Distinctive properties of metastasis-initiating cells

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Primary tumors are known to constantly shed a large number of cancer cells into systemic dissemination, yet only a tiny fraction of these cells is capable of forming overt metastases. The tremendous rate of attrition during the process of metastasis implicates the existence of a rare and unique population of metastasis-initiating cells (MICs). MICs possess advantageous traits that may originate in the primary tumor but continue to evolve during dissemination and colonization, including cellular plasticity, metabolic reprogramming, the ability to enter and exit dormancy, resistance to apoptosis, immune evasion, and co-option of other tumor and stromal cells. Better understanding of the molecular and cellular hallmarks of MICs will facilitate the development and deployment of novel therapeutic strategies.

The majority of cancer deaths is caused by metastasis, when cancer cells manage to escape the primary tumor, survive the treacherous transit through the lymphovascular system, and eventually form secondary tumors in distant organs (Gupta and Massague 2006; Valastyan and Weinberg 2011; Wan et al. 2013). This is a highly challenging process with a tremendous rate of attrition; it is estimated that only <0.02% of disseminated tumor cells (DTCs) are able to successfully seed metastases [Luzzi et al. 1998; Cameron et al. 2000; Chambers et al. 2002]. As a result, although tumor dissemination can occur relatively early in cancer progression [Husemann et al. 2008; Massard et al. 2011; Kang and Pantel 2013], sometimes even at the preneoplastic stage [Rhim et al. 2012], an extended gap time often exists between the formation of the primary tumor and clinical manifestations of metastasis [Yachida et al. 2010; Vanharanta and Massague 2013]. Therefore, the capability to initiate metastatic growth is a major bottleneck during cancer progression and represents an ideal window for therapeutic intervention [Fig. 1A].

Metastasis-initiating cells (MICs), by definition, are cancer cells capable of seeding clinically significant metastatic colonies in secondary organs. Like their primary tumor counterparts, the tumor-initiating cells (TICs), MICs can hijack some of the normal stem cell pathways to increase cellular plasticity and stemness, which provide them with multiple malignant advantages. However, the MICs must possess additional capabilities that allow them to survive the metastatic cascade and function as TICs in an organ microenvironment distinctively different from the primary tumor. These cells form the link between the primary tumor and subsequent metastasis but are exceedingly difficult to identify, track, and characterize. Even the origin of MICs remains elusive; they might exist at the primary tumors or emerge during the journey through the metastatic cascade [where exposure to extreme stress conditions may select for MIC abilities] or may acquire such capabilities only after arriving at the distant site and engaging the stromal components [Fig. 1B]. Such unique challenges in identifying and analyzing MICs demand research tools beyond what are commonly available and used in the study of TICs, such as in vitro tumorsphere assays, in vivo limited dilution tumor initiation studies, and analysis using cancer stem cell (CSC) surface markers. In the past few years, new and emerging technologies have begun to enable the study of MICs in animal and clinical models. Genomic sequencing studies have provided genome-wide comparisons between primary tumors and matched distant metastases from cancer patients and animal models [Campbell et al. 2010; Yachida et al. 2010, McFadden et al. 2014, Gundem et al. 2015]. Gene expression analysis at the single-cell level has become a powerful tool to analyze the population dynamics of tumor cells during metastatic evolution [Lawson et al. 2015]. In addition, lineage tracing and barcode sequencing studies have also been applied to study the interclonal interactions and population dynamics [Maddipati and Stanger 2015; Wagenblast et al. 2015]. Advances in animal modeling of metastasis and detailed analysis of tumor-intrinsic pathways and tumor–stromal cross-talk further provided unprecedented insights into the molecular mechanism of metastasis initiation. Some consensus regarding the hallmarks of MICs has started...
to emerge from these studies, including the maintenance of TIC ability, the flexibility to undergo bidirectional transitions between the epithelial and mesenchymal states, resistance to anoikis and apoptosis, entry into and exit from dormancy, evasion of immune system attack, reprogramming of metabolic activities to adapt to the different nutrient and oxidative stresses, interclonal cooperations, and the ability to build or take advantage of a supportive stromal niche. Underlying all of these myriad properties of MICs is their remarkable cellular plasticity that allows them to survive and thrive against all odds. In this review, we summarize the main tumor-intrinsic hallmarks of MICs and their dynamic interactions with the extrinsic environment to manifest their metastasis-forming activities and discuss the possible strategy of targeting MICs in cancer therapeutics.

**Genomic evolution of MIC traits**

Cancer genome sequencing studies have shown that malignant tumors emerge from the sequential accumulation of mutations in driver genes involved in three core cellular processes during tumor initiation: cell fate regulation, genome maintenance, and cell survival [Vogelstein et al. 2013]. These altered processes favor primary tumor initiation and may still be essential for MICs to seed metastases. However, it was previously unknown whether additional driver mutations are needed for metastasis to occur. Genome sequencing studies have shown high degrees of similarities among mutations in primary tumors and metastases [Yachida et al. 2010]. The most remarkable finding of these studies is that no consistent metastasis-specific mutations have been found other than those that are already commonly found in primary tumors [Bozic et al. 2010, Campbell et al. 2010, Yachida et al. 2010], suggesting that important mutations for metastasis are already present in the primary tumor site. These studies frequently reveal a greater enrichment of clonal populations rather than an acquisition of new mutations, as observed in pancreatic cancer metastasis with amplifications of MYC, RASG13D, and CCND1 [Campbell et al. 2010] and in lobular ER+ breast cancer with ERBB2 mutations [Shah et al. 2009]. A recent study using whole-exome sequencing analysis of experimental metastasis models of multiple cancer types has shown that metastatic competence arises from the selection of pre-existing mutations, such as RASG13D and BRAFG464V in heterogeneous populations without the need for additional mutations [Jacoby et al. 2015]. The selection of these oncogenic pathways favors their prevalence in metastasis, indicating that they are important contributors to metastatic fitness and thus may be required for MICs. Overall, these findings suggest that a large number of metastatic properties may already be forming in the primary tumor through enrichment of existing oncogenic mutations that favor metastasis initiation.

