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# Recent Advances in the Genetics of Metastasis

**H. Ponta, M. Hofmann and P. Herrlich**

## INTRODUCTION

METASTATIC SPREAD of tumour cells appears, at least formally, to be a complex multi-step process. Cells need to detach from their tissue of origin and from neighbouring primary tumour cells, migrate through basement membrane (in the case of epithelial cancer cells) and interstitial matrix, and invade the lymph and blood transport system. In most instances, metastases occur first in lymph nodes and, at some later stage, leave the lymphoid tissue to enter the blood stream. Adhesion to vascular endothelium is thought to lead to extravasation and nesting in new tissue, such as the lung. The complexity of this process suggests the participation of a variety of different proteins, the loss or gain of each possibly accounting for the individual specific step proposed. It is plausible that tumour cells acquire properties by mutation and selection, and that the rare cell that has assembled all the necessary properties will metastasise. However, it is also possible that a genetic programme is elicited by the coregulation of various genes, which may reduce the complexity considerably.

To accomplish metastatic behaviour, matrix degrading pro-

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*Table 1. Genes involved in metastasis formation*

| | Postulated function | Assay* | Reference |
|---|---|---|---|
| Loss of function | | | |
| E-cadherin | Cell–cell contact | a,c | [2], [4], [5], [6] |
| Vinculin | Cytoskeleton interaction | c | [7] |
| Integrin $\alpha_4\beta_1$ | Cell–cell contact | c | [10] |
| nm23 | Signal transduction | b,c | [17, 18] |
| TIMP-1,-2 | Regulation of MMPs | a,b | [23], [24], [25], [26], [27] |
| Gain of function | | | |
| MMP-11 | Matrix degradation | — | [22] |
| MMP-9 | Collagen IV degradation | b | [29] |
| MMP-2 | Collagen IV degradation | a,c | Docherty *et al.*, unpubl. |
| CD44 | Hyaluronan receptor | b | [41, 42] |
| CD44v4–v7,v6–7 | Hyaluronan receptor and others | c | [42a], [43], [44], [45] |
| α(1-2)fucosyl-transferase | Glycosylation | c | [47] |
| Integrin $\alpha_2\beta_1$ | Adhesion | c | [54] |
| Integrin $\alpha_6\beta_1$ | Adhesion | b | [55] |
| mts-1/P9Ka | Signal transduction | c | [72] |
| Tiam-1 | GDP/GTP exchanges | a,b | [74] |

*See Figure 1 for description of assays.

teases, receptors for endothelial cells, matrix binding proteins (to facilitate cell migration), motility factors and their receptors, growth factor receptors (to respond to new microenvironments) and various adhesion molecules have been postulated to participate. Although there is an enormous body of literature on tumour cell properties that correlate with malignancy, only very recently has it been possible to establish causal relationships between the expression of a specific gene product and a specific phenotype. We wish to review these very recent advances. An overview of the genes discussed is given in Table 1.

The methodological basis for these recent advances has been the successful cloning of candidate genes, sometimes using novel techniques, and the manipulation of their expression or of gene product function. The tumour phenotype was tested in one of three assays which measure different steps in the metastatic process (Figure 1). These three most commonly used assay systems are: (i) the spontaneous metastasis assay (Figure 1c): the tumour cells are injected subcutaneously either into syngeneic or immunodeficient animals. This assay most closely resembles the "normal" situation with the exception that the tumour cells are already dissociated and do not have to penetrate the epithelial basement membrane or fibrin layers; (ii) the experimental metastasis assay (Figure 1b): the tumour cells are injected intravenously. In this assay, the capability of tumour cells for extravasation and tissue specific colonisation (mostly in the lung) is examined; (iii) invasion assays (Figure 1a): tumour cells are placed on top of matrix layers, tissues (e.g. chicken heart), or chick chorioallantoic membrane and their migration into this material is followed.

