Template

PROJECT FINAL REPORT

Grant Agreement number: HEALTH-F2-2008-201279.

Project acronym: MICROENVIMET

Project title: "Understanding and fighting metastasis by modulating the tumour microenvironment trough interference with the protease network"

Funding Scheme: Collaborative project (small or medium scale focused research project)

Period covered: from March 1, 2008 to February 29, 2012

Name of the scientific representative of the project's co-ordinator[1], Title and Organisation:

Mélanie Mestdagt, PhD, University of Liege

Tel: 0032-4-3662567

Fax: 0032-4-3662936

E-mail: melanie.mestdagt@ulg.ac.be

Project website address: http://www.microenvimet.eu/

[1] Usually the contact person of the coordinator as specified in Art. 8.1. of the Grant Agreement.

4.1 Final publishable summary report

This section must be of suitable quality to enable direct publication by the Commission and should preferably not exceed 40 pages. This report should address a wide audience, including the general public. The publishable summary has to include **5 distinct parts** described below:

1) Executive summary (not exceeding 1 page).

The formation of metastasis is the most serious challenge for cancer treatment. The development of novel therapeutic strategies to fight metastasis is dependent upon pinpointing molecules that are responsible for initiating and promoting metastatic dissemination of malignant cells. The MicroEnviMet (MEM) consortium used innovative approaches for building a comprehensive understanding of the interplay between cancer cells and their microenvironment both at primary and secondary sites. By interfering with the protease network and by studying the impact of such experimentally manipulated microenvironment on metastasis formation, key molecular pathways underlying early and late steps of metastatic dissemination were explored. The partners characterized different sets of non-metastatic and metastatic cells by proteomic, miRNA, and mRNA profiling.

Several miRNAs that could play a role in the regulation of key modulators of the microenvironment have been identified, opening potential attractive applications to switch off these regulators. Notably, through a collaborative effort between all MEM partners, mRNA and small non-coding RNA (ncRNA) were profiled through a state-of-the-art high-throughput sequencing in the murine MMTV-PymT model, which recapitulates all phases of metastatic breast cancer. This study led to the identification of a set of ncRNAs modulated during the progression of breast cancer.

In addition, molecular pathways governing cancer stem cell (CSC) biology were investigated and the implication of an oncogenic pathway in CSC migration and proliferation is evidenced. Alterations of the tumour microenvironment through genetic ablations revealed a protective effect of some protease members (matrix metalloproteinases) produced by host cells and the contribution of cathepsins in the recruitment of host cells in primary tumours as well as cancer growth and metastasis. These data underline the complexity of the molecular network involved in the elaboration of a tumour microenvironment permissive for or protective towards cancer progression. It also demonstrates the need to get an in-depth understanding of the metastatic process before transferring the data generated from pre-clinical models to clinical settings.

Suitable *in vivo* models for the analysis of the pre-metastatic niche were identified and the impact of governing molecules in the niche was determined. These results are the basis for the identification of molecular pathways describing the nature of the metastatic niche. With the aim to develop an anti-metastatic therapy, the consortium characterized murine monoclonal antibodies neutralizing the murine forms of target proteins and demonstrated their *in vivo* efficacy. Notably, a novel drug delivery system based on ferromagnetic nanoparticles was also developed and improved the efficacy of protease inhibitor in the MMTV-PymT model. The consortium generated human monoclonal antibodies by phage display towards two molecular targets which are currently evaluated as therapeutic drugs. These targets are membrane-bound proteins, one expressed by tumour cells and one produced by stromal cells contributing to the microenvironment.

By sharing of their expertise and the establishment of fruitful collaborations, the partners of the MICROENVIMET consortium have reached major achievements in deciphering the molecular actors orchestrating the complex interactions between cancer cells and their microenvironment. The identification of such actors is crucial for the development of new strategies aimed at treating metastatic dissemination of tumour

2) Summary description of project context and objectives (not exceeding 4 pages).

A hallmark of the malignant process is the acquisition of an invasive phenotype allowing cancer cells to invade surrounding tissue and to disseminate into specific organs forming secondary tumours (metastasis). Metastasis still represents the major death-determinant of cancer patients and causes a significant reduction in their quality of life. Extensive metastasis research is still necessary as this aspect of malignant tumours remained the most serious challenge for cancer treatment.

The traditionally prevailing explanation of metastasis is that during cancer progression, tumour cells acquire, through the accumulation of multiple genetic alterations, the ability to surmount a variety of obstacles including shedding from the primary tumour, intravasation into blood or lymphatic vessels, survival into the circulation, extravasation and growth at a secondary site. For most carcinomas, progression towards malignancy is accompanied by epithelial to mesenchymal transition characterized by the loss of epithelial differentiation and a shift towards a mesenchymal phenotype with exacerbated motility and invasiveness. For long, the emphasis has been to identify tumour features and reveal gene expression signatures of primary tumours that have been associated with their metastatic potential. **The MicroEnviMet (MEM) project** aimed at revisiting the tumour cell-centred view of the metastatic process by taking into account a variety of cellular and non-cellular factors in the microenvironment within the primary and secondary tumour site. The members of this *MEM consortium* foster the concept that the microenvironment is at least as decisive for tumour progression as the intrinsic features of tumour cells.

The importance of the tumour microenvironment is further supported by studies revealing that factors secreted by the primary tumours can induce early changes of the local microenvironment in distant organs leading to the elaboration of a permissive niche for incoming circulating cancer cells. These modifications characterized by the mobilization of non-malignant haematopoietic cells and the deposition of matrix components, precede the influx of metastatic cells. The formation of this so-called "pre-metastatic niche" in distant organs promotes the efficacy of successful colonization of metastatic cells. Validation of this concept, as well as its understanding both at cellular and molecular levels are mandatory for improved future therapeutic intervention. Identification of factors that render an organ more susceptible for metastasis will provide important drug targets allowing interference with the so far devastating and uncontrollable spread of malignant tumours, e.g. at early stages of the

disease, prior and shortly after removal of the primary tumour, as well as in late stages of cancer disease, when new waves of metastatic spread may occur.

The **tumour microenvironment** is a complex ecosystem consisting of cellular and non-cellular components, where a network of proteases and protease inhibitors orchestrates the signals that determine cell fate and functions. The cellular compartment (Figure 1) includes not only tumour cells but also blood or lymphatic endothelial cells, pericytes, smooth muscle cells, (myo)fibroblasts, immune and inflammatory cells. In addition, the cellular compartment also includes a small subpopulation of **cancer stem cells (CSC)**, which are thought to be analogous to stem cells in normal tissue, dividing both to self-renew and to produce progeny that form the bulk of the tumour mass. CSC may be the source of metastatic cells as it is hypothesized that they can easily adopt the invasive genetic programme executed by stem and progenitor cells during normal organ development.

The **non-cellular compartment** consists of the various molecules of the extracellular matrix, whose composition directly and indirectly influences the phenotype of the cellular compartment. The process of cancer progression and metastatic dissemination is now viewed as a change of the homeostasis within the tumour microenvironment towards the accumulation of dissemination-promoting signals at the site of primary tumour formation, as well as in distant organ induced by both genetic and epigenetic stress.

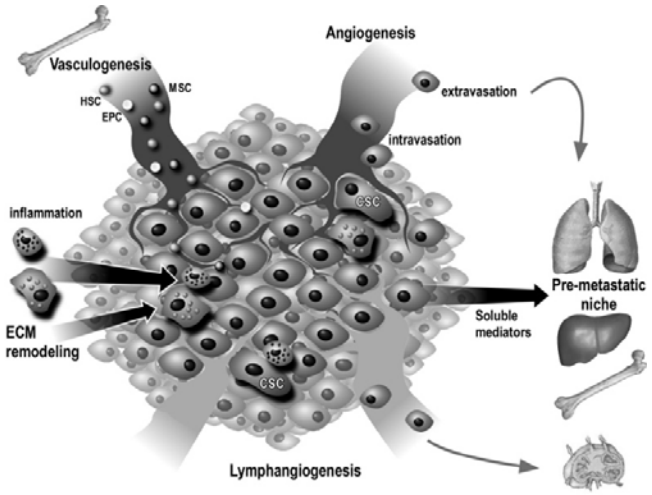


Figure 1: Metastatic dissemination and tumour microenvironment. In addition to the sprouting of neighbouring pre-existing vessels, tumoural angiogenesis is supported by the mobilization of different cell types including hematopoietic stem cells (HSC), endothelial progenitor cells (EPC) and mesenchymal stem cells (MSC). Lymphangiogenesis facilitates metastasis by providing an alternative route of dissemination. Primary tumoural cells could induce the establishment of a pre-metastatic niche preceding metastatic colonization of the targeted organ. CSC = cancer stem cells

The growth and metastatic spread of cancer are related to angiogenesis and lymphangiogenesis, the formation of new blood or lymphatic vessels, respectively (Figure 1).

The formation of these new vessels facilitates metastasis of cancer cells by providing routes for dissemination throughout the body. It is noteworthy that detection of metastatic foci in regional lymph node is a strong indicator of poor patient survival. In contrast to extensive studies in the field of angiogenesis that led to the generation of anti-angiogenic drugs used currently in the clinic, lymphangiogenesis has been overlooked. Despite the identification of main signalling pathways that regulate angiogenesis and lymphangiogenesis, further studies are needed to delineate the key regulatory pathways to be targeted for novel therapeutic approaches.

It is increasingly evident that tumour growth, invasion, and metastatic dissemination involve a set of cells elaborating a permissive microenvironment, both in the primary tumour and in the targeted organ of metastatic dissemination. Alterations in this microenvironment and of the signals emanating from normal cells in the targeted organ are factors that regulate tumour growth, invasion and metastasis. Each cell type (fibroblasts, inflammatory cells, endothelial cells) can communicate with others, or with tumour cells, or CSCs, locally or at distance, through the production and the activation of a variety of signals including growth factors, matrix molecules, chemokines, cytokines, (lymph)angiogenic factors and their receptors. The diverse cross-communications that occur between tumour and host cells, as well as the impact of extracellular signals on the invasive phenotype of CSC are still poorly understood and are the subject of the MEM project.