Beyond realignment of genomic mutations, epigenetic regulation might be a major source of MIC traits, especially in later steps of metastasis. After tumor cells escape the primary site, the epigenome is subjected to microenvironmental signal modulation, conferring cellular plasticity and adaptability to new and inhospitable conditions [Sftor et al. 2006; Hendrix et al. 2007; Tam and Weinberg 2013]. Indeed, multiple studies have unveiled evidence of specific epigenetic pathways involved in the metastatic progression of different cancer types [Cunha et al. 2014; Gu et al. 2015; Okada et al. 2015; Tang et al. 2015]. Therefore, the combination of genetic and epigenetic events during the course of metastasis likely determines the acquisition of MIC traits.

**Cell fate determinants as regulators of MICs**

Adult tissues are hierarchically organized and tightly controlled by lineage-specific transcription factors to regulate growth and differentiation and maintain the homeostasis...
of tissues and organs. During tumorigenesis, the metastatic potential of tumors with different cellular origins (adult stem cells, progenitor cells, or differentiated cells) may be shaped by the dominant lineage-specific cell fate regulators expressed in the originating cells. In addition, alteration or loss of differentiation control may result in dedifferentiation, acquisition of stem cell-like activities, and cellular plasticity that facilitate the development of metastatic traits [Reya et al. 2001; Ben-Porath et al. 2008].

Accumulating evidence supports the notion that loss of differentiation factors leads to dedifferentiation and acquisition of stem cell-like traits that are linked to metastasis initiation properties [Fig. 2; Cao et al. 2011]. Mutation, epigenetic silencing, or reduced expression of luminal differentiation factors in the mammary gland (GATA3 and ELF5) has been shown to promote breast cancer metastasis [Kouros-Mehr et al. 2008; Chakrabarti et al. 2012]. RARRES3, which is involved in retinoic acid-induced differentiation signaling, suppresses breast cancer lung metastasis initiation by promoting tumor differentiation [Morales et al. 2014]. In lung adenocarcinoma, the loss of NKX2-1, a lung lineage-specific transcription factor, increases metastatic seeding [Winslow et al. 2011]. In a recent follow-up study, NKX2-1 was found to work synergistically with other lineage-specific transcription factors (FOXA2 and CDX2) to suppress lung metastasis [Li et al. 2015]. The simultaneous loss of these three lineage cell fate determinants induces dedifferentiation and stem cell-like properties to promote lung metastasis. Two other lung alveolar differentiation transcription factors (GATA6 and HOPX) also cooperatively limit the metastatic competence of lung adenocarcinoma [Cheung et al. 2013]. Similarly, the loss of MITF, a melanocyte differentiation factor, is sufficient to increase metastasis of melanoma [Cheli et al. 2012].

Opposing the function of lineage-specific differentiation factors, the increased activity of stem cell factors has been shown to promote metastasis. For example, the cooperation of mammary stem cell (MaSC) transcription factors SNAI2 and SOX9 induces luminal dedifferentiation toward a stem cell-like state with metastatic seeding abilities [Guo et al. 2012]. In a similar fashion, ID1 [inhibitor of differentiation-1] increases breast cancer lung metastasis [Gupta et al. 2007], and the MaSC marker PROCR is also reported to be involved in self-renewal and metastasis [Spek and Arruda 2012; Wang et al. 2015]. Interestingly, other factors that support tumor initiation activity seem to work only in the malignant context and are not involved in the regulation of normal adult tissue stem cells. For example, MTDH, an essential factor to support tumor initiation and metastasis in breast, prostate, and liver cancers, is dispensable for embryonic and postnatal development [Robertson et al. 2014; Wan et al. 2014a,b]. Such factors will be ideal candidates for therapeutic targeting to prevent metastasis initiation.

Not only are tissue-specific cell fate determinants critical in metastasis initiation, embryonic cell fate regulators also play important roles. With the discovery of the Yamanaka factors—Sox2, Myc, Klf4, Oct4, and others—as potent reprogramming factors, these genes have also garnered much attention in cancer research. Each of these factors has been linked to tumor aggressiveness and poor prognosis [Ben-Porath et al. 2008; Kim et al. 2013; Karetta et al. 2015]. MYC is one of the most thoroughly studied oncogenes [Cole 1986], and KLF4 has also been classified as an oncogene [Leng et al. 2013]. Recently, SOX2 was shown to maintain self-renewal and survival of CSCs in multiple tumor types, including squamous cell carcinoma [Boumahdi et al. 2014]. In medulloblastoma, SOX2 drives the hierarchical organization of the tumors and promotes relapse [Vannier et al. 2014]. Interestingly, during embryonic development, SOX2 specifies cell fate decisions by antagonizing tissue-specific factors involved in metastasis, such as NKX2-1, CDX2, MITF, and others mentioned.

