

# Chapter 2

## The Metastatic Microenvironment

Shelly Maman and Isaac P. Witz

**Abstract** Metastasis is the major killer of cancer patients. Although increased understanding of the metastatic process was achieved in recent years, the mechanisms underlying the progression of cancer cells to form site-specific metastasis are still awaiting complete elucidation. The current consensus is that circulating tumor cells disseminate into future metastatic sites and that these disseminated tumor cells form micrometastasis in these sites. The micrometastases remain in a state of dormancy in these sites until “awakened” to progress towards overt metastases. Whereas the evidence implicating chemokine–chemokine receptor interactions as the mechanism responsible for the targeted migration of tumor cells to future metastatic sites is quite strong, the mechanisms that maintain dormancy of disseminated tumor cells and the mechanisms that awaken these dormant micrometastases, driving their progression towards frank metastasis, are still obscure. It is clear, however, that the metastatic microenvironment plays a major role in these events. Three topics are discussed in this review: Mechanisms that are involved in the targeted migration of tumor cells to future metastatic sites; Specific molecular signatures expressed by metastases and micrometastases and interactions between metastatic and micrometastatic cells with the metastatic microenvironment. In reviewing these topics we focused on studies performed in our lab with neuroblastoma lung and melanoma brain metastasis.

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### Abbreviations

TME	Tumor microenvironment
CAF	cancer-associated fibroblast
CXCL	chemokine (C-X-C motif) ligand
SDF	Stromal cell-derived actor
CCL	Chemokine (C–C motif) ligand
TNF	Tumor necrosis factor
MCP	Monocyte chemotactic protein
CXCR	Chemokine (C-X-C motif) receptor
IFN	Interferon
TEM	Transendothelial migration
CCR	Chemokine (C–C) motif receptor
CTC	Circulating tumor cells
DTC	Disseminated tumor cells
PHOX	Paired-like homeobox
MMP	Matrix metalloproteinase
ERK	Extracellular Signal-Regulated Kinase
PCR	Polymerase chain reaction

## 2.1 Introduction

Stephen Paget, about 120 years ago, conceptualized in his “seed and soil” theory the idea that the tumor microenvironment (TME) plays an important role in site-specific metastasis (Paget 1889). This idea was revitalized in the early seventies of the last century, marking the onset of the post Paget TME research era (Fidler 2001; Onuigbo 1975; Hart and Fidler 1980; Hart et al. 1981; Hart 1982; Weiss et al. 1984; 1988; Nicolson 1988; Pauli and Lee 1988; Pauli and Augustin-Voss 1990; Togo et al. 1995; Kuo et al. 1995). Histological, molecular and cellular studies indicated that the interface between tumor cells and the stroma of the TME is a multi-component interactive arena (Fidler 2001; Jung et al. 2002; Mueller and Fusenig 2002; Lynch and Matrisian 2002; Ben-Baruch 2003; Cunha et al. 2003; Mantovani et al. 2004; Park et al. 2000; McCawley and Matrisian 2001; Rubin 2001; Tlsty 2001; Liotta and Kohn 2001; Fidler 2002; Cunha et al. 2002; van Kempen et al. 2002; Unger and Weaver 2003; Hendrix et al. 2003; Eshel et al. 2002). These studies also indicated that the TME does not operate as a binary neutral growth medium that either supports or does not support metastasis as conceived by Paget, but rather as an active regulator of the malignancy phenotype of cancer cells (Witz and Levy-Nissenbaum 2006).

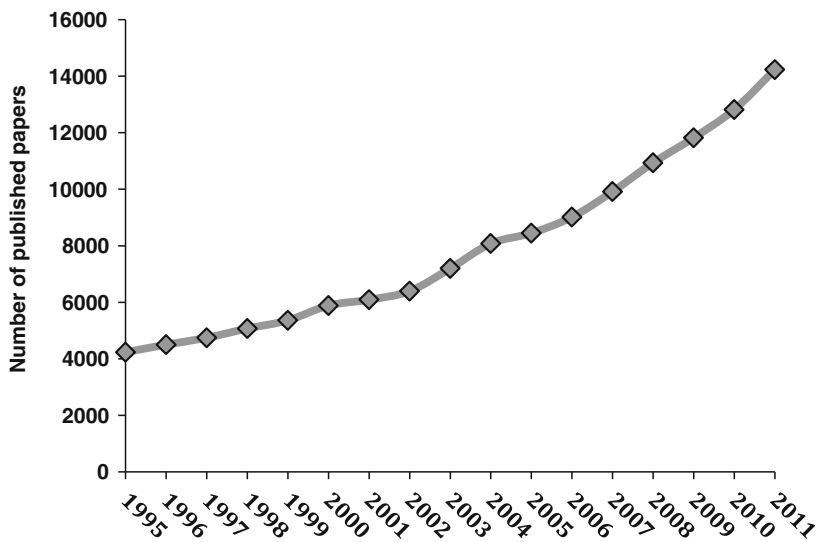
There is a general consensus in the TME field that the progression of cancer towards metastasis is regulated to a large extent by interactions of the cancer cells with non-tumor cells in their vicinity and with soluble factors released or secreted from the cancer cells and from the non-tumor cells in the microenvironment. These tumor-microenvironment interactions are bidirectional and each interaction partner regulates and shapes the phenotype of the other (Witz and Levy-Nissenbaum 2006; Witz 2008b; Kopfstein and Christofori 2006; Weinberg 2008; Gupta and Massague 2006).

