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The Interrelating Dynamics of Hypoxic Tumor Microenvironments and Cancer Cell Phenotypes in Cancer Metastasis

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Abstract The interrelating dynamics of the primary tumor cells and their surrounding microenvironment might determine phenotypic characteristics of disseminated tumor cells and contribute to cancer metastasis. Cytoprotective mechanisms (e.g., energy metabolism control, DNA damage response, global translation control and unfolded protein response) exert selective pressure in the tumor microenvironment. In particular, adaptation to hypoxia is vital for survival of malignant cells in the tumor and at distant sites such as the bone marrow. In addition to the stress response, the ability of tumor cells to undergo certain cellular re-differentiation programmes like the epithelial-mesenchymal transition (EMT), which is linked to cancer stemness, appears to be important for successful cancer cell spread. Here we will discuss the selection pressures that eventually lead to the formation of overt metastases. We will focus the properties of the microenvironment including (i) metabolic and cytoprotective programs that ensure survival of disseminated tumor cells, (ii) blood vessel structure, and (iii) the hypoxia-normoxia switch as well as intrinsic factors affecting the evolvement of novel tumor cell populations.

Keywords Bone marrow · Disseminated tumor cells · Epithelial-mesenchymal transition · Hypoxia · Stem cells · Tumor microenvironment

Abbreviations

ATF6	activating transcription factor 6
CTC	circulating tumor cells
CXCR4	C-X-C chemokine receptor 4
DTC	disseminated tumor cells
EGFR	epidermal growth factor receptor
EIF2	eukaryotic initiation factor 2
EMT	epithelial-mesenchymal transition
ER	endoplasmatic reticulum
HIF-1	hypoxia inducible factor 1
HR	homologous recombination
IRE1	inositol-requiring protein 1
MET	mesenchymal-epithelial transition
NHEJ	non-homologous end joining
PDI	protein disulfide isomerase
PERK	PKR-like ER kinase
PTEN	phosphatase and tensin homolog
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SDF1	stromal cell-derived factor 1
UPR	unfolded protein response

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Introduction

Cancer cells might be characterized by heterogeneous expression patterns of different genes. Within the primary tumor of individual patients different phenotypes of cancer cells exist. Tumor cells in the blood stream or in the bone marrow may differ from the cells of the primary tumor, and they frequently differ from each other. Moreover, cells in solid metastases can exhibit other phenotypes than those of the primary tumor and released tumor cells. It has become obvious that tumor cell populations are not only genetically heterogeneous, but also novel phenotypes dynamically evolve depending on the present microenvironmental conditions. Dynamic

evolvement of novel cell populations affects biomarker discovery, as the manifestation of novel cell populations implies that the prediction capacity based on single biomarker proteins may be limited. Application of biomarkers for clinical detection of tumor cells implies that only phenotypes that are positive for the selected biomarker proteins can be detected, other cells which may contribute significantly to the outcome of patients remain invisible.

Understanding the microenvironmental conditions that favour the evolvement of site specific cell phenotypes can improve the detection of the clinically relevant populations of tumor cells that are responsible for distant metastasis. Cells of particular tumors such as breast or prostate cancer are frequently osteotropic, whereby disseminated tumor cells (DTC) within the bone marrow can be analyzed to elucidate which cell phenotypes are selected to form distant metastases.

In this review, the development of the primary tumor microenvironment potentially influencing the release of cancer cells into the blood and the settlement in the bone marrow will be discussed and underlying mechanisms affecting the evolvement of cell phenotypes under altering microenvironmental conditions will be addressed.

The (dis)regarded Majority of Tumor Cells

Cancer arises from a normal cell that experienced a malignant transformation, followed by invasion of the surrounding tissue through the growing tumor cell population [1]. Having reached the vasculature, tumor cells may be transported by the blood stream to secondary sites where they may form a new tumor cell colony [2]. However, there must be a condition that prevents the formation of millions of metastases in the same patient. The vast majority of tumor cells within the primary tumor and DTC will not form distant metastases, either because they die or remain sedentary [3]. This can be exemplified by a simple calculation. In DTC positive bone marrow samples of 5 ml usually 1–20 DTC are detectable [4–6]. The total volume of human bone marrow is approximately 2500 ml. In this simple scenario the total number of bone marrow DTC would range between 500 and 10.000. Though this expected DTC number is lower than a previous calculation [7], it nevertheless exceeds by far the number of solid metastases in one patient. Using a mouse model and mouse melanoma cells it was demonstrated that cancer cells are able to survive hemodynamic forces after arrest in the microcirculation [8]. Instead, metastatic inefficiency seems to be determined by the failure of solitary DTC or small cell clusters to grow out at secondary sites [9]. Focussing on the fate of single tumor cells the process of metastasis seems to be extremely inefficient, since the majority of tumor cells fail to become a metastasis founding cell [10]. With focus to the tumor cell population as a whole that is ceaselessly subjected to altering microenvironmental pressure, the selection and adaptation process may be regarded as efficient, as the process as a whole is able to create metastases.

Properties of the Microenvironments

Different types of microenvironments are described to be relevant in cancer. The structural composition of normal human tissues and the increasing mass of tumor cells leading to an unphysiological microenvironment affect the tumor development. The human body consists of different microenvironments like the brain, lung or breast tissue, the bone marrow and the endothelium of the blood vessels. In order to reach secondary sites, circulating tumor cells (CTC) have to pass the endothelium of the blood vessels at the primary tumor and DTC might require a second transmigration step at the secondary site [11]. During growth of a tumor colony the tumor cells generate their own local microenvironment within the primary organ (e. g. breast, lung or colon) [12]. Both normal and aberrant microenvironments are characterized by their structural constitution, which is the morphological context for the subsequent dynamic evolvement of tumor cell phenotypes.