The communication between the cellular and non-cellular compartments of the tumour microenvironment is by large mediated by a network of enzymes (**proteases**) able to cleave a large set of proteins. In normal tissue homeostasis, the interacting network of proteases and their natural inhibitors maintain a proteolytic balance. During cancer progression, this balance is disturbed by overexpression of proteases of at least three major families, metalloproteases (MMPs, ADAM and ADAM-TS), serine proteases, and cysteine proteases (cathepsins). This imbalance between proteases and their inhibitors alters the non-cellular compartment, which in turn activates effector molecules leading to the establishment of a milieu permissive for tumour progression, invasion and dissemination. In addition to proteases, **micro RNAs (miRNA)**, a family of small non-coding RNAs have been recently recognized as key regulators of cell fate determination. Profiling these regulatory elements is currently performed allowing the identification of gene-repertoires modulated simultaneously at specific steps of cancer metastasis.

Our experimental approach aimed at elucidating early mechanisms of metastatic dissemination by modulating the production or activities of proteases (metalloproteases,

serine proteases and cysteine cathepsins) both in primary and secondary sites (Figure 2). Proteases are key regulators of interacting molecules that modulate the properties of cancer cells and *CSCs* as well as permissive feature of their microenvironment. Any interference with a protease (gene deletion, overexpression, inhibition) has a profound impact on interacting proteins leading to the modification of the tumour microenvironment and tumour phenotype (Figure 2). The *MEM consortium* study aimed at identifying key molecular pathways, which are fundamental for early steps of metastatic dissemination in both entities (primary tumour and target organ of metastasis). This was achieved by investigating the susceptibility of the experimentally manipulated microenvironment to metastasis.

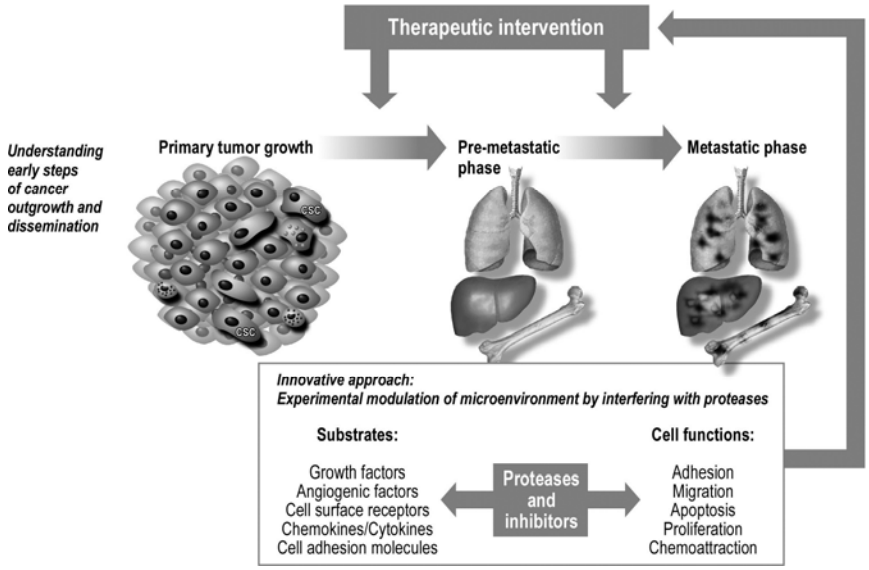


Figure 2: Aims and approaches used by the *MEM consortium*. The study provides new insights into the interplay between cancer cells, cancer stem cells (CSC) and their microenvironment, both at primary and secondary sites. Modulation of tumour microenvironment has been achieved by interfering with proteases that are central mediators of a complex molecular network, as well as key regulators of various cell functions.

The MICROENVIMET project has explored new strategies for treating metastatic dissemination of tumours into lung, lymph node and liver with the specific goals of:

- Improving our understanding of early steps of the metastatic dissemination: matrix remodeling and tissue infiltration, angiogenesis, vasculogenesis, lymphangiogenesis, bone marrow cell recruitment in primary and secondary sites, establishment of pre-metastatic niches.
- Identifying regulatory pathways and cellular events (including intracellular signalling and gene regulation) that coordinate cancer metastasis.

- Investigating the modulation of miRNAs expression during tumour growth and metastatic dissemination, and identifying their target genes at different steps of metastatic spread, as putative targets for therapeutic intervention.
- Improving our understanding of CSC biology and understanding the role of CSC in driving metastatic dissemination: elucidating the microenvironmental cues and molecular mechanisms responsible for CSC mobilization from the primary site, migration and lodging into a distant target organ.
- Identifying critical molecular targets for the development of strategies to delay or eradicate metastatic dissemination.

3) Description of the main S&T results/foregrounds (not exceeding 25 pages).

To achieve its objectives, the MEM consortium has combined the most advanced state-of-the-art technologies for identification of gene signature (mRNA and microRNA microarrays, high throughput sequencing, RT-PCR Taq-Man Low Density Array), *in vivo* and *in vitro* gene manipulation (transgenic mice; viral gene transfer, siRNA, shRNA), proteomic analysis, *in vivo* imaging of metastatic cells, computerized image analysis, production of blocking antibodies against the identified target by phage display or through the immunization of transgenic mice. The work is articulated into the different workpackages:

WP1: Tumour cells and cancer stem cells in the context of metastasis

WP2: The microenvironment in primary tumour

WP3: The pre-metastatic niche

WP4: Anti-metastatic therapy in transgenic cancer models

WP5: Integration and management of microarray information

WP6: Dissemination of results for clinical implications

We will here describe the results generated in the WP1 to WP5. The dissemination of results related to WP6 is presented in the next section.

WP1: Tumour cells and cancer stem cells in the context of metastasis

1. Context and objective

This Workpackage focused on the tumour compartment composed of cancer cells themselves and on selected subpopulation of the so-called "cancer stem cells" (CSC) which is likely resistant to classical cancer treatment. CSC are defined as a minor cell subset in the tumour mass, which is endowed with stemness properties, including the ability to proliferate for an indefinite time (self-renewal) and to generate the tumour mass.

The WP1 goal was to determine the effect of protease web modulation on the metastatic phenotype of murine and human cancer cells. In addition, we aimed at generating a comprehensive expression map of miRNAs and a comparative cell surface proteomic profile in metastatic and non-metastatic cells. These studies were carried out either in the overall cancer cell population of human and murine tumours, or in CSC, derived from primary human and murine tumours, and from cell lines. The protease network in cancer (stem) cells was investigated also in connection with the activity of the HGF-MET signalling system involved in epithelial-mesenchymal transition and invasive/metastatic dissemination. The interplay

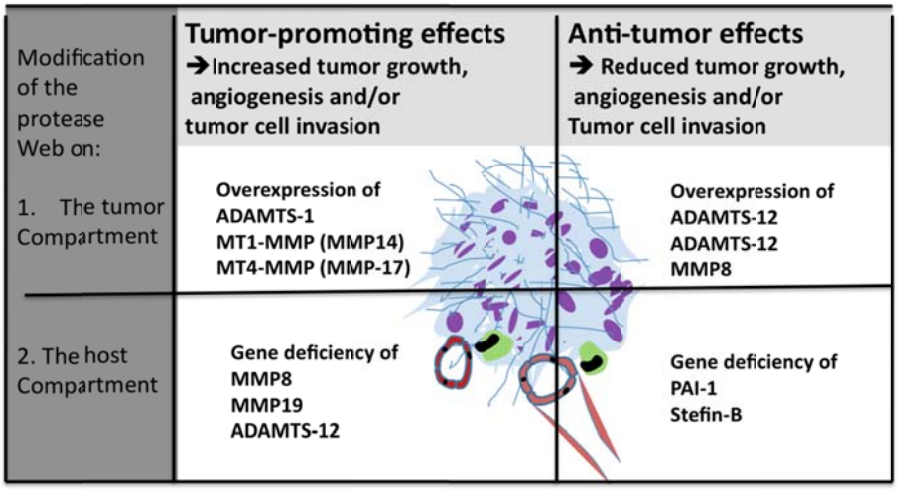
between MET and proteases is likely critical for invasion and metastasis but has been so far not well documented.

MEM partners have first generated several tools that were rapidly available for further collaborative standardized studies in WP1 and WP2. These tools included protocols for CSC isolation, surface proteome identification, cells that can be tracked *in vivo* through luciferase, GFP or LacZ expression as well as cells with modulated expression of proteases.

2. Impact of protease web modulation in the tumour compartment on the metastatic phenotype of murine and human cancer cells

The *in vivo* growth and metastatic features of the different murine and human cell lines engineered to overexpress a protease have been characterized following sub-cutaneous or intravenous injection into immuno competent or immuno-deficient mice. The partners of the MEM consortium contributed to underline the sophisticated multiple functions of proteases during different steps of cancer progression. Several proteases had been identified as pro-metastatic (ADAMTS-1, MMP14, MMP17/MT4-MMP, Cathepsin B, Cathepsin Z), while others exert anti-tumoural action by inhibiting cancer growth and invasion (ADAMTS-12, ADAMTS-15, MMP8, MMP19, Cathepsin L). The source of the enzyme either the tumour cells or the host cells is also an important determinant of the protease effect. The Table 1 summarizes the tumour-promoting effects and the anti-tumoural actions of proteases and their inhibitors demonstrated by the MEM consortium.

Table 1



A special emphasis has been given on pro-metastatic proteases (MT1-MMP and MT4-MMP) and anti-invasive proteases (MMP8, ADAMTS-12 and 15).

Human breast adenocarcinoma MDA-MB-231 cells expressing MT4-MMP and luciferase or their control clones were injected subcutaneously into nude mice. Lung metastasis incidence was clearly increased after subcutaneous injection of cells expressing MT4-MMP compared with control cells (60 % versus 0 to 5 %). In sharp contrast, no significant difference in the incidence of lymph node metastasis was observed between MT4-MMP expressing or control cells. This pro-metastatic effect is related to the capacity of MT4-MMP to affect blood vascular structure and is dependent to its catalytic activity (Figure 3). To investigate whether MT4-MMP produced by host cells could impact tumour progression *in vivo,* tumour cells were into *MT4-MMP* knockout mice or control mice. The tumour growth and angiogenesis were not affected by host MT4-MMP status underlying that this enzyme contributes to the metastatic dissemination when produced at the surface of tumour cells.