Although both the non-tumor cells in the TME as well as the tumor cells themselves are accessories in tumor progression towards metastasis, the tumor is undoubtedly the original perpetuator. On the one hand it evolves into an increasing malignant entity and at the same time recruits non-tumor cells to the TME and programs these cells as well as resident non-tumor cells to promote tumor progression (Chaput et al. 2008; Klebanoff et al. 2011; Goetz 2012). These corrupted non-tumor cells fulfill inductive, adaptive and selective functions. Signals delivered by such cells may direct the tumor towards one or several possible molecular evolution pathways. Many of these pathways may lead to metastasis (Witz 2008b).

Metastasis is the major cause of death in cancer patients (Mehlen and Puisieux 2006). However, only recently did the scientific community demonstrate an increased interest and research efforts in this important aspect of oncology. This point is illustrated in Fig. 2.1, which shows PubMed data on the number of published “metastasis”-related papers in each of the years between 1995 and 2011. Among these publications are numerous informative reviews (Fidler 2001, 2002; Witz 2008b; Kopfstein and Christofori 2006; Gupta and Massague 2006; Mehlen and Puisieux 2006; Weinberg 1995; Ruiz and Gunthert 1996; Lawrence and Steeg 1996; Meyer and Hart 1998; Yokota 2000; Pass 2002; Hunter 2004; Pantel and Brakenhoff 2004; Nguyen 2004; Braun and Naume 2005; Zigrino et al. 2005; Steeg 2005; DiMeo and Kuperwasser 2006; Dai et al. 2006; Palmieri et al. 2007; Langley and Fidler 2007; Nguyen and Massague 2007; Albini et al. 2008; Hu and Polyak 2008; Kumar and Weaver 2009; Joyce and Pollard 2009; Egeblad et al. 2010; Valastyan and Weinberg 2011).

As already recognized by Paget, there is a predilection of tumors to metastasize to specific organ sites. However, the metastatic capacity of a certain tumor is not restricted to a single organ site. For example breast cancer metastasizes to bone, lungs, regional lymph nodes, liver and brain while prostate cancer metastasizes to bones and lymph nodes. Melanoma spreads mainly to lymph nodes, liver and brain. Each tumor type has therefore several different metastatic microenvironments. Since the tumor and its microenvironment regulate and shape each other's phenotype (Witz 2008b), it is to be expected that the metastases arising in one organ site be different from metastases derived from the very same tumor developing in a different organ site. It is also to be expected that different reciprocal signaling cascades take place between metastases and non-tumor microenvironmental cells in different metastatic microenvironments.

Arriving at a secondary organ site, metastatic cells have several possible fates: proliferation, entrance to a dormant state and initiation of apoptosis. It is the



**Fig. 2.1** Number of “metastasis”-related published papers in each of the years between 1995 and 2011 according to Pubmed database

interactions with the microenvironment that will determine whether cancer cells will progress towards metastasis or whether they will stay dormant or disappear altogether. Thus, tumor-microenvironment interactions regulate either anti- or pro-malignancy functions. These and related issues are still in the infancy stage of research and remain to be elucidated. For example: What attracts tumor cells to specific metastatic microenvironment? What sustains the survival of disseminated tumor cells in a particular organ site? What induces them to proliferate? Are the survival and growth factors for metastases in a particular metastatic microenvironment similar or different from survival and growth factors for metastases from the same tumor in a different metastatic microenvironment?

This chapter addresses briefly some of these issues with a particular emphasis to our work on neuroblastoma lung metastasis and melanoma brain metastasis.

## **2.2 Attraction of Tumor Cells to Metastatic Sites: The Role of Chemokine–Chemokine Receptor Axes**

Chemokines are involved in site-specific metastasis (Ben-Baruch 2008; Takeuchi et al. 2007; Fulton 2009; Zlotnik et al. 2011). This involvement occurs at different levels:

1. Secretion of chemokines from tumor cells and from non-tumor cells in the TME

2. Expression of chemokine receptors by tumor cells
3. Expression of chemokine receptors by non-tumor cells in the TME.

Homeostatic and inflammatory chemokines are secreted from a large variety of tumor cells and from non-tumor cells in the TME. Such chemokines can mobilize chemokine-receptor-expressing cells such as myeloid or lymphatic cells to the TME with wide ranging biological consequences manifested *inter alia* by tumor destruction, angiogenesis and metastasis enhancement (Mantovani et al. 1992; Bar-Eli 1999; Wang et al. 2006; Burger and Kipps 2006; Soria et al. 2008; Navarini-Meury and Conrad 2009; Schmid and Varner 2010; Sapoznik et al. 2012; Soria et al. 2012; Umansky and Sevko 2012). Below are some examples of biological activities mediated by TME-derived chemokines.

CAF-derived CXCL12 (SDF-1) enhanced tumor growth through the CXCR4 receptor expressed by breast carcinoma cells. The CXCL12–CXCR4 axis also supported angiogenesis by recruiting endothelial progenitor cells into the carcinomas. Interestingly, the myofibroblastic phenotype and the ability to enhance tumor growth *in vivo* were stably maintained in the CAFs even in the absence of contact between them and the tumor cells (Orimo et al. 2005).