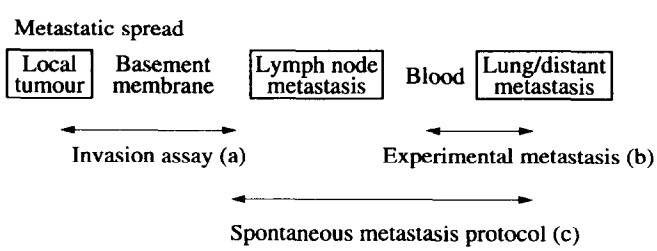


**Figure 1.**

## GENETICS OF CELL DISSEMINATION, LOSS OF FUNCTION OF GENES

Carcinoma cells originate from epithelial stem cells which, during normal differentiation, form well-ordered layers of cells, attached to one another by adhesion plaques, desmosomes and tight junctions. Rous Sarcoma Virus transformation has been known for a long time to disturb the epithelial cell order [1, 2]. Transformed cells round up, obviously losing their cell–cell junctions and this is considered to be one of the necessary steps in metastasis. Recent findings have identified some of the molecules involved in cell–cell adhesion. In adhesion plaques, transmembrane proteins with homophilic extracellular domains such as cadherins, and intracellular proteins that connect the extracellular "anchor" to the cytoskeleton, e.g. catenins and vinculin (reviewed in [3]), are assembled. In a variety of tumours, the expression or function of components of the adhesion plaques is disturbed, suggesting that the malignant phenotype of these tumours might depend on loss of adhesion plaque function. In cell invasion assays, using chick heart explants, direct proof has been obtained that loss of function of E-cadherin (e.g. by inhibitory antibodies) provides cells with invasive capacity [2]. Non-invasive tumour cells acquired the capacity to invade heart muscle spheroids upon reducing the expression of E-cadherin by antisense mechanisms [4]. Further, introduction of E-cadherin expression vectors into E-cadherin-negative metastasising tumour cells suppressed metastatic spreading as judged in invasion assays [5] and in the spontaneous metastasis assay [6]. Similarly, the expression of vinculin in a highly metastatic, vinculin-negative tumour cell led to suppression of metastasis formation [7]. In agreement with the role of catenins, mutations in catenins have been found in gastric cancer [3]. These experiments identify E-cadherin, catenin and vinculin as invasion and metastasis suppressor genes.

A functional role for homophilic intercellular adhesion of melanoma cells has been proposed for the $\alpha_4\beta_1$ integrin which is

one of the fibronectin receptors [8, 9]. $\alpha_4\beta_1$ surface expression was inversely correlated with the invasive potential of murine melanoma cell lines [10]. Introduction of the $\alpha_4$ subunit cDNA into an $\alpha_4^-\beta_1^+$ highly invasive melanoma cell line reduced its invasiveness into matrigel. Furthermore, metastasis formation upon subcutaneous injection, but not upon intravenous injection, of $\alpha_4$ transfected recipient cells was suppressed. Since treatment of $\alpha_4^+\beta_1^+$ cells with $\alpha_4^-$ specific antibodies abrogated homotypic intercellular adhesion and increased matrigel invasion, this supports the conclusion that the $\alpha_4\beta_1$ receptor on these melanoma cells is responsible for homophilic cell interaction, as has been postulated for other cell systems [11–13], and, in this case, does not function as a fibronectin receptor.

An interpretation of the role of the metastasis suppressor genes, discussed so far, is straightforward: all these proteins seem to be required for homophilic cell–cell interaction, and functional loss of these proteins would contribute to the dissemination of cells. Such functional understanding is not obvious for another metastasis suppressor gene: *NM23*. The protein encoded by the *NM23* gene is located in the cytoplasm and carries nucleoside diphosphate kinase activity. Its expression was inversely correlated with metastatic potential in some rodent tumour systems [14] as well as in human infiltrating ductal breast [15] and hepatocellular carcinomas [16]. Metastasis suppressive activity was established in two systems upon transfection of *NM23* expression clones into highly metastatic tumour cells: in mouse melanoma cells as measured in spontaneous and experimental metastasis assays [17], and in a human breast carcinoma cell line measured by subcutaneous injection or injection into the mammary fat pad of immune deficient mice [18]. Interference with metastatic spread may be the consequence of an inhibition of cell migration by nm23; transfection of nm23 reduced the ability of murine melanoma and human breast carcinoma cells to migrate in response to serum or to defined growth factors [19]. Interestingly, the nucleoside diphosphate kinase activity of the nm23 protein appears not to be required for its metastasis-repressing ability [20]. Rather, the nm23 protein seems to participate in signal transduction since phosphorylation of a serine, but not the nucleoside diphosphate kinase activity, was correlated with its metastasis repressive ability.

