CHAPTER 20

Matrix Metalloproteases and Epithelial-to-Mesenchymal Transition: Implications for Carcinoma Metastasis

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General Considerations of the EMT

The epithelial to mesenchymal transition (EMT) is characterized by the loss of epithelial characteristics and the gain of mesenchymal attributes in epithelial cells. It has been associated with physiological and pathological processes requiring epithelial cell migration and invasion. Initially, EMT was observed in embryological and adult development with many well characterized examples including the conversions of epiblast to primary mesenchyme (gastrulation), somite to sclerotome, somite to dermis, myotome to migratory myoblast, dorsal neural tube to neural crest, placodal ectoderm to cranial ganglion precursor, intermediate mesoderm to nephric mesenchyme, lateral mesoderm to connective/muscular tissue, endocardium to cardiac cushion mesenchyme and trophectoderm invasion.[1,2] In addition, evidence is mounting to support an important role of EMT pathways in the progression of carcinoma to metastasis providing epithelial tumour cells with the ability to migrate, invade the surrounding stroma and disseminate in secondary organs.[3-5]

Target Genes of the EMT

A variety of general hallmarks exist for the assignment of epithelial versus mesenchymal phenotype (Fig. 1). Intermediate filament proteins provide a convenient and abundant marker, with keratins indicating epithelium and vimentin indicating a mesenchymal phenotype.[6] This relationship breaks down in early development, where some cells which are clearly epithelial, as judged by junctional and basal lamina criteria, lack cytokeratins and many also possess vimentin. However, in these cases, it is the vimentin positive epithelia, such as the neuroepithelium, which often subsequently undergo dramatic reorganizations, including tissue folding and EMTs.[7,8] Another commonly employed index for the epithelial state is the presence and junctional localization of the classically epithelial homotypic cell adhesion molecule E-cadherin and/or the associated catenins, forming the adherens junctions. These cell-cell adhesion-related criteria are almost entirely absent in mesenchymal cells.[3] Even though keratin / vimentin and E-cadherin have been and are still the widest used markers of the EMT, a variety of other mechanisms and molecules have now been implicated in physiological and pathological EMT pathways, as reviewed by others (reviewed in refs. 2, 4-6, 9). They include a reorganization of other cell-cell contact complexes (tight junctions, desmosomes), a modification of cell-substrate adhesion complexes, the synthesis of extracellular matrix proteins normally expressed by mesenchymal cells such as fibronectin or collagens I/III, and the expression of several proteases including matrix metalloproteases (MMPs)

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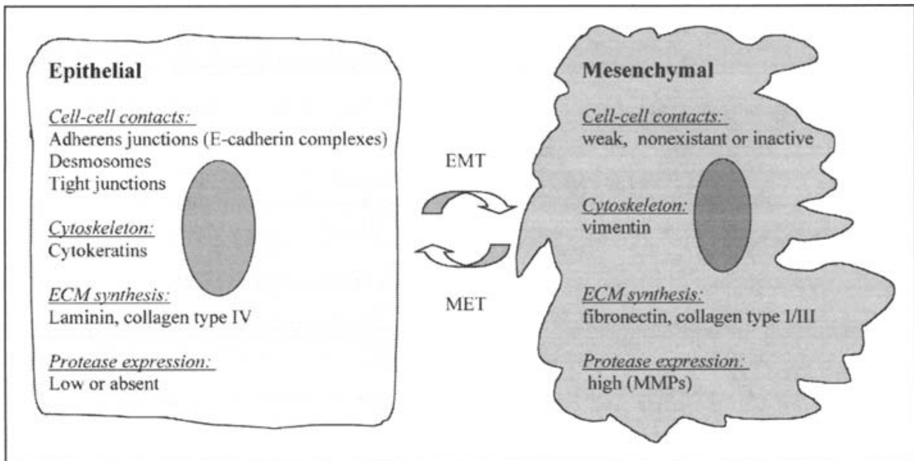

Figure 1. Molecular traits of the EMT. Epithelial and mesenchymal cells are shown schematically, and the differences commonly seen between them is grouped into four major categories: Cell-cell contacts, cytoskeleton, ECM synthesis, and protease expression. Although this is somewhat generalized, most EMT systems show these changes.

which are also predominantly expressed by stromal cells (Fig. 1). These molecular changes confer on epithelial cells the ability to scatter, migrate and degrade ECM components, all properties they do not display in a normal cohesive epithelium.

Inducers and Regulators of the EMT

Several growth factors (epidermal growth factor "EGF", basic fibroblast growth factor "FGF-2", hepatocyte growth factor "HGF", transforming growth factor β1 "TGF-β1") have been documented to trigger or at least modulate EMT phenomena.[4,5,9] The reorganization of cell adhesion molecules (E-cadherin complexes, zonula occludens) has also been shown to trigger EMT changes in different cell systems.[3,10] Also, even though they are rather considered as target genes of the EMT, several ECM components or even MMPs can in some cell systems serve as initiator of EMT changes.[11,12]

The implication of several signaling pathways in the control of the EMT is now clearly established. Thus *ras*, MAPK, PIP3K, *rho*, *rac* and *src* have been shown to control EMT events and regulate EMT target genes (reviewed in refs. 4, 5, 9).

Also, transcription factors of the Snail family (Snail, Slug) and of the ETS family, as well as the transcription factor SIP-1, have been directly implicated in the regulation of EMT target genes.[13-19] This is also well established for β-catenin, which, once relocalized from the membrane E-cadherin complexes, can translocate in the nucleus where it can act as a co-transcription factor and directly regulate gene expression through its binding to TCF/LEF transcription factors.[20]

Many cellular and molecular aspects of EMT have been characterised, and our particular interest has been in the changes associated with a class of extracellular proteases, the Matrix Metalloproteinases (MMPs). The goal of this article is to review the literature and our own data implicating MMPs and their regulation in EMT processes, both normal and pathologic.