Chemokine-driven vicious cycles that enhance tumor progression operate in the TME of mammary carcinomas in mice and in breast cancer in humans. We showed for example that mouse mammary carcinoma cells secreted high levels of CCL2 (MCP-1) known for its capacity to attract monocytes to the TME. Monocyte-derived TNF- $\alpha$  up-regulated CCL2 secretion from the tumor cells, and CCL2 in turn promoted the secretion of TNF- $\alpha$  from monocytes. In this vicious cycle, the tumor cells and the monocytes in the TME promoted each other's ability to express and secrete pro-malignancy factors (Neumark et al. 2003). A similar situation exists in breast cancer in humans (Ben-Baruch 2003). Monocyte chemoattractants CCL5 and CCL2 secreted by breast tumor cells may induce monocyte infiltration to the microenvironment of breast tumors. The resulting tumor-associated macrophages may secrete TNF- $\alpha$ , which induces or up-regulates the secretion of several pro-malignancy factors from the tumor cells such as matrix metalloproteinases. TNF- $\alpha$  also further up-regulates the secretion of CCL5 and CCL2, which drive the merry-go-round for another cycle (Ben-Baruch 2003). It is not unlikely that similar cycles operate also in other types of cancer.

In some tumor types, the CXCR3–CXCL10 axis is considered to antagonize tumor growth and progression (Chakraborty et al. 2008; Agostini et al. 2001). This axis may, however, also engage in pro-malignancy activities (Maru et al. 2008). In a study performed in our lab it was shown that the interaction of the CXCL10 chemokine with its CXCR3 receptor expressed by colorectal carcinoma cells promotes, rather than antagonizes, tumor progression (Zipin-Roitman et al. 2007). It was also indicated that a vicious cycle involving the CXCR3–CXCL10 axis and IFN- $\gamma$  operates in colorectal carcinoma progression (Zipin-Roitman et al. 2007). CXCL10 secreted from CXCR3-expressing colorectal carcinoma cells promotes, by an autocrine mechanism, progression-promoting functions in these tumor cells. CXCL10, at the same time, attracts CXCR3-expressing Th1 cells to the tumor site. The infiltrating

Th1 cells secrete IFN- $\gamma$ , which, in addition to its immune functions, promotes the release of CXCL10 from IFN- $\gamma$  receptor-expressing colorectal carcinoma cells while up-regulating CXCR3 expression. This further promotes the capacity of the colorectal carcinoma cells to respond to CXCL10-mediated pro-malignancy functions. The expression of chemokine receptors by tumors cells enables their targeted migration to specific organ sites expressing the corresponding chemokine ligands. This targeted migration strategy generating site-specific metastasis was “hijacked” from normal migratory mechanisms operating in organogenesis, leukocyte migration and lymphoid tissue neogenesis (extensively reviewed by Zlotnik et al. 2011).

The chemokine receptor CXCR4 is expressed by many cancer types of humans and animals. One of the first studies showing that this receptor is involved in site-specific metastasis was performed by Müller et al. (2001). These authors demonstrated that the expression in the lung of CXCL12 (SDF1), the chemokine ligand of CXCR4, attracts breast cancer cells to this metastatic site.

We evaluated the possibility that neuroblastoma cells, similar to hemopoietic stem cells, use chemokine-chemokine receptor interactions to home to the bone-marrow, a primary metastatic site for such cancer cells. The results of this study demonstrated that CXCR4 expression might be a general characteristic of neuroblastoma cells (Geminder et al. 2001). Such cells express not only CXCR4, but also its ligand, CCL12. CXCR4 expression by neuroblastoma cells is tightly regulated by tumor cell-derived autocrine CCL12, as demonstrated by the ability of neutralizing antibodies against human CCL12 to up-regulate CXCR4 expression on the tumor cells. Conversely CXCR4 expression by neuroblastoma cells was reduced following short-term exposure to recombinant human CCL12. These and additional results strongly suggested that the ability of neuroblastoma tumors to preferentially form metastases in the bone-marrow might be facilitated by a set of complex CXCR4-CCL12 interactions.

Clinical studies supported the above conclusion. It was reported that the clinical outcome in patients with tumors highly expressing CXCR4 was significantly worse than in those patients with a low-expression of CXCR4. It was concluded that CXCR4 expression in neuroblastoma primary tumors is significantly correlated with the pattern of metastatic spread (Russell et al. 2004).

Apart from chemotaxis, chemokine-chemokine receptor interactions have additional functions. They activate various signaling pathways and alter gene expression profiles resulting, for example, in promotion of growth factors of tumor cells (Eshel et al. 2002; Zhang et al. 2010; Richmond et al. 2009). Overexpressing CXCR4 in neuroblastoma cells, we found that gene expression patterns in these cells differed considerably from those in control cells. We hypothesized that these differences were due to an autocrine CCL12-CXCR4 interaction (Nevo et al. 2004).

Fractalkine (CX3CL1) is a chemokine that is expressed either as a soluble molecule or as a membrane-bound molecule, which functions also as an adhesion molecule. Soluble CX3CL1 is capable of attracting fractalkine receptor (CX3CR1)-expressing cells. There is evidence that CX3CL1 and its CX3CR1 receptor are involved in cancer, especially in that of neural origin as well as in prostate, pancreas

and breast carcinoma. Such cancer cells express high levels of CX3CR1, which is involved with migration and site-specific dissemination (Marchesi et al. 2010).