## GENETICS OF INVASION AND MATRIX DEGRADATION

One group of genes that has been suggested for many years [21] to be important in a "metastasis-specific genetic programme" comprises the collagen degrading metalloproteinases. Several members of this group have been cloned including very promising candidate genes for the metastatic process: stromelysin-3 (*MMP-11*) and 72-kDa gelatinase (*MMP-2*) Because of its peculiar expression profile, the experiments leading to the cloning of stromelysin-3 will be briefly summarised. This gene was cloned from a breast cancer cDNA library established from a surgical specimen which contained neoplastic cells and stromal cells [22]. Sequence analysis revealed a similarity with metalloproteinases, especially with stromelysins, and therefore the protein was named stromelysin-3. Surprisingly, *in situ* hybridisation of tumours revealed that mRNA for stromelysin-3 was not detected in tumour cells. Transcripts could, however, be detected in surrounding stromal cells. The expression in stroma decreased with increasing distance from the tumour cells. Since the expression is restricted to invasive breast carcinomas and metastases, stromelysin-3 induction in the stromal cells may well relate to tumour progression.

First evidence for an involvement of metalloproteinases in metastasis was derived from studies using inhibitors. Two inhibitors occur naturally and are coexpressed with metalloproteinases, TIMP-1 and TIMP-2 (tissue-specific inhibitors of metalloproteinase). TIMPs inhibit enzymatic activity of all metalloproteinases. Addition of TIMP-1 to *in vitro* invasion assays or to experimental metastasis assays, performed with metastasising mouse melanoma cells or transformed embryo cells, results in inhibition of invasion and metastasis formation by these cells [23, 24]. Expression of TIMP-1 in highly metastatic murine melanoma cells [25] and in a human gastric cell line, and of TIMP-2 in a transformed rat cell line (upon transfection), also reduced experimental metastasis formation [26, 27]. Furthermore, expression of antisense RNA to TIMP-1 has been found to augment metastasis [28].

Although several members of the metalloproteinase family have been cloned and specific antibodies to them have been raised, only one example has been published where metastatic features of a tumour cell line depended on the expression of one of the metalloproteinases [29]. Rat embryo fibroblasts when transformed by Ha-*ras* or by Ha-*ras* plus v-*myc* are metastatic in nude mice upon tail vein injection. The cells release a 92-kDa gelatinase. Simultaneous expression of the adenovirus E1A protein suppresses metastatic potential and represses the secretion of 92-kDa gelatinase. Transfection of a 92-kDa gelatinase expression vector into these cells restores metastatic capacity. Similar results were obtained after transfection of animal tumour cells, which do not express 72-kDa gelatinase, with 72-kDa gelatinase expression vectors (Cockett, Docherty and Murphy, personal communication). Whereas the parent cells do not colonise the lung upon intravenous injection, the transfectants are highly effective in the experimental metastasis assay.

## GAIN OF NEW ADHESION MOLECULES

For migration of cells to occur, not only is space in the extracellular matrix required, as might be provided by the degradation of matrix components such as collagens, but also new contacts to matrix components, various stromal cells and endothelial cells may have to be established. Such contacts are mediated by cell surface adhesion molecules and are probably especially important for the settlement of tumour cells in new tissue. An important component for the organisation of extracellular matrix is hyaluronan (HA, hyaluronic acid), a large glycosaminoglycan. Originally, hyaluronan was thought to be mainly an inert structural element that provides elastic and water-retaining properties to tissues [30]. Recent data (see below) suggest that hyaluronan plays a far more active role in regulating cells' motility, chemotaxis, invasion, proliferation, shape, and metabolic functions (review in [31]). A crucial role in providing all these different functions is certainly mediated by hyaluronan receptor molecules on the cell surface. Since some of the proposed functions of hyaluronan, such as a support for migration and invasion, require interaction with its receptors, it has been tempting to speculate that expression of hyaluronan receptors might promote metastasis. This assumption has been confirmed convincingly, although in quite an unexpected way.