Normal epithelia—from which cancers evolve—cover the surface of organs, possess few intracellular spaces and lack blood vessels. To endow the cells with elasticity and to maintain the cell shape, epithelial cells express the robust cytoskeleton component of intermediary filaments by which epithelial cells are interwoven with each other to a large planar structure [13, 14]. In addition, epithelial cells show a polarized structure with the basal side connected to the basal lamina. The epithelium is separated from the underlying mesenchyme by a basement membrane, which must be passed by the tumor cells to reach the underlying tissue. The mesenchyme consists of fibroblasts, which secrete the extracellular matrix that can also serve as a storage for growth factors, the intracellular space, blood vessels and cells of the immune system that were recruited from the bone marrow [15]. Since nutrients like glucose and small molecules like oxygen are supplied to the epithelial cells by the blood vessels, short diffusion ways of these substances are essential for the survival of epithelial cells [16]. This passive transport of small molecules to the epithelial cells even in the normal epithelium generates a permanent concentration gradient of these substances from the blood vessels to the epithelial cells. Epithelial cells are adapted to this condition by their

polarized structure. Transmembrane growth factor receptors and proteins for nutrient import are located on their basal side. At this stage normal epithelia are highly structured according to their optimal physiological demands and the overall structure is static.

Genetic or epigenetic alterations in epithelial cells are accompanied by phenotypic changes of the cell like aberrant expression of receptor tyrosine kinases (RTK). Albeit a fluent process, the final result is the conversion of the normal cell to a cancer cell [1]. At that stage the physiological structure of the epithelial cell layer begins to collapse and is being converted to a dynamically changing microenvironment with alterations in different conditions such as pH value, oxygen and nutrient concentration, growth factor concentration or the spatial assembly of cell phenotypes [12, 17]. The aberrant cells become resistant to contact inhibition and overcome their susceptibility to anoikis, which is also a critical condition for the survival of single tumor cells at secondary sites [11]. The growth of the aberrant cell populations into the lumen of the epithelium then leads to an increase of the diffusion ways for growth and anti-growth factors, metabolites and small molecules like oxygen.

Autocrine or paracrine production of growth factors in aberrant cells leads to activation of the intracellular signalling pathways and up-regulation of the protein levels of the growth factor receptors. The overexpression of the growth factor receptors EGFR (epidermal growth factor receptor) and ErbB-2 during tumor progression can serve as an example of a synergistic effect that promotes cellular proliferation under shortage conditions. For example, the increased protein levels of EGFR and ErbB-2 can result from gene amplification, which is a much slower process than upregulation on protein level [18]. Different phenotypic solutions can overcome one restriction for tumor cell growth, which is called the principle of functional equivalence [12]. For example, tumor cell growth and migration is strongly dependent on the activity of the AKT pathway. Activity of AKT can be maintained by overexpression of EGFR, ErbB-2 and ErbB-3, mutations in the PI3K, overexpression of AKT itself or functional disruption of PTEN (phosphatase and tensin homolog).

After microinvasion of the tumor cells into the underlying tissue, which requires the breakdown of the basal membrane, tumor cells are subjected to local shortage in nutrients and hypoxia. Such microenvironmental conditions have been detected in cervical cancer, head and neck cancer and melanomas. In all stages of breast cancer for example the median oxygen partial pressure was detected as 37 mbar, compared with 87 mbar of normal breast tissue. In the normal breast oxygen partial pressures of less than 17 mbar were not detected, whereas in approximately one-third of the breast cancer cases values of less than 3 mbar were observed [19]. Invasion of the local stroma by the tumor cells where nutrient limitation is less may lead to the escape from cell stress [16]. Alternatively, establishment of a local vasculature supports not only the supply with nutrients but also the dissemination from the primary tumor into the blood stream as one of the first steps for tumor cell dissemination [20].

Metabolic and Cytoprotective Programs that Ensure the Survival of the Tumor Cells

Limited supply of growth factors can be compensated by aberrant cells itself, whereas restricted oxygen partial pressure, glucose or amino acid concentration are external factors that limit cellular growth. The transport of these substances is restricted by diffusion kinetics limited to a distance of approximately 150 μm or 10 cell layers [21]. Polar metabolites like glucose diffuse between the intracellular spaces and must be transported actively into the cells by specific importer proteins (e. g. GLUT1 which is regulated by the hypoxia inducible factor 1 alpha HIF-1 alpha [21]). Since the diffusion kinetics of glucose and other nutrients is similar to oxygen, the cells also experience deficiencies in other nutrients [22, 23]. In contrast, the small uncharged molecule oxygen can pass the plasma membrane, so that oxygen can be delivered to dysplastic cell layers directly through the underlying cells.

While the normal oxygen concentration in the atmosphere is 21%, the oxygen partial pressure in well oxygenated tissue ranges between 5–9% and the normal oxygen partial pressure in solid tissues is around 3%. Hypoxia is considered at oxygen partial pressures at 1% and the most extreme pole of oxygen deprivation is anoxia (below 0.01%) [24, 25]. Cells require oxygen not only as a terminal electron pair acceptor in the oxidative phosphorylation, but also for synthesis reactions [26] and respond with different adaptation programs in series to the diminished oxygen concentrations. In tumors Warburg observed a shift in the energy conversion from oxidative phosphorylation to glycolysis mediated ATP production [27]. The underlying molecular principles for this observation were then addressed to a metabolic adaptation program that is induced by HIF-1 alpha, that promotes the glucose metabolism instead of the energy conversion in the mitochondrion as well as the induction of angiogenesis [16, 28, 29]. However, consumption-driven hypoxia, which was most apparent at oxygen concentrations of 1–2%, was observed in HIF-negative cells [16, 30]. These cells exhibited increased mitochondrial function, thereby decreasing the local oxygen partial pressure.

Under even lower oxygen partial pressure the conversion to ATP in the mitochondria becomes limiting. On the one

hand, protein synthesis is required to cope with environmental stress; however, the extreme demand of ATP forces the cells to down regulate their global mRNA translation. Under these conditions, the activity of mTORC1 that controls the global RNA translation machinery is inhibited. As a result, the global protein synthesis is diminished to reduce ATP consumption, leading to selective translation of those mRNAs that are most essential for the survival of the cells [31, 32]. The global protein translation switches from the normal cap-dependent translation to cap-independent translation allowing only the translation of those mRNAs that are most essential for survival [33]. Messenger RNAs that allow the translation via specific 5′ UTR sequences belong to proteins that are essential for cell survival and apoptosis like HIF-1 alpha, VEGF, Grp78, protein disulfide isomerase (PDI), p53, Bcl-2, c-Myc and others [31, 34]. One program that is activated in response to cell stress is the unfolded protein response (UPR) [35, 36]. Proteins in the endoplasmatic reticulum (ER) were malfolded under adverse environmental conditions like hypoxia, altered redox status, glucose deprivation, alterations in calcium homeostasis or excessive protein load. After accumulation of malfolded proteins under microenvironmental stress, chaperones like Grp78 dissociate from receptor proteins that span the ER membrane (activating transcription factor 6 (ATF6), inositol-requiring protein 1 (IRE1) and PKR-like ER kinase, (PERK)) to refold their malfolded protein substrates [33]. The released membrane receptors then become triggered and activate the transcriptional branch of the UPR. The mTOR pathway as a cellular administration programme senses stress indirectly by signal transmitting proteins. Negative regulators of the mTOR pathway are BNIP-3 (which is induced by HIF-1 alpha) that inhibits the positive regulator Rheb–GTP, a small G protein. Activated PERK as a modulator of downstream mTOR signaling phosphorylates the alpha subunit of eukaryotic initiation factor 2 (EIF2) which prevents the EIF2 beta mediated initiation of the protein translation [37].