Transendothelial migration (TEM) of tumor cells is a crucial step in metastasis formation, involving adhesion molecules and chemokines. Since CX3CL1 takes part in both adhesion and chemotaxis and since bone-marrow is the first metastatic site of neuroblastoma, we asked if the CX3CR1-CX3CL1 axis is involved in the transmigration of neuroblastoma cells across bone-marrow endothelium (Nevo et al. 2009). We first demonstrated that functional CX3CR1 and its membrane CX3CL1 ligand are expressed by several neuroblastoma cell lines. It was then demonstrated that CX3CR1-expressing neuroblastoma cells were stimulated by CX3CL1 to transmigrate across human bone-marrow endothelial cells. These results led us to hypothesize that the CX3CR1-CX3CL1 axis participates in bone-marrow metastasis of neuroblastoma.

With a few exceptions, the information about the expression and function of chemokine receptors on melanoma cells and their role in melanoma metastasis is rather fragmented (Richmond et al. 2009; Somasundaram and Herlyn 2009). CCR7 was implicated in lymph node metastasis, CCR9 was shown to be involved in metastasis to the small intestine and CCR10 in metastasis to the skin (Kakinuma and Hwang 2006).

The frequency of brain metastasis in melanoma is increasing and such metastases represent a significant cause of death in melanoma patients. Of all human solid tumors, melanoma has one of the highest risks to develop brain metastasis. More than 40 % of advance stage melanoma patients are treated for complications due to brain metastasis (Denkins et al. 2004).

The mechanisms underlying the targeted migration of melanoma cells to the brain are yet to be discovered. Hypothesizing that melanoma cells employ chemokine receptor-ligand axes to migrate to the brain, we established a chemokine receptor profile of cultured melanoma cells (3 cell lines of cutaneous melanoma and 5 cell lines of melanoma brain metastasis) (Izraely et al. 2010). This profile indicated that cultured melanoma cells express CCR3, CCR4, CXCR3, CXCR7, and CX3CR1. Utilizing cells from newly created variants of human melanoma xenografts, we found that the expression of CCR4 was significantly higher in a brain metastatic variant compared to its expression in the corresponding local variant. AKT phosphorylation patterns in melanoma cells were influenced by exposure of such cells to the CCR4 ligand, CCL22, which is expressed in brain. We hypothesize that CCR4 may be involved in melanoma brain metastasis and that this chemokine receptor may be a novel molecular biomarker for the identification of melanoma cells likely to metastasize to the brain (Izraely et al. 2010).

Concluding this section it is important to note that given the multiple steps in the metastatic cascade, the mechanism for the involvement of chemokine-chemokine receptor axes in site-specific metastasis is undoubtedly considerably more complex than receptor-ligand interactions.

### 2.3 Molecular Determinants of Metastasis

Discovering molecules that could serve as novel biomarkers and therapy targets for metastatic diseases is an important goal. For example, prevention strategies for metastasis could be developed if cells expressing metastatic biomarkers would be identified in the primary tumor. Currently, the availability of *bona fide* metastatic biomarkers is rather limited. Many more molecules associated with tumor progression should be identified and characterized.

Metastasis, a multistep process that requires the coordinated action of many genes, is the primary cause of mortality of cancer patients and in spite of the recent augmented interest in and understanding of this process (Valastyan and Weinberg 2011; Zlotnik et al. 2011; Fidler 2011; Langley and Fidler 2011; Coghlin and Murray 2010; Chaffer and Weinberg 2011; Shibue and Weinberg 2011; Gupta et al. 2005), it is still incompletely understood. The identification of genes that promote or suppress tumor metastasis is an essential requisite for the understanding of this process. The development of microarray technologies had a huge impact on many disciplines of biomedicine including cancer research. Cancer researchers used these technologies to determine the metastatic potential of tumors (Budhu et al. 2005; Adler and Chang 2006; Glinsky 2006; Fingleton 2007; Sarasin and Kauffmann 2008; Sabbah et al. 2008; Woo et al. 2011). However, several investigators expressed concern about results of microarray assays. For example, attempts to link expression profiles and molecular markers to liver metastases in colorectal cancer were not successfully validated as a diagnostic or prognostic tool applicable to routine clinical practice (Nadal et al. 2007). These authors advocated improving reproducibility, increasing consistency, and validating results. In another study, concern was expressed related to the lack of progress in defining markers or gene signatures in metastasis of malignant melanoma (Timar et al. 2010). These authors suggested “that only efficient inter-disciplinary collaboration throughout genomic analysis of human skin melanoma could lead to major advances in defining relevant gene-sets appropriate for clinical prognostication or revealing basic molecular pathways of melanoma progression”.

Some of the above studies can be also criticized for not addressing the issue of organ specificity. After all, metastasis is an organ-specific event. The identification of genes associated with site-specific metastasis was addressed by the group of Massague, who identified groups of genes linked to breast cancer metastasis to various organs (Kang et al. 2003; Minn et al. 2005a, b; Bos et al. 2009). Whereas some of these genes were specifically linked to metastasis in specific organ sites, others were also associated with metastasis to other sites.

Much like other malignancies, neuroblastoma and melanoma metastasis are complex, multistep processes. We elected to study the various gene products involved in metastasis of these tumors by employing xenograft models, which recapitulate the phenotypes seen in the clinic. In order to eliminate “background noise” due to genetic differences between metastatic and non-metastatic cells or between metastases of one organ to those of another organ, our xenograft models consisted of human metastatic



and non-metastatic cell variants of the same genetic background. Such models exist for several types of cancer but none for neuroblastoma or melanoma metastasis. We generated such variants for neuroblastoma lung metastasis (Nevo and Sagi-Assif 2008) and for melanoma brain metastasis (Izraely et al. 2012).

