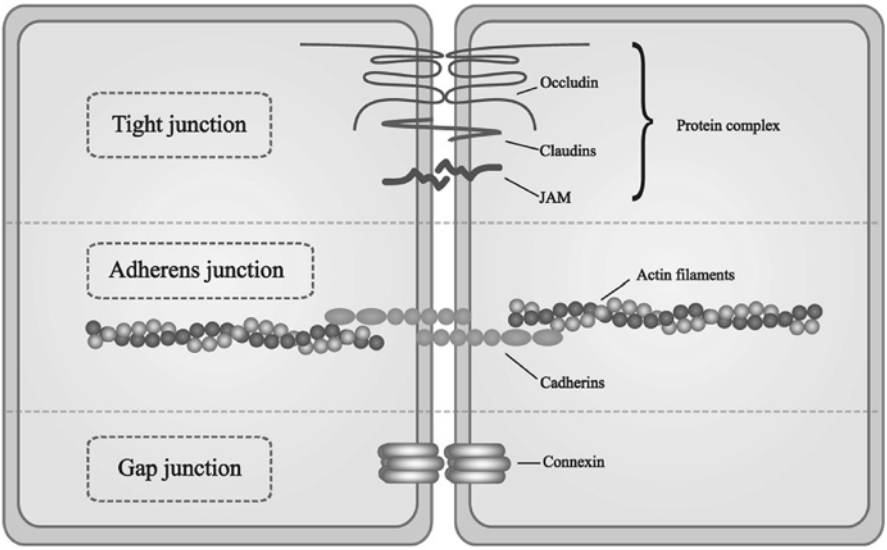

Fig. 10.2 A schematic of three types of junctions connecting two neighboring cells with adherens junctions providing the actual binding between the cells, each represented as a *rectangular box*

gap junctions, serve mainly as communication channels to allow molecules, including nutrients and signals, to pass between cells. An adherens junction consists of cadherin and a number of cytoplasmic proteins such as actin and catenin bound to cadherin, providing the actual intercellular adhesion as shown in Fig. 10.2. The cadherin protein in epithelial cells, specifically *E-cadherin*, is constantly regenerated with a 5-h half-life on the cell surface. As recently reported, reduced expression of this protein allows cells to migrate (Chen et al. 1997). A possible mechanism for this may be that repression of E-cadherin, in conjunction with other factors, can lead to the activation of the EMT (epithelial-mesenchymal transition) pathway since the repression of E-cadherin is crucial to the EMT activation as reported in a recent study (Lee et al. 2006). It has been demonstrated that *SNAIL* is a key regulator in repression of E-cadherin (Peinado et al. 2004; Montserrat et al. 2011). Interestingly, high molecular-weight hyaluronic acid has been reported to have a key role in the regulation of *SNAIL* (Craig et al. 2009), strongly suggesting its roles in repression of E-cadherin, as well as in activation of EMT as discussed in Chap. 6. [*N.B. A mesenchymal cell is a type of stem cell that can differentiate to different cell types and move between different locations*.]

We first briefly introduce the EMT pathway and its associated functions. The EMT pathway is involved in organ formation during embryogenesis. Under physiological conditions, its activation facilitates invasion of the endometrium and placenta placement to enable nutrient and gas exchange to the embryo. Cancer cells have apparently adapted to utilizing this pathway to facilitate their migration and then its reverse pathway, MET, to convert the migrated cells back to the original

epithelial tumor form to become established in the new location(s). It is noteworthy that the two cell types are very different, both functionally and morphologically; yet, they are convertible to each other through the activation of the EMT and the MET pathways.

Briefly, the basic functions of epithelial cells include: (1) protection of the tissues lying beneath them from invasion by pathogens and physical assault; (2) exchange of chemicals between the tissues they separate; (3) secretion of hormones into the vascular system and secretion of sweat, mucus and enzymes that are delivered by ducts glandular epithelium; and (4) transferring sensation such as smell, sound and sight. The epithelial tissue is one of the four major tissue types in humans, with the other three being connective, muscle and nervous tissues as introduced in Chap. 1. In contrast, mesenchymal tissue is a type of undifferentiated loose connective tissue composed of cells that can migrate easily. Generally mesenchymal cells interact with each other via their focal points rather than requiring cell-cell adhesion.

A number of signals have been found capable of activating the EMT pathway, such as the activation of *TGFβ*, *FGF*, *EGF*, *HGF*, *RAS-MAPK*, *WNT* and the *NOTCH* pathway, as well as hypoxia. A recent study reports that the activation of a specific isoform of *CD44* (see Chap. 6), namely *CD44s*, is a necessary condition for the activation of EMT. By integrating the information above and the discussion in Sect. 6.2, one can speculate that the excess production of hyaluronic acid may be a key initiator for abolishing cell-cell adhesion through a sequence of events comprising activation of *SNAIL*, repression of E-cadherin, mechanical stretches induced by hyaluronic acid and interactions between *CD44s* and hyaluronic acid, leading to the disconnection between two cells at the end. Potentially a well-designed computational analysis of transcriptomic data and statistical inference could lead to a detailed model of how cells lose their cell-cell adhesion in specific cancer types.

In addition to cell-cell adhesion, the adhesion between epithelial cells and basement membrane is provided by interactions between integrins on the cell surface and fibronectins of the ECM. While the current knowledge of the regulation of the interactions between such proteins is not complete, it has been observed that the spatial distance between the two is one key regulating factor, namely mechanical forces that stretch the connection can lead to their separation (Li et al. 2008; Schwartz 2010).

Furthermore, cancer cells also need to breach the basement membrane in order to migrate. This is accomplished through assembly and activation of a large complex structure named *invadopodium*, which consists of a dense actin core surrounded by actin-assembly proteins, membrane trafficking proteins, signaling proteins and transmembrane proteinases (Hagedorn and Sherwood 2011; Hagedorn et al. 2013). When activated, invadopodia create tunnels in the basement membrane for delivery of *MMPs* to the desired locations to degrade the membrane, which will be followed by tumor growth into the newly created space as depicted in Fig. 10.3. The current understanding is that the assembly of invadopodia is regulated by pericellular accumulation of excess hyaluronic acid and its interactions with *CD44* and *PKC* (Artym et al. 2006; Hill et al. 2006; Montgomery et al. 2012).