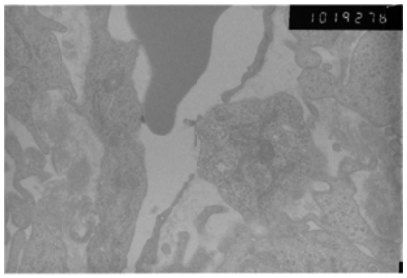


Figure 3: Illustration of the intravasation of a tumour cell frequently observed in MT4-MMP overexpresssing tumours, but not in control tumours.

A protective effect of MMP-8 was observed in both murine and human cell lines. The overexpression of active MMP-8, but not that of an inactive mutant, induces a drastic reduction of the metastatic ability of aggressive tumour cells. In line with these experimental findings, breast cancer patients with increased expression of MMP-8 have a better overall survival and lower incidence of metastasis. Similarly, in a collaborative effort, two MEM partners demonstrated the anti-tumourigenic and anti-angiogenic effects of ADAMTS-12 in different experimental models. Similarly, ADAMTS-15 expression restrained the invasion and tumour growth of colon carcinoma cells. *These data support the tumour protective properties of MMP-8, ADAMTS-12 and ADAMTS-15.*

3. Study of cancer stem cells (CSCs) in invasion and metastasis and the interplay of the protease web and MET pathway

Despite the introduction of new cytotoxic chemotherapies and novel biological agents, such as the monoclonal antibody Cetuximab, which targets EGF Receptor, metastatic colorectal cancer (CRC) remains largely incurable. To investigate the mechanisms underlying CRC

metastasis and envisage new therapeutical strategies, an ample panel of liver metastasis from CRC was directly transferred from patients into immunocompromised mice, and expanded by serial transplantation. These xenografted mice (xenopatients) were shown to be representative models of the corresponding human patients: indeed the xenografts retained the genetic mutations and the histological features of the original tumours, and the same sensitivity to Cetuximab. Then, resistance to this antibody was associated with the presence of mutations of the Ras oncogene, an already known mechanism of resistance in patients, or with HER2 amplification, a new mechanism of resistance discovered with this study. The xenopatients were used to derive cancer stem cells (CSCs), i.e. the subpopulation of cancer cells displaying stem-like properties *in vitro*, which retains tumourigenic potential and metastatic ability *in vivo*. These cells must be characterized at molecular level, as to verify whether they can be the cellular target of conventional and biological agents, and, if so, whether the agent can lead to effective tumour eradication. In CSCs derived from xenopatients, we showed high expression of the Met oncogene, encoding the tyrosine kinase receptor for Hepatocyte Growth Factor (HGF). This factor supports three biological activities crucial for cancer metastasis: proliferation, resistance to drug-induced apoptosis, and invasion. As the liver is a rich source of HGF, Met activation can easily take place in metastatic colorectal CSC, and support metastasis growth and resistance to cytotoxic and biological agents. Based on evidence collected on both cell lines and CSCs, we concluded that Met has an important role in cancer metastasis and drug resistance. Thus, we developed a monoclonal antibody against the extracellular domain of Met (DN-30), which could be proposed as a tool for human therapy. After the first demonstration of activity (2), we showed that DN30 is effective *in vivo* even when administered through an original "gene-therapy" protocol. As an important step of pharmacological characterization, we investigated the mechanism through which DN30 inhibits Met. We found that, upon binding, DN30 unleashes the proteolytic cleavage of the Met extracellular domain by the metalloprotease ADAM-10. Met extracellular domain is thus shed from the cell surface, causing loss of the binding site for HGF. The soluble Met extracellular domain may also exert a decoy-effect, by complexing and blocking HGF bound to the extracellular matrix. We have thus provided evidence of Met activity in supporting metastatic colorectal cancer, and developed a promising inhibitory antibody for human therapy.

4. Transcriptomic (mRNA and miRNA) profiling in tumour cells and CSC with different metastatic features.

The transcriptomic (mRNA and miRNA) profilings of CSC, metastatic and non metastatic cells, or cells overexpressing MMP-8, MT1-MMP (MMP-14), MT4-MMP or ADAMTS-12 have been performed in a collaborative efforts between the MEM partners. Data loaded on Mediante data base were available to all partners for integrated analyses. Validations of set of miRNA or RNA modulated have been validated by qPCR. Functional validations are ongoing. Notably, the analysis of human breast carcinoma cells overexpressing MMP-8 compared to the corresponding control cells has revealed the downregulation of specific miRNAs that could contribute to the anti-metastatic effect of this enzyme. This is to our knowledge the first example of tumour-associated miRNA expression deregulation as a consequence of perturbation in protease expression suggesting that miRNAs may constitute mediators of pro- or anti-metastatic activities of proteases. Thus, MEM partners have provided gene and miRNA profiles specific to CSC or tumour cells overexpressing individual protease that are opening new avenues for unravelling their complex functions during different steps of the metastatic process.

In complement to these *in vitro* studies, similar transcriptomic profiling was conducted on primary tumours induced by tumour cell injection. In addition, because of the well-defined parameters of tumour progression and metastasis of the breast MMYV-PyMT model, the MEM consortium used this transgenic mice model as a prototypic cancer model for miRNA profiling in the primary tumour resected at different stages, as well as in different organs colonized or not by metastatic cell. Such a unique shared study by a whole consortium has generated a comprehensive analysis of miRNA and mRNA modulated at different stages of the cancer progression and during the dissemination to specific organs. Original bioinformatics tools have been generated by one partner to integrate all data and identify key molecular pathways of interest. These are described in WP5.

5. Proteome profiling of metastatic and non-metastatic cells.

As the cell-surface constituents mediate interactions between tumour cells and their environment, the cell surface can provide targets to inhibit cancer growth and metastatic spread. To identify surface proteins characteristic of metastatic cells, one MEM partner has undertaken a quantitative mass spectrometric proteome analysis of metastatic and non-metastatic cell lines This analysis resulted in about 9000 peptides differentially expressed between the metastatic and non-metastatic subclones. The identified proteins display various molecular functions such as catalytic activity, protein binding, and receptor and signal

transduction activity. Some of the proteins are unknown. Through a bioinformatics analysis, key networks of proteins have been identified and on going studies are conducted for functional validation. *This study demonstrates that isogenic human metastasis model combined with cell surface proteomic analysis can be used to reveal novel metastasis-related proteins and protein complexes.*

WP2: The microenvironment in primary tumour

1. Context and objective

With the aim at giving new insights into how the primary tumour microenvironment influences the metastatic process, our first objective was to better understand the mechanisms underlying the elaboration of the primary tumour microenvironment in relation to the metastatic dissemination of cancer cells. In fact, intra-tumoural recruitment and migration of various host cell types provide a permissive microenvironment for tumour expansion and metastatic dissemination. Tumour-associated host cells include local cells, i.e. endothelial cells, fibroblasts and tissue resident innate immune cells, as well as circulating or bone marrow-derived cells, i.e. immune cells, vascular cells and stromal progenitor cells. Signals from the tumour microenvironment are now recognized as key players that lead tumour cells to switch to an invasive state, while the cancer cells are also active in reprogramming their environment in a state that suits them. Over the past years, the work within the *MEM consortium* considerably advanced our understanding of the mechanisms of cancer progression by integrating research on tumour cells, blood and lymphatic vessel wall cells, infiltrating local stromal cells, and recruited circulating and/or bone marrow-derived cells.

2. Establishment of *in vitro* models reconstituting the tumour microenvironment

Molecular investigation of biological processes involving cancer cells and cells of the tumour microenvironment, requires models amendable to reproducible experimentation. *In vitro* cell cultures are a convenient way of experimentation. For example, the MEM partners screened different well established cell lines of both human (HT1080, MDA-MB-231, HeLa, MCF-7, LNCap) and murine (uPARAP+/+ fibroblasts, uPARAP-/- fibroblasts, Lewis Lung, NIH3T3) origin and showed a correlation between the amount of expressed uPARAP and the amount of internalized collagen (Figure 4).

Figure 2.

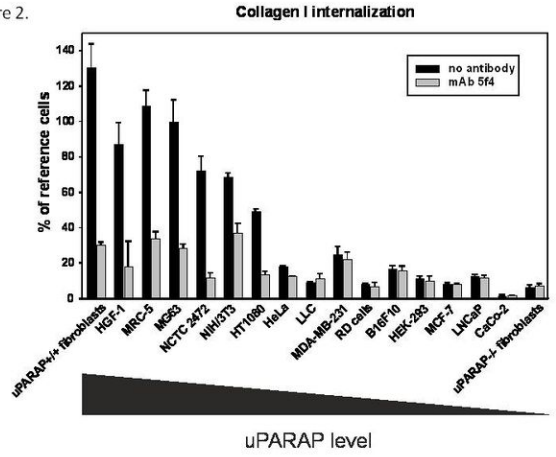


Figure 4: uPARAP/Endo180 mediates collagen internalization in a variety of cell types. Collagen internalization is inhibited by mAb 5f4 for a variety of established cell lines. Collagen internalization was measured for each cell line. Internalization was carried out in the absence of antibody (black columns) or in the presence of 10 µg/ml of mAb 5f4 (grey columns). The cell lines are ranked in the diagram according to their level of uPARAP/Endo180 expression, with the highest expressing cells to the left.

Furthermore, the collagen uptake and internalization is completely blocked by a monoclonal antibody directed against an epitope located to the N-terminal domains 1-3 of uPARAP.