**Figure 2.** Cell fate determinants in development and their influence on MICs. [A] Embryonic and adult epithelial cell lineage transcription factors tightly control self-renewal and lineage-specific differentiation of normal adult tissue stem cells and embryonic stem cells. (OKSM) The Yamanaka factors Oct4, Klf4, Sox2, and Myc. [B] The same transcription factors also influence metastatic behavior of cancer cells and the formation of MICs. Differentiation factors of normal tissues, such as MITF, GATA3, FOXA2, and others, act as tumor suppressors and metastasis inhibitors. On the other hand, dedifferentiation factors, such as the Yamanaka factors or tissue-specific stem cell factors, drive dedifferentiation, plasticity, and metastasis of MICs. Interestingly, these transcription factors constitute a complex network of reciprocal regulation. For example, SOX2 antagonizes multiple tissue-specific differentiation factors. Other factors, such as MTDH, exclusively support TIC and MIC activities and have no known function in normal tissue development.
above [Fig. 2B]. In addition, SOX2 and NANOG have been reported to maintain quiescence programs in DTCs/residual cancer cells and may contribute to metastatic relapse (Sosa et al. 2015). Although SOX2, NANOG, OCT4, and KLF4 have been shown to increase metastasis of bladder cancer, breast cancer, lung cancer, and head and neck squamous carcinoma cells (Celia-Terrassa et al. 2012; Vaira et al. 2013; Lu et al. 2014; Habu et al. 2015), none of these factors has been specifically studied during metastasis initiation. Based on current knowledge, it is tempting to speculate that these factors may also facilitate metastatic initiation by promoting cell plasticity, adaptability, survival, and self-renewal as they do in primary tumors. Therefore, future research should be conducted to study these cell fate regulators during metastasis initiation.

**Epithelial–mesenchymal plasticity and the acquisition of stem cell-like properties**

Cancer cell plasticity is a dynamic state of dedifferentiation, with cells acquiring some characteristics of stem cells. Serious malignant advantages can be acquired when cancer cells hijack developmental processes such as epithelial–mesenchymal transition (EMT) to increase their cellular plasticity. EMT normally occurs during embryonic development and also in pathological conditions such as wound healing and metastasis (Thiery et al. 2009, Nieto 2013). During EMT, epithelial cells lose their polarity and cell–cell adhesions to gain mesenchymal-like properties, such as increased migratory abilities. Cancer cells often undergo EMT to escape from the primary tumor, and mounting experimental and clinical evidence suggests that a reversed process, mesenchymal-to-epithelial transition (MET), is required for the outgrowth of metastatic tumor cells in the secondary organ (Thiery et al. 2009, Korpal et al. 2011; Brabletz 2012; Tsai et al. 2012). Interestingly, besides promoting invasion, EMT can induce stem cell-like properties to promote initiation of primary tumors and accelerate metastasis (Mani et al. 2008; Thiery et al. 2009, Guo et al. 2012).

Whether EMT plays a crucial role in cancer metastasis in human patients and in some animal model systems is still under debate (Ledford 2011; Fischer et al. 2015; Zheng et al. 2015a), largely due to the lack of the ability to track the occurrence of EMT and follow the fate of cells undergoing EMT in clinical settings as well as the diversity of the EMT program that can elude detection using a single EMT marker or reporter in animal models (Li and Kang 2016). Nevertheless, a recent study used rigorous single-cell analysis of breast cancer-derived xenografts to show that MICs indeed display a stem cell program with EMT features at the early phase of metastasis development (Lawson et al. 2015). Metastatic cells from small metastatic lesions have increased expression of EMT and stem cell features and dormancy-associated genes, while such features are often attenuated and replaced with the expression of differentiation and proliferation markers in fully developed macrometastases (Lawson et al. 2015). This finding supports the notion that EMT is required for early seeding of metastasis, while MET is essential for metastatic outgrowth (Tsai et al. 2012). Indeed, other studies have shown that an extreme EMT can lock cancer cells into a terminally differentiated state, depriving them of stem cell-like properties and cell plasticity and reducing tumor growth [Tran et al. 2011, 2014; Celia-Terrassa et al. 2012].

It is thus important to note that EMT is not a binary process; instead, it represents a spectrum of transitional states that can display different degrees of epithelial and mesenchymal features depending on the driver genes and pathways that induce the EMT process. Indeed, distinct EMT programs have been shown to influence different cell populations, and it is proposed that SNAI1 has a stronger effect on TIC generation and metastasis progression than SNAI2, which is crucial for sustaining normal mammary gland stem cells [Ye et al. 2015]. Therefore, it is important to consider distinct EMT drivers and target cell populations when analyzing results from EMT experiments. Further complicating the analysis, the reversion of EMT [MET] can also induce stem cell-like properties and increase metastasis initiation of epithelial-like CSCs, as has been documented in multiple recent studies (Celia-Terrassa et al. 2012, Ocana et al. 2012; Stankic et al. 2013; Beck et al. 2015; Schmidt et al. 2015). Based on these results, CSCs can exist in both an epithelial-like or a mesenchymal-like transitional state, while cells fixed at extreme epithelial or mesenchymal states lose plasticity and the associated stem cell activities [Nieto 2013; Oskarsson et al. 2014]. It is also possible that loss of lineage-specific differentiation factors, as discussed above, can induce CSC properties without the involvement of EMTs.

To provide a unifying model to reconcile all of these experimental observations regarding the link between epithelial–mesenchymal plasticity with CSC properties, a bipotent or hybrid EMT/MET state has been proposed and mathematically modeled as the “window of stemness” [Fig. 3; Jolly et al. 2015a,b]. According to this model, the bipotent state resides within a tiny transitory fraction of the tumor population with both epithelial and mesenchymal features. This “hybrid state” is bidirectional and displays a gradient of partial states toward either extreme EMT or extreme MET [Fig. 3]. This model has been supported by a recent analysis of CSC markers in breast cancer [Liu et al. 2014]. In this study, mesenchymal-like breast CSCs [BCSCs] characterized as CD24−CD44+ primarily quiescent with high invasive ability, while epithelial-like BCSCs are ALDH+, highly proliferative, and less invasive. Breast cancer cells with dual expression of both sets of markers have the highest degrees of plasticity [Liu et al. 2014]. However, more research is needed to evaluate the proposed hybrid state hypothesis in other cancer types and model systems and determine its importance for metastasis and MICs.