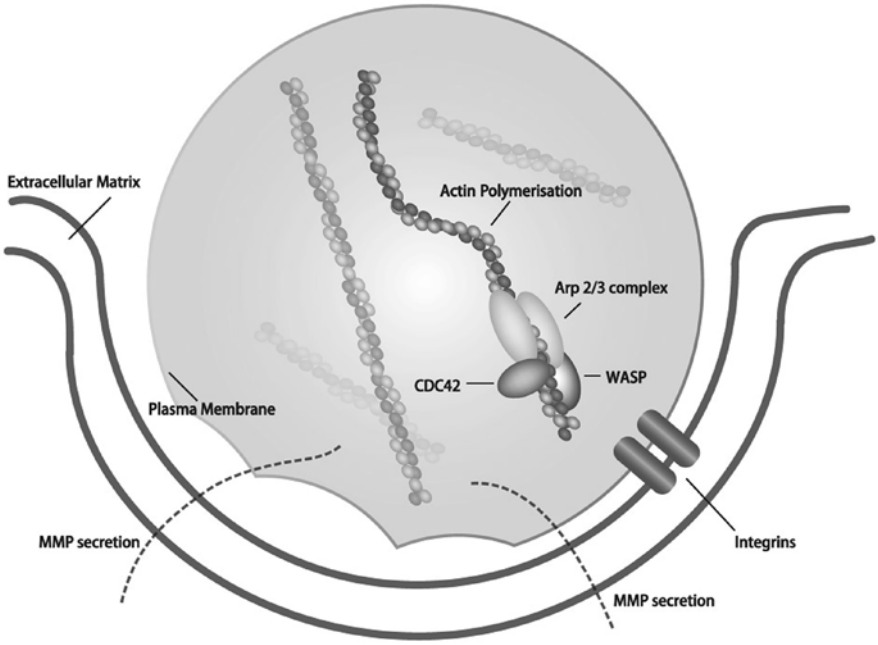

Fig. 10.3 A schematic of an invadopodium complex in action to break an extracellular matrix, where the *ARP2-3* protein complex has a key role in the regulation of the actin cytoskeleton; *CDC42* is involved in the regulation of cell cycle; and *WASP* is related to the **W**iskott–**A**ldrich **S**yndrome

The stiffness of a basement membrane has been found to have a key regulatory role in promoting the activity of invadopodia (Alexander et al. 2008; Parekh et al. 2011), in addition to its role in stimulating cell proliferation as discussed in Chap. 8. As reviewed in the earlier chapters, the stiffness of a basement membrane is mainly determined by the relative concentrations of collagen, elastin and laminin (Alberts et al. 2002; Owen and Shoichet 2010). Interestingly, hyaluronic acid fragments may have an important role in determining the relative concentrations of these macromolecules, hence the stiffness of the matrix. Specifically, hyaluronic acid fragments are able to up-regulate collagen-encoding gene (Chung et al. 2009) and enhance the synthesis of matrix elastin (Kothapalli and Ramamurthi 2009; Kothapalli et al. 2009). In addition, hyaluronic acid has also been linked to the production of laminin in various diseases such as cirrhosis (Lindqvist 1997). Overall, increased stiffness results in an increased concentration of invadopodia, as well as increased activities by invadopodia via the myosin II-*FAK*/*CAS* (Crk-associated substrate) pathway (Alexander et al. 2008), which could be triggered by the increased production of hyaluronic acid, ultimately induced by increased ROS levels as discussed later in this chapter.

To ascertain how these distinct components may functionally cooperate to initiate the metastatic process, one needs to focus on one specific protein family, *TGFβ*, as it may be the thread that connects all these pieces. The *TGFβ* proteins are a well-studied family of growth factors and are known to control cell proliferation and differentiation in most cell types. They exhibit regulatory roles in: (1) the activation of apoptosis; (2) cell cycle control by blocking cell-cycle advance from the G_1 to the S phase; and (3) inhibiting lymphocytes and monocyte-derived phagocytes from formation. The family has three known members, *TGFβ1*, *TGFβ2* and *TGFβ3*, all having been implicated in tumor invasion and metastases of multiple cancer types. Interestingly, these proteins serve different roles in early stage *versus* advanced stage cancers as discussed in Chap. 6. Specifically, they are anti-proliferative factors in the early stage of tumorigenesis but become oncogenes in advanced cancers (Prime et al. 2004; Seoane 2006). *TGFβ* is synthesized as a latent protein complex with *LTBP* (latent *TGFβ* binding protein) and *LAP* (latency-associated peptide), and secreted into the extracellular space. The first step in its activation is that of release from the complex. After its release, the protein can be activated by multiple factors under different conditions, such as integrins, *MMPs*, the tissue-injury responder protein *TSP1* (thrombospondin 1) (Rifkin and Sheppard 1999; Yu and Stamenkovic 2000) and even by changes in the ROS (Barcellos-Hoff and Dix 1996) and pH levels (Lyons et al. 1988). A particular mechanism is most relevant here, that of mechano-chemical signaling through integrin-αvβ5 (Wipff et al. 2007), strongly suggesting that tissue growth may play a role in the activation of *TGFβ*.

To examine if hyaluronic acid may have a role in the breaching of the basement membrane, an analysis of transcriptomic data of three cancer types, namely brain, liver and lung, was conducted. The statistical analysis revealed that, when *TGFβ* is activated, key hyaluronic acid synthesis and export genes, e.g., *HAS2* and *ABCC5* (see Chap. 6), tend to be expressed as shown in Fig. 10.4, suggesting that *TGFβ* may be a regulator of hyaluronic acid synthesis. Interestingly, multiple studies have reported that *TGFβ* can indeed increase the synthesis of hyaluronic acid (Wang et al. 2005; Nataatmadja et al. 2006), hence providing strong supporting evidence to the hypothesis.

In addition to its role in promoting hyaluronic acid synthesis, *TGFβ* can also activate the EMT pathway through multiple mechanisms. One pathway involves the activation of *SMAD2-3*, which then forms a complex with *SMAD4*, together serving as a transcription factor to trigger the EMT pathway (Miyazawa et al. 2002; Derynck and Zhang 2003; ten Dijke and Hill 2004; Gui et al. 2012), where *SMAD*s are a family of proteins that transduce extracellular signals from *TGFβ* ligands to the nucleus. Another pathway does not involve the *SMAD* proteins, instead through the activation of *ERK MAP* kinases, *RHO GTPases* and the *PI3K/AKT* pathway (Derynck and Zhang 2003; Xu et al. 2009; Zhang 2009). Overall, the current understanding is that *TGFβ* can activate both *SMAD* and non-*SMAD* pathways, which crosstalk with various signaling pathways to trigger EMT and possibly other pathways depending on the specific context.