Most traditional cell culture models are on flat 2D surfaces that do not mimic the spatial 3D organization of cancers and their stroma. Thus MEM researchers were involved in development and wide-spread application of so called 3D cell culture models. In this context, various MEM partners employed 3D *in vitro* culture model to assess the capacity of different human breast tumour cell lines to grow and invade 3D gels composed of type I collagen or Matrigel. By using these assays, it was shown that the more aggressive cell lines (MDA-MB-231, Hs578T) proliferate and invade the gels whereas the poorly aggressive ones (MCF-7, ZR-75-1) were unable to either proliferate, invade the gels and died by apoptosis. These models have also been used to assess pericellular proteolysis and invasive growth of primary cancer cells derived from the MMTV-PyMT mouse model of metastasizing breast cancer with deficiencies for several proteases.

A novel model of 3D cultures of lymphatic capillaries, the lymphatic ring assay that is analogue to the aortic ring assay has been set up. Small fragments of mouse lymphatic thoracic duct are embedded in a collagen gel and lead to the outgrowth of lymphatic vessels containing a lumen (Figure 5). Ultra-structural analyses revealed the suitability of this model

to mimic lymphatic endothelial cell migration observed in pathological conditions. This model has also been proved suitable for the screening lymphangiogenic factors and the phenotyping of transgenic mice. For instance, this lymphatic ring assay has allowed the unexpected identification of MMP2 as a key regulator of lymphatic endothelial cell sprouting. The novel lymphatic ring assay and the more classical aortic ring assay are unique complementary tools suitable to unravel and compare cellular and molecular mechanisms of lymphangiogenesis and angiogenesis, respectively.

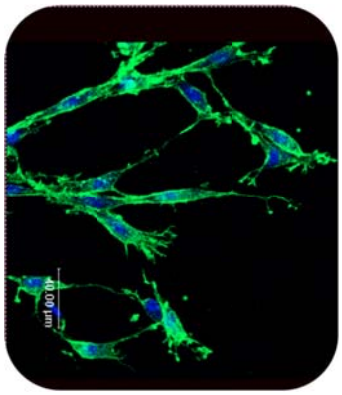


Figure 5: Illustration of lymphatic endothelial cells spreading from a lymphatic vessel fragment. Endothelial cells are labeled in green (with an anti-LYVE-1 antibody) and nuclei are stained in blue. This model reproduces the different steps of the lymphangiogenic process observed during cancer progression, both at primary site and secondary sites of tumour growth.

3. Tumour growth and metastasis in the transgenic models of breast and skin cancer

Besides the 2D and 3D cell culture models, the MEM consortium has widely used mouse animal models of human cancers to address the interactions of primary cancer cells and cells of the surroundings. The consortium utilized K14-HPV16 transgenic mice as a mouse model of epidermal carcinogenesis (Figure 6). These mice exhibit a series of discrete neoplastic stages, including hyperplasia by 1-month of age and development of dysplasias between 3- and 6-month of age. These precursor lesions undergo malignant conversion into squamous cell carcinomas (SCCs). In addition to epithelial alterations, neoplastic progression in HPV16 mice is characterized by chronic inflammation regulating microenvironmental remodelling and the activation of angiogenic programs in premalignant tissue. The MMTV-PymT mouse model of breast cancer shows a variety of distinct pathological stages from normal mammary gland tissue to premalignant lesions and, later, invasive ductal carcinoma. With regard to lung metastases in the PymT model, a pre-metastatic phase can be distinguished from a micrometastatic and macrometastatic phase.

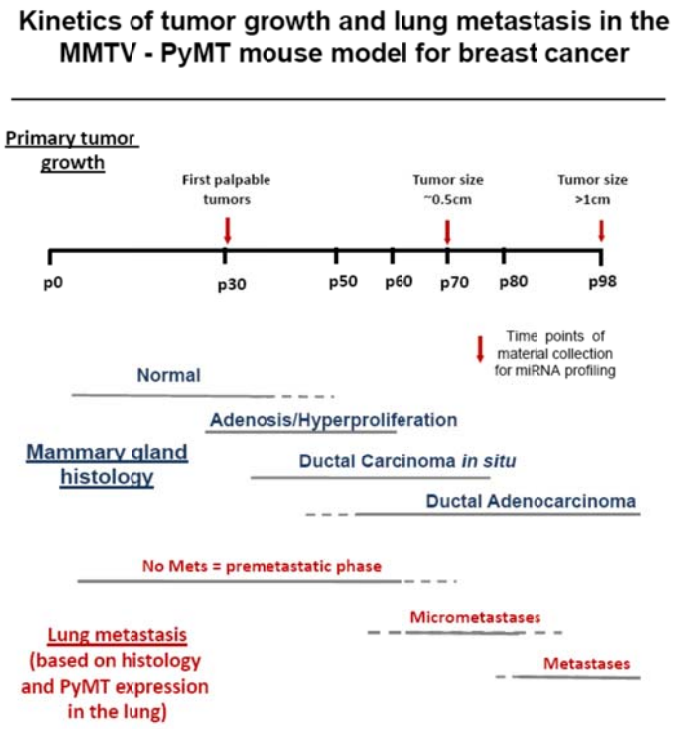


Figure 6: Kinetics of tumor growth and metastasis formation.

A main strategy was to cross these mouse models to mouse lines deficient for various proteins suspected for their tumour-promoting and stroma-activating potential. A major focus of the *MEM consortium* was at the proteases and their naturally present inhibitory proteins, such as MMP3, MM8, MMP13, cathepsin B, Z, and L, stefin B, uPA, and PAI-1.

The success of the mouse genetic studies also depends on the generation and phenotyping of new gene-deficient mouse models. In this respect, the collaboration established between different MEM partners led to the generation and phenotyping of novel mouse lines deficient for the metalloprotease ADAMTS-12 and the cysteine protease cathepsin Z. These new tools were essential for identifying surprising anti-tumourigenic functions exerted by ADAMTS-12. Indeed, both in *in vitro* models (aortic ring assay) and *in vivo* models (transplantation chamber assay, matrigel plug assay), invasion and migration were exacerbated in mice deficient. A stronger invasion characterized by a deeper extension of tumour cells was observed in ADAMTS-12-/- mice. In addition, blood vessels migrated roughly towards tumour cells leading to increased overlapping area of tumour cells and blood vessels. Altogether these data demonstrate a protective effect of ADAMTSs-12 towards cancer

progression. In the absence of ADAMTS-12, both the angiogenic response and tumour invasion into host tissue were increased.

4. Bone marrow-derived cell recruitment and inflammation

Tumour-promoting inflammation and induction of angiogenesis are hallmark characteristics of activated tumour stroma. The recruitment kinetics and activation state of various myeloid cell types to the microenvironment has been studied in the tumour models available to the MEM consortium with a variety of sophisticated methods such as FACS of tumour-tissue and quantitative histometry of tissue sections. For example, the overexpression of human cathepsin B in MMTV-PyMT mice resulted in increased recruitment of B cells and mast cells to the tumour stroma. Cathepsins derived from macrophages were shown to promote the formation of micrometastases and the colonization in the MMTV-PymT breast cancer model (Table 2).

Table 1 Tumor-associated immune cells

Cell type	*Marker*	*PymT$^{+/0}$;wt* *% of total ± s.e.m.*	*PymT$^{+/0}$;Tg(CTSB)$^{+/0}$* *% of total ± s.e.m.*	
CD4 + T cells	CD4	0.60 ± 0.17	0.61 ± 0.23	NS
CD8 + T cells	CD8	1.05 ± 0.15	1.79 ± 0.47	NS
B cells	CD19	0.51 ± 0.07	1.47 ± 0.55	$P<0.05$
Macrophages	F4/80	2.00 ± 0.30	2.83 ± 0.66	NS
Neutrophils	7/4 Antigen[a]	1.77 ± 0.41	2.25 ± 0.75	NS
Mast cells	CAE	18.8 ± 3.1[b]	31.7 ± 1.5[b]	$P<0.01$

Quantification of distinct immune cell types, including T and B cells, macrophages and neutrophils as percentage of total cells by flow cytometry ($n = 8$–12 per genotype). Mast cells were identified by CAE histochemistry and the number of CAE + cells per mm² of tumor stroma was calculated from three independent sectional planes ($n = 4$ per group). Data are presented as means and standard errors; statistical analysis was done by Student's *t*-test.
[a]Detected by anti-mouse neutrophils/Clone 7/4; rat IgG2a (Cedarlane, Burlington, Ontario, Canada).
[b]Number of mast cells per mm² tumor stroma.

In addition, MMP13-deficiency resulted in impaired pathological angiogenesis. In a collaborative effort, two MEM partners demonstrated the key contribution of Mesenchymal Stem cells (MSC) derived from the bone marrow in this MMP13-mediated angiogenic phenotype.

5. Lymphangiogenesis, angiogenesis and blood vessel maturation

The mechanisms leading to the formation of new blood vessels during physiological and pathological processes are well documented, but how migrating LEC organized into new lymphatic vessels is not known. Although lymphatic vessels are enclosed in a matrix structure mainly composed of collagens, the extent and importance of extracellular matrix remodeling in lymphangiogenesis is unclear. The formation of neovessels was studied by Transmission Electron Microscopy (TEM) in two *in vivo* models (lymphangiogenic corneal assay and

lymphangioma) and in the *in vitro* models of lymphatic ring assay. In both *in vivo* models, migrating lymphatic endothelial cells extended long processes exploring the neighbouring environment and organized into cord-like structures. Signs of intense extracellular matrix remodelling were observed extracellularly and inside the cytoplasmic vacuoles. The formation of intercellular spaces between endothelial cells led to lumen formation. The different steps of lymphangiogenesis observed *in vivo* are recapitulated *in vitro* in the lymphatic ring assay and include: (1) endothelial cell alignment in cord like structure, (2) intracellular vacuole formation and (3) matrix degradation as depicted in the following figure 7.

