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Using a Systems Biology Approach to Understand and Study the Mechanisms of Metastasis

Ngoc-Han Ha and Kent W Hunter

Laboratory of Cancer Biology and Genetics, National Cancer Institute, Bethesda, MD, USA

Abstract

Metastasis remains the main cause for cancer-related deaths due to the lack of effective therapy. The clonal selection model has long been thought to be the primary mechanism of metastatic progression but many different mechanisms have been hypothesized for the progression from tumorigenesis to the successful dissemination and expansion of tumor cells at the secondary site. MicroRNAs, germline polymorphisms in combination with the tumor microenvironment are few of the different pathways to explain the metastatic cascade. Technological advances for high-throughput screening of cells such as expression profiling, next generation sequencing as well as global network analyses have advanced the studies of these mechanisms. Combined with new insights into the various mechanisms of metastasis a systems biology approach has also been shown to be useful in identifying metastasis-specific gene signatures as well as predicting disease outcome. Furthermore, the results of these studies have been relevant for identifying biomarkers for metastatic disease.

Introduction

Metastasis is a complex process and it remains the main cause for cancer-related deaths in the United States. The progression from primary tumor development to its final stage of metastasis involves the detachment of selected cancer cells from the primary tumor to the intravasation into the bloodstream followed by extravasation, colonization and expansion of tumor cells at the secondary site¹. Since the metastatic cascade involves many complex steps, it is generally considered to be an inefficient process and yet, when metastasis does occur, it is almost always fatal to the patient. For these reasons, increased understanding of each step in the metastatic cascade would allow for the development of better therapeutic interventions.

Research in the field of metastasis has been ongoing for decades and many different mechanisms of metastasis have been suggested, all of which have added another layer of complexity to the metastatic cascade. Recent technological advances such as high-throughput genomic, proteomic and metabolomic analyses have enabled better tools for studying this complex disease on a whole system level. The incorporation of multiple systems allowed for better biomarker discoveries for metastatic disease and it is hoped that they can aid in the development of more suitable and individualized targeted therapy. In this

review, we will touch upon the different mechanisms of metastasis and give examples on how using a systems biology approach could further the understanding of this complex disease. Finally, we will highlight how some of the approaches have been used to identify relevant networks, pathways and possibly biomarkers in metastasis.

Mechanisms of Metastasis

Clonal selection Model

The commonly accepted mechanism of metastasis dates back to Nowell who initially described the clonal selection model². The establishment of metastatic tumor at the secondary site involves many steps, each of which needs to be successful. Therefore it is thought that cancer cells acquire traits that allow them to initiate invasion, survive within the blood stream, extravasate, and grow in a foreign tissue environment, which are all needed for successful dissemination¹. The clonal selection model bases its theory on this: genomic instability leads to stochastic mutations that endow primary tumor cells with attributes that give them the ability to disseminate and expand at the secondary site²⁻⁴. This provides an explanation for why only a subset of cells succeeds in forming lesions at the secondary site. Therefore, the clonal selection model is an attempt to explain the heterogeneity observed in primary tumors as well as the inefficiency of the metastatic process.

Fidler and Kripke were first to demonstrate clonal selection by showing in animal models that a small proportion of tumor cells were capable of successfully disseminating to the distant organ⁵. Using a parental mouse tumor, they showed that the cells varied in their metastatic potential suggesting that metastatic heterogeneity exists within the primary tumor. To further demonstrate that successful dissemination to the secondary site was not due to random selection, they induced unique chromosomal rearrangements by irradiation and found that a certain subset of cells were able to metastasize⁶. Therefore, they concluded that the metastatic potential within primary tumor cells was due to clonal and not random selection.