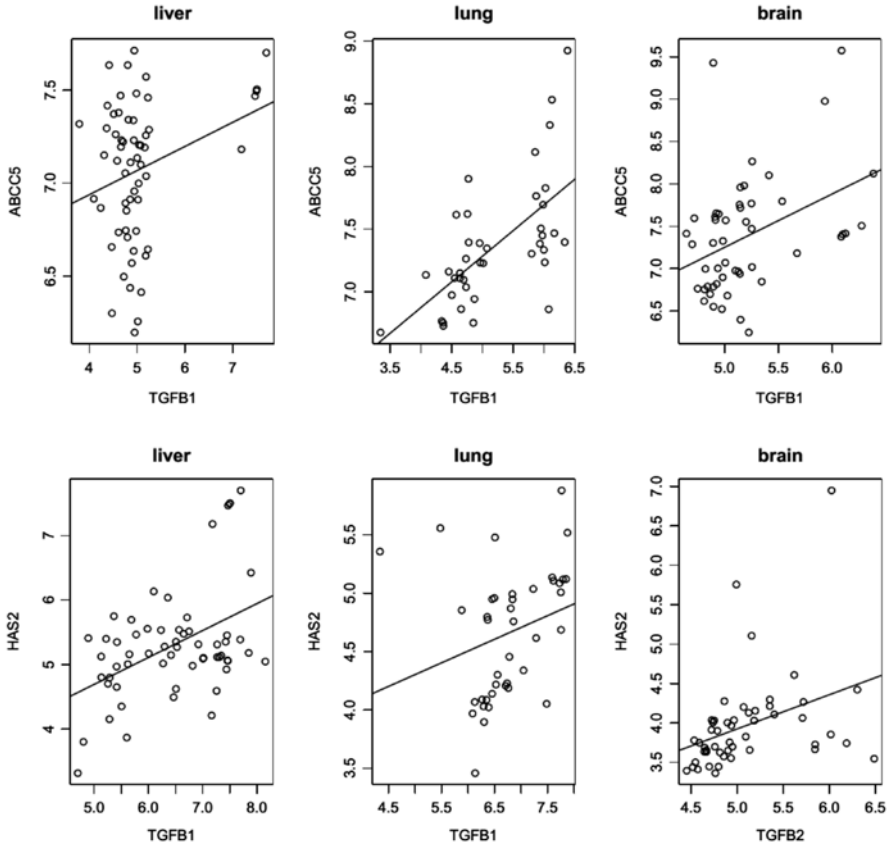

Fig. 10.4 Gene expression of *TGFβ versus* genes responsible for hyaluronic acid synthesis across multiple tissue samples of three types of advanced cancer, where *HAS2* and *ABCC5* are hyaluronic acid synthesis and export genes, respectively (data from the GEO database)

Joining the above information and discussion, one can postulate the following sequence of events that could lead to the breach of the basement membrane by a growing tumor. The continuous growth of a tumor, coupled with inflammation, may create mechanical forces that promote the activation of integrin-αvβ5, which in turn activates *TGFβ*, leading to the synthesis and export of hyaluronic acid. The increased production and export of hyaluronic acid can further increase the aforementioned mechanical forces, further increasing bioactive integrin-αvβ5 and *TGFβ*, which, in conjunction with the repressed E-cadherin due to hyaluronic acid, can ultimately lead to the activation of EMT, as well as increased mobility and invasiveness of the cancer cells. The actual breaching of the basement membrane is accomplished by *MMPs* delivered to the right locations through the assembly and activity of invadopodia, which seem to be initiated by the production and degradation of hyaluronic acid. Overall, hyaluronic acid exerts an essential role in making this sequence of events possible.

10.1.2 Interactions with Stromal Cells

The second key event during local invasion of tumor cells is that they enter the stromal compartment where they can interact directly with stromal cells, namely fibroblasts and pericytes, which are the supporting cells to the parenchymal cells in an organ. The local immune cells are also considered as stromal cells because of their supporting roles.

The main physiological functions of fibroblasts are to synthesize and export ECM proteins, glycoproteins and glycosaminoglycans, and to function in wound healing. Multiple diseases are closely related to excess production, deposition and contraction of the ECM, such as diabetic nephropathy, liver cirrhosis, arteriosclerosis and rheumatoid arthritis. The current understanding is that *TGFβ* can induce not only the synthesis by fibroblasts but also the contraction of an ECM. Specifically, it induces a specific form of fibronectin, *EDA* (ectodysplasin-A), which together with *TGFβ1* can trigger the enhancement of α-SMA (alpha-actin-2) and accelerate the contraction of fibroblasts (Ina et al. 2011), a major cue for activation of latent *TGFβ1* (Wipff et al. 2007). The information here complements the above model for mechanical force-induced activation of integrin-αvβ5, ultimately leading to the activation of EMT. Moreover, these two processes may interact, sending signals to each other and together accomplishing EMT activation. Well-designed analyses of transcriptomic data for various advanced stage cancers may lead to the establishment of a self-consistent model for the entire process of EMT activation in the microenvironment of advanced cancers. In addition to this role, cancer associated fibroblasts are also known to release a variety of proteases such as *MMPs*, hence further facilitating remodeling of the ECM needed by cancer invasion and metastasis.

Pericytes are contractile cells that surround the endothelial cells of capillaries. Their physiological function is to regulate capillary blood flow and clearance of cellular debris. Previous studies have discovered that pericytes serve as a gatekeeper in preventing cancer cells from spreading as it has been demonstrated that pericyte-deficiency in mice increases cancer metastasis (Xian et al. 2006). A report on diabetic retinopathy may provide a strong clue as to why cancer tissues tend to have decreased numbers of pericytes as has been observed. The study concludes that the activation of the angiopoietin-2 protein leads to a reduction in pericyte population (Hammes et al. 2004). Hence, one can infer that the increased expression of angiopoietin-2, triggered by the need for angiogenesis in a tumor microenvironment, results in a decrease in pericyte population, hence gradually losing its safeguard against metastasis. This model is well supported by the transcriptomic data collected on a large number of tissue samples of four cancer types. Specifically the pericyte concentration decreases in tumor samples as a cancer advances, as reflected by the decreased expressions of pericyte marker genes: *ACTA2* (actin, aortic smooth muscle), *CSPG4* (chondroitin sulfate proteoglycan 4), *ENPEP* (glutamyl aminopeptidase) and *ANPEP* (alanyl aminopeptidase), as well as by increased expression of *ANGPT2* (angiopoietin-2) as shown in Fig. 10.5. It can be expected that data mining and statistical inference on larger datasets in a more systematic manner could lead to the development of a detailed model for this hypothesis.

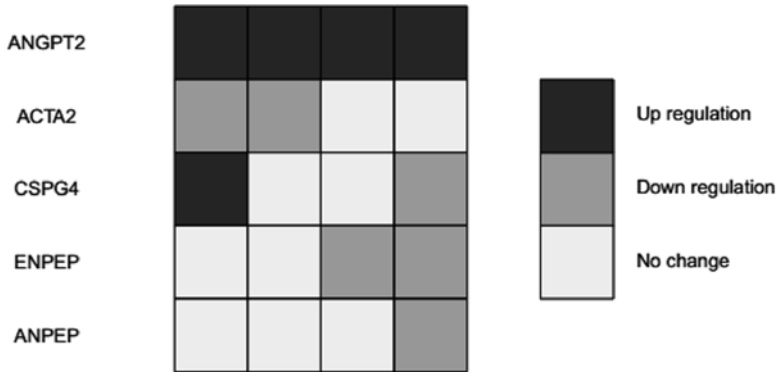

Fig. 10.5 Expression-level changes of five genes related to pericyte population in cancer *versus* control samples. Each *column* represents one cancer from *left* to *right*: renal cell carcinoma, leukemia, liver, and lung adenocarcinoma, and each *row* represents a gene. *Black* and *gray* denote increase and decrease in gene-expression levels, respectively, while *white* is for no change. The detailed dataset used here is given in Appendix

In addition, there seems to be a partner relationship between tumor cells and the tumor-associated stromal cells from an energy-metabolism perspective. As discussed in Chaps. 5 and 8, cancer cells produce high concentrations of lactic acid in their microenvironments, which is an incompletely used energy source. The lactate acid can be further used by the stromal cells, which do not necessarily use glycolysis for ATP generation as tumor cells do after its oxidation back to pyruvate. A proposal has been made with supporting data that the regenerated, excess pyruvate in stromal cells can also be released and reused subsequently by the cancer cells (Martinez-Outschoorn et al. 2011). This model is also partially supported by the observation that tumor-associated stromal cells tend to have low expression levels of their glucose transporters, indicating the possibility of low glucose uptake, thus enabling tumor cells greater access to glucose. In addition, epithelial cancer cells have been found capable of inducing the expression of metabolic genes in the neighboring fibroblasts and enhancing their output for the production of energy-rich metabolites (Pavlides et al. 2009; Migneco et al. 2010; Martinez-Outschoorn et al. 2011), hence making these two cell types adopt a close "host-parasite" relationship.