Chronologically the neuroblastoma metastasis model was developed first. An orthotopic implantation of human neuroblastoma cell lines into the adrenal gland of athymic nude mice yielded local adrenal tumors, as well as lung metastases. After repeated cycles of in vivo passages, local adrenal and lung metastatic variants were generated. The human origin and the metastatic phenotype of these variants were confirmed (Nevo and Sagi-Assif 2008). The melanoma metastasis model was developed pretty much along the same scheme (Izraely et al. 2012) except that the inoculation of the melanoma cells to nude mice was via the intra cardiac route, which is used by other investigators studying brain metastasis (Weil et al. 2005; Palmieri et al. 2006).

The various human tumor and metastasis variants generated in our lab, comprising tumor cells propagating in the local, orthotopic site (adrenal gland for neuroblastoma and skin for melanoma) and 2 corresponding metastatic sites (lung for neuroblastoma and brain for melanoma), share a common genetic background. Genetic, proteomic and transcriptomic differences between the variants may thus be ascribed to their differential malignant phenotype and to the different (local versus metastatic) microenvironments they reside in. These reproducible models can also serve as an unlimited source of biological material to be used in various types of investigations facilitating, for example, the identification of novel metastasis biomarkers and targets for therapy.

Analyzing gene expression of cultured cells will obviously reveal only genes that preserved their expression during and after the transition from the in vivo metastatic microenvironment to culture conditions. The possibility cannot be excluded that the expression of certain genes requires constant signaling from the particular in vivo microenvironment and that the expression of these genes will fade away following explantation. However, several studies indicated that the downstream effects of exogenous signals could endure for extended periods of time or even be permanent (Hardy et al. 2010; Matsumiya and Stafforini 2010; Khoo et al. 2011).

*Neuroblastoma lung metastasis.* Neuroblastoma is the most commonly occurring extracranial tumor in children. It is initiated most frequently in the adrenal gland and accounts for approximately 8 % of all malignancies in patients younger than 15 years (Brodeur and Castleberry 1997). More than half of these patients have a metastatic disease at diagnosis. Children older than 1 year with a widespread metastatic disease or with a large, aggressive, localized tumor, have an extremely poor prognosis (Modak and Cheung 2010; Mullassery et al. 2009). The lung-metastasizing human neuroblastoma variants described above exhibited an aggressive and metastatic phenotype in vivo and a malignant phenotype in vitro (Nevo and Sagi-Assif 2008).

A robust gene-expression based classifier, which reliably predicts neuroblastoma tumor behavior and can aid physicians in choosing the most appropriate form of first-line treatment, was developed several years ago (Oberthuer et al. 2006).

This neuroblastoma-specific oligonucleotide-array utilizing several platforms of gene-expression data comprises of 10,163 (11 K) probes for the 8,155 Unigene Cluster considered to be important in the development and progression of neuroblastoma. Aiming to identify molecular correlates of neuroblastoma metastasis and to determine the clinical relevance of these molecules, the genetically identical local and lung metastasizing human neuroblastoma variants described above were subjected to genome-wide expression profiling using the neuroblastoma-specific array (Nevo et al. 2010).

Our filtering and statistical comparison criteria revealed 112 genes that were differentially expressed in local and lung metastatic variant. These differentially expressed genes were intersected with genes differentially expressed in stage 1 and stage 4 primary tumors of neuroblastoma patients. By using the same gene-expression platform, molecular correlates associated with metastatic progression in primary neuroblastoma tumors were identified. The resulting smaller gene set was clinically relevant as it discriminated between high- and low-risk neuroblastoma patients, suggesting that these genes could be used as therapy targets or prognostic markers in neuroblastoma (Nevo et al. 2010).

*Melanoma brain metastasis.* Patients with malignant melanoma have a very high risk to develop brain metastasis. Greater than 40 % of advance stage melanoma patients have such metastasis (Denkins et al. 2004; Soffiatti et al. 2002). Treatment options for melanoma patients with brain metastasis are limited (Bafaloukos and Gogas 2004). Tumor cells with the potential to metastasize to and colonize the brain may express distinctive metastasis-promoting molecular determinants. The results of gene expression profiling experiments performed in our lab (Izraely et al. 2012) demonstrated that about 40 genes were differentially expressed in brain-metastasizing human melanoma variants and in the corresponding local, sub-dermal variants. The functional significance of the genes differentially expressed in the brain-metastasizing and the sub-dermal melanoma cells, to brain metastasis is investigated at present. For example, Claudin-1, a tight junction protein, whose expression was significantly higher in the sub-dermal melanoma cells compared to the brain metastasizing cells, turned out to be a melanoma metastasis-suppressor gene.

A recent report identified a group of about 20 genes linked to breast cancer brain metastasis (Bos et al. 2009). We found that several of these genes were more highly expressed in brain metastasizing melanoma cells than in the corresponding cutaneous variants. The existence of a molecular signature of brain metastasis common to several types of cancer may thus be postulated.