While these studies provide compelling evidence that clonal selection exists within the primary tumor, they do not rule out other mechanisms of metastasis. Support for this comes from reports showing that metastatic gene expression signatures can be derived from expression profiles of the primary tumors^{7,8}. These studies were able to successfully classify patients as having good or poor prognosis based on a 70-gene signature that was derived from primary tumors¹⁰. If metastatic disease depended only on clonal selection of a small subset of cells alone, it is unlikely that their gene expression profile would be detectable by expression profiling of the bulk tumor. Therefore it has been suggested that the observed inconsistency could be due to other mechanisms of metastasis such as germline polymorphisms, microRNAs and the tumor microenvironment (reviewed below), all of which could contribute to the metastatic gene expression signatures. It is now clear that metastatic disease is more complex than initially thought and that the clonal selection model cannot account for all of the steps in the metastatic process^{9,10}.

Genetic Susceptibility to Metastasis

Metastatic disease has been mainly viewed as a consequence of somatic mutations but genetic susceptibility cannot be discounted. Similar to the differences in eye color, responses to drugs etc., germline mutations could also hold true for the susceptibility of an individual to develop metastasis¹¹. Studies in our laboratory along with others, have demonstrated that germline polymorphisms modify cellular properties leading to tumorigenesis, invasion, and metastasis^{12–14}.

Genetic susceptibility to metastatic disease was first observed in inbred mice of distinct genetic backgrounds varying in susceptibility to lung metastasis. Subsequent to these observations, experimental strategies were conducted in which female mice of various inbred strains (such as FVB, NZB AKR, DBA, etc.) were crossed to male FVB mice transgenic for the mouse mammary tumor virus promoter driving mammary tissue specific expression of the polyoma middle T antigen (MMTV-PyMT) oncogene. While all female progeny with the MMTV-PyMT transgene developed mammary tumors, the degree of lung metastasis varied depending on the genetic background. These findings led to the hypothesis that genetic differences played a role in the susceptibility to metastasis¹⁵. Mapping of quantitative trait loci (QTL) have enabled to determine regions and genes on the genome that could be significant for the metastatic phenotypic differences observed in mice. For example, the first polymorphic gene identified in our laboratory was *Sipa1*, whose differential expression was able to influence pulmonary metastasis. Furthermore, a polymorphism was found in human breast cancer samples that could predict metastatic progression in Estrogen receptor-positive mammary tumors¹⁶. We have recently expanded our studies and found other genes whose differential expression can influence metastatic susceptibility^{17,18}. These and other studies provide evidence that genetic polymorphisms can influence disease progression such as metastasis.

Metastatic Gene Regulation by MicroRNAs

Tumorigenesis and metastasis are processes in which the normal genome of cells becomes perturbed leading to an increase in growth potential, a resistance to cell-programmed death and the ability to leave the primary tumor and disseminate and grow at a distant site. Given that these processes involve the regulation of genes, it is logical to study the implication of microRNAs. MicroRNAs (miRNAs) are short nucleotide sequences (17–20 nt) of non-coding RNAs that are capable of binding to numerous mRNA targets via sequence specificity. miRNAs regulate the transcription of target genes by binding to their 3' untranslated region and depending on their degree of complementarity, miRNAs can regulate mRNA transcript abundance or expression by degradation or translational repression¹⁹. Perfect complementarity to target genes is not required for the regulation of gene expression and consequently, it is thought that a single miRNA is capable of influencing the expression of hundreds of genes^{20,21}. For these reasons, miRNA have been demonstrated to play a very important role in regulating cellular processes in maintaining homeostasis as well as disease states such as cancer and metastasis^{20–22}. As such, it was demonstrated by the group of Weinberg that miR10b can be regulated by a genes involved in EMT, such as TWIST²³. They showed that overexpression of TWIST induces EMT and subsequent cellular invasion of tumor cells, upstream steps of the invasion-metastasis

cascade. TWIST-mediated activation of miR-10b leads to the downregulation of HOXD10, initiating the transcription of various pro-invasion genes including the derepression of RhoC which enhances cellular motility. Identifying miR-10b as a target of TWIST regulation, in addition to other studies, demonstrated that miRNAs are significant contributors to the initiation of metastasis.

Furthermore, miRNAs have been shown to be involved in the metastatic process via regulation of pro-metastatic genes as well as genes involved in creating a favorable tumor microenvironment for cancer cell dissemination (see below).