The results of interactions between cancer and immune cells can be very complex as different immune cells may have different roles, including both anti- and pro-cancer, at different stages of cancer development. In addition to the direct involvement of immune cells in tumorigenesis, they also serve as a selection process for those tumor cells that elude destruction by immune cells, as discussed in detail in Chap. 8.

10.2 Traveling Cancer Cells

While cancer cells can travel to distant locations through both blood circulation and the lymphatic system, clinical data suggest that the majority of cancer cells travel through the circulatory system (Wong and Hynes 2006; Eccles and Welch 2007). Hence, we focus on the fate of cancer cells in blood circulation in this section.

10.2.1 Intravasation

The first step for the invading cancer cells to enter circulation is to cross the endothelial cell barrier that forms the wall of capillaries. Innate immune cells are known to have essential roles in promoting cancer cell intravasation, analogous to their roles during cancer initiation as discussed in Chap. 7. Specifically, tumor-associated macrophages (TAMs) promote tumor angiogenesis through release of *VEGFs* to stimulate the formation of tumor blood vessels, which tend to be leaky compared to normal blood vessels, hence allowing cancer cells to enter the circulation more easily.

10.2.2 In Circulation

It is estimated that for each gram of tumor tissue accessible to blood circulation, about one million tumor cells actually escape into the circulation, and they typically remain there for just a few hours before they lodge on the inside wall of the blood vessels or are destroyed. Another estimate suggests that 1 out of 10,000 cancer cells in circulation can survive and ultimately settle in a distant location.

Circulating tumor cells (*CTCs*) need to overcome a number of challenges to survive in circulation, including mechanical forces and immune destruction. In addition, they also need to overcome a programmed cell death, called *anoikis*, which is self-induced when cells leave their original habitats and become anchorage-free (Douma et al. 2004; Gupta and Massague 2006). The mechanism(s) by which the CTCs avoid this programmed cell death is poorly understood, but the following information provides some hints about this. It is known that tumor cells can continue to thrive in an anchorage-independent manner through a signaling process mediated by cell-surface hyaluronic acid to avoid anoikis. However, it is not known whether the CTCs have any hyaluronic acid on their surfaces. While determination of whether this is the case can be done through metabolic analyses of cells using techniques such as mass spectrometry, no data are currently available in the public domain to give a direct answer to the question.

Recent findings suggest that protein *TRKB* (tyrosine receptor kinase B) may have an important role in rendering tumor cells anoikis-resistant (Kim et al. 2012), where *TRKB* is a growth factor that can induce cell survival and differentiation pathways,

upon binding with and being activated by its cognate ligand such as *BDNF* (brain derived neurotrophic factor). Another study has reported that the hyaluronic acid tetrasaccharide can increase the expression of *BDNF* and *VGEF* in an *in vitro* experiment (Wang et al. 2012). Furthermore, a study on tissue regeneration has shown that high molecular-weight hyaluronic acid can serve as a scaffold for *BDNF* during tissue regeneration (Takeda et al. 2011). Together this information suggests the possibility that hyaluronic acid may be generated by CTCs, which trigger the expression of *BDNF*, possibly along with other factors, that in turn activates *TRKB* and provides the CTCs with anoikis-resistance. Clearly this possibility requires further experimental validation.

While in circulation, CTCs tend to aggregate into clusters with platelets (Cho et al. 2012a). Such a formation should give CTCs an advantage for their survival against the mechanical forces of the blood flow, the shear force and immune attack in circulation. Platelets seem to have an essential role in transporting the CTCs and maintaining their viability as multiple studies have demonstrated that platelet depletion, or even an inhibition of tumor cell-induced platelet aggregates, diminishes metastasis (Gasic et al. 1968; Gasic 1984; Amirkhosravi et al. 2003; Palumbo et al. 2005). While the detailed binding mode between CTCs and platelets has not been thoroughly elucidated, it has been proposed that the interaction is through binding of integrins on the cell surfaces of the two types to common fibronectin or collagen, or through binding of *PAR*s (protease activated receptors) on the two cell surfaces to common thrombin (Gay and Felding-Habermann 2011). Note that integrins are a family of transmembrane receptors for providing linkages among cells or between cells and macromolecules in the ECM as discussed earlier.

An analysis of gene-expression data of breast cancer CTCs in the public domain (Molloy et al. 2012) revealed that genes encoding integrin-α2bβ3, integrin-α2β1, *GPI* receptors (responsible for platelet adhesion), *ADP* (adipose) receptors and the "platelet aggregation plug formation" pathway are all up-regulated, hence providing strong evidence in support of the above proposal.

10.2.3 Extravasation

Tumor extravasation is the process through which the CTCs lodge to the inner wall of a blood vessel of a distant organ and then penetrate the wall to settle in the stromal compartment of the organ. Little is known about the mechanism of tumor extravasation, but it has been speculated that it is probably similar to that of leukocyte extravasation into inflammatory tissues during immune responses (Strell and Entschladen 2008).

Briefly, the extravasation process of leukocytes, such as T-cells, natural killer cells, neutrophil granulocytes and monocytes, consists of the following steps: (1) *rolling*: the vascular endothelial cells recruit leukocytes through the protein selectin on cell surfaces, which bind with selectin ligands such as *SELPLG* (p-selectin glycoprotein ligand-1, also known as *CD162*) on the surfaces of leukocytes, forming

loose interactions, where selectins are a family of cell-adhesion glycoproteins. Because the interactions tend to be relatively loose, the recruited leukocytes have rolling motions in response to the blood flow, hence the name; (2) *adhesion*: integrins are activated on both leukocytes and endothelial cells during the rolling step, and their binding gives rise to tight adhesion between the cells; and (3) *transmigration*: leukocytes transmigrate through the endothelium without irreversibly impairing its integrity as they tend to move through the endothelial monolayer between the endothelial cells (Hofbauer et al. 1999).