Two major classes of hyaluronan receptors have been described that are distinctly different in amino acid sequence: CD44, a pleomorphic receptor that, in addition to hyaluronan, also binds to fibronectin, collagen, laminin, and possibly other ligands; and RHAMM, which stands for receptor for hyaluronic

acid mediated motility [32]. Proteins of both classes of receptors contain hyaluronan-binding motifs, which are also found in hyaluronan-associated proteins like link protein, aggrecan, T6G, versican or hyaluronectin [33]. As judged by antibody-blocking experiments, RHAMM appears to be required for locomotion of cells under the influence of an activated *ras* oncogene or TGF-$\beta_1$ [34, 35], or in the process of wound healing. Interestingly, some of the cells examined expressed both RHAMM and CD44. In these cells, locomotion depended exclusively on RHAMM. Although locomotion is certainly a property of tumour cells no experiments have yet been published that connect RHAMM with tumour progression.

CD44 designates a family of proteins that differ in their glycosylation profile and in their primary amino acid sequence. Whereas the N-terminal and C-terminal parts of all known proteins of the CD44 family have the same amino acid composition, they differ in a membrane-proximal region in the extracellular portion through alternative usage of ten exons (v1–v10), [36, 37]. In the most ubiquitously expressed isoform, the standard form (CD44s) sequences encoded by these ten exons are all excised, whereas various combinations of alternating exons give rise to a multitude of CD44 variants (CD44v).

CD44s was first found as a leucocyte antigen, and has been postulated to be involved in "lymphocyte homing", a process of transendothelial migration from post-capillary venules into lymphatic tissue [38, 39]. Since metastatic cells leave the blood stream by a similar process of transendothelial migration [40], CD44 has been proposed to mediate part of these migrations. Interestingly, a role of CD44s in the colonisation of the lung by tumour cells has been documented. The Burkitt lymphoma cell line, Namalwa, has only weak tumorigenic activity in the experimental metastasis assay, and these cells express neither CD44v nor CD44s. However, overexpression of CD44s (but not of a HA-binding deficient form of CD44v8–v10) increased the metastatic potential of these cells when tested according to the experimental metastasis protocol [41]. Lung colonisation could be blocked by soluble CD44s-immunoglobulin fusion protein and not by CD44v8–v10–Ig HA-binding negative fusion protein, suggesting a specific interaction between CD44s and a HA-like ligand [42].

CD44 was independently discovered as a metastasis-associated antigen [43] and this work yielded convincing support for a metastasis-promoting role for CD44. Aiming at the identification of metastasis-specific genes, specific monoclonal antibodies directed against surface molecules were generated and screened for the presence of epitopes on a metastasising tumour cell line and absence on a non-metastasising isogenic cell line. Some of the antibodies reacted with a CD44-related protein, expressed on the surface of the metastatic line, which has now been identified as an isoform of CD44. It turned out that this protein is a new splice variant of CD44 that differs from the so-called standard leucocyte form of CD44 (CD44s) by additional amino acids in the extracellular domain [43]. The original cDNA isolate contains sequences corresponding to the variant exons v4–v7 (CD44v4–v7). This splice variant is expressed in metastatic pancreatic and mammary carcinoma cell lines, whereas the CD44s protein is expressed quite ubiquitously on most transformed cells, including both the non-metastatic and metastasising cell lines [43].

A causal involvement of CD44v proteins in the metastatic process was tested in two different ways, blocking its function and overexpressing it in the non-metastatic cell line. Blocking experiments were performed using the spontaneous metastasis assay and simultaneous intravenous injection, together with the metastasising tumour cells, of antibodies specific for the CD44v protein. The antibody injection was repeated every other day for 3 weeks [44]. This protocol led to considerable retardation in tumour growth and, in more than half of the animals, to complete repression of metastatic spread. Expression of CD44v4–v7 or CD44v6,v7 (another isoform detected in the same metastatic cell line [45]), from either a Simian virus 40 promoter or the cytomegalovirus promoter in non-metastasising tumour cells, provided these cells with metastatic properties in the spontaneous metastasis assay. Overexpression of CD44s did not alter the behaviour of these tumour cells [43, 45, 46].

