When a collective outcome triggers a rare individual event: a mode of metastatic process in a cell population

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Abstract

A model of early metastatic process is based on the experimentally assessed role of a protein, PAI-1, which at high enough extracellular concentration promotes the transition of cancer cells to a physiological and morphological state prone to migration. This transition is described at the single cell level as a bi-stable switch generically associated with a subcritical bifurcation. In a multilevel reaction-diffusion scenario, the microenvironment of the tumor is modified by the proliferating cell population so as to push the concentration of PAI-1 above the bifurcation threshold. The formulation in terms of partial differential equations fails to capture spatio-temporal heterogeneity. Cellular-automata and agent-based simulations of cell populations support the hypothesis that a randomly localized accumulation of PAI-1 can arise and trigger the escape of a few isolated cells. Away from the primary tumor, these cells experience a reverse transition back to a proliferative state and could generate a secondary tumor. The proposed role of PAI-1 in controlling this metastatic cycle would partly explain its well-documented role in cancer progression.

Running title: Multilevel metastatic escape model

Keywords: cell population, metastastic escape, multilevel modeling, reaction-diffusion, agent-based simulation, multi-stability.

Abbreviation. PAI-1: Plasminogen-Activator-Inhibitor-1 (a protein, either intra-cellular (subscript i), soluble in the extracellular space (subscript s), or matrix-bound (subscript m)).

1 Introduction

Due to lethal consequences of metastatic spreading of cancer, understanding and controlling the processes underlying the formation of metastases is a major challenge, remaining largely open. Several modes of metastatic spreading (letting aside surgical dissemination) were identified: (i) transport in lymphatic circulation, (ii) transport in blood circulation, and (iii) a mode involving a specific migration mechanism, the *amoeboid migration* (Friedl, 2004). The present paper focuses on this latter mode. Taking place at the cell scale, it appears less pervasive than the first two ones, where circulation-facilitated transport spans the whole organism. However it is less dependent on the anatomical features of the location of the tumor and is a candidate for the early events of the metastatic spreading, before metastatic cells reach the lymphatic or the blood circulation. It might well be an essential preliminary step common to all metastatic processes.

A difficulty comes from the fact that early events involved in the escape of a cancer cell from the primary tumor are rare events, too rare to be easily observed or experimented in

varying conditions. Only a small fraction of tumor cells provides the seeds for secondary tumors. Accordingly, experimental protocols are restricted to indirect investigations, mainly genetic and biochemical analyses of metastatic cells compared to those of the primary tumor (Witz, 2008), or statistical tracking of the number, location, and genetic lineage of secondary tumors (Albini, 2008). An increasing number of experiments focus on the biochemical analysis of the surrounding microenvironment (Taylor et al., 2008), the morphological signature of potentially metastatic cells (Vincan et al., 2007) and the reproduction *in vitro* of the epithelial-mesenchymatous transition and the mesenchymatous-amoeboid transition which affect the morphology and the proliferative and migratory capacities (amoeboid migration) of cells of epithelial origin (Malo et al., 2006). These complementary experiments have shown that the metastatic process involves jointly genetic determinants (accumulation of specific mutations (Gerstung and Beerenwinkel in this issue)), biochemical factors (triggering new pathways or switching existing ones, leading to modifications in the cell state and metabolism), and requirements about the state and geometry of the microenvironment —that is, the extracellular space and matrix— of the tumor cells. However there is no integrated understanding of this process so far. The difficulty comes from the different natures, locations, and time scales of the potential causal factors, and is strenghtened by the rarity of metastatic events. Modeling is then essential to articulate the different partial and indirect experimental results, and to validate their interpretation in an integrated scenario, bridging molecular, cellular, extracellular, and cell population levels.