It has been speculated that while cancer cells may use a similar mechanism for extravasation, they may use a different set of selectin ligands in different metastasis types to accomplish the initial loose binding with endothelial cells, such as *HCELL* (an E- and L-selectin ligand), *CD44*, *ELAM1* (E-selectin ligand-1, also known as *CD62E*) for bone metastasis and *CEA* (carcinoembryonic antigen) for colon metastasis (Dimitroff et al. 2005; Strell and Entschladen 2008; Thomas et al. 2008; Dallas et al. 2012; Hiraga et al. 2013). The adhesion between cancer and endothelial cells may be accomplished via binding between integrins as in the case of leukocytes discussed above, but possibly by subgroups different from those used by leukocytes, specifically the β*2* subgroup. A recent study has observed that the α*4* subgroup of integrins is used in some cancers (Okahara et al. 1994; Garofalo et al. 1995; Bendas and Borsig 2012). For the transmigration step, cancer cells may have evolved a strategy different from the one used by leukocytes as they tend to be highly destructive by damaging the endothelium, possibly because of their substantially larger sizes compared to those of leukocytes and no restraint being placed on them for not impairing the integrity of endothelium. No specific genes have been implicated for this, but it is expected that computational analyses of transcriptomic data on multiple metastasis types may lead to candidate genes.

The CTCs that reached the new locations are referred to as *DTCs* (disseminated CTCs) in the literature, which specifically refer to the direct progeny of the primary cancer rather than highly transformed metastatic cancer. Publicly available gene-expression data of *DTCs* originating from prostate cancer have been analyzed. It was found that the following gene groups are up-regulated in DTCs in comparison with the corresponding CTCs: cell cycle related genes such as the G_1-phase and S-phase genes, actin-cytoskeleton remodeling genes, *WNT*-signaling pathways, DNA synthesis, glucose metabolism, steroid metabolism and sphingolipid metabolism. These data strongly suggest the following: (1) these DTCs are in a state of proliferation; and (2) these cells are under oxidative stress.

For (1), it is worth noting that a DTC population tends to remain stable for months or even years in their new habitats, hence possible dormancy of these cells having been suggested (Meadows 2005; Wang et al. 2013). Clearly this hypothesis was not supported by the above results from data analysis. One possible explanation for these two pieces of seemingly conflicting information is that DTCs may initially be in a proliferation state but gradually stop proliferation to remain in a growth-arrest state in the cell cycle, possibly triggered by their incompatibility with the new microenvironment. Such incompatibility may include: (1) attacks from the local immune cells that have not become associated with the cancer cells; (2) limitation in the blood supply, which is designed only to support the local normal cells before

the establishment of tumor angiogenesis; (3) toxic effect by the increased O_2 level in comparison with their previous habitats (see Chap. 11 for details); and (4) altered ROS and pH levels, both of which will be quite different from their original sites, hence possibly causing substantial changes in the cellular metabolism of the DTCs if not killed by the altered ROS and pH. Another possibility is that the majority of the proliferated cells from the DTCs may be destroyed due to their incompatibility with the new microenvironment (see Chap. 11 for a detailed discussion). Therefore, the observed population stability of the DTCs may represent a dynamic equilibrium, rather than no proliferative activities. Potentially both possibilities may be true for some metastatic cancers.

For (2), the possibility is an interesting one that has not received much attention in the cancer literature. A detailed analysis of the DTC transcriptomic data revealed that a number of *CYP* genes, all encoding anti-oxidant P450 enzymes, are up-regulated along with increased sphingolipid metabolism *versus* those in CTCs. Similar expression patterns are also observed between metastatic cancer and the matching primary cancer tissues (see Chap. 11 for details). These data strongly suggest that the DTCs are under increased oxidative stress and the plasma membranes are damaged. One possible explanation is: the DTCs have just migrated from a highly hypoxic condition where they had been residing for an extended period of time, possibly up to 10 or 15 years, and the cancer cells may have evolved to become nearly anaerobic. When they are suddenly exposed to an oxygen-rich condition, their cellular responses to the new stress induced by the increased O_2 level include the activation of *CYPs* and other anti-oxidant genes. In addition, the plasma membrane damage, suggested by the increased sphingolipid metabolism, may be the result of increased and continuous lipid peroxidation produced by the increased O_2 level as discussed in Chap. 11, where the implications of these observations are also offered.

Overall, one can see that cancer cells leave their primary bases and travel through the circulatory system, with help from multiple local environmental factors such as hyaluronic acid and stromal cells. This journey occurs in various steps, including disconnection from the original site and other cells, protection while in circulation and establishment in distant organs. The multi-faceted roles played by hyaluronic acid highlight this class of molecules as probably a most important facilitator throughout the whole process of cancer development (see Chap. 6). The relatively simple data analyses as done here have revealed interesting and previously unknown information about the activities of stromal cells and DTCs as they leave their base and seek establishment in the new location(s). The following section discusses how the DTCs survive the new environment.

10.3 Adaptation to the New Microenvironment

As mentioned earlier, it typically takes just a few hours for the CTCs to adhere to the endothelial cells along the blood vessels after leaving their primary sites, but they may remain dormant for weeks, months or even years before they begin to actively

proliferate (Meng et al. 2004; Alix-Panabieres et al. 2008). During this period, these cells must overcome a number of obstacles to remain viable and to retain the ability to achieve reactivation.

The questions we are interested in understanding here are: (a) *What challenges must the metastatic tumor cells overcome in their new microenvironment*; (b) *What changes must these cells make in their microenvironment before they begin proliferating again*; and (c) *What determines the rate of proliferation of a metastatic cancer, knowing that some metastatic cancers grow substantially faster than others*?

To address these questions, it is prudent to first review a hypothesis proposed by British surgeon Stephen Paget over 100 years ago when he observed that breast-cancer patients tended to develop secondary cancer in their livers. Since then, it has been widely observed that cancers from different origins have propensities to different destinations. At the heart of the Paget hypothesis, different organs, in terms of their microenvironment, may have different levels of compatibility with specific metastasizing cells, the so-called "seed and soil" hypothesis (Fidler 2003; Fidler and Poste 2008). The hypothesis has recently regained some momentum and is being considered as a good model for distant metastasis because of the finding that the expression patterns of genes involved in mediating the metastasis of breast cancer to bone are rather different from those that direct the metastasis to lung (Langley and Fidler 2011). While validating the "seed and soil" hypothesis experimentally may prove to be tricky, it can potentially be computationally validated (or rejected, refined) through comparative transcriptomic data analyses of primary *versus* matching metastatic cancer samples across multiple cancer types, particularly samples with the same type of primary cancer that has metastasized to different organs. This can be accomplished by checking if primary cancers that metastasized to different organs tend to share similar expression patterns of some to-be-identified genes among those that spread to the same organs, which are not shared by those metastasized to different organs. Similar analyses can be carried out on cancers that have metastasized to the same organ but from different origins.

Interestingly, if one compares the microenvironments of wherever the primary cancer is located and wherever it may spread to, the difference between the old microenvironment and the new one is substantial and multi-faceted. The low compatibility between the metastatic cells and their new environment can make the survival of the new settlers very challenging. One key piece of information that the suggested analyses above could potentially reveal is: which aspects in the cell-microenvironment compatibility are the most essential factors in determining if DTCs can remain viable and develop in a specific new location? An answer to this question could potentially have a profound impact on our understanding of metastatic cancer and identifying possible ways to slow their growth. In the following, we discuss the adaptations the DTCs must make in order to survive in their new environment.