These cellular adaptation strategies efficiently enable tumor cells to survive under adverse environmental conditions like severe hypoxia combined with glucose and/or serum deprivation. They can survive even anoxia (0.01% of oxygen) for 24 h, which only blocks their proliferation. However, the critical limit is reached after exposure to 0.01% of oxygen for 72 h which reduces the amount of viable cells to 20–30% [38].

In tumor cells the metabolism can be affected by the activity of oncogenes [16, 29]. One of the most striking examples is the ErbB-2 mediated increase of HIF-1 alpha expression [39]. Interestingly, ErbB-2 signaling increases the translation of HIF-1 alpha and does not affect the half-life of the HIF-1 alpha protein. Further downstream signalling proteins of ErbB-2 have been identified to increase HIF-1 alpha activity in breast and prostate cancer, including phosphatidylinositol 3-kinase, AKT or PTEN [39, 40]. The AKT dependent translation of HIF-1 alpha was then assumed to be dependent on mTOR activity [41]. Similar to the AKT pathway, activity of the mitogen-activated protein kinase pathway also leads to accumulation of HIF-1 alpha protein [42]. In this work it was also shown that HIF-1 alpha subsequently increased hypoxia response element activity, which overlaps with other cytoprotective strategies like the UPR and the mTOR signalling [33]. Moreover, HIF-1 alpha can accumulate by loss of function of the p53 tumor suppressor in cancer cells, which further links the angiogenic switch to DNA damage response [43].

Emergence of Novel Phenotypes

Tumor cell progression can occur not only within primary tumors, but also independently at secondary sites implicating an early dissemination of tumor cells from the primary tumor [2, 44]. For this model a continuous release of cells from the primary tumor occurs with sequential formation of metastases at distant sites is proposed. DTC with the capacity for metastatic outgrowth that are detected at the time of diagnosis would then form solid metastases many years later. It is also possible that low proliferating, Ki-67 negative or dormant DTC that have been disseminated in early stages contribute to this delayed metastatic outgrowth [45, 46]. Indeed, DTC/CTC seem to be heterogeneous with regard to the expression of growth factor receptors, adhesion molecules, proteases and their inducers and receptors, major histocompatibility complex antigens or signalling kinases [47–49].

In these models the term “early” describes a later process than one would assume on the first look. Considering a tumor diameter of approximately 1 cm at the time of diagnosis, the primary tumor has existed for approximately 10–12 years [20, 44]. Hence, in these models the tumor has already existed 6–9 years until the proposed “early” dissemination has been implicated as a potential mechanism for metastasis [20, 44, 50]. Even though “early” dissemination reduces the time for malignant transformation of the cells, it is conceivable that within this time span the cancer cells are able to colonize secondary organs [2, 44]. Importantly, the concept of “early” dissemination explicitly includes the possibility of tumor cell dissemination closer to the time of primary tumor diagnosis, so that early disseminated cell are not necessarily the exclusive source of later metastases [11].

On the other hand, due to the exponential growth of the primary tumor the amount of cells that can contribute to dissemination is strongly reduced at early dissemination. Metastasis is an inefficient process [10], which further

reduces the number of DTC that may contribute to distant metastasis. The number of proliferating cells at secondary sites was only around 3% as detected by Ki-67 staining in mouse experiments, indicating that the majority of the cells remain dormant [8]. Cancer stem cells have been proposed as candidates for a cell subpopulation that is able to grow out at secondary sites [51]. Cancer stem cells as a subpopulation were implicated for leukaemia and multiple myeloma, but until now the best defined system are the $CD34^{+}$ $CD38^{-}$ cells in acute myeloid leukaemia [52]. Moreover, the ability of cancer cells to transit between epithelial and mesenchymal traits like loss of E-Cadherin with nuclear accumulation of beta-catenin has been implicated in cancer metastasis [53]. Since the majority of the DTC pool seems to consist of dormant cells, metastases may arise from the pool of low proliferating or quiescent cells [54]. The concept of tumor cell dormancy has been extended by the postulation of tumor mass dormancy [55]. Tumor mass dormancy describes a stage where cancer cells are able to proliferate but the net growth of a tumor cell colony is restrained through equilibrium between proliferation and apoptosis. The observation that metastatic relapse in breast cancer patients can occur more than 10 years after diagnosis and resection of the primary tumor [56] challenges any model of cancer metastasis. If metastatic relapse results from the pool of the dormant cells, a mechanism is required that confers the re-initiation of cell growth, which is currently in focus of research activities. Approaches point towards the impact of the microenvironment at secondary sites. Possible mechanisms involve interleukins, the colony-stimulating factor 1, or activation of the tumor cells via uptake of the stromal cell-derived factor 1 via the C-X-C chemokine receptor 1 [17]. Further, there is ample preclinical evidence that the angiogenic switch may contribute to re-activation of tumor cells after dormancy and the subsequent formation of metastases [57]. The latter mechanism suits well to the assumption that the hypoxic microenvironment of the bone marrow may be considered as a reservoir for DTC as precursors for later metastatic outgrowth [2]. In particular the persistent exposition to hypoxia may be one microenvironmental factor that can lead to the acquisition of DNA damage in DTC, which is discussed below.