Influence of the Tumor Microenvironment

While the initial focus on tumor progression and metastasis was on tumor-intrinsic factors, it is now widely accepted that non-tumor autonomous dynamics are very important in these processes²⁴. As mentioned in the above concepts, the involvement of cells surrounding the tumor play an important role for successful dissemination and colonization of cancer cells at the distant site. The tumor microenvironment consists of the untransformed cell types in the immediate surroundings of tumor cells including myoepithelial cells, endothelial cells, lymphocytes, myeloid cells, and fibroblasts. In addition to various cell types, the tumor environment also includes the extracellular matrix which is important in keeping cells place. During initiation of metastasis it has been shown that interactions between the tumor and the stroma lead to degradation of this extracellular matrix allowing tumor cells to intravasate^{25,26}. For example in a study by Peinado et al.²⁷, the authors demonstrated the importance of tumor-secreted exosomes in recruitment of bone-marrow- derived cells to the tumor microenvironment leading to a more pro-metastatic flora for successful metastasis. Specifically, the authors analyzed exosomes from cells with varying degree of metastatic capability and found that more metastatic cells had an increase in MET protein within the secreted exosomes. The presence of MET subsequently re-educated bone marrow-derived cells to promote a suitable tumor microenvironment as well as pre-metastatic niche for colonization of melanoma cells. During tumorigenesis and metastatic progression, therefore, aberrant signaling from stromal cells can stimulate tumor cells to disseminate as well as prepare the secondary site for successful colonization by the disseminated tumor cells²⁴.

Systems Biology Approach to Metastasis

Using Systems Biology to Study Metastasis

The mechanisms of metastasis mentioned here demonstrate that the metastatic process is a very complex system with many different aspects. As seen in the brief review above, metastasis involves many genes, pathways and cell types. Recent advances in technology have now made it possible to study the aforementioned theories on metastasis as a whole system, rather than analyzing single genes. Many of these technologies can be applied to study the various mechanisms of metastasis. For example, to better understand the concept of clonal selection, a study by Ding et al.²⁸ used second generation sequencing to analyze a single patient's peripheral blood, primary basal-like breast tumor, and matched brain metastasis. The authors found a wide range of mutations in the primary tumor supporting genetic heterogeneity within the sample. Subsequent analysis of the metastasis showed an

enrichment of a subset of mutations, suggesting this particular cell population within the primary tumor metastasized to the brain. The xenograft derived from the patient's primary tumor was shown to contain a mutational profile that overlapped with the metastasis, further supporting the notion that a minority population of cells arose within the primary tumor with an enhanced metastatic capability²⁸.

Furthermore, it has been shown that genomic instability contribute to tumor heterogeneity and subsequent metastatic advantage, however, it cannot account for all the mechanisms of successful dissemination. For example, using a single-cell PCR gene expression analysis approach, Dalerba et al.²⁹ studied the evolution of colon cancer cells both in normal as well as malignant tissue. By first analyzing different cellular populations within the normal colonic crypt via single-cell PCR, they identified several distinct cell populations, some of which were stem cell-like. The authors analyzed single cells from colon cancer and demonstrated that malignant cells were similar to the differentiated normal cells. To determine whether tumor heterogeneity is due to lineage differentiation or clonal selection of tumor cells, a single cell from the isolated subpopulation was injected into mice. Analysis of the resulting tumors suggests that the transcriptional diversity of colon cancer cells was due to their multilineage differentiation because the bulk tumor consisted of various subpopulations that must have differentiated from the single cell²⁹. Studies such as these have therefore been able to expand the original findings that Fidler and Kripke observed. Employing high-throughput technology and patient samples, these studies demonstrated on a genome level that there exists a heterogeneous cell population and that only a subset of cells is able to disseminate and subsequently form the secondary tumor at the distant site.