Matrix Metalloproteases

The MMP family currently comprises 23 human homologs.[21-24] MMP protein structure is made of specific domains some of which are common to all MMPs. These conserved domains include a "pre" domain which directs the MMPs to the endoplasmic reticulum, a "pro" domain which maintains the MMP in an inactive form and a "catalytic" domain (Fig. 2). Most MMPs

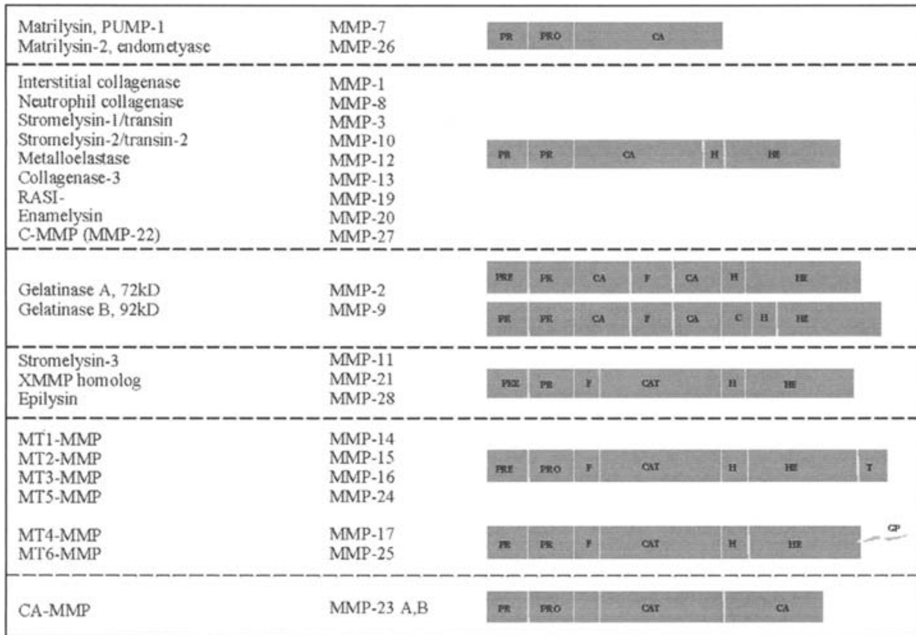

Figure 2. Schematic diagram showing the grouping of MMPs based on domain structure. Drawing courtesy of Dr. Neeracha Ruangpanit.

also contain a c-terminal hemopexin-like domain, which mediates interactions with substrates and in some cases, directs substrate specificity and participates in substrate binding. This is attached to the catalytic domain by a flexible linker termed the hinge region. A specialized, fibronectin-like gelatin binding domain is found in the two gelatinases (MMP-2, MMP-9) and facilitates binding to type IV collagen. The generation of active enzymes, known as the activation process, requires the cleavage of the "pro" domain. In some cases, this is effected constitutively by furin-like enzymes which cleave a consensus sequence near the end of the prodomain. MMP activity can be regulated by specific tissue inhibitors of MMPs (TIMP-1 to -4).

Collectively, MMPs can degrade virtually every component of the ECM. Initially, MMPs were thought to predominantly degrade specific components of the ECM thereby providing new substrates facilitating migration and invasion. Since then, it has become clear that by degrading ECM components, MMPs can also modulate signaling pathways from the ECM and modulate the bioavailability of growth factors. Furthermore, others substrates have now been identified, the cleavage of which is also involved in increased migratory and invasive properties. Thus, cell adhesion molecules (E-cadherin, CD44, αv integrin)[25-27] or growth factor receptors (FGF receptor 1, members of the EGF receptor family HER2 and HER4, c-met)[22,28-30] can be processed by MMP-dependent proteolysis. Particular attention has been paid to the gelatinases MMP-2 and MMP-9 (gelatinase A and B, respectively), previously denoted type-IV collagenases (72 kDa and 92 kDa type IV collagenases, respectively), since they specifically can degrade the type IV collagen. This forms a major component of the basement membranes normally segregating epithelial tissues from surrounding mesenchyme. Loss of the basement membrane is one of the most reliable signs of poor prognosis in most carcinoma systems.[31,32] Like most other MMPs,[23,33] MMP-2 is secreted in a latent form, requiring activation. This is effected on the cell surface by a membrane-associated subclass of MMPs called the membrane-type MMPs (MT-MMP).[34] Six MT-MMPs have been identified so far (MMP-14, 15, 16, 17, 24, 25), all of which except for MMP-17 have pro-MMP-2 activation function.[34,35] MT1-MMP was the first MT-MMP identified as an activator of

pro-MMP-2.[36] Activation of MMP-2 by MT1-MMP is particularly well documented and involves the formation of a ternary complex between MT1-MMP, pro-MMP-2 and TIMP-2. Besides their implication in MMP-2 activation, MT-MMPs can also contribute directly to cell migration and invasion by degrading specific substrates. Thus MT1-MMP can cleave several specific substrates including collagens (I, II, III), laminin and fibronectin. Through their anchoring at the plasma membrane, MT-MMPs, and particularly MT1-MMP, have been shown to play a key role in the pericellular proteolysis associated with cell migration and invasion.[21,34,37,38]

MMPs in general have been implicated in many steps of malignancy, including primary tumour growth, angiogenesis, invasion of the basement membrane and stroma, and metastatic progression.[22,24,35] With the notable exception of MMP 7/matrilysin,[39] the consensus view is that MMPs are in general not produced by epithelial cells but rather by the surrounding stromal cells.[33,40] However, we will discuss here data leading to the conclusion that expression of "stromal" MMP's is one of the major attributes that epithelial cells acquire after undergoing the EMT.