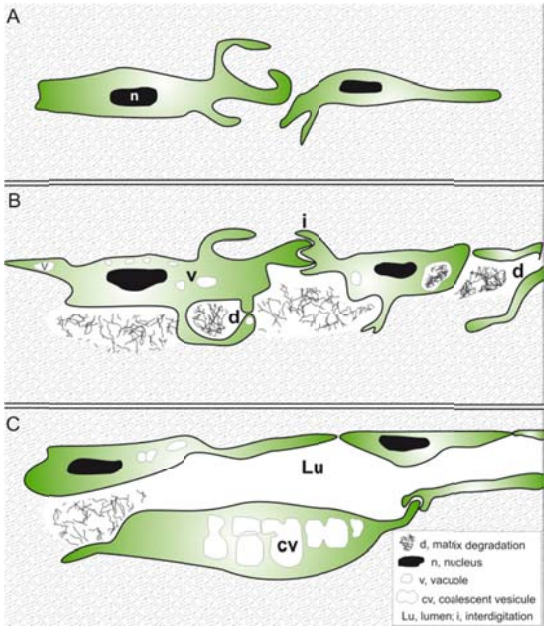


Figure 7: Model of formation of new lymphatic vessels during the process of lymphangiogenesis (see description in the text).

The aortic ring assay and the lymphatic ring assay are unique complementary tools suitable to unravel and compare cellular and molecular mechanisms of angiogenesis and lymphangiogenesis, respectively. These models have been applied to different KO mice including MMP2, MMP8, MMP9, MM13, ADAMTS-12, and PAI-1-deficient mice. The results clearly establish a differential impact of the proteases and protease inhibitors on angiogenesis and lymphangiogenesis.

A new aspect of this research has emerged with the involvement of miRNAs in the regulation of the hypoxic and angiogenic response in different cell types. Interestingly, the "hypoxia-miR", miR-210 appears as a key regulator of the hypoxia-inducible transcription factor (HIF) activity in tumour cells with potential consequences on cell metabolism, invasion

and angiogenesis. By contrast, another miRNA, miR-21 has demonstrated anti-angiogenic functions in endothelial cells.

WP3: The pre-metastatic niche

1. Context and objective

In this work package the consortium explored the contribution of the tumour-manipulated microenvironment on metastatic dissemination. We first challenged the validity of the recently proposed concept of the 'pre-metastatic niche'. This concept is an expansion of the 'seed and soil' hypothesis from the 19th century that already had pointed at the importance of the microenvironment in the target organ of metastasis for tumour cell tropism and colony formation. The concept of the pre-metastatic niche proposes that the primary tumour releases soluble factors, including growth factors and cytokines, which distribute in the body via the blood circulation and act at distant organs such that these tissues become well susceptible sites for the colonization by tumour cells disseminating from the primary tumour. This pre-conditioning action of tumour-released factors involves the activation of supporting scouts, namely cells from the bone marrow that lead the path for traveling tumour cells.

Prior to the creation of the *MEM consortium*, the concept of the pre-metastatic niche was only shown in lungs and lymph node. Some factors were proposed which rendered the distant organs more susceptible to metastasis by changing its gene expression profile and by inducing activation of bone marrow cells which infiltrate this prospective target organ of metastasis. Our aim was to validate this concept and to explore whether it has more general value. In addition, we asked whether proteases, their natural inhibitors, and functionally closely related factors play a role in this process.

2. Identification of the niche-establishing phase in new mouse models

The aim of this WP during the initial phase of the MEM project was to validate in a collaborative effort, the emerging concept of pre-metastatic niches in different target organs in metastasis models where this concept had not been described before. This consortium was able to validate and expand the concept of the pre-metastatic niche to a great variety of syngeneic murine and xenogeneic murine and human metastasis models in immunocompetent and nude mice, respectively. These models used by the different MEM partners included carcinomas (breast, lung, colon), melanomas, fibrosarcomas, and lymphomas. Notably, the

MEM consortium expanded the analyses of the niche from lung to the liver in spontaneous and experimental metastasis assays as well as spontaneous tumour models with modified oncogene expression. The detection of metastases was routinely improved by genetic tagging (lacZ marker gene) of the used tumour cells, allowing detection at the single cell level, as well as by RT-PCR.

In the aggressive B16F10 syngeneic melanoma model, the kinetic of metastasis was found highly variable offering a too short window for molecular analysis of the pre-metastatic niche. The limitation imposed by the heterogeneity of metastasis formation in this model was overcome with a different experimental set up using syngeneic T-cell lymphoma cells. The pre-metastatic niche was either induced by systemic elevation of the hypothesized niche-inducing factor in the mice, followed by analysis of the niche before tumour cell inoculation. Alternatively, a non-metastatic but tumour-forming variant of this tumour (genetically modified to over-express the hypothesized niche inducing factor) was inoculated into the skin of mice, followed by analyses of the niche. At certain time points, the respective pre-metastatic niches were challenged with a metastatic variant of the tumour. The spontaneous MMTV-PymT transgenic mouse model for breast cancer used by several MEM partners and applied to wild type mice or mice deficient for stefin B or cystatin C showed a pre-metastatic phase that could be distinguished from a micrometastatic and macrometastatic phase. The human MDA-MB-231/MT4-MMP xenograft model provides a window of 1 to 20 days after implantation of tumour cells for pre-metastatic niche analysis. With the exception of the contribution of the T-cell repertoire, this model can be used to investigate the pre-metastatic niche in a model of human cancer cells.

2. Identification of parameters establishing a pre-metastatic niche in lungs and livers

After establishing the kinetic of pre- and post-metastatic phases in these models, a molecular characterization of pre-metastatic organs was performed. For this purpose, gene expression profiles in the different target organs of metastasis were assessed by quantitative RT-PCR-based Low Density Array (LDA) or microarray technology, including both mRNA and miRNA expression analysis as well as protein analysis in situ as well as in biochemical assays. These studies were complemented with histological analysis of immune cell infiltration into the target organs of metastasis.

Our search for molecular candidates involved in the elaboration of a pre-metastatic niche was oriented by paradoxical clinical data showing that high levels of tissue inhibitor of

metalloproteinases-1 (TIMP-1) were associated with a bad prognosis for cancerous patients, although it inhibits pro-metastatic proteases. Thus, MEM partners postulated that TIMP1 might be a niche-inducing factor. To assess this hypothesis, TIMP-1 levels were systemically increased in mice by overexpression in the primary tumour or by systemic elevation in functional genetic recipient mice after adenoviral gene transfer.

The three major parameters that determine the pre-metastatic niche are a) the tumour-released factors, b) the bone marrow-derived cells (BMDC) which react on these factors and migrate to the tissue sites of prospective metastasis formation, and c) the expression signature (of the mRNA/proteins or regulatory RNAs (micro RNAs)) at the site of metastasis which may favour establishment of metastatic colonies. In close interaction, the consortium was able to elucidate many of these parameters.

a) Tumour-released factors

Based on the previous observation by one partner that elevated levels of the natural broad spectrum metalloproteinase inhibitor TIMP-1 in mice promote liver metastasis by inducing Met-signalling, it was further elucidated that TIMP-1 can indeed act as a typical inducer of a pre-metastatic niche and that many different tumour cell lines can take advantage of this niche. These findings for the first time explain the clinical paradox that a protease inhibitor (TIMP-1), which was thought to be anti-metastatic, correlates with bad prognosis. This sheds more light on the importance of the proteolytic network for the understanding of the complex cancer disease and points at the necessity of very specific and careful application of new anti-metastatic agents.

b) Reacting bone marrow-derived cells:

One MEM partner provided evidence that bone marrow-derived MSC promoted lung metastasis of Lewis Lung carcinoma and that these cells are induced by tumour cells to produce several proteases and a protease inhibitor known to promote invasion of tumour cells. Employing specific depletion experiments, neutrophils were identified as likely carriers of the metastasis scatter factor HGF, which was hypothesized to contribute to the Met-associated metastasis scattering in the liver. In this context it is important to note that highly specific inhibition of metalloproteinases (such as MMP-9) can also act on bone marrow-derived cells and induce them to secrete elevated levels of interleukin-6, causing circulating tumour cells to metastasize more efficiently. Macrophages closely associated with experimental lung metastases in the MMTV-PymT model show elevated expression of cathepsin B, suggesting

that cathepsin B expressed by metastasis-associated macrophages has a pro-metastatic effect. In a collaborative effort, two MEM partners provided evidence that Cathepsin Z, in combination with cathepsin B, may have synergistic contributions in those processes. Transfer of cathepsin L-deficient bone marrow into the K14-HPV16 mouse model of skin cancer did also not affect cancer progression suggesting a role for tumour cell-derived cathepsin L or cathepsin L expressed by non-myeloid cells of the tumour stroma.

c) Relevance of the pre-metastatic niche-associated signatures

In the different tumour models, the partners involved in this consortium confirmed the gene expression signature that had been described for the pre-metastatic niche in the lung and found very similar patterns in the liver including marker genes of neutrophils and many others, which can promote metastasis. Ongoing studies are unravelling the functional implication in metastasis of this large set of genes identified through microarrays and highthrough-put sequencing.

A key contribution of the *MEM consortium* is the demonstration of the functional implication of several factors induced by elevated systemic levels of TIMP-1:

- Host-derived plasminogen activators (uPA, tPA) are important executor molecules in the metastasis-promoting TIMP-1-modulated liver microenvironment.
- TIMP-1-upregulated cysteine cathepsins do not seem to be crucial as systemic overexpression of their natural inhibitor cystatin C in the host were found not inhibit the TIMP-1-induced niche effect. One partner generated new mouse models with the alternative cathepsin inhibitor stefin B expression that will be employed to determine the role of this inhibitor in the pre-metastatic niche.
- TIMP-1-induced stabilization of the presence of the Met tyrosine kinase caused by inhibition of its sheddase Adam-10 seems to be a crucial mechanism for metastasis scattering. The fruitful collaboration of two MEM partners led to the demonstration that Met can be shed by Adam-10, a metalloproteinase that can be inhibited by TIMP-1. In fact, pharmacological inhibition of Met signalling by a Met-specific antibody was found to be dependent on the activity of Adam-10 and would be curtailed in the presence of TIMP-1.
- Interestingly, tumour cells can only efficiently take advantage of the TIMP-1-induced metastasis-promoting microenvironment when they are able to produce sufficient

amounts of Hypoxia-inducible factor-1 α (HIF-1 α). Tumour cell-derived HIF-1α enhances the metastatic potential by a survival-independent mechanism.