Under abnormal microenvironmental conditions a vicious cycle of complicit interaction between aberrant cells and their microenvironment evolves, thus accelerating the malignant progression [58]. One driving force for the emergence of novel tumor cell phenotypes in the primary tumor is the competition of the cancer cells with each other, which accelerates the malignant progression [16, 58]. In contrast, bone marrow DTC exist as single tumor cells embedded into a matrix of normal bone marrow cells [54, 59], so in the early onset of bone marrow metastases, intercellular competition between tumor cells does not contribute to malignant progression. Instead, factors like SDF1, RANKL, TNF-related apoptosis-inducing ligand or VEGF are critical for the survival of the tumor cells and for the induction of the vicious cycle of bone metastasis [45, 59, 60].

Citri and Yarden have underlined on the example of the ErbB signalling network that robust systems consist of modules that enable a system to locally contain inflicted damage, as well as to promote evolvability. These components offer alternative ways to generate an output in the face of severe perturbations [61]. The ability of signalling pathways to self organize a functional structure may be one potential mechanism that supports functional plasticity that contributes to the survival and evolvement of novel tumor phenotypes.

An interesting extension of the hypothesis that DTC are able to recirculate via the bloodstream into other distant organs and even to the primary tumor [54] is the idea of tumor self-seeding [62]. In this concept CTC derived from the primary tumor can settle down in the primary tumor again after a passage through the blood stream. Alternatively, CTC that are released by metastases may be able to return to its place of origin. Even though this concept implies the evolvement of novel tumor cell phenotypes by site-specific metastasis, the continual exchange of tumor cell phenotypes between the tumor cell colonies tends to equalize the molecular profiles of the primary tumor and its metastases [62, 63]. Experimental evidence for the existence of tumor self-seeding was provided by mouse models, which also showed that the tumor-derived cytokines IL-6 and IL-8 act as CTC attractants and for example the actin cytoskeleton component fascin-1 mediates the CTC infiltration into the mammary tumors [64].

The Wall: Structure of Blood Vessels in Tumor Cell Dissemination

Outside of the first microenvironment relevant in cancer—the primary tumor—the composition of the blood vessels affects tumor cell metastasis. Metastatic tumor cells pass at least twice the endothelial cell layers to grow out at secondary organs. In the first crossing the cells leave the primary tumor into the blood stream and during extravasation at secondary sites they pass the endothelium the second time. Cancer cells can be arrested in the capillaries of secondary organs like the liver or lung by size restrictions, which may lead to deformation of the cells under the local blood pressure [10]. The high survival rates of such trapped cells in the lung and the liver suggest that cancer cells can persist under this mechanical stress for several days [8, 9].

The structure and penetrability of endothelial cell layers varies massively within the human body, so that there is a

site specific barrier for the release and infiltration of tumor cells [65]. Arteries have thick walls, whereas the walls of the veins are thin, and both arteries and veins are lined with continuous nonfenestrated endothelium. In contrast, the capillaries show a large degree of organ specific composition. Skin, brain and lung capillaries are continuous and nonfenestrated as well, whereas the capillaries of the endocrine glands (e.g. pancreas) are continuous and fenestrated [65]. The capillaries of the liver and the bone marrow are discontinuous, which means that the capillaries show larger fenestrations or even gaps. In these capillaries, the basement membrane is marginally formed. Fenestrae are intercellular openings that increase the intracellular transport. Normal fenestrae posses a diameter of around 70 nm, and the sinusoid fenestrations in the liver have a diameter of 0.1 to 0.2 μm [66]. Since haematopoietic cells are released from the bone marrow into the blood stream, the gaps in the bone marrow sinusoids even allow transendothelial migration of cells and allow the infiltration of tumor cells without the process of extravasation [63, 67]. This structure of the bone marrow capillaries may allow the settlement of a subpopulation of immature DTC in the bone marrow [68].

In contrast, the blood brain barrier provides a sealed microenvironment for tumor cell extravasation. Apart from the absence of fenestrae, the endothelial cell structure with the presence of a basement membrane, which is composed of nidogens, laminins, collagen and heparan sulfate proteoglycans, provides a barricade for the extravasation of tumor cells [63, 69]. In case of breast cancer the tumor cells first pass the breast endothelium into the blood stream. However, both the breast tissue and the breast vasculature may be subjected repeatedly to drastic changes in the life cycle of the mammary gland [70]. In particular, the regressing of the vascular network after weaning of the child results in extremely thin diameter capillaries with blind-ended stumps. Moreover, the microvascularization of the lobules and the ducts differ considerably. Lobules show a low number of microvessels with large diameter and sinusoidal shape, whereas ducts are surrounded by a high number of microvessels with the appearance of typical small capillaries [71]. It has been shown that cells of ductal carcinoma in situ require increased glucose uptake supplied by the vasculature for invasion, which contributes to the correlation of increased amounts of functional capillaries with a shortened survival of the breast cancer patients [72, 73].

Hypoxia-normoxia-hypoxia: The Hard Way Out from the Primary Tumor to the Bone Marrow

One cellular program that is activated after nutrient deprivation and cell stress is induction of HIF-1 alpha induced and VEGF mediated induction of angiogenesis [74]. Novel blood vessels support the supply of the tumor cells with nutrients and oxygen whereas microenvironmental stress occurs in the less perfused microenvironments of a tumor (ischemia). Structural abnormalities of the tumor vasculature, including altering blood vessel diameters, blind ends, arterial-venular shunts or temporal occlusions (acute hypoxia, followed by reoxygenation) can also be found [58].

As an alternative, during tumor hypoxia migratory active cell phenotypes evolve which share some attributes of “migratory stem cells” or epithelial mesenchymal transition (EMT) passed cells that are able to escape from the primary tumor into the blood stream [20, 75]. After their passage through the blood followed by settlement into the bone marrow, a subset of DTC are assumed to be able to survive in the hypoxic microenvironment of the bone marrow even for decades [76]. It is possible that these cells can then undergo the reverse reaction of EMT—mesenchymal epithelial transition (MET)—, remain as epithelial phenotypes or acquire a dormant state in the bone marrow and can contribute later to the pool of bone marrow metastases [2, 77].