## 2.4 Micrometastasis and Dormancy

Circulating tumor cells (CTC) were described for the first time in the middle of last century (Romsdahl et al. 1960). CTC are capable of disseminating primarily to regional lymph nodes and bone-marrow and persist in these organs in a state of

dormancy for long periods. It is postulated that “awakening” of the dormant disseminated tumor cells (DTC) or micrometastases would lead to full blown, overt metastasis (Balic et al. 2010; Riethdorf et al. 2008; Alix-Panabieres et al. 2008). The research on micrometastasis became intense in the eighties and nineties of last century, when epithelial cells were detected in the bone-marrow of patients with epithelial cancers such as colorectal, breast and lung cancer (Dearnaley et al. 1981; Schlimok et al. 1986; Schlimok et al. 1991; Schlimok and Riethmuller 1990; Schlimok et al. 1990; Riethmuller and Johnson 1992; Lindemann et al. 1992; Pantel et al. 1993a, b; Cote et al. 1991). Nowadays micrometastasis has become an integral phase of the metastatic cascade (Riethdorf et al. 2008; Alix-Panabieres et al. 2008; Goss and Chambers 2010; Hedley and Chambers 2009). Micrometastatic cells remain as solitary cells or as small, steady state cell clusters, either due to a balance between proliferation and apoptosis or due to cell cycle arrest (Chaffer and Weinberg 2011; Chambers et al. 2002). For example, micrometastatic cells of breast cancer are in a state of dynamic dormancy, i.e., cell division and cell death are balanced (Meng et al. 2004). In view of the strong possibility that such cells are precursors for metastasis, it has been proposed that these cells could serve as targets for therapy (Goss and Chambers 2010). It is therefore logical to search for specific molecular targets on micrometastatic cells (Hedley and Chambers 2009; Ringel 2011; Vera-Ramirez et al. 2010).

The existence of micrometastatic cells could also be used to evaluate cancer outcome. For example, in a recent study the authors identified a dormancy-associated gene signature in breast cancer determining that tumors that exhibited a high dormancy score showed a significant correlation with low metastasis, since these tumors were more likely to undergo prolonged dormancy before resuming metastatic growth (Kim et al. 2012).

As mentioned above, regional lymph nodes and bone-marrow are major target sites for DTC (Balic et al. 2010). If micrometastasis indeed progress towards frank metastasis in a given organ site, it is logical to assume that micrometastases are present in this particular organ site. However, with some exceptions e.g. Yokoyama et al. (2012) the experimental evidence to support this assumption is rather limited. A possible reason for that is that detection of micrometastasis represents a great technical challenge (Riethdorf et al. 2008). Employing the xenograft models of human neuroblastoma lung and melanoma brain metastasis described above (Nevo and Sagi-Assif 2008; Izraely et al. 2012) which consisted of local and metastatic variants with an identical genetic background, we detected the presence of dormant micrometastases that formed spontaneously in lungs and brain following an orthotopic inoculation of neuroblastoma (Edry Botzer et al. 2011) and melanoma (Izraely et al. 2012) cells respectively. These systems allowed for a comparison of characteristics between metastatic cells in a specific organ and micrometastatic cells appearing in the same organ. Both metastatic and micrometastatic cells of the two tumor systems generated local tumors when implanted in the orthotopic sites, demonstrating that the intrinsic autonomous proliferative capacity of these cells remained intact except in the corresponding metastatic microenvironment.

A comparative *in vitro* characterization of metastatic and micrometastatic neuroblastoma cells revealed similarities and differences. Micrometastatic, but not metastatic, neuroblastoma cells expressed the minimal residual disease markers PHOX2B and tyrosine hydroxylase. The metastatic neuroblastoma cells demonstrated a higher migratory capacity, an elevated MMP secretion, and a higher constitutive ERK phosphorylation than micrometastatic cells (Edry Botzer et al. 2011). A preliminary comparative *in vitro* characterization of metastatic and micrometastatic melanoma cells demonstrated that the gene expression pattern of both cells was in general similar (Izraely et al. 2012). However, in view of the biological differences between these 2 types of brain-localizing melanoma cells, a thorough comparative analysis between these cells is warranted.

Concluding this part of the review, it is our opinion that studying metastases and micrometastases developing in the same organ site may lead to a better understanding of the role of the metastatic microenvironment in tumor dormancy, to solving possible mechanisms underlying the transition of micro- to macro-metastases and to finding ways to induce or prolong tumor dormancy.

## 2.5 Cross-Talk Between Tumor Cells and the Metastatic Microenvironment

Different subsets of cells in the primary tumor are genetically pre-destined to metastasize to specific organs (Dai et al. 2006; Kang et al. 2003; Ring and Ross 2005). Genes coding for: growth and angiogenesis factors; adhesion molecules and receptors for such molecules and for capacities to migrate to and invade specific organ target sites are responsible for site-specific metastasis. In addition to the genetic makeup of the cancer cells, the microenvironment of the organ to which cancer cells metastasize (referred hereafter as the metastatic microenvironment) plays a crucial role in the establishment, maintenance and further progression of metastasis (Pratap et al. 2011; Croci 2007; Kaplan et al. 2006; Harlozinska 2005; Cairns et al. 2003; Radinsky 1995; Radinsky and Fidler 1992). However, and although recent studies shed some light on the contribution of the metastatic microenvironment to site-specific metastasis (Lorusso and Ruegg 2012; Spano et al. 2012; Koh and Kang 2012; Lukanidin and Sleeman 2012; Sleeman et al. 2012; Spano and Zollo 2012; Taylor et al. 2011; Mathot and Steninger 2012; Friedl and Alexander 2011; Cirri and Chiarugi 2012), there is still quite a lot to discover.