From biological facts about the mesenchymatous-amoeboid transition and amoeboid migration, we wish to explain how permissive conditions for the mestastatic escape of a few cancer cells might be collectively induced at the cell population level. Our claim is that proliferation-induced modifications of the tumor microenvironment could produce a feedback localized in a few privileged individual cells, selected by a complex conjunction of stochastic and history-dependent molecular events. Our working hypothesis, supported by experimental results (Malo et al., 2006), is the central role of the protein, PAI-1, synthesized at high rate by the cancer cells and released in their immediate environment. We shall describe how this molecule could mediate an interplay between intra-cellular, extracellular, and cell population features, switching a few cells into a state prone to escape and migration, then switching them back into a proliferative state at a distance of the primary tumor (this is the process of metastasis). This scenario is rooted in a generic description of the single cell state in bifurcation theory, supported by *in vitro* experiments. Its spatially extended formulation at the cell population level is achieved in a reaction-diffusion model, implemented either in the standard framework of partial differential equations or in the one of cellular automata and agent-based simulations. The predictions of our study motivated and guided new experiments proving the existence of the reverse change from the amoeboid to the mesenchymatous state and backing up the dynamic nature of this transition. Finally, we resort to catastrophe theory to suggest a possible path towards the cancer stage where amoeboid state and migration can be observed.

2 Biological Setting

Considering epithelial cells, a first transition towards a cancerous state is observed, originating in accumulating mtuations and leading to the so-called *mesenchymatous state* (see

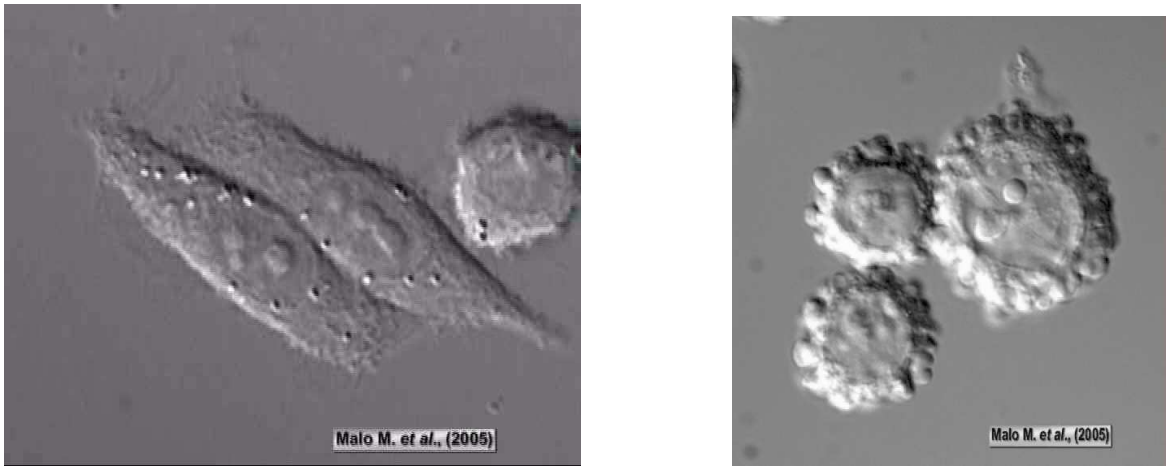

Figure 1: Epithelial cancer cells (colon cancer). (left) Mesenchymatous state responsible for the destabilization of epithelium and prone to proliferation. (right) Amoeboid state characterized by a blebbing morphology and by modified adhesion leading to a special migratory ability. The mesenchymatous-amoeboid transition (as experienced by the rightmost cell in the left hand side picture) is likely to play a key role in early metastatic escape.

Figure 1left). In this state, cell-cell junctions are no longer established and the epithelium is destabilized. This state has moreover a strong proliferative capacity, hence the transition to this mesenchymatous state is generally associated with the appearance of a well-defined tumor (Thiery, 2002). In invasive epithelial tumors, it is the default state of the cells (Gavert and Ben-Ze'ev, 2008) and it will be the default state of the cell population in our model.

A second transition may occur towards the so-called *amoeboid state* (see Figure 1right) identified by a specific and persistent "blebbing" morphology (round shape with dynamic actin rings visible at the cell periphery). This mesenchymatous-amoeboid transition is associated with a change in adhesion properties (more precisely, adhesion becomes integrin-independent, the actin cytoskeleton reorganizes and a modification of one of its regulatory pathways, RhoA-pathway, occurs: it now involves an auxiliary protein, ROCK, while there is no longer proteolysis of the extracellular matrix (Friedl, 2004; McCarthy, 2009). Due to its peculiar features, a blebbing cell can move fast and progress by exploiting interstices of the substrate with no need of matrix proteolysis. Accordingly, amoeboid migration is a very efficient mode of migration in a tissue, encountered in normal conditions during some developmental stages (Thiery, 2002); in a pathological context, it was suggested as a privileged mode of metastatic migration (Friedl and Wolf, 2003; Berx et al., 2007).