One of the major implications of this review is thus to counteract the notion that MMPs are exclusively produced by the peri-tumoural stroma. In contrast, we suggest that under the appropriate stimuli, genetic or epigenetic, certain epithelial cells will undergo EMT-like changes, and exhibit MMP production. We will summarize observations regarding MMP alterations and regulation in a number of EMT systems, both normal and neoplastic. Although our review will focus primarily on the MT-MMP/ MMP-2 axis, other MMP associations from our own work and the published literature will be summarized. The relationships which exist between the EMT and MMP-2-activation, especially those which are common to different systems, may provide insights into the implication of MMPs in EMT pathways and in their ability to modulate the migratory and invasive phenotype of epithelial cells.

MMPs in Carcinoma Model Systems for the EMT

In Vitro Observations

There are many reports of the expression of MMPs in epithelial tumour cell lines. We will only discuss here studies reporting MMP expression in epithelial cell lines in relation to well defined EMT changes. Many data have been generated by comparing different cell lines of the same origin showing different invasive potentials. Also, some cell lines have been shown to be inducible for EMT changes, either by exogenous factors or by migration opportunity in in vitro wound assays. Using these in vitro models, it has become clear that the EMT is a dynamic process and that different intermediate phenotypes can be observed. Also, whether only one EMT pathway exists is still unknown, and it can thus be considered that different EMT pathways could generate different phenotypes. Accordingly, variation may also be observed in the overall migratory/invasive behavior. For instance, based on signal transduction criteria, cell scattering can be considered to be different from, or a part of, full EMT events.[41] As such, it does not necessarily correlate with increased motility and migration. Vice-versa, active cell migration does not necessary imply comprehensive cell scattering. For instance, the migration of some tumour cell types or the archetypal developmental EMT and cell migration, such as that of the neural crest (see Chapter 3), when viewed with time lapse in situ, shows that (former) epithelial cells can migrate as dynamic groups and are not always scattered individuals.[42,43] The in vitro observations discussed below are summarized in Table 1.

NBT-II Cells

Perhaps the oldest and most studied carcinoma model of the EMT is the NBT-II rat bladder carcinoma system (reviewed in ref. 9). These cells show EMT changes to a variety of specific stimuli including collagens, HGF, FGF-1 and TGFα. Responses to FGF-1 have been mapped to a splice variant of the FGFR-2,[9,44] and shown to be mediated, in part at least, by the Snail family mesenchymal-inducing transcription factor Slug.[15] Regarding MMP expression, one of the most rapidly detected changes in stimulated NBT-II cells is the secretion of MMP-9 and MMP-2, some of which appears in the media in the active form,[45,46] which further suggests MT1-MMP expression.

Table 1. MMP expression and regulation in in vitro EMT models

Origin of the Cell Line	Inducer of the EMT/ Comparison of Different Lines with Different Invasive Degrees	EMT Traits	MMP Expressed in EMT
Rat bladder carcinoma NBT- II cells	Numerous inducers (bFGF, HGF, collagens,...)	Cell scattering, reorganization of E-cadherin	MMP-2[45]
Madin Darby canine kidney (MDCK)	Numerous inducers (bFGF, HGF, oncogenes...)	Cell scattering, reorganization of E-cadherin, tube formation in collagen gels	MT1-MMP[38,105,106]
Human breast cancer cell lines	comparison	Loss of E-cadherin, vimentin expression, invasiveness	MT1-MMP MMP-2 activation[54-57]
Human breast MCF10A cells	Oncogenes (v-Ha-ras and erbB2)	Loss of E-cadherin, vimentin expression, invasiveness	MMP-2[63]
Human MCF10A breast cell line	Wound assay	E-cadherin reorganization, vimentin expression, migration	MT1-MMP[66]
Mouse mammary SpC-2 cells	MMP-3	Loss of E-cadherin, vimentin expression, invasiveness	MMP-3, MMP-9, MMP-13[12]
Mouse mammary NMuMG cells	TGF-β	Transformation to extremely spindle morphology from cobblestone appearance, branching tubules in 3D collagen gels.	MT1-MMP Sato, unpublished data
Mouse mammary tumour cell lines	Comparison	Vimentin expression, Invasiveness	MMP-2 activation[76]
Rat mammary carcinoma cell lines	Comparison	Epithelial (E) and Mesenchymal (M) variants of the same tumour based on morphology	MMP-3, MMP-9, MMP-13[78]
Human bronchial cell lines	Comparison	Lack of E-cadherin, vimentin expression, invasiveness	MMP-2, MT1-MMP[53,79]
Human bronchial epithelial cells	Wound assay	Vimentin expression, migration	MMP-9, MMP-3, MMP-11[113, 114]
Human prostate cancer cell lines	Comparison	Lack of E-cadherin, vimentin expression, invasiveness	MT1-MMP Williams & Thompson, unpublished also[81,82]
Human cervical cell lines	Human Papillomavirus 33+ Ha-ras	Lack of E-cadherin, vimentin expression, invasiveness	MT1-MMP, MMP-2 activation[84]
Squamous cell carcinoma A431	snail	Loss of E-cadherin, vimentin expression	MMP-2[85]