Detection of all kinds of gene and genome aberrations, like subchromosomal allelic losses, double strand breaks or chromosome over replication shows that DNA damage significantly contributes to the evolvement of novel DTC genotypes [68, 78, 79]. Tumor hypoxia has been associated with genetic instability, increased metastasis and poor patient outcome [79–81]. The accumulation of DNA damages itself may be due to affected DNA damage sensing and repair mechanisms [82]. Large DNA aberrations are caused by DNA double strand breaks, which are repaired by homologous recombination (HR) and non-homologous end joining (NHEJ). It has been shown for breast cancer cell lines that the protein of the HR BRCA1 is suppressed by hypoxia, whereas the proteins of NHEJ remained unaffected [83]. Using the cell line HepG2 it was shown that the genes of NHEJ *Ku70*, *Ku80*, and *DNA-PKcs* were induced by hypoxia, which was confirmed on protein level [84]. Diminished homologous recombination may lead to genetic instability by shifting the balance between the high-fidelity homologous recombination and the error-prone NHEJ pathways of double-strand break repair in primary tumor cells [83]. It was recently demonstrated that CTC that were released from the primary tumor show an altered phenotype compared to the parental tumor cells [85]. These CTC show a hypoxic phenotype with overexpression of HIF-1 alpha, asparagine synthetase and GLUT1. In addition, these CTC exhibited greater aggressiveness in the mouse model. As Kallergi et al. published, CTC from patients with metastatic breast cancer are frequently HIF-1 alpha positive. This could explain the metastatic potential of these cells and may provide a therapeutic target for their elimination [86].

A special aspect for tumor cell dissemination from hypoxic areas of the primary tumor is their transfer into the well oxygenated microenvironment of the blood [11, 25], which then may result in cell damage that is called reoxygenation injury [87]. From that point reoxygenation induced DNA damage is of special importance for the selection of viable DTC in the bone marrow, since such DTC are quickly confronted in the blood stream with reoxygenation. Recently the presence and generation of reactive oxygen species (ROS) attracted increased interest as a microenvironmental factor as part of reoxygenation affecting the survival of tumor cells. ROS are oxygen molecules or ions that have been reduced incompletely by one-electron reduction. Among others, the hydroxyl radical OH^- has been made responsible for DNA damage like genomic instability, DNA strand breaks and point mutations, [88, 89]. Upon reoxygenation, cells started apoptosis, in which increased apoptosis rates were observed for cells with a functional p53 protein [90]. Chan et al. have proposed a model in which hypoxic or anoxic cells of the primary tumor acquire HR defects like down regulation of Rad51 which then leads to unrepaired single strand breaks after reoxygenation [91]. When hypoxic cells experience reoxygenation, ROS induced DNA damage may result in apoptosis [90, 92]. Indeed the half-life of CTC ranges between 1 to 2.4 h, and after 24 h the number of CTC detection events reaches background levels [76]. However, the hypoxic microenvironmental conditions of the bone marrow fit well to an observed hypoxic CTC phenotype [46, 86, 93]. Hence, hypoxic CTC may have a higher probability to survive in the sanctuary of the bone marrow than in the toxic microenvironment of the blood stream.

Interestingly, the vast majority of bone marrow DTC appears to persist in a nonproliferating state, as shown by a Ki-67 staining, which was interpreted as tumor cell dormancy [3, 94]. This cellular adaptation strategy enables tumor cells to persist in patients over decades after primary tumor diagnosis [76]. Later metastatic outgrowth can be supported by expression of the C-X-C chemokine receptor 4 (CXCR4) which enables the binding of the pro-survival chemokine stromal cell-derived factor 1 (SDF1) [63].

Intrinsic Factors Affecting the Evolvement of Novels Tumor Cell Phenotypes

The phenotypic heterogeneity of tumor cells can be attributed to the evolvement of novel phenotypes during the process of malignant progression as a reflection of microenvironmental pressure in an adaptive landscape [12]. The phenotypic heterogeneity of primary tumor cells and thus of DTC is also influenced by cell intrinsic development and differentiation programmes [20]. As a result, the evolvement of metastasis consists of at least four factors, in which all factors interrelate with each other. The first aspect is the development of the microenvironment in the primary tumor, the second is the microenvironment of the secondary site (including the blood stream), the third is the development/selection of tumor phenotypes in response to all microenvironmental factors and the fourth are cell intrinsic differentiation programmes.

Models for cellular differentiation programmes based on histopathological and molecular observations are essential tools for investigation of cancer phenotypes. First, using a set of biomarker proteins, primary tumors can be classified into definite cancer subgroups with their own specific clinical outcomes [95, 96]. However, for breast cancer it has been shown that the probability for late-onset bone metastasis (relapse >5 years after cancer diagnosis) is associated with a gene expression signature of c-Src activation independent of the hormone receptor status of the primary tumor [45]. Cancer cell survival in the bone marrow environment was supported by Src facilitating CXCL12-CXCR4-AKT signalling and conferring resistance to TRAIL [45]. Second, cell lines—especially MDA-MB-231 cells in breast cancer—with their own specific differentiation status and metastatic potential [97] were frequently used as cellular models, e.g., for cellular adaptation strategies and site specific dissemination [60, 85, 98, 99]. These experiments additionally show that differentiated cells are one source to produce tumors. Third, marker proteins indicative for epithelial differentiation like cytokeratins [100] or EpCAM are most frequently used for the detection of CTC and DTC in patient samples from a variety of tumor entities like breast, lung, prostate or colon cancer [2]. For that reason fundamental scientific and clinical conclusions are extremely sensitive to the choice of the most suitable set of biomarker proteins.

In breast cancer the development of different cellular subgroups can be considered according to model for the phylogenic breast differentiation applied to cancer cells [101, 102]. In this model breast cancer stem/progenitor cells are characterized by expression of cytokeratin 5/6 and expression of the epidermal growth factor receptor EGFR together with low expression of the cytokeratins 8 and 18. In this model basal carcinoma arises from cancer stem/progenitor cells or from intermediary glandular cells, and can express both cytokeratin 5/6 and the cytokeratin 8 and 18. Luminal carcinomas derive from glandular cells and can be detected by expression of cytokeratin 8 and 18, whereas cytokeratin 5/6 expression is low in this subgroup. Assuming breast cancers derive from cancer stem cells, Korsching et al. proposed a model in which basal carcinoma (EGFR overexpressing), ErbB-2 overexpressing carcinoma and luminal carcinoma may derive from aberrant transit amplifying cells [13]. This model also implies that