In the mesenchymatous state, migration is quite inefficient while proliferation is very active. Proliferation is mostly controlled by cell density for obvious steric reasons. In the amoeboid state, cell migration is very efficient. Roughly, *proliferation occurs at high rate in the mesenchymatous state whereas migration occurs more efficiently in the amoeboid state*, in agreement with the current wisdom that proliferation and migration are mutually exclusive processes in a given cell.

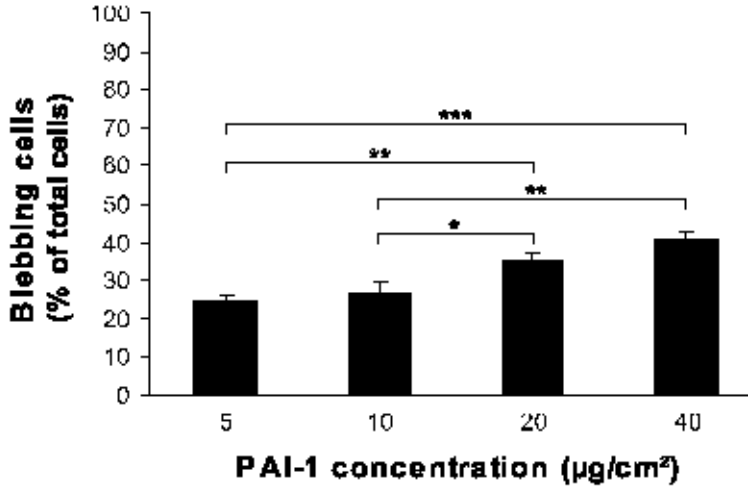

Figure 2: *In vitro* observation of the effect of the concentration of substrate-bound PAI-1 on cell morphology and the mesenchymatous-amoeboid transition. The proportion of blebbing MDA-MB-231 breast cancer cells is measured at fixed PAI-1 concentration (values 5, 10, 20, 40 μg/cm^2); $* \; p < 0,05$; $* * \; p < 0,01$; $* * * \; p < 0,001$.

Recent observations *in vivo* hint at a key player in amoeboid migration, metastases, and more generally cancer progression: the plasminogen-activator-inhibitor protein of type 1, henceforth termed by its acronym PAI-1. It is an ubiquitous species involved in several pathways and functions, among which some aspects are relevant for metastatic process. It is found in the surroundings of the most invasive tumors (Pedersen, 2005; Wilkins-Port and Higgins, 2007; Wilkins-Port et al., 2007) and considered as a marker of bad prognosis (Jänicke e al., 2001; Look et al., 2002; Castello et al., 2007; Biermann et al., 2008). PAI-1 is encountered under several forms: the newly synthesized molecule in the cell (*internal PAI-1*), as a soluble form in the extracellular medium (*soluble PAI-1*). This latter form can either diffuse in the extracellular medium, or bind to the extracellular matrix (*matrix-bound PAI-1*), or be trapped on the cell surface and deactivated, or be internalized and degraded with no further known consequence on the state of the cell. On the contrary, internalization of matrix-bound PAI-1 occurs through the formation of a tripartite complex with a membrane receptor, uPAR, and a molecule, uPA. Its role in modifying the cell physiology (specifically, in modifying the activity of RhoA pathway) is acknowledged (Chazaud et al., 2000). Strikingly, when cancer cells are placed on artificial substrates with high concentration $c_m > c_m^*$ of matrix-bound PAI-1, they experience the above-mentioned mesenchymatous-amoeboid transition (Malo et al., 2006). In this respect, matrix-bound PAI-1 can promote cancer cell migration, at least *in vitro* (Friedl and Wolf, 2003). Moreover, these experimental results, presented on Figure 2, indicate that the mesenchymatous-amoeboid transition is not due to some mutations but is rather a dynamic transition between two different states of the cell, controlled by its environment.