It is also possible that sequential transitions—first EMT and then MET—may be required to achieve an optimal CSC state in MICs. Recent studies using nonmalignant cells suggest that sequential EMT and MET events increase the pluripotent state in keratinocytes and fibroblasts [Liu et al. 2013; Unternaehrer et al. 2014]. Other studies have validated this in mammary epithelial cells
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Metabolic reprogramming

Different organs and tissues of the human body have specific metabolic characteristics. Therefore, it is likely that MICs may require a high metabolic adaptability and metabolic stress resistance. The aerobic glycolysis observed in the primary tumors—the Warburg effect—is frequently replaced in metastasis by other routes of energy acquisition. One of the main causes of this phenomenon may be the detachment of tumor cells from the extracellular matrix (ECM) during metastasis, which impairs glucose uptake and shuts down the Warburg effect, thereby requiring alternative ways to circumvent deficiencies by using mitochondrial metabolism and peroxide signaling [Weber 2016]. Although EMT has been shown to induce aerobic glycolysis to promote the growth of basal-like breast cancer [Dong et al. 2013], another study showed that disseminated invasive cells do not use aerobic glycolysis. Instead, PPARGC1A [PGC1α] mediates mitochondrial oxidative phosphorylation and biogenesis to facilitate lung colonization [LeBleu et al. 2014]. Accordingly, a recent study reported that extreme mesenchymal-like prostate cancer cells are dependent on the function of the mitochondrial respiratory chain function [Aguilar et al. 2016], which is typically associated with slowly proliferating cells [DeBardinis et al. 2008]. Therefore, the metabolic differences observed in cells undergoing EMT seem to be more dependent on their proliferation rates than the mesenchymal-like or epithelial-like phenotype. In addition, quiescent DTCs can sustain their metabolic fitness by autophagy as an alternative source of energy during nutrient deprivation [Liang et al. 2007; Sosa et al. 2014]. However, to reinitiate metastatic growth and engage proliferation, MICs may display marked metabolic plasticity in order to obtain energy from multiple substrates and pathways, as has been shown for highly metastatic breast and prostate cancer cells [Chen et al. 2007; Aguilar et al. 2016].

Different host organs also have different nutrient and oxygen conditions. The brain and lungs have high levels of glucose and oxygen, which may grant easier colonization of metastatic cells using aerobic glycolysis [DeBerardinis et al. 2008] or oxidative phosphorylation [LeBleu et al. 2014; Weber 2016]. An adaptive metabolic mechanism critical for brain metastatic cells is the ability to co-oxidize acetate and glucose in the citric acid cycle as main fuels to support the bioenergetic demands of rapidly proliferating cells [Mashimo et al. 2014]. In contrast, the liver has lower levels of oxygen and irregular glucose availability, and therefore MICs in the liver need to adapt to such metabolic stresses. Due to low oxygen levels, liver metastatic breast cancer cells activate HIF-1α with a concomitant increase of the pyruvate dehydrogenase kinase-1 (PDK1), switching their metabolism to glycolysis [Fig. 4A]. Such metabolic adaptation is essential for breast...
cancer cells to efficiently colonize the liver (Dupuy et al. 2015). Another recent study has demonstrated the necessity of scavenging energy from the extracellular environment to overcome liver metabolic stress (Loo et al. 2015). In this study, colon cancer cells, by down-regulating miR-483 and miR-551, derepress and secrete creatine kinase, brain-type (CKB) into the extracellular space to convert creatine and ATP into phosphocreatine. The phosphocreatine is then imported into the MICs to serve as an ATP source for growth functions (Fig. 4A; Loo et al. 2015). In metastatic ovarian cells, fatty acids secreted from adipocytes are imported by FABP4 in MICs to colonize the intra-abdominal fat (Nieman et al. 2011). Under nutrient deprivation conditions, mitochondrial HSP90 chaperones, including TRAP1, overcome metabolic stress and promote metastasis by limiting the activation of the nutrient sensor AMPK and preventing autophagy (Caino et al. 2013).

Exposure to new inhospitable environments and drastic metabolic reprogramming cause high levels of metabolic stress. Indeed, redox signaling pathways are often up-regulated in metastasis (Pani et al. 2010). For example, it was recently shown that metastatic melanoma cells adopt detoxifying mechanisms, such as producing NADPH detoxifying enzymes of the folate pathway, including ALDH1L2 and MTHFD1, to withstand oxidative stress at the metastatic sites (Piskounova et al. 2015). This is in contrast to previous studies suggesting reactive oxygen species (ROS) as prometastatic effectors (Wu 2006; Ishikawa et al. 2008; Nishikawa 2008; Porporato et al. 2014). Such contradictory observations may be due to the fact that these earlier studies focused on the action of ROS on the primary tumor site, which may promote cancer progression by generating genomic instability. ALDH enzymes are well-established CSC markers in several cancer types (Ginestier et al. 2007; Medema 2013). Accumulating...
functional in vivo studies have also shown that different ALDH members can increase metastasis in breast and other cancers [Rodriguez-Torres and Allan 2016], which may be linked to their aldehyde and ROS detoxification functions.

In general, MICs can use oxidative phosphorylation in oxygenized organs and generate potent mechanisms to protect themselves from ROS and oxidative stress. In other organs with low oxygen and nutrients, such as the liver, MICs are able to use the creatine cycle to scavenge ATP or activate β-oxidation. Therefore, metabolic reprogramming is an essential hallmark for MICs to survive at distant sites.

**Resistance to anoikis and apoptosis**

Cancer cells often encounter multiple apoptotic death signals in the new environment. It is likely that DTCs already acquire anti-apoptotic mechanisms, such as elevated expression of caspase inhibitors and other anti-apoptotic genes, at the primary tumor sites [Su et al. 2015]. Nevertheless, new extrinsic apoptotic signals are often present at the metastatic sites that present new challenges for MICs and coerce MICs to adopt different protective mechanisms. A good example is the protective action of anti-PA serpins to prevent proteolytic activation of the proapoptotic ligand FasL, produced by reactive astrocytes in brain metastasis [Fig. 4B; Valiente et al. 2014].