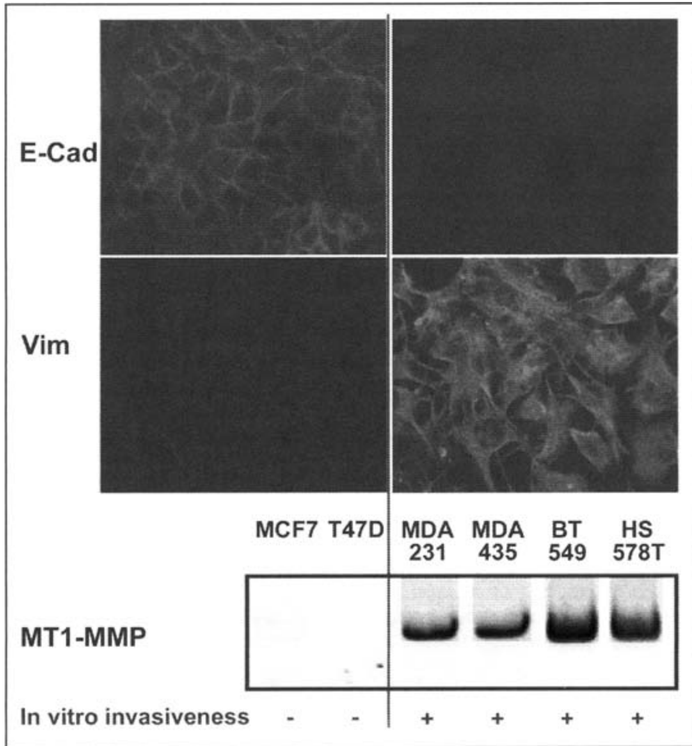

Figure 3. EMT in human breast cancer cell lines characterized by vimentin (Vim) expression, MT1-MMP expression and loss of E-cadherin (E-cad) associated with invasive properties.

Human Breast Cancer Cell Lines

Considerable circumstantial evidence exists in human breast cancer (HBC) cell lines in support of both the occurrence of EMT-like changes and their association with a more aggressive phenotype (also reviewed in refs. 47, 48). Cell lines which are invasive in vitro, many of which also metastasize in immuno-compromised hosts, show mesenchymal tendencies. They express vimentin, show reduced cytokeratins, and lack E-cadherin, in contrast to poorly invasive HBC cell lines which lack vimentin, express keratin abundantly, and in some but not all cases express functional E-cadherin.[49-53] We have also found that the vimentin-positive HBC cell lines express *c-ets*-1, a member of the ETS transcription factor family usually expressed by mesenchymal cells and largely implicated in MMP regulation.[18] When we examined proteases shown to be regulated by *c-ets*-1, we saw differential expression of MMP-1, but no expression of MMP-3, as well as differential induction of the plasmin-generating axis.[54] Also, invasive vimentin-positive HBC cell lines were found to express MT1-MMP (Fig. 3) and activate MMP-2[55] in response to ConA or collagen type I which subsequently increased MT1-MMP mRNA and protein.[56,57] Interestingly, all but one of the invasive HBC cell lines also expresses mRNA for MT3-MMP, while none expressed MT2-MMP mRNA, and both some non-invasive and some invasive lines expressed MT4-MMP.[57] A very comprehensive survey of MMP expression by numerous HBC cell lines has been published.[58,59]

Co-expression of MMPs in conjunction other mesenchymal markers in human breast cancer cell lines was also verified by independent gene array analysis by Zajchowski et al, who found that a set of 24 gene products could be used to predict the degree of invasiveness of untested cell lines.[60] Higher expression of typically epithelial gene products (e.g., keratin-18,

keratin-19, plakoglobin) was seen in the less invasive cells, while high expression of mesenchymal markers (e.g., integrin α3, TIMP-2, TIMP-3, BIG-H3, PAI-1, FRA-1, vimentin, osteonectin, TSP-1, collagen α1(VI) and α2(I), and thrombospondin-1) typified the highly invasive cell lines. It was notable that MT1-MMP was co-expressed with these markers in the invasive cells. Similarly, Jechlinger et al performed microarray analysis on mammary cells undergoing an EMT following ras transfection and in the list of genes upregulated, they found MMP-2, MMP-13 and MMP-12.[61]

We have examined the potential involvement of the EMT in two other human mammary systems that were developed to model various stages of malignancy. Transformation of the A1N4 human mammary epithelial cells with SV40 middle T antigen and v-Ha-ras, or v-mos and v-Ha-ras, but not either oncogene alone, conferred dramatically increased invasiveness and stellate Matrigel outgrowth properties on the cells. Although vimentin expression was apparent in all A1N4-derived cultures, the proportion of vimentin expression increased in the invasive transformants.[62] No differences in MMP-2 or MMP-9 secretion were observed, however MT1-MMP status or collagen/ConA-induced MMP-2-activation potential was not examined. A parallel analysis was performed with various oncogene transformants derived from the MCF10A human mammary system, where again a combination of v-Ha-ras and erbB2, but not either oncogene alone, conferred a highly invasive phenotype.[63] In this case, MMP-2 expression was increased, and TIMP-2 decreased, however again, the MMP-2 activation/MT1-MMP expression was not examined. Other studies also demonstrated an inducible EMT with a temporary expression of vimentin in MCF10A cells migrating to fill a monolayer wound, and indicated a functional role for vimentin in the migratory phenotype using antisense technology.[64] In this system, vimentin expression also associates with a reorganization of E-cadherin/β-catenin complexes and β-catenin transactivation pathway.[65] Corresponding to these EMT changes, an induction of MT1-MMP was also found exclusively in migratory cells undergoing an EMT but not in the stationary ones.[66]

Rodent Mammary Systems

A surprising twist to the relationship between EMT and MMP came from studies in the laboratories of Bissell and Werb, with observations that stromelysin-1 (MMP-3) could initiate EMT changes in SpC-2 mouse mammary cells.[12,67] This extends considerable observations of MMP-3 expression during mammary gland morphogenesis (for review see refs. 68), and accelerated ductal development in transgenic MMTV-MMP-3 mouse mammary glands over-expressing activated MMP-3.[69,70] Such over-development can progress to carcinoma in the WAP-MMP-3-transgenic mammary gland.[70] Further studies have shown that MMP-3 is critical for mouse mammary cell invasion in vitro,[71] and that malignant mouse mammary cells, as opposed to normal counterparts, show transcriptional regulation of MMP-3 similar to that seen in fibroblasts.[72] MMP-2 and MMP-11 are also upregulated with mouse mammary gland development,[69] and although stronger in humans than mouse, so is MMP-7.[39,73] The capacity of MMP-3 to initiate the EMT in the SpC-2 mouse mammary system is unprecedented, and MMP-expression is usually placed downstream of the EMT in the same cell type. In the case of the mammary gland, however, it is predominantly stromal cells which produce the MMP-3[68,69] and this in turn could influence EMT-like changes in the neighboring mammary epithelial cells. However, MMPs, including MMP-3, also appear to be downstream targets of the EMT since endogenous MMP-3, MMP-9 and MMP-13 are upregulated following EMT induction with MMP-3.[12]