10.3.1 Challenges to Cancer Cells in the New Microenvironment

A key challenge for the arriving metastatic cancer cells is survival in the new microenvironment. A fundamental difference between the new and the old one is that the original environment is a tumor environment that is pro-cancer growth while the new one is a normal microenvironment in a healthy organ, which is anti-cancer. This difference could be substantial. Using pH as an example, the pH level in the new microenvironment will be higher than that of the old one as discussed in Chap. 8; furthermore, the environment is not lactate-rich as in the original one. Hence, T-cells in the new environment may be much more aggressive against the new settlers, compared to the T-cells in their old habitats where they have become less active against cancer cells due to the lactate-rich environment. More generally, the original microenvironment offers a variety of pro-cancer signals, such as anti-apoptotic, angiogenesis, cell survival and proliferation signals, but these will not be available in the new location. Similarly, their supporting stromal cells, such as TAMs, will not be available upon their arrival. Another key challenge is that, unlike the highly hypoxic environment typically associated with primary cancers, the new environment is rich in oxygen. Having the tumor cells in the new environment is analogous to putting anaerobic cells in an aerobic environment; it may either kill the new comers or yield substantial changes, for example through regulation or selection of specific mutations, in their cellular states to protect them against the toxic oxygen. A similar argument can be made about the need to adapt to the local redox states by the new settlers. Further discussion along this line is given in Chap. 11.

Another key factor that may affect the viability of the arriving tumor cells is their interactions with the ECM. As discussed in Chaps. 5 and 6, such interactions play essential roles in both the transformation and viability of the tumor cells in their original habitat. One obvious difference now is that the old ECM is pro-growth, and hence possibly very stiff, while the new one is clearly not. This may be one of the reasons for the relatively slow growth during the early stage of the new comers. Other than this, very little is currently known about the differences between the physicochemical properties of the new *versus* the original ECM. In addition, cell-cell competition will be a key factor that may affect the fate of the new settlers (as discussed in Chap. 8), knowing that they continue to proliferate as revealed by our analyses of gene-expression data of DTCs and shown in Fig. 10.6. One possibility could be that the long dormancy time may represent the preparation time needed by a metastatic cancer to select the fittest cells for the new environment through rounds of proliferation and cell-cell competition (see Chap. 8 for the details). It is worth noting that cell-cell competition does not change the overall biomass of a tumor but only serves as a selection process for more robust cells in the new environment, as discussed in Chap. 8. Overall, one can imagine that, for the cancer cells to become established and thrive in the new environment, they must undergo substantial adaptations to make the cell population strong enough to survive the new environment.

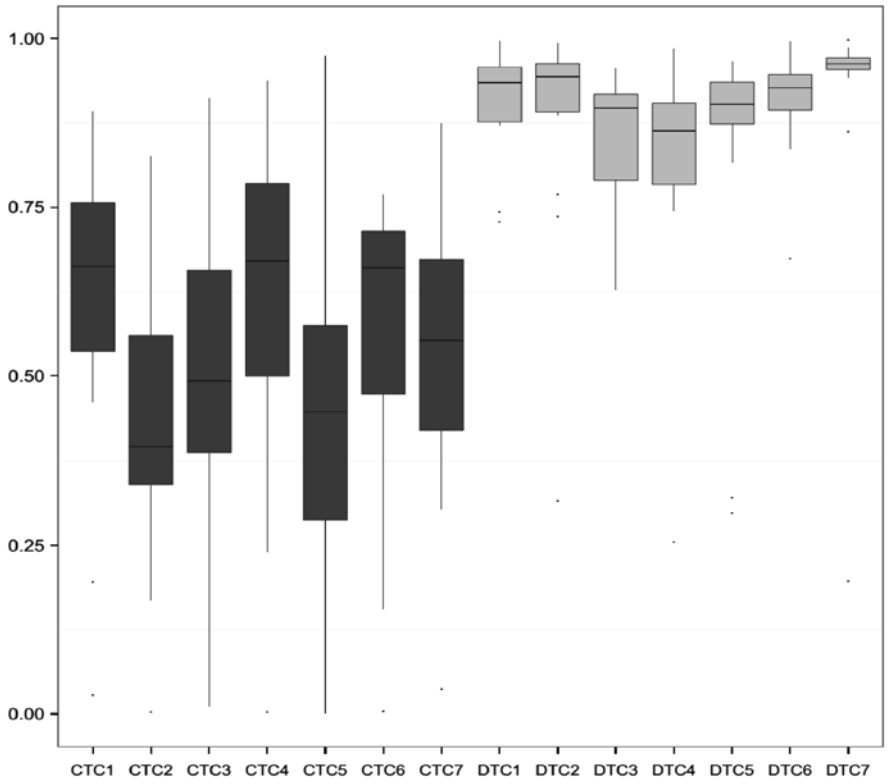

Fig. 10.6 The rankings of 13 cell-cycle related genes, namely *CCNA2* (cyclin A2), *CCNB1*, *CCNB2*, *CCND2*, *CCNE1*, *CCNF*, *CDH1* (cadherin 1, type 1, E-cadherin), *E2F1*, *MCM2* (mini-chromosome maintenance complex component 2), *MCM3*, *MCM4*, *MCM5*, *MCM6* among all *N* human genes in terms of gene expression levels, where *N* is set at 20,000 here. For each of the seven CTC samples (on the *left*) and the seven DTC samples (on the *right*), a gene's normalized expression rank is calculated as: (*N* − rank of the gene's expression)/*N*. Each *dot* in a box plot is the normalized rank for 1 of the 13 above genes in a specific sample

10.3.2 Changing the Microenvironment

One proposed mechanism by which the new settlers alter their new environment to enhance their chance for survival is through the release of exosomes. *Exosomes* are derived from cancer cellular endosomes through a process termed *inward budding*, where cytoplasmic RNA molecules and functional proteins are encapsulated into exosomes and then secreted through a process driven by *RAB* (Rab escort protein 1) *GTPases* (Hsu et al. 2010; Ostrowski et al. 2010). The tetraspanin–integrin complex enables the binding of exosomes to the target cells that express adhesion molecules such as *ICAM1* (intercellular adhesion molecule 1) on the cell surface. Such adhesion molecules can be activated by pro-inflammatory signals. Cancer cells *in situ*

have been found to exchange proteins endowed with oncogenic activities with each other through exosome-mediated transfer for their survival (Kahlert and Kalluri 2013). A number of studies have reported cases where cancer cells change their environment by releasing exosomes into the extracellular space. For example, exosomes from breast cancer cells have been found to convert mesenchymal cells to myofibroblasts via a *SMAD*-mediated pathway. Myofibroblasts, a less differentiated form of fibroblasts, are a key source of matrix-remodeling proteins within the tumor microenvironment and participate in tumor angiogenesis (Vong and Kalluri 2011; Cho et al. 2012b). Another example is that melanoma-derived exosomes enhance the lung endothelial permeability and increase lung metastases in mice (Peinado et al. 2012).