Another major apoptosis-inducing stress that cancer cells typically encounter during metastasis is detachment from ECM during dissemination, which often leads to anoikis. In a recent study, transcription factor ATF4 has been shown to be part of the integrated stress response (ISR) and protects MICs against death via anoikis [Dey et al. 2015]. Another critical pathway promoting cell survival and anoikis resistance is the AKT signaling pathway [Vanharanta and Massague 2013; Wan et al. 2013]. Various mechanisms converging on AKT activation have been reported to favor survival at different organs. In DTCs, elevated expression of neurotrophic receptor NTRK2 [TrkB] inhibits anoikis by activating the PI3K–AKT pathway [Douma et al. 2004]. In breast cancer bone metastasis, SRC kinase mediates activation of AKT, which is required for CXCL12/SDF-mediated cell survival and resistance to TNFSF10 (TRAIL)-mediated apoptosis [Zhang et al. 2009]. In lung metastasis, macrophages support the survival of breast tumor cells by activating the VCAM1–Ezrin–PI3K/Akt survival pathway via the counter-receptor α4-integrin [Chen et al. 2011]. Pancreatic cancer metastasis has also been associated with STAT3-induced anoikis inhibition [Fofaria and Srivastava 2015]. Another mechanism of anoikis resistance involves cell metabolic reprogramming to overcome an ATP deficit, as we discussed above [Schafer et al. 2009; Weber 2016].

**Co-option of the metastatic niche**

Distant tissues are normally hostile environments for the newly arriving tumor cells. Most of the DTCs will either die by anoikis/apoptosis due to lack of survival signals or energy resources or be killed by death signals from the incompatible stromal and immune cells of the host tissue [Hanahan and Coussens 2012; Quail and Joyce 2013; Piskounova et al. 2015]. The ability of tumor cells to manage and re-educate their new stromal partners in the host microenvironment is a critical event in colonizing a distant tissue. Cancer cells can compete for, corrupt, or co-opt the existing normal tissue niches and recruit additional stromal cells to generate a supporting niche for MICs. Indeed, tumor cells and the host stroma coevolve during metastasis initiation and progression [Joyce and Pollard 2009; Polylak et al. 2009; Barcellos-Hoff et al. 2013]. Additionally, emerging evidence shows that tumor-stromal interactions do not occur only after DTCs arrive at a distant tissue. Indeed, the primary tumor can systematically influence the metastasis milieu before and after MIC colonization [McAllister and Weinberg 2014]. An example of systemic influence by the primary tumor is the instigation of a supportive stroma enriched in EGF and IGF1 to activate indolent breast cancer DTCs [Castano et al. 2013]. In addition, primary tumors can secrete cytokines and exosomes and mobilize bone marrow-derived cells (BMDCs) to form premetastatic niches, which facilitate metastatic colonization by DTCs [Kaplan et al. 2005; Peinado et al. 2012; McAllister and Weinberg 2014; Zhou et al. 2014; Fong et al. 2015; Hoshino et al. 2015]. The induction of a receptive microenvironment increases the metastatic efficiency of MICs and enriches the century-old “seed and soil” hypothesis formulated by Stephen Paget [Fidler 2003].

MICs may require a specific niche environment at the target organ to survive and eventually form overt lesions. Following this concept, many studies have focused on finding evidence of the metastatic niche. One of the first relevant studies showed that prostate cancer DTCs displace hematopoietic stem cells (HSCs) from their natural niche to colonize the bone marrow [Fig. 4C; Shiozawa et al. 2011]. Recently, the osteogenic niche in bone has been proposed to induce mTOR signaling in DTCs via heterotypic adherens junctions and promote the initial outgrowth of micrometastases in bone [Fig. 4C; Wang et al. 2015b]. To successfully form macrometastasis, cancer cells express VCAM1 to recruit preosteoclasts and stimulate osteoclast differentiation, leading to the eventual formation of a “vicious cycle” of metastatic tumor growth, osteoclast-mediated bone lysis, and the release of tumor-promoting growth factors [Fig. 4C; Lu et al. 2011; Sethi et al. 2011; Weilbaecher et al. 2011; Ell and Kang 2012].

Another important niche location in many organs is the vasculature. Endothelial cell sprouting abolishes inhibitory growth signals in dormant tumor cells to allow metastasis of breast cancer cells in different organs [Ghajar et al. 2013]. Of note, in different types of cancers, certain microRNAs suppress or promote metastasis by influencing endothelial recruitment to form the metastatic niche [Pencheva and Tavazoie 2013]. In the lung, bone marrow-derived endothelial progenitor cells drive the angiogenic switch to promote lung metastasis of luminal
breast cancer cells (Gao et al. 2008). In the brain, vascular co-option of breast cancer cells through L1CAM-mediated adhesion facilitates MIC access to nutrients and oxygen, while tumor-derived anti-PA serpin protected MICs from FasL death signals from astrocytes [Fig. 4B; Valiente et al. 2014]. MICs can also generate their own niche by building a supportive ECM in distant organs. For example, breast cancer cells secrete tenasin C, an ECM protein, in lungs to a stimulate stemness and favor metastasis [Fig. 4D; Oskarsson et al. 2011].

In order to cultivate a supporting “soil” at secondary organ sites, tumor cells can also activate other nonniche cells, such as fibroblasts in metastatic sites, and turn them into cancer-associated fibroblasts [CAFs] with metastasis-promoting functions [Kalluri and Zeisberg 2006], such as producing ECM niche components peristin (POSTN) and tenasin C [Fig. 4D; O’Connell et al. 2011; Malanchi et al. 2012]. In addition, TGF-β released from co-locreal cancer cells stimulated CAFs to secrete IL-11, which feeds back to tumor cells to activate STAT3 signaling, favoring the survival of metastatic cells in the liver [Calon et al. 2012]. In the brain stroma, reactive astrocytes also mediate important cross-talks with MICs to enhance their proliferation, survival, and metastasis [Kodack et al. 2015]. Astrocytes promote stem cell-like traits to breast cancer cells by activating Notch signaling in the brain [Fig. 4B; Xing et al. 2013]. A recent study demonstrated how PTEN expression is suppressed in MICs by the interaction with astrocytes. In this study, astrocyte-derived exosomes transfer the PTEN targeting miR-19a to the MICs. PTEN repression increases NFκB-dependent CCL2 secretion and recruitment of myeloid cells to promote survival and growth of MICs in the brain [Fig. 4C; Zhang et al. 2015].