NMuMG cells, another mouse mammary epithelial cell line, undergoes a rapid and striking mesenchymal transdifferentiation in response to TGF-β, mediated by the TGF-β type I receptor/Alk-5 and SMAD proteins.[74,75] This EMT is indicated by dramatically altered cytoarchitecture, reorganization of the actin cytoskeleton to stress fibers, and down regulation of E-cadherin and β-catenin. Although MMP-2 activation analysis has not been performed on these cells after TGF-β treatment, MT1-MMP expression is seen in these cells when they form branching tubular structures in collagen gels (Sato, unpublished data).

We have recently examined another syngeneic mouse mammary cancer model system comprising a panel of cells lines clonally isolated from a naturally occurring mouse mammary tumour. These cell lines show differential metastatic potential (4T1 >> 66Cl4 >> 67NR), and further single cell cloning has yielded a variant which spontaneously metastasizes to bone from the mammary fat pad (4T1.2[76]). We have characterized whether the EMT-like patterns accompany metastatic potential in these cells, and find that while all four cell lines express vimentin and show stellate Matrigel outgrowth, the metastatic lines show much increased Boyden chamber chemoinvasion, and selectively activate MMP-2 (4T1.2>66Cl-4). This was surprising since all of the progression variants showed similar levels of MT1-MMP expression.[77] Thus, we believe that all of these cell lines have undergone some level of EMT, as indicated by their expression of vimentin and stellate outgrowth, but those which are metastatic have a more complete set of mesenchymal traits, while the non-metastatic variants either lost, or never developed, some molecular traits critical for increased MMP-2-activation and in vitro invasion. It is noteworthy that the metastatic 4T1.2 cells do not show a particularly mesenchymal appearance on plastic culture, being rather rounded.

Studying a metastatic variant of a rat carcinoma cell lines, Martorana et al also found that it has undergone an EMT and expressed higher level of MMP-3, MMP-9 and MMP-13. Furthermore, cross regulation of MMP expression was observed between the epithelial cells and the mesenchymal variants.[78]

Bronchial Cell Lines

An association between fibroblastoid features (vimentin expression, loss of E-cadherin) and increased invasiveness was also reported in human bronchial epithelial tumour cells. Indeed, the 16HBE14o-vimentin-negative, E-cadherin positive cell line formed cohesive clusters and was not invasive whereas BZR vimentin-positive cells displayed a high dispersion ability and were highly invasive in the Boyden chamber assay. Correspondingly, BZR cells synthesized MMP-2 and MT1-MMP in contrast to 16HBE14o-.[53,79] In this system, a direct link was also made between E-cadherin/β-catenin complex reorganization and MMP regulation. Treatment of non-invasive, MMP-negative 16HBE14o- cells with a soluble fragment of E-cadherin (sE-cadherin), known to be generated by protease activity and known to enhances epithelial cell migration,[25] resulted in enhanced MMP-2, MMP-9 and MT1-MMP expression.[53] This also enhanced in vitro invasiveness of the cells. On the other hand, transfection of an intact E-cadherin cDNA into invasive BZR cells clearly diminished MMP-1, MMP-3, MMP-9, and MT1-MMP. Using different in vitro (cell dispersion, modified Boyden chamber) and in vivo assays (human airway epithelial xenograft), it was also shown that E-cadherin transfectants displayed decreased invasive abilities.[80]

Prostate Cell Lines

We recently analyzed human prostate carcinoma cell lines for the same parameters as described above for the breast cancer model systems (Williams and Thompson, unpublished observations). Indeed, we found that cell lines known to be metastatic in immunocompromised mice showed increased Boyden chamber activity and a tendency towards more extensive outgrowth in matrigel, although the differential was not as clear as seen in HBC cell lines. The invasive cells however clearly express vimentin, and show high levels of MT1-MMP mRNA and protein. Consequently, they activate MMP-2 readily when stimulated with ConA. Selective expression of MT1-MMP by the more invasive prostate cancer cell lines was also reported by others.[81,82]

Squamous Carcinoma Cells

EMT events (E-cadherin loss and expression of vimentin) have also been observed in several human squamous carcinoma cells. This has been shown in a model of cervical cell lines generated by transfection of human papillomavirus type 33.[83] Only cell lines which expressed vimentin

were highly invasive in the Boyden chamber assay, and these were found to synthesize MT1-MMP and were able to activate MMP-2 following ConA induction.[84]

In other established squamous cell carcinoma cell line systems, an EMT phenotype was also correlated to high levels of the transcription factor snail.[85] Furthermore, transfection of snail in vimentin-negative, E-cadherin-positive A431 cells resulted in induction of vimentin and MMP-2, and loss of E-cadherin.

From all these in vitro data, it is clear that MMP expression in epithelial cells associates with EMT processes. Among MMPs, the MT1-MMP/MMP-2 axis appear as a general pathway activated in most of the in vitro systems described above.

In Vivo Observations

Studying EMT in tumour biopsies remains a major challenge. Indeed, if one considers a "full" EMT as a dynamic phenomenon resulting in the acquisition of migratory and invasive properties by epithelial tumour cells, it is very likely to be discrete and affect only a small proportion of cells in the tumour mass. Also, if the dynamic nature of the EMT is clear, the sequence of events is still not well defined and may vary. Different phenotypes might thus be observed in a tumour mass. Accordingly, the expression of EMT genes such as E-cadherin and vimentin is very heterogenous within a given tumour.