10.3.3 From Proliferation to Dormancy

Generally, DTCs enter a period of dormancy after becoming lodged in their new locations. This could result from the limited availability of blood supply (referred to as *angiogenic dormancy*), immune surveillance and attack on fast growing cells, or by cellular quiescence triggered by the incompatibility with the new environment, where growth may be arrested in the G_0-G_1 phase of the cell cycle. The duration of dormant time varies substantially across different cancers, even cancers of the same type. One observation is that the less differentiated (i.e., more stem-cell like) tumor cells tend to have shorter dormancy times and become more aggressive in their renewal to the proliferative phase (Aguirre-Ghiso 2007; Wikman et al. 2008). The overall level of understanding of cancer dormancy is quite limited at this point, partially due to the reality that very limited experimental data on such cells have been collected, possibly due to the challenging nature in identifying these cells *in vivo*.

One proposed tumor-dormancy model is that after the DTCs arrive and adhere to the local cells, their metastasis suppressor genes may regulate cell dormancy in response to the stresses invoked by the new environment, which will protect them from detection by the immune system (Horak et al. 2008). Another study has suggested that interactions between the arriving tumor cells and the local ECM may play a key role in sending the tumor cells into dormancy. Specifically, the study found that melanoma cells are growth arrested at the G_1/S checkpoint when they are in contact with polymerized fibrillar collagen. In comparison, alteration of the collagen formation to that of denatured collagen activates the cell cycle and moves to the S phase (Hansen and Albrecht 1999). This may represent another problem that can benefit from comparative statistical analyses of transcriptomic data across multiple types of early metastatic cancers, which could lead to discoveries regarding the validity of the model or whether it is true for only certain types of metastatic cancers.

A number of genes have been implicated in executing the growth inhibition of metastatic tumor cells, hence referred to as *metastasis suppressor* genes. Such genes have the ability to prevent proliferation as the tumor cells are becoming established in their new environment by inducing dormancy or apoptosis. *KISS1* (kisspeptin) is one such gene.

The Kisspeptins, processed by *KISS1*, can bind with *GPR54* and possibly regulate cellular cytoskeletal reorganization to block cell proliferation through the induction of dormancy (Nash et al. 2006; Paez et al. 2012). *KAI1* (R2 leukocyte antigen) is another metastasis suppressor gene. Its encoded protein can form a complex with integrins, and together they inhibit cell proliferation through induction of tumor-cell growth arrest. Other known metastasis suppressor genes include *MKK4* (an activator of *MAPK*, *P38* and *JNK*), *BRMS1* (inhibitor of angiogenesis by suppressing the *NFκB* activity) and *CTGF* (a regulator of cell adhesion, proliferation and differentiation). A recent study suggests that some ECM components may have important roles in maintaining metastatic dormancy (Barkan et al. 2010).

One should be able to design and carry out computational analyses of relevant transcriptomic data of early metastatic cancers to validate, refine or reject this hypothesis. One thing is clear, however, during dormancy the cancer cells continue some of their cellular activities, including proliferation as discussed earlier. It can be hypothesized that some of these activities are related to a change(s) in their metabolic state for their adaptation to the new microenvironment, which can also be computationally validated, refined or rejected when transcriptomic data for metastatic cancers at the early stages are available.

10.3.4 Reactivation to Proliferation from Dormancy

The remodeling pathway of the ECM is believed to have a key role in the reactivation of cancer growth from dormancy. Specifically, it has been reported that dormant cancer cells have a distinct cytoskeletal organization, which has only transient adhesion to the ECM (Barkan et al. 2010). Changing the components of the ECM, such as an increase in the fibronectin composition and hence the structure as well as the physical properties of the matrix, can reactivate the dormant tumor cells. In addition, type I collagen has been found to exhibit reactivation roles of dormant tumor cells, suggesting that it may not be specific molecular types, but rather the shape and the physical properties, such as stiffness of the matrix, that can reactivate dormant cells.

A few studies have been published that focused on the detailed molecular mechanisms of the reactivation process. One such investigation on bone metastasis found that local inflammation increases the expression of the *VCAM1* (vascular cell adhesion molecule 1) protein in cancer cells in a bone microenvironment. When *VCAM1* sheds from the cell surface, the soluble *VCAM1* molecule attracts osteoclast progenitors to the cancer cells through binding with the cognate receptor integrin α4β1, leading to adhesion of the progenitors to each other and ultimately resulting in an increase in osteoclast activity and the escape of dormancy (Lu et al. 2011). One possible cause of the local inflammation could be the result of certain renewed activities in the dormant cancer cells, which triggers the immune response and also alters the ECM properties.

As discussed throughout this chapter, hypotheses like the above can be computationally examined by determining changes in the expression patterns of genes believed to be involved in the aforementioned processes. Then, an assessment of the correlations between the expression changes of these genes and those possibly linked to the reactivation of cancer cells from dormancy could lead to a firm validation or rejection. The key for doing such analyses is the availability of gene-expression data of metastatic cancer cells in dormancy *versus* such cells that are exiting dormancy, which are currently lacking. Complementing such studies, it should be possible in time to also access proteomic data to determine protein content directly, including post-translational events.

A study on the transition from quiescence to proliferation of metastatic breast cancer showed that it is the cytoskeletal reorganization with *F-actin* (a protein that can form a linear polymer microfilament, relevant to cell mobility and contraction) that leads to actin stress fiber formation and reactivation of proliferative growth (Barkan et al. 2008). This study also showed that the ECM and tetraspanins play critical roles in enabling cell survival, proliferation and cytoskeletal changes required for the switch from dormancy to proliferation and invasion. Clearly this hypothesis can also be examined computationally as discussed earlier.

10.4 Hyaluronic Acid Is a Key Facilitator of Metastasis

Like the roles played by hyaluronic acid in cancer initiation, this glucosaminoglycan seems to also serve as a major facilitator of cancer metastasis and the initial development after migration (see discussion in the previous sections). Intuitively this makes sense since human cells have evolved in such a way that the ECM, of which hyaluronic acid is a part, serves as the main signal source for cell survival, proliferation and mobility among other cellular state transitions as introduced in Chap. 1. In these capacities, hyaluronic acid continues to serve cancer cells by facilitating their migration and survival in their new environment(s).

10.4.1 Motility

A question of interest here is: *Are there thresholds of some conditions beyond which primary cancers start to metastasize*? Clearly, various pericellular or intracellular environmental factors can be considered, such as the level of hypoxia, oxidative stress, extracellular pH or anything that can potentially lead to the increased production, and hence export, of hyaluronic acid as there are multiple lines of evidence suggesting that this molecule is a key to initiate the metastasis process. Here some discussion is provided on the accumulation of ROS and its role in increased hyaluronic acid production.