- Specific systemic ablation of one target of TIMP1 (MMP-9) in the host impacts on the bone marrow as it induces interleukin-6 elevation in the serum and promoted metastasis of circulating tumour cells.

In addition to these achievements, the cooperation of all members of the consortium led to global scale signatures of mRNA and miRNA that could be associated with defined steps in the establishment of the pre-metastatic niche and the formation of metastatic colonies.

In summary, this working package supports the concept of the pre-metastatic niche with some important extensions by identifying the niche also in the liver. An important link has been established between the pre-metastatic niche and the regulatory functions of proteases, their natural inhibitors and their substrates. The impact of governing molecules in the niche such as TIMP-1, cMet, Adam-10, uPA/tPA, cysteine cathepsins, Hif-1α, MMP-13 emerged from this research. Importantly, we could reveal two paradigm-shifts. Firstly, we could explain the paradoxical observation from the clinic that elevated TIMP-1 levels correlate with bad prognosis of cancer patients. In this context we could reveal the pleiotropic function of TIMP-1 to render the liver more susceptible to metastases. The mechanism of this function is short from being elucidated, but we have already revealed that stabilization of Met tyrosine kinase at the cell surface through inhibition of its sheddase Adam-10 by TIMP-1 is one aspect of it. Secondly, we could demonstrate that one key pathway of metastasis, executed by the Met tyrosine kinase, can be inhibited by employing a Met-specific antibody. However, Adam-10 activity is a pre-requisite of the function of this antibody, pointing at the fact that some proteases (Adam-10) are crucial for therapeutic success of a Met-directed antibody.

WP4: Anti-metastatic therapy in transgenic cancer models

1. Context and objective

Studies using gene-deficient mice performed in WP1 and WP2 have revealed the importance of several proteases and other proteins involved in extracellular matrix remodeling for cancer invasion, progression and metastatic dissemination. As a model for therapy, our aim was to inhibit the activity of these proteins *in vivo* in the adult animal by administration of either inhibitory murine monoclonal antibodies (mAbs) against the murine proteins or small molecular inhibitors. The murine mAbs against the murine proteins were generated by

conventional hybridoma-technology using immunization of knockout mice with the autologous protein. The antibody-mediated effects have been compared with those of the gene knockouts, providing experimental evidence for similarities or changes between acute disruptions of function *versus* genetic disruption *in utero*. The effect of blocking cysteine cathepsins with a small molecule inhibitor was investigated in a mouse breast cancer model. Finally, the effect of blocking angiopoietin-1 and 2 and their receptor on primary tumour growth and metastasis was analysed in xenograft models.

2. Inhibitory murine mAbs against uPA

Two mAbs against the murine form of the protease urokinase plasminogen activator, muPA were shown to have inhibitory effect on plasminogen activation. Anti-muPA mAb mU1 prevented both plasmin-mediated pro-muPA activation and uPA-mediated plasminogen activation, while mU3 only blocked the latter of these reactions. The *in vivo* efficacy of mU1 and mU3 was determined using uPA-activable anthrax pro-toxin, because mice deficient in uPA are insensitive to treatment with this pro-toxin. Wild-type mice treated with mU1, but not with mU3, survived showing that mU1, but not mU3, has *in vivo* efficacy in blocking uPA activity.

From studies with uPA deficient mice, it has been demonstrated that uPA plays a role in skin wound healing as well as in fibrin clearance in the liver. When mU1 was administered to mice deficient in the other plasminogen activator (tPA), a dose-dependent increase in the healing time was observed. At the highest dose the mean healing time of the wound was not significantly different from that of the double deficient mice (Figure 8).

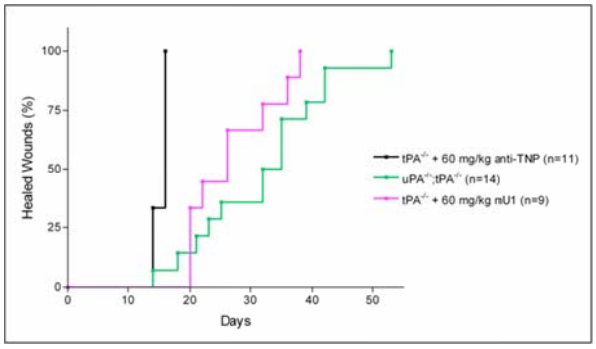


Figure 8: mU1 retards wound healing, demonstrating the efficient blocking of uPA function in a physiological invasive process *in vivo*.

Double-deficient $uPA^{-/-};tPA^{-/-}$ mice spontaneously develop hepatic fibrin deposits. Systemic treatment with mU1 resulted in fibrin accumulation in the livers of the tPA-deficient

mice, being indistinguishable from those observed in $uPA^{-/-};tPA^{-/-}$ mice. *These data further support the suitability of the blocking antibodies generated by the MEM consortium as therapeutic tool.*

3. Inhibitory murine mAbs against uPARAP

The uPARAP (also designated Endo180) is a collagen internalization receptor. When added to fibroblasts in culture, one of the mAbs, mAb 5f4, inhibited the cellular internalization of collagen. Interestingly, in a purified system the same antibody did not prevent the binding of uPARAP to collagen. Thus, the capacity for blocking collagen internalization may be related to another interesting characteristic in that the antibody down-regulates the expression of uPARAP on the cell surface of mouse fibroblasts and human MG63 osteosarcoma cells.

4. Murine mAbs against mMMP14

Even though the MMP14 knockout mice have a severe phenotype and a short life span, they have successfully been used for generation of anti-mMMP14 mAbs. None of the six anti-mMMP14 mAbs obtained have an inhibitory effect on the collagenolytic activity of mMMP14. Interestingly, however, one of the mAbs was capable of stimulating the MMP14 dimerization step on the cell surface that is necessary for MMP14-mediated activation of the soluble gelatinase, MMP-2. *These antibodies provide new tools to study and block the multiple functions of MMP14 that is recognized as an important therapeutic target.*

5. **Inhibiting cysteine cathepsins**

Cysteine cathepsins are involved in cancer development and upregulated in tumour stroma cells. It was shown that JPM-565, a small molecule, broad-spectrum inhibitor of cysteine cathepsins could be very potent in the treatment of pancreatic islet of cancer cells in a Rip1-Tag2 mouse model, whereas it lacked any efficacy in the MMTV-PyMT mouse breast cancer model due to its poor bioavailability. In order to improve this, we have developed a **novel delivery platform** based on magnet-sensitive lipidated nanoparticles that can target the tumour microenvironment. The core of the system represents ferrimagnetic iron oxide nanoparticles that could be used for magnetic targeting and, in addition, possesses superior MRI contrast properties, enabling non-invasive drug distribution monitoring. As such, successful tumour microenvironment targeting and uptake of cargo administered by ferri-liposomes were visualized *in vivo*. Finally, to improve the delivery of the inhibitor a novel

nanosized MRI-visible drug delivery system based on ferromagnetic nanoparticles encapsulated into lipid vesicles was developed. This delivery system was loaded with JPM-565 and administered to mice with one orthotopically transplanted mouse mammary tumour. A drastic reduction in tumour growth was obtained in the group of mice treated with magnetic targeting of ferriliposomes loaded with JPM-565 to the tumour compared to control untreated mice or treatment with JPM-565 alone (Figure 9). Thus, the improved bioavailability of cysteine cathepsin inhibitors identifies them as promising cancer therapeutics.

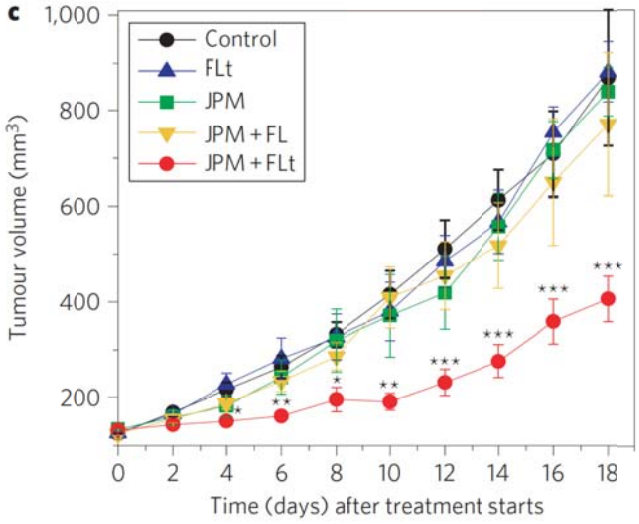


Figure 9: Anti-tumour effect of magnetically targeted ferri-liposomes containing JPM-565. Tumour volumes for each treatment day for the different treatment groups. Mice were treated with ferri-liposomes without (FL) and with magnetic targeting (FLt), and JPM-565 (JPM) combinations as represented by the '+' sign.

4. Soluble forms of Tie1 and 2 and Angiopoietin 1 and 2 as inhibitors of tumour angiogenesis, lymphangiogenesis and metastatic dissemination.

The angiopoietin-1 (Ang1)/Tie2 signalling pathway is known to play an important role in the regulation of vascular maturation and maintenance of vessel integrity. Systemic Tie2 inhibition using a soluble Tie2 receptor (sTie2) had no effect on intra-tumoural blood or lymphatic vessel density. However, expression of sTie2 reduced the number of infiltrating tumour homing macrophages. Soluble Tie1 (sTie1) administration to mice resulted in significant reduction in primary tumour growth compared to control treated mice. The metastatic spread of cancer cells from the primary tumour was not inhibited by sTie1 to a significant extent in the SCID mice but a pronounced inhibition of distant metastasis was seen in nude mice. This implies that background-specific immunological factors may contribute to the degree of the inhibitory effect of sTie1 on the metastatic dissemination. Treatment with adenoviral Ang1 promoted tumour cell metastasis into the lungs. Adenoviral expression of

Ang2 increased lymph node and lung metastasis in tumour xenografts, whereas Ang2-blocking antibodies reduced lymph node and lung metastasis, as well as tumour lymphangiogenesis. Ang2-blocking antibodies improved endothelial cell-cell junctions. Taken together this indicates that blocking Ang2 inhibits metastatic dissemination in part by enhancing the integrity of endothelial cell-cell junctions.