The discovery of miRNAs has expanded the field of tumor progression and metastasis by providing a new mechanism of gene regulation. Undoubtedly, they have been shown to play a very important role in gene expression and hence diseases like cancer and metastasis; however, because miRNAs have the ability to bind to many targets, it is currently difficult to identify all targets and what their individual involvement in the metastatic cascade is. In addition to studying the evolution of tumor cells, genome-wide studies can also be employed to understand other possible mechanisms for metastatic disease. miRNA expression profiling has become a valuable tool to identify miRNAs that are involved in disease progression. For example, these expression studies have been able to identify novel miRNAs which play a role in lung^{30,31}, breast³², ovarian cancer³³ as well as colorectal cancer³⁴. While miRNA regulation in the metastatic cascade has been shown to be primarily tumor-autonomous^{19,35,36}, their involvement in other processes cannot be discounted. For example, in a recent study by Zhang et al.³⁷, the authors demonstrated the role of miRNAs on the tumor microenvironment to inhibit the metastasis of breast cancer stem cells. Through miRNA expression profiling, the authors observed several miRNAs whose expression correlated with metastatic potential of normal breast and cancer cells. Choosing to further study miR-126 and its pair miR-126*, the authors identified Sfa-1a, a chemokine involved in recruiting mesenchymal stem cells to the tumor microenvironment, as the miRNA's target. Subsequently, they showed that through this downregulation, fewer mesenchymal and inflammatory monocytes were recruited to the tumor stroma and therefore inhibited lung metastasis³⁷. This study substantiated the role of miRNAs in not only regulating the expression of genes involved in tumor-autonomous functions in metastasis but that they can

also play a role in influencing the surrounding tumor microenvironment and subsequently inhibit or enhance metastasis.

Genetic mutations have also been shown to be a possible mechanism of metastasis. Understanding that these factors have a significant contribution to disease adds another layer of complexity to the metastatic process. Many studies, including those in our laboratory, demonstrated the influence of germline polymorphisms on the susceptibility to metastatic disease^{13,17,18}. With recent advances in global transcript analysis, further investigations of gene networks and their role in metastatic progression became possible³⁸. Understanding the interactions of genes and how they are involved in metastatic progression is a powerful tool since it highlights the pathways at a global gene level. As such, using the mouse genetic crosses as well as human data as the basis, a study from our laboratory showed that gene networks can predict metastasis-free survival in human breast cancer cohorts. Interestingly, the data in this study suggest that different gene networks could predict outcome for the different subsets of breast cancer. In specific, it was shown that Estrogen receptor-positive breast cancers rely on tumor-autonomous factors while Estrogen receptor-negative breast cancers were influenced by host-derived stroma³⁹. Identifying the genes within these networks will not only allow for further analysis on how they contribute to the metastatic process but it also indicated the complex interplay of various cell types.

While germline polymorphisms have been shown to influence metastatic susceptibility, a recent study by Quigley et al.⁴⁰ demonstrated that these influences become perturbed as the tumor progresses. Using integrated linkage and genomic analysis in normal, benign and malignant skin tumors, the authors identified that germline polymorphisms were able to change gene expression to influence tumor susceptibility, as previously shown. However, the authors discovered that as skin cancer progressed, somatic mutations and changes in the tumor environment perturbed the influences seen with germline polymorphisms. This study therefore showed that tumor progression and possibly metastasis is quite complex and that various factors, host- and tumor-specific, can influence disease susceptibility.

Biomarkers for Metastasis

Although advanced technologies can be used to study the genetic make-up of primary and secondary tumors and therefore study the possible mechanisms of metastasis, they can also be employed to identify disease-specific genes as well as predict outcome. Understanding the various aspects of metastasis and identifying genes and pathways involved, along with high-throughput technology, allows for better diagnosis and prediction of disease outcome.

As mentioned, miRNAs have been found to play an important role in gene regulation via gene silencing or degradation¹⁹. While miRNA expression profiling can be used to identify relevant miRNAs in metastasis, it can also be employed for identification of gene signatures that could be helpful in classifying cancer subtypes as well as predicting metastasis-free survival outcome. For example, Lerebours et al.⁴¹ employed miRNA expression profiling using patient samples to identify a miRNA signature that could predict disease phenotype in inflammatory breast cancer (IBC), a subset of breast cancer for which no distinct diagnostic tool exists and that has a low rate of survival. In this study, using miRNA expression profiling the authors identified 18 miRNAs that were differentially expressed in IBC versus

non-IBC patients. Furthermore, they were able to predict the IBC phenotype using five of the miRNAs (miR-421, miR-486, miR-503, miR-720 and miR-1303) with 89% accuracy in IBC patients.