In attempts to comprehend the role of the metastatic microenvironment on site-specific metastasis, one should consider the following possible scenarios. Since the microenvironments of different organs differ in their cellular and molecular composition, it is to be expected that different interactions will take place between tumor cells metastasizing to organ A with the corresponding microenvironment, and the tumor-microenvironment interactions of cells from the same tumor that metastasize to organ B. The consequence of these differences would be the generation of a different phenotype of metastases from the same tumor in different

organ sites. Using this scenario, one could imagine that cells from different tumor types metastasizing to the same organ site would share certain genetic and/or phenotypic traits. Contributing to the complexity of the interactions taking place in the metastatic microenvironment is the fact, that the microenvironment is an ever changing milieu; At times, it will enhance the malignancy of metastasizing cancer cells, and at other times, it will inhibit tumor progression (Witz 2008; Klein et al. 2007; Lin et al. 2009).

Above we summarized the role of chemokine–chemokine receptor axes in the targeted migration of tumor cells to selective organ sites. The mechanisms that sustain tumor cells in these sites and those promoting their progression and further dissemination to additional sites are still incompletely deciphered.

Focusing on the earliest steps in site-specific metastasis, it was demonstrated (Peinado et al. 2011) that cancer cells from the primary tumor communicate with bone-marrow-derived hematopoietic progenitor cells. This cross-talk is mediated by cytokines and chemokines secreted from the tumor cells and/or by tumor-derived exosomes. These tumor-derived factors are involved in the recruitment of bone-marrow-derived hematopoietic progenitor cells to future metastatic microenvironments by up-regulating the expression of fibronectin, matrix metalloproteinases or S100A8 and S100A9 proteins in these microenvironments. These and other molecules are those which directly mediate this recruitment. The recruited hematopoietic cells generate a pro-angiogenic, pre-metastatic niche, which supports the sustainability of cancer cells in this niche (Peinado et al. 2011). It should be noted that bone-marrow-derived cells could be detected in the pre-metastatic niche prior to the arrival of tumor cells at that niche (Psaila et al. 2006).

How do factors derived from the primary tumor select a particular organ to serve as a future organ-specific metastatic site? This is still an open question. A possible answer to this question is that cancer cells released into the circulation from non-metastatic primary tumors disseminate to future metastatic sites but are unable to progress further towards metastasis. Such disseminated cells, which may be dormant and therefore hard to detect, could be those that release the factors which will subsequently recruit the bone-marrow-derived cells, forming the pre-metastatic niche hospitable for colonization by subsequent waves of released tumor cells and for the propagation of already present tumor cells (Bidard et al. 2008).

Results obtained in our lab may support this possibility. Working with the xenograft models of human neuroblastoma lung and melanoma brain metastasis, we asked if non-metastatic neuroblastoma or melanoma cells inoculated orthotopically (neuroblastoma into the adrenal gland and melanoma subcutaneously) to nude mice would disseminate to the corresponding metastatic sites. No overt metastasis was formed and standard detection methods failed to detect disseminated tumor cells in these organ sites. However, if the lungs of neuroblastoma-inoculated mice or the brains of melanoma-inoculated mice were cultured *in vitro* for a few weeks, human neuroblastoma and melanoma cells could be observed in the corresponding organ culture. Real-time PCR using human-specific probes confirmed the organ culture results (Izraely et al. 2012; Edry Botzer et al. 2011). These results indicate that tumor cells may disseminate to future metastatic sites

and persist in these sites as undetectable “sleepers” without progressing to frank metastasis. Whether these micrometastatic “sleepers” are able to attract hematopoietic progenitor cells to the corresponding organ sites is unanswered as yet.