WP5: Integration and management of microarray information

1. Context and objective

This WP first aimed at standardizing procedures used by all partners in order to allow the comparative studies and the exploitation of data obtained in different metastatic models used by all partners. A WIKI system of communication has been developed in order to integrate and exchange information between the partners, in particular for expression and miRNA profiling data. Finally, dedicated bioinformatics tools have been developed in order to integrate miRNA-related pathways with expression data.

2. *Standard Operating Procedures (SOP).*

We first established SOPs to determine and compare the impact of protease web modulations on tumour growth, inflammation, angiogenesis, lymphangiogenesis, and metastatic dissemination. These methods have been spread among the consortium via our wiki information system and include a list of SOPs for immunohistochemical analysis as well as for analysis and quantification of metastasis.

3. *System of information.*

This WP has integrated microarray (at both the mRNA and miRNA levels) data obtained in all other work packages in order to identify key molecular pathways regulating different steps of metastatic dissemination. This data bank has strongly facilitated the access of partners to all data generated in different *in vivo* models of metastasis. The database Mediante has been developed in order to contain detailed experimental settings, the mRNA and/or miRNA profile, crude and comparative data generated in one experimental model applied to different transgenic mice or upon different treatments. This platform is in compliance to the standard recognized by international and industrial partners (ISO 9001 v.2000). The development of new tools has been mainly devoted to (i) Integration of data from miRNAs/pan-genomic

microarrays and recently High Throughput Sequencing (HTS) profiling, (ii) *In silico* and experimental miRNA target identification tools.

4. *Data integration.*

A large serie of microarrays (expression and miRNAs) and HTS experiments have been performed and analysed. Associated data are discussed in WP1, WP2 and WP3 sections. Since the beginning of the MEM project, the partner in charge of microarrays and HTS sequencing has processed more than 200 samples from other MEM partners (40 miRNA microarrays, 100 whole genome microarrays and 80 small RNA sequencing). Associated data are available to all MEM partners through the Mediante database.

5. *Development of miRNA dedicated bioinformatics tools.*

In the majority of experiments, a parallel measurement of mRNAs and miRNAs has been performed, and one key biological question has been to evaluate the impact of miRNA on transcriptome modulations. The underlying question concerns more globally the identification of miRNA targets. Indeed, the interaction between the miRNA and its targets stems from a short stretch of 6-8 nucleotides located 5' of the miRNA, termed the "seed sequence" and one miRNA can theoretically target hundreds of mRNAs. Because several miRNAs can also target the same transcript, the miRNA regulatory network appears amazingly complex. In order to facilitate the work of biologists, several bioinformatics prediction algorithms, ie: TargetScan, miRBase Targets and Pictar have been integrated into our system of information. If most of them use the "seed match" as the main rule, these methods often lead to very distinct predictions. Some reasons for this imperfect overlap includes the precise treatment of the seed match, the allowance of GU wobble pairs or the length of the seed match (6, 7 or 8 nt), the potential contribution of 3'-supplementary pairing, the degree of conservation of the site across species, the 3'UTR context or the use of different 3'UTR database. Moreover, additional "home-made" bioinformatics tools have been developed and were available for all partners. The miRSuite web portal provides original tools for the study and the comprehension of miRNAs action. This portal is written in Java and is based on information stored in a PotgreSQL relational database. Three main programs have been developed: (i) MicroCible, a miRNA target predictor that scans transcripts sequences for the presence of "miRNA seed" complementary sequence; (ii) SnipMiR, for the search of putative miRNA binding sites within polymorphic regions, notably within mRNA variants associated with disease susceptibility and that has been used successfully to identify a site for the miR-196

family in a protective variant of the IRGM transcript, previously associated with Crohn's disease; (iii) miRonTop that uses mRNA expression profiles to identify miRNAs targets, as described below.

6. Combining *in silico* and experimental tools to identify miRNAs targets.

Because the bioinformatics prediction tools give a high percentage of false positive results, several experimental approaches may be performed to improve these predictions. MiRonTop uses paired expression profiles of miRNAs and mRNAs and provides a complement to computational predictions. It has been used in several studies developed in the context of MEM, notably for the identification of key targets of miR-210 in the context of tumour hypoxic microenvironment and represents the routine method for the search of miRNA targets in the ongoing characterization of the MMTV-PymT model. Recently, this tool has been used to explore a large data set of miRNA-overexpressed microarrays experiments in order to identify miRNAs with overlapping regulatory functions. A close relationship between two miRNAs from distinct families, hsa-miR-147b and hsa-miR-210 has been found. Following this preliminary observation, further studies have contributed to better define the important pairing and molecular rules controlling their binding and to investigate their cellular function.

7. Small RNA profiling using high throughput sequencing.

Major improvements in nucleic acid sequencing have occurred during the last ten years, with sequencers of the latest generation being able to read more than 100 gigabases in one run. This renders feasible sophisticated experimental approaches on nucleic acids that were simply out of reach one or two years ago. A large renewal of our conceptions regarding gene expression, genome organization, population genetics and species interactions can thus be clearly anticipated in the years to come after applying these new approaches on well characterized biological models. In that context, the MEM consortium has found critical to have access to this technology, in particular regarding the characterization of the PymT mouse model. The Nice Sophia Antipolis Functional Genomics Platform associated with Partner 6 has acquired a high throughput sequencer (SOLiD™, Applied) in October 2008. One the first available application has concerned the sequencing of small non-coding RNAs. Small RNA sequencing provides additional information that are not available using classical microarray or qPCR experiments, such as mutation, editing or catabolism of the sequences of interest and information about new classes of small RNAs (piRNAs, fragments of snoRNAs or tRNAs, etc…) for which no prior information was available. The library preparation techniques have

been optimized and development of the use of different barcodes has now been fully mastered, allowing the use of up to 48 commercial barcodes in the last version (SOLiD v4). The development of these new protocols has been coupled with bioinformatics developments regarding de novo transcriptome assembly, or integration of gene expression data into our database Mediante web server. This became accessible to all MEM partners of high-troughput sequencing which has already been applied to the PyMT model selected by the consortium as a prototype model of breast cancer metastasis, as described below.

4) Potential impacts (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results (not exceeding 10 pages).

After cardiovascular diseases, cancer is the main cause of death in developed countries. **Metastasis** leads to a significant reduction of the quality of life, is the main reason for the pains, and still represents the major death-determinant for cancer patients. Thus, novel and effective anti-metastatic therapies are urgently needed. Diverse clinical approaches have failed to alleviate metastatic spread of neoplastic disease, indicating that the process of metastasis is not sufficiently understood. Accordingly, the design of novel anti-metastatic treatment strategies depends on improved knowledge of molecular mechanisms underlying tumour cell scattering and spreading in ectopic tissue microenvironments in patients with cancer.

The MEM consortium thus addressed one of the most urging questions in cancer research: How can we better understand the process of metastasis, i.e. the devastating feature of malignant tumours that is the major cause of death in cancer patients? The consortium focused mainly its interest on **metastasis into lung, liver and lymph nodes**, the major organs in which secondary cancer foci develop. Since breast cancer is the most common form of women cancer in the EU, we especially employed several transgenic and cellular models of breast cancer. Breast cancer represents the most often diagnosed female cancer. Its incidence increases sharply between the ages of 30 to 50 meaning that the disease affects many women in the prime of life. The MEM partners have also used other models of cancer to extend knowledge to various types of cancer. Improvements in the treatment and management of cancers would have impact on both health and economy.

The MEM project was initiated by considering the novel emerging concepts of metastasis. It used an innovative approach focusing on the **interface between tumour cells and their microenvironment**. This approach consisted of the modulation of the tumour microenvironment by interfering with proteases, which are key regulators of a complex molecular network, to test for the role of this protease-web on the control of early and late steps of cancer progression and dissemination. This original approach was

successful and expanded our insight into the protease landscape. Only by altering specific components of this network, it has been possible to identify proteases with pro-tumourigenic or pro-metastatic functions, as well as proteases with tumour-defying properties. The fact that proteases might have opposite functions, either promoting or inhibiting cancer progression provides an explanation to the failure of MMP inhibitors used in the past in the clinic. Notably, in some cases, the administration of MMP inhibitors led to increased cancer development rather to the expected reduced growth. The recent identification of mutation in protease genes in melanoma and colorectal cancers highlights the growing list of MMPs with protective functions against tumour development. ***Altogether, these findings underline the requirement of a better understanding of the multifaceted aspects of the molecules to be targeted with new drugs. They warrant against a premature and therefore unsuccessful transfer of promising therapies into the clinic.***

The MEM research led also to an important validation of the emerging concept referred to the "pre-metastatic niche". According to this concept, some factors produced by the primary tumour induce important changes in distant organs promoting thereafter the colonization of disseminating cancer cells. Such validation has direct impact on future therapeutic intervention. In fact, a molecule identified as being able to render an organ more susceptible to metastasis represents an important drug target. The consortium was able to expand the concept of the pre-metastatic niche to a great variety of different tumor types and identified conditions of pre-metastatic niche formation in another organ, the liver. This organ is of exceptional relevance as this organ is affected by most cancers and liver metastasis offers extremely poor therapeutic options. Therefore, the MEM consortium findings open new avenues to interfere with the so far devastating and uncontrollable spread of malignant tumours, e.g. at early stages of the disease, prior and shortly after removal of the primary tumour and in late stages of cancer disease, when new waves of metastatic spread occur.

Importantly, the MEM consortium reveals two paradigm-shifts. Firstly, it provides an explanation for the paradoxical observation from the clinic that elevated TIMP-1 levels correlate with bad prognosis of cancer patients. In this context, MEM partners demonstrate the pleiotropic function of TIMP-1 to render the liver more susceptible to metastases. Secondly, they demonstrate that one key pathway of metastasis, executed by the oncogenic MET tyrosine kinase, can be inhibited by employing a MET-specific antibody. However, ADAM-10 activity is a pre-requisite for the function of this antibody, pointing

at the fact that some proteases are crucial for the therapeutic success of drugs targeting an oncogene.