Furthermore, the identification of miRNAs involved in tumorigenesis and metastasis has prompted research to target them for therapeutic developments. In a study by Ma et al.⁴², miR10b antagomirs were synthesized and treatment of tumor-bearing mice was shown to inhibit breast cancer metastasis. Since the treatment was well tolerated *in vivo*, the authors suggest that antagonizing miRNA could be used as targeted therapy against metastatic disease. While these studies have not been tested in patients, the identification of miRNA and their targets seems to be a promising field especially since miRNA expression profiles could be implemented in the clinic if they can eventually be used as biomarkers for metastatic disease. As such, in a study led by Baffa et al.⁴³, several primary tumors and their matched metastases representing different cancer types were analyzed using a miRNA expression array. The goal was to identify a specific miRNA signature which could help identify miRNAs involved in metastatic progression as well metastatic organotropism. The data presented in this study identified several miRNAs, some of which have already been shown to play a role in specific cancers suggesting that miRNA expression profiling could be useful to determine cancer origin, especially in cases for which the primary tumor origin is unknown.

Proteins are the functional results of gene expression and therefore it is important to study their role in cancer progression and metastasis. While genome-wide studies are valuable in understanding gene regulation and expression, some of the functional outcomes could be perturbed in these studies. Therefore, proteomic approaches could also aid in the identification of proteins involved in the metastatic process. High-throughput techniques such as 2-D gel electrophoresis in combination with mass spectrometry (MS) have been successful tools to study the proteome of cancer cells. In a recent study by Jin et al.⁴⁴, a proteomic analysis of the secretome of four breast cancer cell line derivatives revealed a profile of proteins involved in homing cancer cells to metastasize to bone. Employing 1-D electrophoresis with liquid chromatography-MS/MS, they identified CST6, a cysteine protease inhibitor, to be one of the proteins that could suppress breast cancer cells to metastasize to bone. This study is an example of how proteomic analyses of tumor as well as metastatic cancer samples could provide an insight into how proteins influence organotropism. Furthermore, such studies could possibly bring forth biomarkers for disease progression.

Conclusion

The process of metastasis has been studied for decades and the complexity of this multidimensional disease has been difficult to completely understand. Many mechanisms of metastasis have been suggested over the years, each of which provided experimental evidence for the possible pathways. The birth of genomic technologies since the turn of the century has allowed for a more global study of the genes and pathways involved. With the ability to look at tumor and metastatic cancer cells at a whole genome level, it has furthered the understanding of a disease that includes many different aspects such as tumor and

population specificity. Systems biology can be used to study and understand the various mechanisms of metastasis. The ability to perform high throughput sample analyses has provided many insights into the various mechanisms of metastasis. It is now clear that metastasis is not only a consequence of somatic mutations and subsequently due to clonal selection of the primary tumor, but it also involves the interplay of cells within the tumor microenvironment (Fig. 1A). Furthermore, studies have demonstrated that an individual's genetic make-up can also influence metastatic susceptibility (Fig. 1B). In addition to using a systems biology approach to study and understand the metastatic process, it can also be employed for gene expression profiling as well as network analyses to identify gene signatures for diagnosis and for outcome prediction (Fig. 1C). Finally, it is hoped that these can aid in the identification of new biomarkers for metastatic disease (Fig. 1D).

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Fig. 1. A Systems Biology Approach for Metastasis

- (A) Understanding that metastasis is a complex disease involving many pathways and cell types.
- (B) Using genetics and next-generation sequencing to understand and study metastasis.
- (B) Employing expression profiling and network analyses to find disease-specific signatures
- (D) Identifying biomarkers to predict disease and outcome