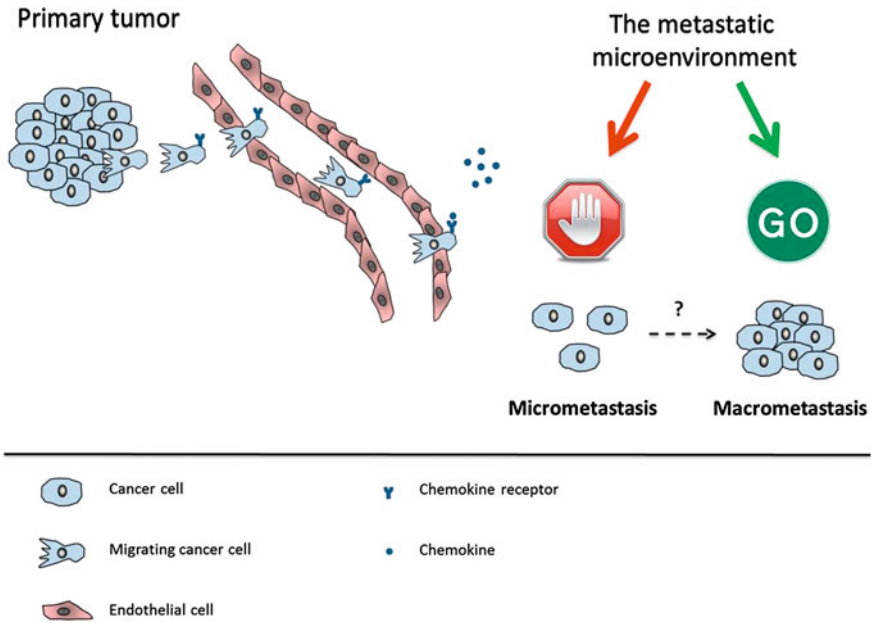
Interactions between cancer cells metastasizing to a specific organ site and the microenvironment of that site is subject to active research efforts. The working hypothesis of all these studies is that interactions between the microenvironment and tumor cells determine metastasis formation at this organ site (Langley and Fidler 2011; Sleeman et al. 2012; Rowley 2012; Cuiffo and Karnoub 2012; Krishnan et al. 2012; Nishimori et al. 2012; Reddy et al. 2012; Korkaya et al. 2011; Achyut and Yang 2011; St Hill 2011). Indeed these studies and others demonstrate that the microenvironment of the metastatic organ functions at several levels to facilitate metastatic growth of tumor cells that disseminated to that site. These functions include the creation of a pre-metastatic niche (Psaila et al. 2006), the delivery of site-specific chemo-attractants for tumor cells (Zlotnik et al. 2011) and the formation of a favorable milieu to sustain metastatic cells and promote their propagation by providing survival and proliferation signals (Langley and Fidler 2011; Rowley 2012; Cuiffo and Karnoub 2012; Krishnan et al. 2012; Nishimori et al. 2012; Reddy et al. 2012; Korkaya et al. 2011; Achyut and Yang 2011; St Hill 2011).

Based on the assumption that the microenvironment of future metastatic sites exerts far reaching influences on the ability of tumor cells to metastasize to that site, employing the human melanoma brain xenograft model developed in our lab (Izraely et al. 2012), we assessed the influence of brain-derived soluble factors on several malignancy traits of melanoma cells (Klein et al. 2012). It was found that brain-derived soluble factors enhanced the migration of melanoma cells metastasizing to the brain, but did not affect the migration of melanoma cells growing locally under the skin. This differential influence on brain-metastasizing cells could enhance the generation of new metastases from existing ones (metastasis-derived metastasis) (Langley and Fidler 2007).

Brain-derived factors also up-regulated the expression of the chemokine receptor CCR4 on melanoma cells. This finding is interesting in view of our previous findings that the CCR4-CCR4 ligand axis may be involved in the targeted migration of melanoma cells to the brain (Izraely et al. 2010). It is not unlikely that CCR4 ligands secreted from the brain interact with the CCR4-expressing melanoma cells, thereby directing them to the brain. Brain-derived soluble factors also enhanced the transmigration of melanoma cells growing locally under the skin across human brain endothelium. This activity could promote the capacity of these cells to metastasize to the brain.

An interesting finding was that brain-derived soluble factors, while enhancing the viability of melanoma cells growing locally, caused an S phase arrest followed by apoptosis of brain-metastasizing melanoma cells (Klein 2012). This represents another example of the fact that the TME may exert yin-yang activities i.e. opposing functions on interacting tumor cells (Witz 2008).

Asking what keeps micrometastatic neuroblastoma cells residing in the lungs from progressing to overt metastasis, we hypothesize that the lung microenvironment



**Fig. 2.2** A suggested model for the influence of the metastatic microenvironment on cancer progression and dormancy. Cancer cells expressing chemokine receptors detach from the primary tumor and metastasize to distant organs expressing the appropriate chemokine ligands. Once lodged in a secondary organ, the interactions with the metastatic microenvironments will determine whether these metastasizing cancer cells remain dormant or progress towards frank metastasis. It would be interesting to find out if the breakdown of this microenvironmental control mechanism is responsible for the awakening of dormant micrometastasis

contains factors that restrain the propagation of such cells. It was indeed found that a lung-derived soluble factor (or factors) caused a G0-G1 arrest followed by a decrease in cell viability of neuroblastoma lung metastases. This cytotoxic effect was significantly greater on micrometastatic lung-residing neuroblastoma cells. The fact that the lung contains a factor that restrains the proliferation of neuroblastoma cells, may explain the fact that lung metastasis in neuroblastoma is a late event in the progression of this disease (Cowie et al. 1997; Kammen et al. 2001). The fact that normal organs express metastasis-restraining factors may constitute a hitherto un-described manifestation of intercellular surveillance, or microenvironmental control (Klein et al. 2007; Flaberg et al. 2011; Flaberg et al. 2012; Allen 2011; Bissell and Hines 2011). We suggest that such a mechanism (Fig. 2.2) would explain micrometastasis dormancy.

## 2.6 Conclusion

Metastatic tumor cells are endowed with characteristics conferring upon them the general capacity to migrate and disseminate to distant organs. Different types of cancer have multiple favorite metastatic organ sites. For example breast cancer

metastasizes to bone, lungs, liver and brain, while colorectal cancer metastasizes mainly to liver and also to lungs. Each tumor type may therefore encounter several different metastatic microenvironments. It is therefore to be expected that the cross-talk between tumor cells infiltrating a certain secondary organ site be different from the cross talk between cells originating in the same tumor but infiltrating a different secondary organ. Since the microenvironment regulates and shapes the phenotype of tumor cells (see above), the result of this difference may be the emergence of multiple metastatic variants each expressing a different phenotype. The impact of this variability on cancer therapy is still largely unknown and should be pursued further.

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