A main threat to MICs is the immune cells present at the new organ sites. The immune system is believed to prevent the formation of >80% of all primary tumors [Hanahan and Weinberg 2011]. Even if DTCs have successfully evaded the immune system at the primary tumor site, they are likely to encounter new, hostile immune cells with the ability to recognize and kill them in the circulation and at metastatic sites. Indeed, the plasticity of MICs to readily fluctuate between EMT–MET states might facilitate the immune evasion during metastasis. EMT transcription factors have been shown to have immunosuppressive functions. SNAI1 induces CD4+ CD25+ Treg immune-suppressive cells and impairs dendritic cell activity [Kudo-Saito et al. 2009]. Moreover, ZEB1 repression of miR-200s up-regulates its target PD-L1, a known immune checkpoint regulator of CD8+ T cells [Chen et al. 2014]. The secretion of TGF-β from tumor cells can repress the production of cytolytic and proapoptotic factors by CD8+ CTLs [Thomas and Massague 2005]. Therefore, mesenchymal-like DTCs, which often have elevated expression of TGF-β, may escape attack by CTLs upon arrival in distant tissues. In contrast, BMP4, another member of the TGF-β family, functions as a metastasis suppressor in breast cancer by blocking G-CSF-induced expansion of myeloid-derived suppressor cells [MDSCs] [Cao et al. 2014]. As another mechanism to compromise the function of innate immune cells during metastasis, melanoma cells express FcyRIIB that negatively regulates B-cell recognition and humoral immunity to promote liver metastasis [Cohen-Solal et al. 2010].

Alternatively, some immune cells can be subverted by DTCs to promote their metastatic growth. For example, activated M2 macrophages can promote metastatic colonization of different cancers by supporting growth, survival, and vascularization while impairing immunogenicity [Qian and Pollard 2010; Quail and Joyce 2013]. In the lungs, breast cancer cells can interact with macrophages to activate the PI3K–AKT pathway and protect the cancer cells from apoptotic signals [Chen et al. 2011]. In fact, ablation of macrophage activation by blocking CSF-1R or CCR2 is a promising strategy to prevent macrophage instigation of metastasis outgrowth [Quail and Joyce 2013].

Taken together, MICs have evolved multiple mechanisms to turn a potentially hostile environment in a secondary organ into a supportive niche. This can be achieved by releasing systemic growth and survival signals from the primary tumor to foster a premetastatic niche, competing for existing normal stem cell niches, and engaging and converting the stromal cells to thwart death signals and immune attack.

**Exit from dormancy**

Metastatic dormancy is a frequent occurrence in many cancer types, with distant relapse occurring many years after the successful treatment of an early-stage primary tumor and initial complete remission. Dormant DTCs have been defined with three main features: growth arrest, survival, and therapy resistance [Ghajar 2015]. Furthermore, their entry into dormancy and reactivation not only is triggered by intrinsic programs but is also dependent on specialized microenvironmental niches, extrinsic signals, and immune effects [Giancotti 2013; Quail and Joyce 2013; Sosa et al. 2014].

Due to technical limitations, it is impractical to follow a single cell for years and witness its awakening from dormancy to initiate metastatic outgrowth, especially in clinical settings. Consequently, little has been known about how dormant cells escape growth arrest to initiate metastasis. Some studies propose different mechanisms for different organ-specific metastases [Sosa et al. 2014]. In bone metastasis, elevated expression of VCAM1 induced by inflammatory pathways in tumor cells promotes the transition from indolent micrometastasis to overt metastasis [Lu et al. 2011]. In lung metastasis, BMP signaling from the parenchyma restrains breast DTCs from exiting a dormant state by repressing self-renewal and inducing differentiation [Gao et al. 2012]. Production of BMP inhibitors, such as Coco, by tumor cells can release them from latency, prevent differentiation, and promote metastasis initiation. Thus, the ability of dormant DTCs to overcome such anti-growth signals is what turns them into active MICs. Other signals from the stromal niche can also induce the reactivation of growth and self-renewal pathways, such as ERK, Wnt, and Notch [Giancotti 2013]. For example, an
ECM component of the metastatic niche, tenasin C, can activate Notch and β-catenin signaling [Oskarsson et al. 2011], while POSTN can present Wnt ligands to activate WNT/TCF signaling [Fig. 4D; Malanchi et al. 2012]. Moreover, the perivascular niche can reactivate metastatic growth of dormant DTCs by endothelial sprouting and secretion of POSTN and TGF-β [Ghajar et al. 2013]. Therefore, MICs can overcome dormancy by activating self-renewal and stem cell-related pathways, such as Wnt, Notch, and TGF-β.

We discussed above how EMT or MET can generate stem cell properties in cancer cells and how mesenchymal-like cancer cells are less proliferative than epithelial-like cancer cells [Brabletz 2012; Liu et al. 2014]. According to paradigm, it has been proposed, but not yet proven, that mesenchymal-like TICs remain in a dormant state upon arrival in a distant organ and need to undergo MET in order to reactivate and initiate metastasis [Giancotti 2013]. In this scenario, both processes of EMT and MET would be critical for metastasis: EMT for entering dormancy, promoting survival, and drug resistance and MET as the mechanism to reactivate proliferation and self-renewal to initiate metastasis. This could also explain the pathological observation that metastases display epithelial traits rather than mesenchymal characters [Chaffer et al. 2007; Korpal et al. 2011; Tsai et al. 2012; Chui 2013].