Cancer cells synthesize more PAI-1 than normal cells do; PAI-1 molecules are then se-

creted in the extracellular medium and can bind to the extracellular matrix at tumor boundary regions, where the matrix is not fully occupied by cells. Hence, concentration of the matrix-bound PAI-1 in the border region of the tumor is expected to be higher than around normal cells. This was observed experimentally (Look et al., 2002; Chazaud et al., 2002). Cancer cells also produce more uPA and have an increased number of uPA membrane receptors uPAR, directly involved in the internalization of matrix-bound PAI-1. The net result is an *increased internalization flux J_i in cancer cells*, hence an amplification of the ensuing cell metabolic and morphological changes compared to normal cells.

From these experimental facts, we shall start from the fact that given cancer cell, with regards to its metastatic potentialities, can be in two different states, the mesenchymatous and the amoeboid one. We adopt the leading pattern according to which the abrupt mesenchymatous-amoeboid transition of a cell is controlled by its internalization flux of matrix-bound PAI-1. The coupled dynamics of several cells and extracellular medium have then to be considered. Indeed, proliferation in mesenchymatous state turns a single cell into an aggregate collectively contributing to the concentration of matrix-bound PAI-1. We shall therefore embed the dynamics of a single cell in a spatially extended population model, focusing on the spatio-temporal varying internalization flux of the matrix-bound PAI-1, considered as a marker of the metastatic potentialities of the cells.

3 Model

3.1 Challenges

Let us first avoid a possible misunderstanding: what we term a *model* is a way to check the consistency of several experimental facts and partial mechanisms (possibly occuring at different levels), to put forward control parameters, thresholds, switch behaviors and quantitative hints on the major determinants of the fate of the system. It should allow us to derive testable consequences of the hypotheses and suggest novel protocols. A model is a necessary intermediary step between qualitative understanding and further experimental evidence and can be seen as a way of hypothesis testing. .

The guideline of our investigations is the multilevel and intricate mechanisms by which PAI-1 could play a role in the fate of cancer cells and in the metastatic process. As shown in Section 2, these mechanisms have functional consequences at four different levels:

- *at the molecular (intra-cellular) level*: internalization of matrix-bound PAI-1. The internalization of soluble PAI-1 has no intra-cellular consequences and amounts to a mere degradation;

- *at the cellular level*: mesenchymatous-amoeboid transition. The experimental fact that the concentration c_m of matrix-bound PAI-1 is a major determinant of the mesenchymatous-amoeboid transition (Figure 2) will be formulated in the framework of bifurcation theory at the single cell level, with a control parameter related to c_m. The molecular analysis of intra-cellular pathways and morphological transformations shows that a more straightforward determinant of the mesenchymatous-amoeboid transition is the internalization flux J_i of the cell. Our hypothesis is that intra-cellular dynamics

determining the overall cell state shows a bifurcation revealed by a switch between two markedly different mesenchymatous and amoeboid states, at some threshold value $J_i = J_i^*$.

- *at the cell population level*: the mutualized secretion of PAI-1, feeding extracellular PAI-1 species. Cell growth and division are considered at this level;
- *at the microenvironment level*: soluble PAI-1 diffusion in the extracellular medium and binding on the cell-free matrix, thus turning into matrix-bound PAI-1.

The point is to explain how the interplay between the various forms of PAI-1 and the various levels at which they are produced, controlled, or used, can trigger the mesenchymatous-amoeboid transition and more generally can explain the metastatic process. Complementary modeling approaches are essentially needed to capture the multilevel determinants and mechanisms at work. In order to bring out a robust explanatory scenario, we devise *the most parsimonious model*. Such models often need collective variables and effective parameters, accounting in a bottom-up and already integrated way of a wealth of elementary mechanisms (here, the use of average densities and pseudo-first order kinetics, see below). In complex systems, they also involve effective inputs or boundary conditions, accounting in a top-down way of the influence of the system emergent features or structures and surroundings on elementary parts and mechanisms (Lesne, 2008b). The main quality of a parsimonious model is the robustness of its predictions with respect to small changes in the microscopic ingredients, because they will only slightly affect the value of the effective parameters without modifying the general form of the model (Lesne, 2008a). For instance, a bifurcation (here the mesenchymatous-amoeboid transition) will still be observed with possibly only a shift of the bifurcation location. The confrontation with experimental observations would validate the leading principles and scenario. It is then another part of the work, involving in general different data and computational tools, to substantiate the minimal model with underlying mechanisms and explicit ingredients in order to derive the exact numerical values of the effective parameters, interactions and reactions. The use of a minimal model is here all the more essential that no experimental access to the values of e.g. kinetic parameters is today possible, nor a direct experimental investigation of the metastatic process.