vimentin expressing breast cancer cells rather derive from bilinear glandular/myoepithelial breast progenitor cells than being an indicator of EMT [13, 103]. Since normal differentiated breast cells have a limited life span, the conversion of such cells to cancer cells requires the acquisition of self-renewal capabilities, thus the acquisition of cancer stem cell attributes [104]. Assuming that cancers derive from cancer stem cells solves the problem how cancer cells become immortal and explains how metastases form out of one single cell. However, similar to the evolvement of different phenotypes under adverse microenvironmental conditions, the cancer stem cell concept implies the evolvement of different subpopulations, but in this context novel phenotypes arise in the first line through an intrinsic differentiation programme. Cancer stem cells are a self-renewing subpopulation within the bulk of tumor cells, which also have a differentiation component and are required for initiation and maintenance of tumor growth [105, 106]. The content of cancer stem cells within tumor depends on the marker proteins applied (e. g ALDH1, $CD44^+$ 24^- in breast cancer) leading to varying detection rates for cancer stem cells. In case of breast cancer the content of cancer stem cells ranges between 12 to 60 percent, whereas in acute myeloid leukaemia the stem cell population (detected as $CD34^+$ $CD38^-$ cells) in patient samples ranges around 0.2% [52, 104].

The cancer stem cell concept predicts not only the presence of semi-autonomous subpopulations like for the cell populations under microenvironmental stress, but also hierarchical order of cells in a tumor. The progenitors within a heterogeneous population of tumor cells—in which any of the cells may acquire stem cell attributes—are committed to their immortal precursor [107]. Even though it is expected that this acquisition should be difficult to detect due to its transient nature, the experimental data that were acquired for one breast cancer patient support this concept [108]. Under the condition that the observed $CD44^+$ $CD24^+$ and $CD44^+$ $CD24^{-/low}$ cells in fact correspond to different stages of the cellular differentiation programme, in this particular patient a cell subpopulation has obtained self-renewal capacity to contribute to the extended stem cell population [109]. The gain of genetic and epigenetic alterations leading to a cancer stem cell or an aberrant cancer stem cell can result in a survival advantage of this particular cell over the other tumor cells [107]. Similar to the influence of the tumor microenvironment, this mechanism then again leads to the survival of the fittest [110]. As a final consequence, cancer stem cell attributes may be regarded as a phenotype responsible or associated with tumor progression driving evolutional advantages [104]. Hence the gain and loss of cancer stem cell attributes might be treated in similar fashion like the gain and loss of a hypoxic phenotype.

Alterations in the microenvironmental conditions activate cellular adaptation programmes like hypoxic adaptation strategies, which also are able to affect the phenotype of the cell. In particular, hypoxia was identified to promote the transition from an epithelial to a mesenchymal phenotype [111]. One link between mesenchymal stem cells, epithelial differentiated cells and EMT seems to be provided by HIF, since one of the proteins induced by HIF-1 is vimentin, which is upregulated after exposition to hypoxia, in which hypoxia promotes a dedifferentiated phenotype in breast cancer [29, 112, 113].

Vimentin expression is one of the hallmarks in EMT and is considered to be one indicator of a more mesenchymal phenotype. We interpret in this manuscript the term “EMT” and its association with cancer in accordance with most recent reports [20, 75]. However, from the histopathological view, this denotation might be considered as an unlucky term in the context of cancer, since “EMT” in the embryonic development and cancer is not exactly the same [114]. EMT is a morphogenetic process in which cells lose their epithelial characteristics and acquire mesenchymal-like migratory phenotype during tumor progression, endowing cells with invasive properties, thereby contributing to the formation of metastases [53]. Mesenchymal cells do not show the apical-basolateral organization of epithelial cells, but rather show a spindle-like fibroblastic shape in cell culture. During acquisition of mesenchymal traits, epithelial cells start to detach from each other. As a result, mesenchymal cells contact only focally resulting in degradation of adherens junctions like nectin and desmosomes (desmocollin, desmoglein). In cancer, induction of EMT can be accompanied by the oncogenic receptor tyrosine kinase EGFR leading to downstream activation of Ras, Rac and Rho [115]. Importantly EMT results in disassembly of the F-actin cytoskeleton, a phenomenon that may be comparable with the induction of breast cancer metastasis upon EGF stimulation leading to cell migration [116]. Interestingly, induction of cell migration including actin reorganization was mainly observed via tyrosine 1248 activation of ErbB-2 leading to increased activation of AKT in which gelsolin serves as actin depolymerising factor [116, 117]. Knockdown of gelsolin expression by siRNA in mammary epithelial MCF10A cells resulted in activation of the EMT program, including activation of AKT and Snail [118]. EMT in cancer results in diminished cell adhesion, promoting cell motility with the potential of the cells to metastasize and establish novel cancer cell colonies at secondary sites [119]. Moreover, one mechanism that is suggested to re-initiate proliferation in dormant cancer cells, the angiogenic switch, can be supported by EMT [57, 120]. However, EMT is not necessarily a prerequisite for tumor cell dissemination because CTC with clear epithelial markers such as EpCAM and cytokeratins are frequently found in blood of cancer patients [54].

The homology between stem cells and EMT was further underlined by the observation that $CD44^+$ $CD24^-$ cells show overexpression of the EMT inducing transcription factors such as Twist1, Twist2 and Snail [121]. The pathways that are implicated in the regulation of breast cancer stem cells show some degree of overlap with the pathways identified in the oncogenic activation of cancer cells [122]. One of these pathways is the axis from ErbB-2 via PI3K to AKT, including PTEN. Further pathways that were implicated in the maintenance of stem cell attributes are the canonical Wnt pathway, Notch and Hedgehog, which are also suspected to be involved in EMT. In addition—but not restricted to—TGFβ-signalling, RTK-signaling, endothelin A receptor signaling and integrin signaling has been implicated in the EMT process [75]. Analogous to the cytokeratins and CD44/CD24, which may serve as marker proteins for epithelial differentiation and the stem cell phenotype, respectively, negative testing for E-cadherin expression together with nuclear β-catenin accumulation can be used to identify EMT. These and other observations recently attracted increasing interest, so that currently attempts were made to find overlapping principles between EMT and the stem cell phenotype [20, 75, 123].