Drug resistance of MICs

A close correlation between metastasis and treatment resistance is frequently observed. Metastatic tumors are invariably more chemoresistant than primary tumors, as evidenced by the marked decrease of chemotherapy response rate in metastatic settings as compared with neoadjuvant settings [Gonzalez-Angulo et al. 2007]. Conversely, poor response to neoadjuvant chemotherapy often correlates with earlier metastatic recurrence and shorter survival, indicating that chemoresistant tumors are prone to metastasize [Gonzalez-Angulo et al. 2007]. Therefore, the generation of MIC properties may be phenotypically linked to enhanced drug resistance capacities. MICs enriched with CSC-like features may benefit from resistant mechanisms of CSCs, such as a stronger DNA damage response [Wang 2015], elevated expression of efflux drug pumps [Schinkel et al. 1994; Zhou et al. 2001; Dean et al. 2005], and ALDH detoxifying enzymes [Honoki et al. 2010; Rausch et al. 2010]. Therefore, inhibitors of pathways involved in CSC regulation, such as antibodies against NOTCH, FZD, ILE6R, and other relevant signaling pathway receptors, may also have a therapeutic impact on MICs [Brooks et al. 2015].

Importantly, EMT induction is well known to increase chemoresistance [Thiery et al. 2009; Yu et al. 2013; Zheng et al. 2015b] and recently has been shown to induce chemoresistance in lung metastases using an EMT lineage tracing system in breast cancer [Fischer et al. 2015]. These studies help explain why conventional treatments like gemcitabine or cyclophosphamide usually do not affect mesenchymal-like cells. Therefore, the existence of dormant mesenchymal-like clones at a distant site could resist many conventional treatments [Giancotti 2013; Kang and Pantel 2013] and require novel therapeutic strategies targeting EMT-related pathways and features. For example, tumor cells undergoing EMT become resistant to EGFR inhibitors due to the activation of AXL kinase, which may be blocked with specific kinase inhibitors [Zhang et al. 2012]. The inhibition of PKCa and FRA1 can suppress tumor initiation by mesenchymal-like CSCs and is therefore a potential target for mesenchymal-like MICs [Tam et al. 2013]. However, dormant cancer cells can also escape existing cancer treatments because of their quiescent status or niche protection [Braun et al. 2000; Naumov et al. 2003]. Therefore, dormancy-specific treatment strategies should be designed to target the dormant cells [Sosa et al. 2014; Ghajar 2015]. Furthermore, other MIC-associated features, such as metabolic reprogramming and activation of survival pathways, are additional candidates for developing new treatment options [Holohan et al. 2013; Loo et al. 2015].

Besides these MIC-intrinsic properties, tumor-associated stroma has also been found to severely increase resistance to traditional cancer therapies [Gilbert and Hemann 2010; Sun et al. 2012]. In metastasis, primary tumor-associated endothelial cells produce TNFa to increase CXCL1/2 in cancer cells. These attract myeloid cells at the metastatic site, which produces S100A8/9 to feed back to metastatic cells and stimulate increased chemoresistance [Acharyya et al. 2012]. The tumor–stromal niche interactions discussed earlier provide additional opportunities to disrupt a prosurvival niche for MICs and sensitize them to anti-cancer agents [Wan et al. 2013].

MIC heterogeneity: clonal or polyclonal metastasis

Primary tumors are heterogeneous masses of cells containing multiple subclones that are genetically and epigenetically different [Marusyk et al. 2012]. Primary tumors are considered to arise from single TICs capable of both self-renewing and producing heterogeneity [Hanahan and Weinberg 2011; Greaves and Maley 2012]. In metastasis, the classical view also considers a single tumor cell as the origin of metastases, based on chromosomal analysis [Talmadge et al. 1982]. However, circulating tumor cells (CTCs) have been found to be genetically and phenotypically heterogeneous [Stoecklein et al. 2008; Kang and Pantel 2013; Yu et al. 2013], raising the possibility of polyclonal seeding and metastases. Until now, little was known about the clonal population dynamics throughout the different steps of metastasis leading into the formation of overt metastases. However, recent studies using lineage tracing, barcode sequencing, and whole-genome sequencing are shedding light on this question and have demonstrated a mostly polyclonal nature of metastasis [Fig. 5; McFadden et al. 2014; Gundem et al. 2015; Maddipati and Stanger 2015; Wagenblast et al. 2015].

Mutation analysis between the primary tumor and the metastatic lesions indicated polyclonal metastatic spread in the lymph nodes but not in the liver [McFadden et al. 2014; Gundem et al. 2015; Maddipati and Stanger 2015; Wagenblast et al. 2015].
Another whole-genome sequencing study analyzed 51 tumors of 10 prostate cancer patients, including primary tumors and multiple metastases in the same patients, and revealed the coexistence of multiple clones in the metastases, including those from metastasis-to-metastasis spreads (Gundem et al. 2015). Molecular barcoding offers another effective method to track clonal populations in experimental animal models of metastasis, and this approach has recently been used to analyze metastasis heterogeneity generated by the 4T1 mouse mammary tumor cell line (Wagenblast et al. 2015). In this study, orthotopic injection of barcoded cells generated metastases composed of multiple, different clones in various tissues, although it cannot be ruled out that independent metastatic nodules in the same organ might be seeded monoclonally.