3.2 Reaction-Diffusion Model

We first consider a description (termed *mean-field-like description* in statistical physics for interacting many-body systems) in which soluble PAI-1, internal PAI-1, and matrix-bound PAI-1 species are described by means of smooth deterministic concentrations. The cell population is described by a smooth deterministic cell density. The experimental observations are formalized in terms of chemical kinetics, diffusion, and growth, but the discrete nature of cells and molecules, and the stochasticity of the elementary processes are no part of the mean-field description. The set of essential variables includes the concentrations $c_m(\vec{r},t)$ of matrix-bound PAI-1, $c_s(\vec{r},t)$ of soluble PAI-1, and $c_i(\vec{r},t)$ of internal PAI-1 at time t and location $\vec{r}$, and a smooth variable $\sigma(\vec{r},t)$ accounting for the presence of cells at the considered position $\vec{r}$. According to the standard continuous-medium approximation (Landau and Lifshitz, 1959), the element $d\vec{r}$ has to be large enough to contain a large number of molecules, so that concentrations are smooth and deterministic, but not too large so as to

remain infinitesimal at the scale of the system. The evolution of the concentrations is then ruled by chemical kinetic equations (mass action law) and diffusion equation (Fick law). In a similar spirit, the variable σ is an homogenized version of the Boolean function $\sigma_0(\vec{r},t)$ with $\sigma_0(\vec{r},t) = 1$ if a cell is present in $\vec{r}$ at time t and 0 otherwise. There is need neither to count cells nor to care much for the boundaries of the cell population in a discrete setting, and the resulting cell density $\sigma(\vec{r},t)$ is a continuous field with $0 \leq \sigma \leq 1$. In particular, cell growth and cell division can be treated similarly, both producing a spreading of the support and a local increase of the field $\sigma(\vec{r},t)$. This description is mean-field-like insofar as correlations between the fluctuating numbers of molecules and cells at various locations and times are neglected and only their local averages are considered (Lesne, 2007). The overall dynamics is described in a spatially extended kinetic model, as illustrated on Figure 3, accounting for:

- *cell division and growth*, continuously enlarging the region occupied by cells (the region where $\sigma > 0$). This expansion of the cell population is measured by a rate k_g and a unimodal kernel $\Gamma(.)$ of finite range: cell growth and division induce a continuous spreading of the continuous regions with short-range increments weighted with Γ. As mentioned, there is no need to consider separate contributions for growth and division. This kernel is isotropic ($\Gamma(\vec{r})$ depends only on r), time-independent, normalized by setting its integral over the whole space equal to 1. Its width corresponds typically to the cell radius;

- a source term describing the *synthesis of PAI-1* inside the cell. It describes the net result of the protein synthesis following gene expression, its degradation right after formation, and possibly a negative self-regulatory effect on the expression of PAI-1 gene; this is accounted for by an effective term $f(c_i)$ such that $f(0) > 0$ and monotonously decreasing to 0 as the concentration c_i of internal PAI-1 increases. Prescribing a more detailed form for f is not possible given the limited biological knowledge and it would give an illusory precision; we thus limit ourselves to well-assessed general features of f, that will appear to be sufficient for deriving qualitative and robust conclusions.

- the *release of soluble PAI-1* when cells are present, feeding on their content in internal PAI-1. It is described by a pseudo-first-order kinetics, accounting only for the species of interest and the simple proportionality of the secreted amount with respect to c_i. The influence of possible additional factors and species other than PAI-1 (whose explicit description would obscure the dominant scenario that we want to explore) is implicitly taken into account in the effective rate k_s:

- soluble PAI-1 then *diffuses* with a diffusion coefficient D. The fact that it diffuses only in the extracellular medium is accounted for by using a space-dependent diffusion coefficient $(1-\sigma(\vec{r},t))D$ which vanishes at maximal cell density $\sigma = 1$;