The most reductionist viewpoint is that EMT describes a process while stemness describes a cellular state. Both the EMT passed phenotype and the cancer stem cell phenotype show some degree of similarity with observations made in the embryonic development [14] (but note refs [13, 114]). Activation of a cellular program that shifts a cell away from the indifferent state induces cellular differentiation. Hence, the transition from mesenchymal cells to an epithelial differentiated phenotype describes the process to reach a differentiated state. Consequently, there exist only three different cell types: stem-like cells, differentiated cells and fluent graduations between. Cancer cells are able to both acquire and switch between stemness and differentiation. For example aberrations on nucleic acid and protein level leading to aberrant activation of signaling pathways may aberrantly induce cellular differentiation programs. External factors are for example oxygen, glucose, growth factors and cytokines, interaction with other cells and the structural composition of the microenvironment [14, 75].

The degree of stemness of tumor cells in an adverse microenvironment has interesting implications for the dissemination of tumor cells into the blood stream and their detection. If the most prominent site of malignant progression is the hypoxic core within a primary tumor, then we need models to address how these cells reach the blood stream [58]. Hypoxia mediated EMT of differentiated cells for example may confer increased migratory capacity [112]. Alternatively, the accumulation of DNA aberrations may lead to amplification of oncogenes like EGFR or ErbB-2, followed by increased production of growth factors as part of the malignant transformation process leading to increased invasive and migratory capacity [116, 124]. In other instances selection pressure leads to the evolvement of phenotypes with increased motility as a result of the completion for nutrients and space [12]. Since cell migration requires large amounts of ATP for the actin cytoskeleton assembly [125] this ATP cannot be used for synthesis reactions. However, if cancer cells are able to reach the surrounding stroma beyond the ischemic regions of the tumor they can have access to increased amounts of nutrients like glucose that can be metabolized both for synthesis reactions or further cell migration [12]. Apart from increased access to substrates, increased oxygen concentration and diminished acidification in the surrounding stroma result in decreased microenvironmental stress. Finally, a metabolically healthy cancer cell close to a blood vessel that has experienced the typical stages of malignant progression [1] may disseminate. As a result one would observe different but functional equivalent phenotypes: a primitive phenotype with at least partial loss of epithelial characteristics, a malignant RTK overexpressing phenotype or a phenotype as a result of an emergent process.

The protein expression profile of stem-like CTC/DTC has implications for their detection in the blood and the bone marrow. Since current CTC isolation systems (i.e. CellSearch® system, CTC-Chip, Maintrac®, Adnagen) almost completely rely on the expression of epithelial-specific cell surface markers like EpCAM, only CTC with a detectable expression of such proteins are isolated. Thus, the putative subset of EpCAM CTC might escape most current technologies. The phenotype of this cell population might resemble a $CD44^+$ $CD24^-$ phenotype with expression of TWIST1 TWIST2, SNAIL, fibronectin, vimentin, SLUG, and carbonic anhydrase IX [121, 126]. In breast cancer patient samples, subsets of CTC and/or DTC with some properties of this stem-like phenotype have been detected which are suspected to be metastatic precursor cells [127–129]. Since a broad range phenotypic determination of single cells is still difficult to perform, the stem-like/EMT CTC/DTC phenotype remains to be identified. At this stage application of cell lines as working models might be an option. Sheridan et al. and Solakoglu et al. have carried out valuable groundwork on this field of research [6, 130]. Using cell lines that were directly generated from DTC of breast cancer patients properties of such a primitive phenotype could be collected [131, 132]. These experiments showed a DTC phenotype with low expression on protein level of cytokeratins and ErbB-2 with concomitant high expression levels of vimentin. More recently, this DTC phenotype was further characterized as $CD44^+$ $CD24^{-/low}$ with no detectable expression of EpCAM and low levels of the RTK proteins EGFR, ErbB-2 and ErbB-3 [133]. In