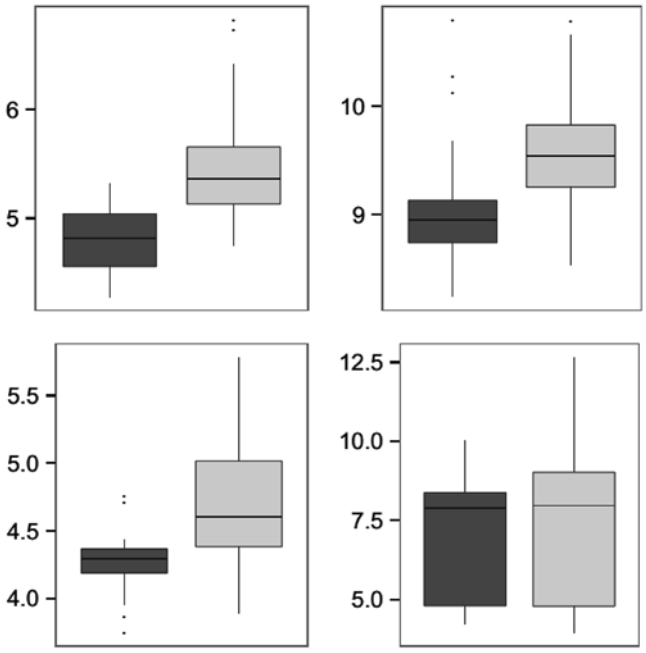

Fig. 10.7 Ranges of ROS level fluctuation, reflected by expression levels of ROS responsive genes, GSS (*top*) and GCLC (*bottom*) in normal (*dark gray*) *versus* cancer (*light gray*) in two datasets: GSE13195 consisting of 25 pairs of gastric cancer *versus* matching control tissues (the *panels* on the *left*) and GSE19804 consisting 60 pairs of lung cancer *versus* matching control tissues (the *panel* on the *right*)

It is known that ROSs such as the superoxide radical, hydrogen peroxide and hydroxyl radicals have an important role in cancer development. The general understanding has been that ROS tends to accumulate as a cancer evolves, leading to increased DNA damage (Waris and Ahsan 2006), faulty antioxidants, activation of cancer-related transcription factors such as *NFκB* and a gradual change in the cellular redox state (Gupta et al. 2012). A recent study even suggests that cancer metastasis is a cancer cell's escape from oxidative stress in their primary sites (Pani et al. 2010). Figure 10.7 shows a general trend of ROS levels in two cancer types as they progress, measured in terms of the expression levels of ROS stress-responsive genes, indicating that when the ROS levels fluctuate inside cells, their transient maximum ROS level tends to be higher in cancer *versus* matching controls.

Previous studies have reported that ROS can induce *TGFβ* (Barcellos-Hoff and Dix 1996; Jain et al. 2013), which can serve as an oncogene in advanced cancers and trigger the synthesis of hyaluronic acid by activating both hyaluronic acid synthases *HAS1* and *HAS2* (Liu and Gaston Pravia 2010). Actually it has been widely observed that advanced stage cancers tend to have increased hyaluronic acid production, which we posit is the result of the increased accumulation of ROS. In addition, it has been well established that an elevated level of hyaluronic acid

increases the motility of tumor cells, facilitating their escape from the primary tumor site and starting the metastatic process. One study, for example, has shown that excess hyaluronic acid synthesis and processing directly promotes metastasis of prostate cancer (Bharadwaj et al. 2009). Hence it is reasonable to speculate that this may be related to the mechanical forces generated by the increased content of hyaluronic acid around the cancer cells, leading to the disconnection between the host cells and other cells, as well as between the cells and their basement membrane (discussed in Sect. 10.1).

10.4.2 Prevention from Programmed Cell Death

Analyses of gene expression data of CTCs of breast cancers (see Table 10.3) show that their hyaluronic acid synthesis gene, *HAS2*, and exporter gene, *ABCC5*, are both up-regulated compared to the levels in their primary counterparts, indicating that hyaluronic acid is being synthesized, exported and possibly used in circulation. Hence, one can hypothesize that these circulating cells may use hyaluronic acid on their cell surfaces to prevent activation of the programmed cell death by anoikis as discussed earlier. This possibility clearly requires experimental validation.

10.4.3 Helping Adaptation to and Change of the New Microenvironments

Gene expression data of newly-arrived cancer cells in a secondary site show that the hyaluronic acid synthesis genes, *HAS1* and *HAS2*, and the exporter gene, *ABCC5*, are further up-regulated compared to CTCs, indicating that hyaluronic acid, in addition to its role in preventing programmed cell death, is also serving in a key role to assist the integration of the cells into the local environment. Previous studies have shown that hyaluronic acid and its cell-surface receptor *CD44* are important in changing the local environment to a more pro-metastatic environment by promoting the generation of various growth factors, e.g., *FGF* and *VEGF* for growth and tumor angiogenesis, respectively (Misra et al. 2011; Ween et al. 2011).

10.5 Concluding Remarks

Rapid progress has been made in the past decade in our overall understanding of the process of cancer metastasis, such as elucidation of the functional roles played by the EMT pathway and by the ECM compositional changes in metastasis. Building on this knowledge, numerous hypotheses have been developed regarding some important causal relationships in the overall process of cancer metastasis. Such

knowledge, in conjunction with the increasing pool of *omic* data collected on metastases at different developmental stages, including primary cancers with different levels of local metastasis, CTCs, DTCs, micro-metastasis and full metastatic tumors, provides unprecedented opportunities for computational cancer biologists to develop and computationally validate causal models. Such models can, in turn, be directly validated experimentally, significantly accelerating research progress in gaining a full understanding of cancer metastasis.

By integrating the information provided in this chapter and the one in Chap. 5, one can possibly develop a full model in which hyaluronic acid and fragments serve as the information backbone for providing instructions for stress-responses and possibly guiding a cancer to evolve. This seems reasonable since the whole purpose of cancer cells is growth as Otto Warburg pointed out in the 1960s (Warburg 1966). For a growing machine, like cancer, living in a rapidly changing and highly stressful environment, all they need for survival is to interact with hyaluronic acid and its fragments, which already encode all the "instructions" related to tissue remodeling and repair under different conditions, and well-tuned through millions of years of evolution. It is this well-developed instruction set that guides the evolving cancer cells to find their means to survive and proliferate. This is the fundamental difference between our view and the current genome-centric views about cancer. That is: survival guided by a set of well-developed instructions *versus* survival by selecting genomic mutations that are offered to them by chance.

Appendix

Table 10.1 Data used for gene-expression analysis of circulating tumor cells

Data set	Tissue	Platform	#Samples
GSE31364	Breast	GPL14378	72
GSE18670	Pancreatic	GPL570	24

Table 10.2 Data used for gene-expression analysis for Fig. 10.5

Data set	Tissue	Platform	#Samples
GSE36895	Kidney	GPL570	76
GSE31048	Leukemia	GPL570	221
GSE41804	Liver	GPL570	40
GSE30219	Lung	GPL570	85

Table 10.3 Data used for gene-expression analysis for Fig. 10.6

Data set	Tissue	Platform	#Samples
GSE31364	Breast	GPL14378	72
GSE18670	Pancreatic	GPL570	24

The breast cancer set contains seven DTC samples, and the pancreatic cancer dataset has seven CTC samples

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