- the *fixation* of soluble PAI-1 on the matrix when no cell is present, producing matrix-bound PAI-1 with a rate $(1-\sigma(\vec{r},t))k_m$ which vanishes at maximal cell density $\sigma = 1$;

- the *deactivation* of soluble PAI-1 or its internalization; this process is much different from the internalization of matrix-bound PAI-1 with regards to cell physiology: it has no signaling role and does not trigger any pathway, having finally no consequence on the overall state of the cell; hence it should not be taken into account in the internalization

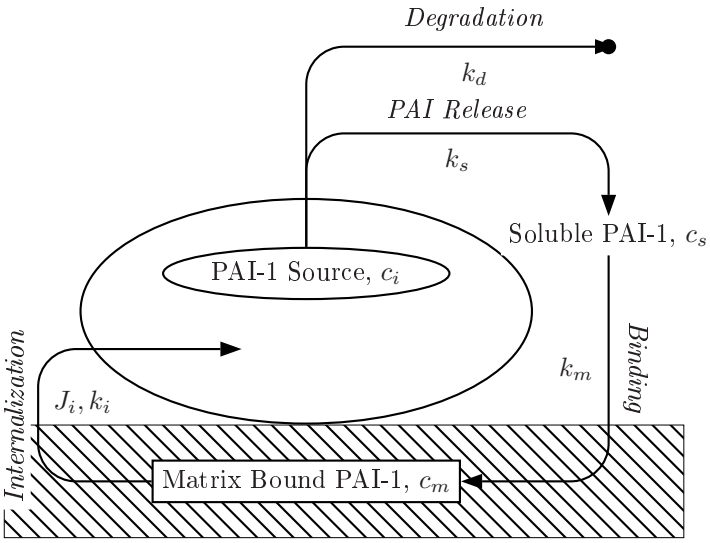

Figure 3: Fluxes and transformations of the three PAI-1 forms considered in the model, introducing the notations k_s, k_m and k_i for the kinetic rates used throughout the paper.

flux J_i introduced as a control parameter of the mesenchymatous-amoeboid transition. Both deactivation and internalization merely amount to a degradation of soluble PAI-1 and are jointly accounted for by a first order kinetic $-\sigma k_d c_s$. They correspond to the only possible fate for soluble PAI-1 at maximal cell density, when $\sigma = 1$ and diffusion or matrix-binding are no longer a possible option.

- the *internalization of matrix-bound PAI-1* with a rate k_i when a cell is present. This process generates the internalization flux J_i per cell, related according to an effective single-order kinetics $J_i = k_i c_m$. As in first-order kinetic terms introduced above, the rate k_i is an effective parameter possibly depending on factors not explicitly described in this basic model; only matters here the proportionality between the concentration c_m and the flux J. This flux triggers mesenchymatous-amoeboid transition when it exceeds a threshold J_i^*. Internalized PAI-1 molecules are degraded after having influenced internal pathways controlling the transition of the cell state.

In terms of regular deterministic fields, these different processes, individually well-established experimentally, together lead to the system:

$$\begin{cases} \partial\sigma/\partial t &= k_g\, H(1-\sigma) \int_{\mathbf{R}^3} \sigma(\vec{r}')\Gamma(\vec{r}'-\vec{r})d\vec{r}' & (1)\\ \partial c_i/\partial t &= \sigma f(c_i) - k_s\sigma c_i & (2)\\ \partial c_s/\partial t &= (1-\sigma)D\Delta c_s + k_s\sigma c_i - k_m(1-\sigma)c_s - k_d\sigma c_s & (3)\\ \partial c_m/\partial t &= k_m(1-\sigma)c_s - k_i\sigma c_m & (4)\\ J_i &= k_i c_m \qquad \text{for } \sigma > 0 & (5) \end{cases}$$

where $H(.)$ is the Heaviside step function such that $H(z) = 1$ if $z > 0$, and $H(z) = 0$ otherwise. Due to intra-cellular synthesis and degradation of PAI-1, there is no conservation of the total amount of PAI-1 in the system and Eq. (2) to (4) describe more than a mere interchange between three different forms of the same molecular species.

Here arise a major problem for modeling: the difficulty, if not total impossibility of getting any insight about the actual values of the kinetic rates; this would require single-cell measurements out of reach with the sensitivity of the technologies of today. For this reason, we adopt a semi-quantitative viewpoint aiming at providing robust proof-of-principles of the dominant mechanisms, despite this lack of quantitative biological data.