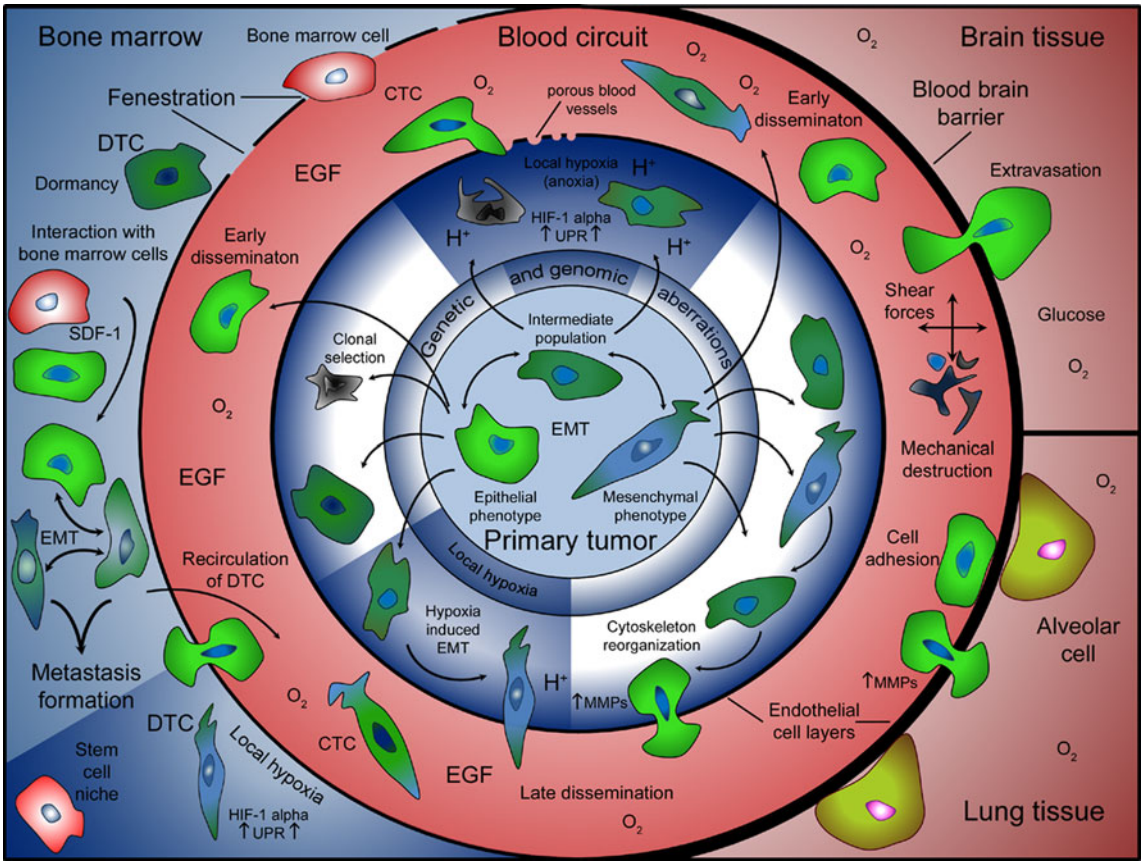

Fig. 1 Schematic overview of tumor cell phenotypes involved in breast cancer metastasis. The expansion of the tumor cell population within the primary tumor can be steered by cancer cells with the mesenchymal phenotype (cancer stem cells) or cells that have passed EMT. Alternatively, the bulk of tumor cells with epithelial differentiation push the tumor progression. These are the disseminated tumor cells that are detected by their high expression of cytokeratins. All these phenotypes can acquire genetic or epigenetic alterations, which then are subjected to clonal selection resulting in clonal evolution of the tumor cell population. At any stage of the tumor progression tumor cells can be released into the blood stream (circulating tumor cells, CTC). In addition, dynamic exchange of cancer cell populations can occur through tumor self-seeding. Early dissemination can be promoted by expression of pro-migratory factors like Twist and later dissemination can be mediated ErbB-2/ErbB-3 heterodimer mediated induction of cell migration. During the expansion of the primary tumor local fluctuations in the microenvironment (hypoxia, acidification (H^+), nutrient deprivation) can create tumor cell subpopulations that are specifically adapted to their microenvironmental conditions (e.g. induction of HIF-1 alpha, VEGF, lactate dehydrogenase). Persistent cell stress may lead to induction of cytoprotective programmes like the unfolded protein response (UPR) together with the mTOR mediated down regulation of the global protein synthesis. Cell stress together with impaired expression of DNA repair proteins can then further promote genetic and genomic aberrations or cell death (necrosis). Acidic conditions can corrode the endothelial cell layer of the blood vessels leading to an easy access of tumor cells to the blood stream without the maneuver of invasion. During the passage to secondary sites CTC are confronted with high oxygen partial pressure, shortage of growth factors and mechanical destruction diminishing the pool of CTC. The fenestrated capillary structure of the bone marrow sinusoids provides easy access to the bone marrow stroma, whereas the sealed structure of lung capillaries and the blood brain barrier requires the expression of specific proteins like matrix metalloproteases and receptor tyrosine kinase mediated cell migration. Having reached the bone marrow, DTC can fall into a non proliferative state (dormancy). Alternatively, DTC are able to undergo productive interactions with the bone marrow cells via the extracellular matrix, direct-cell to cell contacts or by uptake of factors like SDF1 via its receptor CXCR4. Cells of the growing tumor cell population are then able to undergo EMT generating migratory active DTC that are able to invade the bone marrow, which may lead to metastases. Since the bone marrow is a hypoxic microenvironment with regions of lowest oxygen partial pressure in the haematopoietic stem cell niche, hypoxia adapted DTC populations might have a selection advantage in these hypoxic areas. In analogy to hypoxia mediated EMT in the primary tumor DTC can undergo this process in the bone marrow

addition, an association with a stemness/EMT DTC phenotype with high levels of UPR chaperones and oxidoreductases was observed, suggesting that UPR mediated cytoprotection from microenvironmental stress might provide a survival advantage of such DTC in the hypoxic microenvironment of the bone marrow. It has been suggested that the microenvironment of the bone marrow consists of an uneven distribution of local oxygen concentrations up to regions with almost anoxia [93]. Interestingly, the hematopoietic stem cell niche is located in the region of the bone marrow with the lowest oxygen partial pressure and least perfusion [134]. In analogy to the uneven

distribution of hematopoietic cells within the oxygen partial pressure gradient, it might be possible that DTC of the bone marrow are also spatially distributed along the oxygen partial pressure gradient. DTC with high expression levels of the UPR proteins might be located in more hypoxic regions than low UPR protein expressing DTC [133].

After dissemination into the bone marrow, this DTC population with a mesenchymal-like migratory phenotype might undergo a mesenchymal-to-epithelial transition with a regain of epithelial properties like cytokeratin expression as an intermediate step to start metastatic outgrowth [75, 77]. As a consequence, these DTC would be already detected by epithelial markers (i.e. EpCAM and cytokeratins) and contain the pool of the well known DTC population with their clinical significance [11]. This hypothesis might be an approach to the understanding why in some studies a higher incidence of DTC was observed than for CTC when both bone marrow and blood samples from the same breast cancer patients were investigated with epithelial differentiation markers [94, 135]. Alternatively within the CTC, pool selection of the most viable cells might be still in progress, so that CTC detection is just a snapshot of the struggle for survival, whereas DTC in bone marrow might comprise the cell population that has already reached the next selection level towards the formation of distant metastasis.

Conclusion

If the majority of DTC were able to start metastatic outgrowth there would be thousands of metastases in cancer patients, which is clearly not the case [136]. Instead, only a subpopulation of DTC is able to start metastatic outgrowth; however, it is still unclear which phenotypic characteristics enable DTC to do so. Figure 1 summarizes the potential mechanisms and processes relevant for this complex process. Survival programs include the induction of HIF-1 alpha, the Warburg Effect, UPR, induction of tumor cell dormancy or DNA-damage response pathways like activation DNA-PK_{CS}. Together with the protein mediated survival programs, hypoxia induced DNA damage like double strand breaks or point mutations can result in the evolvement of novel genotypes. The plasticity of epithelial cells allows the conversion of tumor cells from one state to another by the processes of EMT and the reverse reaction of MET. These programmes can be promoted by intrinsic factors like oncogenic aberrations in the cell signalling modules or by environmental stress. During the recursive development of primary tumor cells and their microenvironment under selection pressure novel tumor cell populations emerge. Iteration of mutation and selection in an adaptive landscape may lead to emergent behaviour with generation of novel tumor phenotypes. This might lead to tumor cell phenotypes having the capacity to start early metastatic outgrowth, independent from their classification as stem cells, EMT passed cells or differentiated cells. Moreover, the concept of tumor self seeding predicts a dynamic exchange of cancer cells between tumor cell colonies in the human body. There is an urgent need for reliable biomarkers for the detection of the CTC/DTC phenotype that lack the common epithelial marker proteins to get insight to the entire pool of released tumor cells.

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