Nevertheless, the existence of EMT phenomena in tumour biopsies is now well demonstrated. For instance, considering the two well known markers of EMT (E-cadherin reorganization and vimentin expression), numerous studies have reported the reorganization of E-cadherin complexes or vimentin expression in a variety of cancer types. Regarding MMPs, a mass of data have shown the implication of several MMPs in tumour progression (reviewed in refs. 21, 22). From these data, it is clear that the peritumoural stromal cells are the major source of MMPs in most cancers. However, even though it has rarely been studied in relationship to other EMT genes, the expression of MMPs in tumour cells has been also reported in many cancers.[86] This has particularly been shown for MT1-MMP in carcinomas including cervical,[87] lung,[88,89] prostate,[90] colorectal,[91] gastrointestinal,[92] oesophageal,[93,94] larynx,[95] breast,[96,97] oral,[98] ovarian,[99,100] head and neck,[101] hepatocellular and pancreatic[102] carcinomas.

Given the associations presented above from a number of in vitro model systems, it seems possible that the EMT in vivo may contribute to the expression of several MMPs in epithelial tumour cells and this results in tumours that do poorly. However, this is not proven, and the possibility remains that this is due to a more reactive stroma. It is interesting to note, however, the documentation in breast carcinomas of MMP-11 expression in metaplastic breast carcinoma cells which have undergone a degree of EMT-like changes.[103] The authors speculate that this may be related to the poorer prognosis associated with such carcinomas. Similarly, Trudel et al reported the expression of MMP-2 in both stromal and epithelial tumour cells of prostate cancer.[90] Importantly, MMP-2 expression by stromal cells was not associated with progression whereas MMP-2 expression by >50% of malignant epithelial cells was associated with decreased disease-free survival.[90] MMP-3 is also upregulated in a model of experimental mouse skin carcinogenesis, when squamous cell carcinomas progress to spindle cell carcinomas.[104]

While the inherent genetic deviations and implicit uniqueness of individual cancer systems make it difficult to draw general conclusions, the fact that so many different systems show EMT like capacities which are associated with increased migration, invasion, and metastasis does offer a degree of confidence in projecting an important role for this process in carcinoma progression.

MMPs in Developmental EMT Systems

Even more supportive is the parallel observations of MMP expression in EMT systems of normal development such as kidney morphogenesis modeled with MDCK cells and avian heart development, and studies on trophoblast invasion and wounded epithelia.

Kidney Morphogenesis and Disease

Madin Darby canine kidney (MDCK) epithelial cells are non tumourigenic cells and provide a well-accepted model for many aspects of epithelial cell biology. Under basal conditions in culture, they can polarize and differentiate. Following induction with different inducers including basic fibroblast growth factor (bFGF/FGF-2), hepatocyte growth factor (HGF), collagens and oncogenes (v-src and $erbB_2$), they can easily undergo dynamic EMT changes (see Fig. 4 for $erbB_2$ example). This often correlates with the loosening of cell-cell contacts, cell scattering and the capacity to invade 3D-collagen gel cultures and form branching tubules, thus mimicking a physiological renal morphogenesis rather than a pathological process. When induced by hepatocyte growth factor (HGF) to form branching tubules in collagen gels, an induction of MT1-MMP is observed[38,105] (see also Table 1). Furthermore, antisense RNA inhibition of the induced MT1-MMP abrogates the branching morphogenesis response to HGF. Also, TIMP-2 and BB-94, which inhibit both MMP-2 and MT1-MMP, block invasion or tubular formation in collagen gel. In contrast, TIMP-1, which inhibits MMP-2 but not MT1-MMP, fails to interfere with branching, implicating MT1-MMP rather than MMP-2 in the branching morphogenesis. Interestingly, we also see induced MT1-MMP after src transformation of MDCK cells,[106] and this is accompanied by overexpression of TIMP-1.[107] MT1-MMP induction is also seen after erbB2 transfection in MDCK cells (Fig. 5).[108] Although we have not examined vimentin expression in the MDCK cells undergoing branching morphogenesis, the *src* transformed cells show loss of cohesive cobblestone morphology in cell culture, and orientate as stellate single cells spread on the culture surface. They further acquire tumourigenesis in both the subcutus and kidney of athymic mice, and are metastatic to the lung when inoculated orthotopically.[106]

In vivo, and in organ culture systems, MMP-2 and MMP-9 have been demonstrated in conjunction with mouse renal tubulogenesis, however antibodies against MMP-9 only, and not MMP-2, blocked renal morphogenesis. [109] Although this study did not directly correlate this MMP expression with EMT, the EMT is known to be important in kidney morphogenesis. In contrast, MMP has been directly associated with the EMT changes seen in the tubular epithelium during kidney fibrosis associated with diabetic nephropathy.[110,111] Here, a surprising role of MMP-2 has emerged, being necessary and sufficient to induce the EMT in renal cell models.[112]

Wounded Bronchial Epithelium

Considerable attention has been paid to the MMP expression in a system where confluent human surface respiratory epithelial cells are wounded, and the cells undergo EMT-like changes (vimentin expression) to migrate into the naked culture surface. In this system, we see focal expression of MMP-9,[113,114] MMP-3 and MMP-11[115] in the cells undergoing migration at the wound edge, and this migration can be blocked with MMP-inhibitors.[114]

Avian Heart Development

EMT events have been well characterized in the transformation of endothelial cells lining the atrio-ventricular canal of avian heart to form progenitors of the septa and heart valves.[116] An induction of MT1-MMP and/orMMP-2 have been shown in EMT regions[117-120] together with an induction of Slug and c-*ets*-1, transcription factors known to be involved in EMT events and to regulate MMPs.[121,122]