The MEM project has identified key molecular pathways that play a crucial role in metastatic dissemination of cancers. Notably neutralizing human and murine antibodies directed against selected key targets were generated by using state-of-the-art biotechnologies. The value of such neutralizing antibodies as therapeutic drugs has already been demonstrated to treat different pathologies. Compared to small molecular inhibitors, the antibodies possess an amazing selectivity and have a long half-life in circulation. The Consortium is providing novel blocking antibodies that are new tools for exploring the multiple complex functions of proteases and their partners. These antibodies also provide a basis for the rational design of novel anti-metastatic treatment strategies that are under investigation by the different MEM partners. An in-depth investigation of the mechanisms of action of these antibodies and their putative side effects is mandatory before transfer to clinical assays.

The MEM partners have demonstrated that the inhibition of cysteine cathepsins could be a potent strategy for treating cancer. The first study on the use of the broad-spectrum small molecule cysteine cathepsin inhibitor, JPM-OEt, in cancer mouse models demonstrated significant anti-tumour efficacy in three distinctive trial designs: prevention, intervention and regression. Unfortunately, there might be some limitations for the use of small synthetic probes in the clinic, primarily because of their pharmacokinetic properties, such as relatively short circulation half-life and poor bioavailability. To overcome this limitation, a system for targeted drug delivery based on magnetic nanoparticles and biocompatible lipid shell, forming ferri-liposomes, has been developed and validated by the MEM consortium. This offers new options for drug delivery into the tumour microenvironment.

The major impacts of the MEM consortium findings are listed below.

Technological impacts

- Available biotechnological platforms allowing the identification of set of genes regulated during cancer progression and metastatic dissemination (miRNA microarray, “Small RNA” and “Whole Transcriptome” High-throughput sequencing, LDA arrays).
- Available bio-informatics tools for the identification of miRNA targets.
- Therapeutic molecules (neutralizing monoclonal antibodies) with specific action on tumour or tumour microenvironment.

- Safe system of drug delivery based on magnetic nanoparticles (ferri-liposomes) to target the tumour microenvironment.
- Improved *in vitro* 3D models mimicking different steps of cancer progression (cell invasion, (lymph)angiogenesis, interstitial matrix degradation…) for pre-clinical studies on other cancers.
- Concerted generation of novel animal models for pre-clinical studies on other cancers.
- Validation of knock out mice for the generation of neutralizing antibodies.
- Demonstration that acute abruption of function in the adult animal using neutralizing monoclonal antibodies can mimic phenotype of knockout mouse.

MICROENVIMET outputs

- Advance in the knowledge of tumour-host interface.
- Better understanding of the basic mechanisms of early and late steps of cancer progression.
- Validation of the pre-metastatic niche concept.
- Extension of the pre-metastatic niche to the liver, an organ often colonized by metastatic cells with poor treatment options.
- Identification of relevant molecular pathways regulating early steps of metastatic dissemination.
- Advance in the knowledge of cancer stem cell biology in the context of metastasis and identification of the MET pathway as key mediator of cancer stem cell fate.
- Identification of TIMP1 as a key regulator of the pre-metastatic niche.
- Providing an explanation of a clinical paradox (high levels of a protease inhibitor being associated with poor prognosis for cancer patients).
- Providing an explanation on why MMP inhibitor failed in clinical trials.
- Generation of a platform of mice transplanted with samples of metastatic colorectal cancer, to be used as a preclinical model for testing compounds active on metastatic cancer cells.
- Elucidation of the mechanism of action of therapeutical anti-Met antibodies and demonstration that some proteases (ADAM) are essential for the antibody activity.
- Design of a novel, safe and efficient therapeutic strategy using magnetic nanoparticles (ferri-liposomes) to target the tumour microenvironment and to fight metastasis. By

improving drug efficacy and reducing side effects, such an approach is expected to improve **quality of life** during and after patient treatment.

- Generation of mRNA and miRNA profilings for the identification of molecular targets in primary or secondary tumours. This set of data provides an important source of information whose current exploitation will increase our knowledge on the mechanisms underlying metastatic dissemination.
- Acquisition of new know-how that can be applied to other fields and diseases such as those associated with abnormal (lymp)angiogenesis (age related macular degeneration, graft rejection, lymphedema) or aging.
- Identification of novel candidate proteins associated with metastatic spread of cancer.
- Identification of novel miRNAs candidates associated with metastatic spread of cancer.
- Demonstration that miRNAs may constitute mediators of microenvironment modifications.
- Providing evidence that inhibition of protease activity with monoclonal antibodies has efficacy *in vivo.*

In addition to these scientific achievements, the MEM project has also a great impact on the **competitiveness of Europe** regarding cancer research and cancer therapy. Indeed, it leads to a better understanding of the basic mechanisms underlying cancer dissemination and to the validation of novel concepts of metastasis. The MEM project has helped in keeping **Europe on the leading edge of Cancer Research**. The complexity of the topic research requires a high degree of networking between experts in the field. The MEM consortium represents an easily manageable network with a critical mass of complementary expertise. It has created a coherent and competitive European scientific network with unique expertise on functional and pre-clinical validation on *in vitro* and *in vivo* tumour models. **The MEM partners have demonstrated their exceptional capacity to conduct a complex shared transcriptomic study on a dedicated multi-stage tumor animal model**. Indeed, in a collaborative effort involving all MEM partners, transcriptomic (mRNA and miRNA) profilings of primary and secondary tumours issued from PyMT mice have been performed. Therefore, the MEM project represents an **European added value** because no single group could have performed such a study alone. The integration of research efforts performed during the MEM project has been an important stimulator of increased competitiveness.

The MEM consortium has also contributed to structure the European Cancer Research in the field of cancer cell invasion and metastasis. Contacts have been established with other consortia and teams focusing on metastasis. However, this momentum will not be maintained due to the disturbing absence of new calls on basic research on metastasis and even on cancer in 2011 and 2012. The recent failure of clinical trials using MMP inhibitors as well as the limited effects of anti-angiogenic compounds in clinic, should provide enough evidence that it is mandatory to increase our basic knowledge of selected targets that might display multiple effects with sometime unanticipated opposite impacts on cancer progression. The data generated by the MEM consortium require further analyses for functional validation, which cannot be achieved by individual partners alone. In the absence of European support, the major opportunity to upgrade the collaborative efforts generated during the MEM will be irreversibly lost as no national support could substitue the lack of European support. **As a consequence, the absence of basic cancer research funding is expected to limit the competitiveness of the European life science and biotechnology sector in a very short time, and to make Europe falling behind the main competitors such as the USA.**

To ensure dissemination and exploitation of results emanating from MEM consortium, a system of information has been established to allow constant and tight communication within the consortium (**internal communications**) and with the scientific and clinical communities (**external communications**). With this aim a ***MICROENVIMET web site (***http://www.microenvimet.eu/) has been set up and maintained during the course of the project. The Internet portal was split into a private and a public area. This web site was extremely helpful to provide a platform for dissemination of knowledge generated throughout the project, as well as to maintain an updated basis for the data generated.

Internal dissemination of project results

Through the MICROENVIMET web site, all partners have a restricted access to the following relevant information:

- Scientific information:
  - project information: objectives, summary of WP, list of deliverables, list of milestones.
  - library of publications
  - reports
  - minutes of meetings.

- Technical information: - Standard Operating Procedures
  - lists of tools, models and models.
- Relevant contacts: - links to partners and partner Institutions.

In addition, a secured **wiki server** was created to improve contacts between partners. Members were able to add information, remove, edit and change content. The interest of this system is the ability to rapidly and easily create and up-date pages. This wiki server was only opened to the MEM members after registration.

External dissemination of project results and activities

The continuous dissemination activities are listed in the annexed tables and concerned the following issues:

- Publications in peer-reviewed journal.
- Presentations of results at national and international meetings (posters, oral presentations): > 80 talks given by the different partners.
- **A Research Topic on « Tumour microenvironment in primary and secondary sites: impact on metastasis » has been edited on Frontiers Pharmacology.** Five reviews written by the MEM partners are updating our understandings of tumour microenvironment and cancer stem cell biology in the context of metastasis with an outline on clinical implication. These reviews will be freely accessible on line.
- a satellite conference organized at mid-term and devoted to present and discuss data emerged from the MICROENVIMET project has been organised as a satellite meeting of the EMBO Molecular Medicine Workshop “Invasive Growth: a Genetic: a Programme for Stem Cells and Cancer”, organized by Paolo Comoglio and Carla Boccaccio.
- A “Joint Meeting” has been organized on the topic of Non-Coding RNAs (“microRNA and small non coding RNA: new actors in physiopathology") in October 2009 in Nice by Bernard Mari. Members from two other FP7 supported miRNA projects were represented : SCIROCCO (O. Voinnet and A. Harel-Bellan, http://www.sirocco-project.eu/) and ONCOMIRS (JC Marine, coordinator, http://www.dmbr.ugent.be/oncomirs/index.html).
- An international “MicroEnviMet Symposium” organised at the end of the project by Agnès Noel, on February 24, 2012 in Liège, Belgium. All MEM

partners presented and discussed data and concepts emerging from MEM project.

- Contacts with other consortia working on metastases have been established at several occasions. For instance, two MEM partners presented their data at the international meeting on "New concepts in cancer metastasis" organized by J. Sleeman on June 25-28, 2011 in Lisbon.

Public awareness

- Project information (summary) is posted on the MICROENVIMET web site.
- Several Press releases have been performed by different partners when the project started and during the course of the project.

Exploitation of project results

Although a policy of wide dissemination of project findings and results has been pursued throughout the course of the project, care has been taken in implementing activities of dissemination to ensure that possibilities for Intellectual Property Protection are not compromised. A Consortium Agreement has established the procedures relative to Intellectual Property Rights (IPR) management, knowledge dissemination, and clarifies the points relative to pre-existing knowledge and to the management of new discoveries (exploitation, patenting and publications). This document provides the foundation for a comprehensive knowledge management process, with the aim to transform knowledge into economic value.