CD44 proteins differ not only in amino acid sequence, due to alternative usage of the variant exons, but also in their glycosylation status. The degree of glycosylation and the type of glycosylation seem to vary with the cell type in which the isoforms are expressed [43, 45, 47]. For one type of modification, a contribution to tumour progression has been established. Antisense inhibition of the expression of $\alpha(1–2)$ fucosyltransferase, leading to a reduced masking of galactosyl residues by fucose linked in $\alpha(1–2)$, converted a highly metastatic colon carcinoma cell line into a more benign phenotype upon subcutaneous injection into syngeneic animals [47]. The modification mediated by $\alpha(1–2)$ fucosyltransferase was observed predominantly on only one surface protein - a CD44 splice variant. Although definitive proof is pending, this result could mean that the fucosyl structures on CD44 could modulate its function, and might even determine whether it acts as a metastasis promoting protein.

CD44v is not expressed on resting lymphocytes. Interestingly, we have found expression of CD44v transiently on T- and B-cells and on macrophages, upon antigenic stimulation [48, 49]. We have hypothesised from this observation that metastasising tumour cells make use of a genetic programme that lymphocytes and macrophages, the only migratory cells in normal adult animals, require during activation [50]. It is tempting to speculate that additional molecules, originally detected on lymphocytes, might also be important in metastatic spreading of tumour cells, such as L-selectin and LFA-1. The integrin family illustrates an interesting case. Tumour cells appear to change the composition of integrins on their surface during progression. Integrins are surface receptors composed of two different subunits, $\alpha$ and $\beta$. Several different $\alpha$ and $\beta$ subunits exist and their combination allows the generation of a variety of different receptor structures which all differ in their biochemical features. Integrins are ubiquitously expressed molecules. If a change is found for one tumour, generalisation is a reasonable possibility. As part of the gain of motility, the integrin VLA4 ($\alpha_4\beta_1$) is downregulated.

In individual tumours, upregulation of other integrins has been found and their expression has been correlated with an increase in malignancy [40]. More direct evidence for an involvement of integrins in the metastatic process has been derived from experiments in which their interaction with ligands was blocked by the peptide RGD. This sequence is present in many extracellular matrix components, and is crucial for interaction with some integrins. RGD not only blocked *in vitro* invasion [51], it also inhibited experimental metastasis formation [52, 53]. Direct proof for the involvement of integrins in the metastatic process has been established for the $\alpha_2$ subunit, which is not expressed in human rhabdomyosarcoma cells of low metastatic capacity. Transfection of the $\alpha_2$ gene permits the assembly of $\alpha_2\beta_1$ (VLA2), and results in enhanced

metastasis formation in both experimental and spontaneous metastasis assays in nude mice [54]. Another example of gain of function by integrins in the metastatic process is the $\alpha_6$ subunit which is a component of the $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins, which share affinity for laminin. They are expressed on lymphocyte progenitors and on highly metastatic melanoma and lung carcinoma cells of mice. The metastatic potential of these cells was tested by intravenous injection and colonisation of the lung [55]. When the experimental metastasis assay was performed in the presence of antibodies that specifically block $\alpha_6$ function, lung colonisation was strongly inhibited. Since the tumour cells bind to lung endothelium and this binding is again abolished by $\alpha_6$-specific antibodies, it is plausible that the integrin molecule participates in the metastatic process not only via its laminin binding property, but also by interaction with endothelial cells.

Surface and transmembrane molecules, such as CD44 or integrins, do not only contact with other cells and with components of the extracellular matrix, but also signal to the interior of the cell upon ligand binding. Two concepts are currently proposed for CD44 and for integrins, which might merge as our understanding improves. One concept suggests that a signal is transmitted upon ligand interaction, which ultimately leads to reorganisation of the cytoskeleton. This may affect cell shape, motility and internal cellular architecture. The cytoplasmic domains of integrins, as well as CD44, do indeed interact with cytoskeletal components [56–61]. Alternatively, a receptor-like pathway for ligand-mediated signalling is proposed. The receptor would make use of signal-transducing pathways and exert changes in the genetic programme. Evidence for this proposal has been obtained by the observation that ligand binding to integrins leads to enhanced tyrosine phosphorylation [62, 63]. In addition, it has been proposed that CD44 possesses structural features of a GTP-binding protein [64]. Furthermore, expression of several genes has been found to be increased following activation of either integrins [65, 66] or CD44 [67]. Both concepts, cytoskeletal association and transfer of signals, could be relevant for tumour metastasis formation.