Placental Implantation

Another area in which a well described EMT has been studied with respect to MMPs is the trophoblast implantation (reviewed in refs. 123, 124). Regarding MMP expression, MT1-MMP has been observed in invading trophoblasts in vivo[125] and both MMP-2 and MMP-9 in the trophoblastic columns.[126] In vitro, a clear requirement of MMP-9 for Matrigel invasion of cytotrophoblasts has also been demonstrated and correlated with the abundance of MMP-9 in the first trimester.[127]

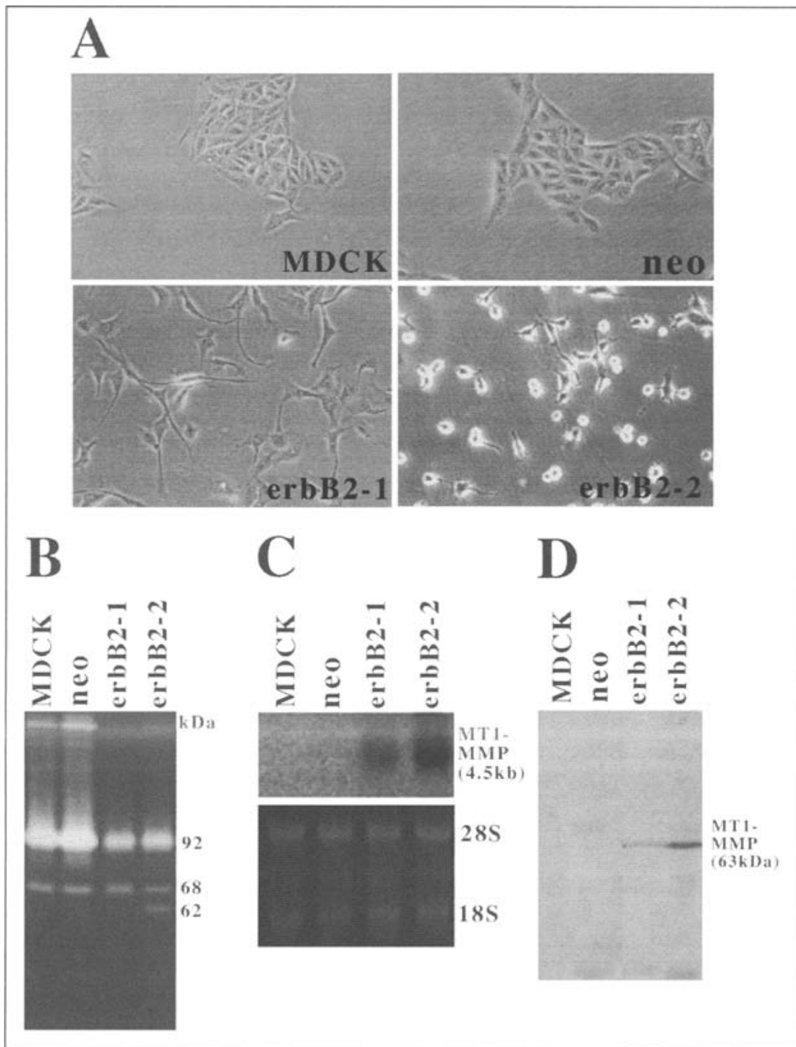

Figure 4. Induction of MT1-MMP expression by erbB2. A) Subconfluent cultures of MDCK cells (MDCK), MDCK cells transfected with control plasmid (neo), and two clones of MDCK cells transformed with erbB2 gene (erbB2-1, and erbB2-2, respectively) are shown. B) Gelatin zymography. Aliquots of culture supernatants from confluent MDCK cells (Lane MDCK), MDCK cells transfected with control plasmid (Lane neo), and two clones of erbB2-transformed cells (Lanes erbB2-1 and erbB2-2, respectively) were subjected to gelatin zymography analysis. C) Northern hybridization analysis. Total RNAs (10 μg) from MDCK cells (Lane MDCK), MDCK cells transfected with control plasmid (Lane neo) and two clones of erbB2-transformed cells (Lanes erbB2-1 and erbB2-2, respectively) were electrophoresed in a gel, stained with ethidium bromide (bottom), and then probed after Northern blotting with 32P-labeled erbB2 (top) or MT1-MMP cDNA fragments. D) Western analysis of MT1-MMP expression. MDCK cells (Lane MDCK), MDCK cells transfected with control plasmid (Lane neo), and two clones of erbB2-transformed cells (Lanes erbB2-1 and erbB2-2, respectively) were solubilized and subjected to SDS-PAGE. After blotting onto nitrocellulose filters, MT1-MMP protein was detected with a monoclonal antibody against MT1-MMP (113-5B-7).