Qualitative insights about the dynamic behavior captured by the above coupled partial differential equations can be gained from a mere inspection of their form and terms. Eq.(2) and features of the function $c_i \to f(c_i)$ show that $\partial c_i/\partial t$ is strictly positive for c_i close to 0 and decreases monotonously as c_i increases, reaching negative values $\partial c_i/\partial t < 0$ for c_i large enough; hence we expect to observe a stationary value $\bar{c}_i$ where cells are persistently present ($\sigma > 0$). This $\bar{c}_i$ is determined as the unique solution of $f(\bar{c}_i) = k_s\bar{c}_i$. In Eq. (3), the degradation term ensures that c_s remains bounded. In regions where $\sigma < 1$, the diffusion term describes how the soluble PAI-1 produced by the tumor cells diffuses in the extracellular space toward the tumor boundary and beyond, in the outer shell, where it binds the extracellular matrix and increases the concentration of matrix-bound PAI-1, while c_s tends to 0 far away from the tumor in the regions not reached yet by the diffusion. Eq. (4) shows that $\partial c_m/\partial t < 0$ in regions where cells are dense (σ close to 1) while $\partial c_m/\partial t > 0$ at the tumor outer boundary, that is, in a region almost devoid of cells (σ close to 0) but not too far from the cells producing PAI-1, so that $c_s > 0$. We expect that $c_m = 0$ in the tumor bulk while reaching its maximum value in the tumor outer shell, before decreasing to 0 far away from the tumor: matrix-bound PAI-1 is mainly located in the tumor boundary regions where both σ and $1-\sigma$ are non zero. An obvious steric argument (core tumor cells can neither escape nor even move) reinforces the claim that *only tumor boundary regions are involved in the metastatic escape process.*

J_i given by Eq. (5) is defined as an internalization flux *per cell.* As the mesenchymatous-amoeboid transition occurs at the cell level, its relevant control parameter should correspond to a cell as a whole, notwithstanding its spatial extent. The biological relevant question is to determine whether this flux J_i could overwhelm the transition threshold in some cells, that is, $J_i > J_i^*$ with $J_i^* = k_i c_m^*$. Such an occurrence requires the conjunction of soluble PAI-1 production by tumor cells, diffusion of soluble PAI-1 in the vacant extracellular space, binding of a large amount of soluble PAI-1 at a given empty location of the extracellular matrix,

and growth or division of a cell to cover this location, internalize the underlying matrix-bound PAI-1 and experience the mesenchymatous-amoeboid transition. This sequence of events should follow a coordinated time ordering, namely soluble PAI-1 binds the matrix and c_m reaches a high level *before* a cell invades the corresponding location. This heuristic analysis shows the importance of the precise spatio-temporal geometry of the tumor, the history of its growth and the relative timing of the various events occurring at its boundary. Accordingly, consistency and relevance of the approximations leading to Eq. (1-5) must be reevaluated. Such a feedback of the predictions of the model onto its very design provides internal quality assessment (another assessment of quality relies on experimental data).

In this deterministic setting, the most rigorous way of accounting for the constraint that soluble PAI-1 diffuses only in the extracellular medium would be to impose boundary conditions on the bare diffusion equation (that is, involving a bare diffusion term $D\Delta c_s$) delineating the space available for diffusion. However, cell division is a random event at the cellular level of description so that the precise tumor boundary geometry is itself random and highly irregular. A precise account of the boundary conditions would require a full description of the random growth of the tumor and history of the cell population, hence the extension to a stochastic setting. On the contrary, Eq. (1-5) provide an homogeneous description which involve an average version σ of the actual Boolean function indicating precisely where cells are present, and accounts for the geometrical constraints through an *effective diffusion coefficient* $(1-\sigma)D$. A flaw of this average description is to ignore inhomogeneities whereas they are likely to play a central role, being reinforced by the heterogeneous growth dynamics and the geometry-dependent interplay between intra-cellular and extracellular processes. This would prevent the solution for c_m to reach very locally the very high level required to exceed the mesenchymatous-amoeboid transition threshold. A rough estimate from Eq. (2-4) in the outer shell where $\sigma \approx 1-\sigma \approx 1/2$ yields the stationary and spatially uniform concentrations $c_i \approx \bar{c}_i$, $c_s \approx \bar{c}_s = k_s\bar{c}_i/(k_m+k_d)$ and $c_m \approx \bar{c}_m = k_m\bar{c}_s/k_i$: c_m would either always or never exceed the threshold c_m^* of the mesenchymatous-amoeboid transition, depending on the values of the kinetic rates in Eq. (1-5) . Either all cells or none would display the mesenchymatous-amoeboid transition and escape. That does not correspond to the metastatic escape where only a very few cells emerging spontaneously manage to escape.