Lineage tracing using fluorescence markers is another robust method to study polyclonal metastases in animal models. Combining the multicolor “confetti” mouse model for multiclonal tracking with the K-ras\textsuperscript{LSL,G12D/+}; p53\textsuperscript{R172H/+}; PdxCre (KPC) mouse model of pancreatic ductal adenocarcinoma (PDAC), a high frequency of polyclonal metastasis was revealed, including 11%–14% in the lungs and liver and 80% in the peritoneum and diaphragm. Interestingly, during the metastatic outgrowth to overt lesions, the clonal diversity usually decreases, leading to formation of monoclonal or polyclonal expansions that appear to depend on the metastatic site (Maddipati and Stanger 2015). Taken together, emerging evidence suggests that MICs are heterogeneous, and different clones are often involved in seeding and forming overt metastasis. Depending on the interaction of MIC clones and the conditions of the host organ, the metastatic outgrowth can remain polyclonal or become monoclonal (Fig. 5A). Additionally, the demonstration of polyclonal metastasis suggests the contribution of different heterotypic interactions among different tumor clonal subpopulations to initiate metastasis.

Clonal cooperation

The importance of tumor heterogeneity in cancer evolution has led to the idea that tumors may function as ecosystems of interactive populations within an environment. Consequently, several studies have started to focus on the ecological cooperation or competitive interactions between tumor populations (Merlo et al. 2006; Moreno 2008; Neelakantan et al. 2015; Tabassum and Polyak 2015). In primary breast tumors, recent research using mouse models has characterized the polyclonal origin of certain tumor types and the interclonal cooperation between multiple subclones (Cleary et al. 2014). Small subpopulations can drive the growth of other non-cell-autonomous clones through paracrine interaction and microenvironment modulation (Marusyk et al. 2014). In the metastatic context, this phenomenon was first reported by coingrafting nonmetastatic cells with metastatic cells to increase the metastasis of the former (Miller 1983). Cooperation can be promoted by endocrine and exosome signaling between different clones (Martorana et al. 1998; Neelakantan et al. 2015). ECM proteins, such as SPARC, also serve as messengers of cooperation to enhance invasion and metastasis [Fig. 5B; Mateo et al. 2014]. In addition, heterotypic interactions among EMT and non-EMT cells have also been demonstrated to increase metastasis progression of hamster cheek pouch carcinoma cells (Tsujii et al. 2008) as well as in xenograft models of prostate cancer metastasis [Celia-Terrassa et al. 2012]. In the latter study, both clonal populations seeded distant organs, although only the non-EMT clonal population—enriched in epithelial-like CSCs—expanded to overt metastases. Interestingly, the different clones presented complementary essential properties for metastasis, invasion in the mesenchymal-like clones and self-renewal/proliferation in the epithelial-like clones. As a result, the combination of clones enhanced metastasis, including new organs never colonized by either clone alone. This kind of
cooperation has been further characterized in other models proposing a leading invasive cell followed by “opportunist” cells (Fig. 5B; Chapman et al. 2014; Westcott et al. 2015).

This synergy is in agreement with the observation that CTC clusters have dramatically increased metastatic potential compared with single CTCs [Aceto et al. 2014] and the polyclonal nature of metastases [Gundem et al. 2015; Maddipati and Stanger 2015]. Therefore, MICs might be “opportunist” cells, which benefit from the establishment of heterotypic interactions with other DTCs with complementary abilities that can be exploited by MICs [Fig. 5B].

The polyclonal origin of metastases and cooperative interactions between different clonal populations in metastasis may be highly relevant to the understanding of the so-called polygenic drug resistance (Holohan et al. 2013). Heterogeneous polyclonal metastases confer a great diversity of drug resistance. Furthermore, hierarchically organized populations are reported to stochastically transition between phenotypic states to balance cancer cell populations in any direction, even from non-stem-like cells to stem-like cells [Gupta et al. 2011]. Such a high degree of plasticity of MICs, combined with the polyclonal nature of metastasis, presents a major challenge for conventional therapy.

Concluding remarks and perspectives

In this review, we enumerate a compendium of relevant hallmarks of MICs. Many of these hallmarks are represented within the same MIC population; however, it should be kept in mind that not all of them need to be possessed by the same tumor cell to initiate metastasis. A great degree of diversity, including the requirement of different assortments of MIC hallmarks, exists among MICs in different cancer types and subtypes and in different metastatic organ sites. Furthermore, many of the MIC features are required only at specific windows of metastatic progression. Importantly, the discovery of polyclonal metastasis introduces an additional layer of variability, as different clonal populations may cooperate to collectively seed metastasis, and even non-MIC populations can provide important functions to complement MICs in enhancing their metastatic competency. Therefore, the ability to form a metastatic lesion may not be the privilege of a specific tumor cell population with the requisite molecular and functional hallmarks of MICs. Instead, metastasis initiation may be the culmination of a highly fluid process involving multiple iterations of transitional cellular states, dynamic interactions between clonal tumor populations, and both short-distance and long-range interactions between tumor cells and the host organs. Despite all of these ambiguities in defining MICs, a central core property of MICs is their cellular plasticity, which underlies almost all other MIC hallmarks [Fig. 6]. This most fundamental hallmark of MICs may therefore represent a potential Achilles’ heel of cancer that can be exploited in developing new treatments. Future research should address these key questions: Is cellular plasticity really crucial for metastatic initiation, and how can we target cellular plasticity? Cellular plasticity targeting treatments are unlikely to be cytotoxic when applied as single agents in treatments and therefore are likely to fail standard clinical trials that often rely on reduced tumor burden as an indication of effectiveness. Instead, such treatment may demonstrate efficacy only when combined with other treatments to induce stress on potential MICs and in adjuvant settings. These will be major hurdles to advance cellular plasticity targeting treatment through the traditional drug development and clinical trial pipelines. Nevertheless, as technical innovations continue to bring about major breakthroughs in the study of MICs in clinical settings and experimental models, our newly developed insights into the mysterious process of metastasis initiation will undoubtedly lead to improved prevention and treatment of metastatic diseases.

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Molecular mechanism of metastasis initiation


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Molecular mechanism of metastasis initiation

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Distinctive properties of metastasis-initiating cells

Toni Celià-Terrassa and Yibin Kang

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