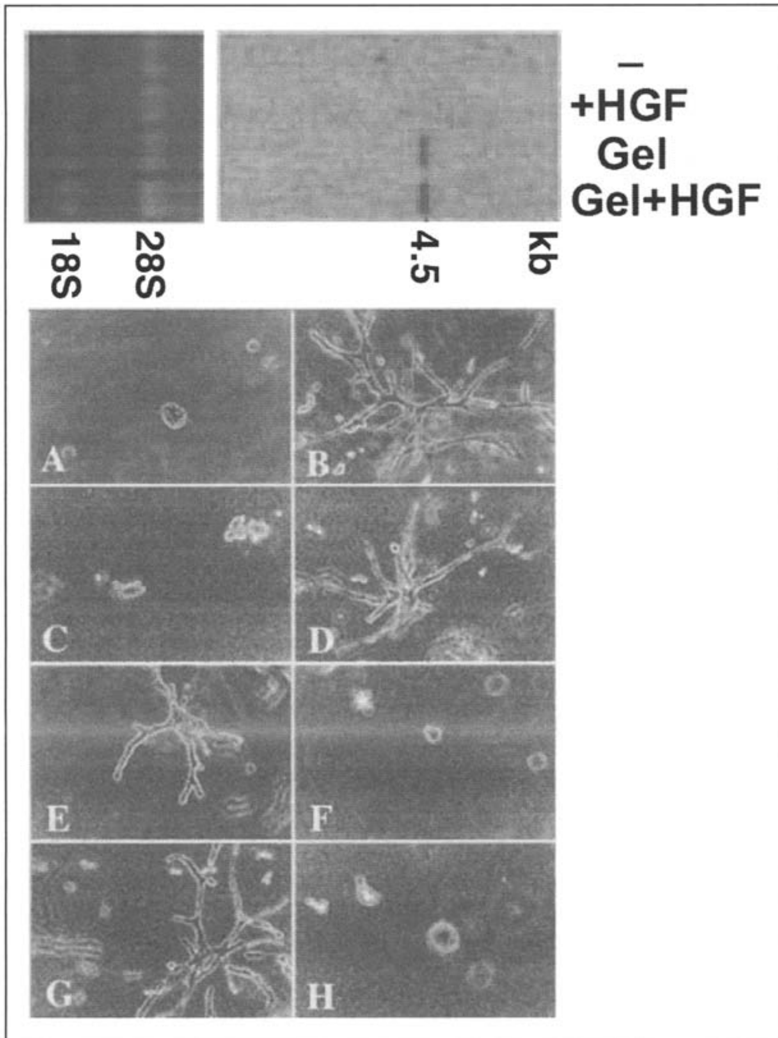

Figure 5. Upper panel) MT1-MMP induction in MDCK cells requires 3-dimensional gel. Lower Panel) Effect of MMP inhibitors on tubulogenesis. MDCK cells were cultured in collagen gel matrix for 7 days in the absence (panel A) or presence of 50 ng/ml HGF (panel B). MMP inhibitor BB-94 (1.0 x 10-7 M) was added to HGF-collagen gel culture (panel C). MDCK cells stably transfected with control plasmid, TIMP-1 or TIMP-2 gene were cultured in HGF-collagen gel as described above (panels D, E and F, respectively). Recombinant TIMP-1 (50 ng/ml) and TIMP-2 (50 ng/ml) were included in HGF-collagen gel culture of MDCK cells (panels G and H, respectively). Magnification = 100x.

Neural Crest Migration

The migration of neural crest cells in vertebrate embryogenesis is initiated by EMT of the dorsal most cells of the neural epithelium, and is perhaps the most intensively studied developmental EMT and cell migration[2] (see Chapter 3). This EMT is normally executed via stimulation by BMP-4 and crucially involves expression of the zinc-finger transcription factor genes Slug or Snail.[128] As in the heart, *c-ets-1* transcription factor is also expressed during the neural crest EMT.[129] Neural crest cells just prior to and during migration express genes for a number

of extracellular proteases. MMP-8 is expressed in neural crest cells during EMT in mouse embryos.[130] Experiments in chicken embryos reveal the expression and importance of MMP-2 in these dynamic processes.[131] It is expressed when neural crest cells detach from the neural epithelium during EMT, but switched off as the cells separate. A similar scenario is seen in the sclerotome and in the dermis, where MMP-2 expression is seen when the EMT is initiated and maintained during the migration of the cells, but down-regulated once the cells cease movement. Interestingly, MMP-2-specific knockdown with morpholino antisense oligonecleotides, and global MMP- inhibition using BB-94, confirm that MMP-2 is required for neural crest EMT, but these do not inhibit migration if applied after the EMT. In contrast, MMP-2 inhibition during mesenchyme production from the somite blocks both EMT parameters and cell migration.[119] A full audit of MMP expression in relation to neural crest cell morphogenesis has not been undertaken, so the possibility of redundancy has not been addressed.

Regulation of MMPs by EMT-Associated Transcription Factors

If the data presented above clearly demonstrate that MMP expression is associated with EMT changes, several studies have also shown a direct regulation of MMPs by EMT-associated transcription factors. Thus, MMP-2 regulation by Snail has been demonstrated in squamous carcinoma cells.[85] Also, even though they do not necessarily establish a link with EMT processes, the regulation of MMPs by transcription factors of the ETS family in a variety of carcinoma cells has been clearly established (review in refs. 132, 133). Accumulating data also demonstrate the implication of the β-catenin co-transcriptional activity in the regulation of MMP. Thus MMP-7,[134-136] MT1-MMP,[137] MMP-26[138] are targets of the β-catenin/TCF pathway.

Summary and Conclusions

The EMT appears as a sequence of changes which can lead to the expression of migratory and invasive properties by epithelial cells. As shown in numerous tumoural and non tumoural in vitro systems, but also in vivo on tumour biopsies and on developmental models, the expression of MMPs (and particularly of MT1-MMP) is clearly part of EMT processes. Accordingly, the regulation of several MMPs by transcription factors (snail, ETS, β-catenin), known to regulate EMT pathways, have clearly been established. Consequently, MMPs are rather considered as target genes of EMT pathways and MMP expression as a late event of the EMT. This may be due, in part, to direct regulation of MMP gene transcription by the factors which drive EMT. Nevertheless, some MMPs (such as MMP-3) have been shown to initiate EMT changes. Also, several MMPs have been shown to be able to cleave E-cadherin, thereby inducing E-cadherin complex fragility and EMT changes. At present, the MMP field is undergoing a re-evaluation stage following the relatively poor results seen with clinical trials of MMP inhibitors.[139,140] One major factor often raised in this re-evaluation is our lack of precise knowledge regarding which specific MMPs are mediating pro-tumoural processes. Those associated with EMT are clearly contenders for this category, and may warrant specific targeting. A number of MMPs have been associated, as detailed above, with the EMT, however, MT1-MMP stands out in this regard. Although stromal cells are a major source of MMP in tumours, expression of MMPs (particularly MT1-MMP) by parenchymal cells is a clear EMT step which can ensure a pericellular proteolysis of basement membrane components but also other substrates, and thereby facilitate migration and invasion.

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