## CHANGES IN INTRACELLULAR SIGNALLING

Two very recent publications describe the identification of intracellular proteins that are specifically expressed in metastasising tumour cells, and for which a causal involvement in the metastatic process is very likely: mts-1 and Tiam-1. The proteins presumably participate in signalling. The *mts-1* gene codes for a calcium binding protein, isolated from a metastasising murine mammary carcinoma cell line by use of differential screening techniques [68]. mts-1 is highly homologous to the rat p9Ka protein [69], differing by substitution of only two amino acids. In accordance with the data obtained in mouse tumours, the rat *p9Ka* gene is also expressed at high levels in rat metastatic mammary tumour cell lines [70], but not in benign tumours [71]. One such benign tumour cell line was used for transfection experiments to compare metastatic properties of *p9Ka* overexpressors and of mock- or *ras*- transfected cells [72]. Metastasis formation was examined in the spontaneous metastasis assay in syngeneic animals. It appears that considering all the evidence, the overexpression of *p9Ka* increases the metastatic potential of the recipient cell while *ras* expression has no influence.

Tiam-1 (*T*-lymphoma *I*nvasion *a*nd *M*etastasis) belongs to the family of RHO-like GDP-GTP exchange proteins [73]. These proteins participate in signalling through cytoskeletal structures. Tiam-1 was identified by analysis of proviral insertion in a T lymphoma cell line and subsequent *in vitro* selection for invasiveness into fibroblast monolayers [74]. Highly invasive clones containing retroviral integrations were examined for metastasis formation upon intravenous injection. The invasive virus-infected cell clones produced metastases in liver, kidney and spleen at elevated frequency over the non-invasive parental cells. An invasion-related proviral integration cluster was cloned, and coding sequences in this cluster determined, with the respective gene and protein termed Tiam-1. It appeared that the expression of this gene was enhanced in all clones that had been selected with proviral integration. Furthermore, integration of proviral DNA resulted in truncations of the protein in such a manner that both N-terminal and C-terminal fragments were generated. Interestingly, expression of either one, as shown by transfecting an appropriate cDNA expression clone, enhanced invasiveness. The interpretations are not straightforward, but nevertheless, the existence of intracellular pathways affecting invasion and metastasis has been made plausible.

The advances described above will certainly have an impact in clinical medicine in the future. For instance, consider the promising "experiment" reported recently: a mouse monoclonal antibody directed against a tumour surface protein of unknown function, injected after surgery, substantially improved the disease-free survival of colon cancer patients [75]. There is certainly more to be discovered, including other surface molecules, perhaps involved in the tissue preference of metastatic growth, as well as the mechanisms by which tumour cells acquire new properties. The history of the last few years has set the stage.


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# Genetics of Growth Arrest and Cell Death: Key Determinants of Tissue Homeostasis

**P.A. Hall and D.P. Lane**

## INTRODUCTION: THE PROBLEMS OF BEING METAZOAN

BEING A multicellular organism poses considerable practical problems! In some way the information encoded as a linear array of four nucleotides within the genome must be transformed into a complex, dynamic arrangement of cell types that make up the three-dimensional complexity of organisms. This enigma can be broken down into three specific (but inter-related) problems. The first is the problem of cell type specification – the differentiation problem — which involves the processes that control the phenotype of cells. The second problem concerns the control of the number of these cell types — the quantity problem. And the final problem relates to the regulation of the spatial relationship of the various numbers of the different cell types — the morphogenesis problem. It is the second of these problems, the issue of regulation of cell number, that we will consider here. This is of obvious relevance to an understanding of cancer, since this set of diseases represents conditions in which the mechanisms that control cell number become deranged. It is also, however, of relevance to a wide range of non-neoplastic disease states.

In the past, considerable attention has focused upon those regulatory processes that control the generation of new cells i.e. the cell cycle and its control. In many cases, the mechanisms that have been studied have involved those factors that positively control proliferation including the many dominantly acting oncogenes and the pathways in which their products participate.

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