Moreover, as the basic mechanisms involve only few cells, statistical fluctuations of the total number of cells are likely to play a key role. In particular, the invasion by a new cell of an extracellular matrix site coverred by PAI-1 is mainly a random event, whereas we consider here that it is fully controlled by an homogeneous and deterministic spreading of a smooth field $\sigma(\vec{r},t)$. Even the description of molecular diffusion and transformations in terms of deterministic concentrations is questionable and lead us to investigate the role of the fluctuations of the total number of molecules, and the stochasticity of their motion and interactions.

Hence partial differential equations model are ill-suited to take into account the geometry of the tumor boundary, in the first place the heterogeneous accumulation of the matrix-bound PAI-1. Although this model provides a concise and tractable description to identify elementary mechanisms involved in the metastatic process, its resolution does not yield a relevant picture of the resulting biological process. If an explicit description of the occupied region were preferred to the homogenized description by the field σ, curing the singularities introduced by the sharp distinction between cells and extracellular medium would require

a recourse to regularizing kernels. Moreover, consistency in the level of details requires an explicit stochastic model for cell growth and division. These two refinements would introduce a large number of arbitrary parameters, to the detriment of the robustness of the model, its significance, and its discriminating power. For this reason, we rather turn to a numerical modeling using complementary cellular-automata and agent-based simulations, both accounting for the full random history of tumor growth and tumor boundary location, better suited to account for the interplay between the evolving geometry of the tumor boundary and the accumulation of matrix-bound PAI-1 with its related stochastic aspects.

3.3 Cellular-Automata and Agent-Based Simulations

In order to circumvent the aforementioned inadequacies of the partial differential equations model, we implemented two complementary numerical approaches: cellular-automata and agent-based simulations.

The cellular-automata simulation implements the same mechanisms as partial differential equations, see Figure 3, but explicitly accounts for the cell discreteness and stochasticity of cell division. Its main goal is to provide a proof-of-principle that stochasticity in cell division and tumor growth are sufficient to produce heterogeneous accumulation of matrix-bound PAI-1 at the tumor boundary. The agent-based simulation provides a phenomenological description accounting for stochasticity not only in cell division but also in the diffusion and transformations of PAI-1 molecules; it also includes vitronectine (a molecular species) and comprises more cell states. This makes the agent-based model closer to the biological reality but could obscure its interpretation; we rather use it as a complement of the cellular-automata simulation in order to check that its conclusions are not affected when supplementing the scheme of Figure 3 with auxiliary species, reactions and cellular processes.

Both simulations predict a heterogeneous accumulation of matrix-bound PAI-1, yielding supra-threshold concentration peaks, leading in turn to high internalization fluxes of PAI-1 in novel cells reaching these very specific locations; in the ensuing step (not included in the simulations) these few cells would experience the amesenchymatous-amoeboid transition and be plausible candidates for the metastatic escape. We shall first describe the cellular-automata simulation then discuss the additional insights provided by the agent-based simulation.

3.3.1 Cellular Automata

The cellular automata simulation accounts for stochasticity of cell division and spatial constraints on tumor growth: the tumor boundary will now evolve in a random and history-dependent way. The simulation aims at investigating the possible heterogeneous features in the distribution of matrix-bound PAI-1, in particular whether c_m could locally reach the transition threshold c_m^*. The simulation takes place on a 2-dimensional grid, where the spatial extent of each cancer cell corresponds to a unit grid cell (to avoid confusion between numerical cells of the simulation grid and biological cells of the tumor, we use the term grid cell for the former and simply cells for the former). The concentrations c_i, c_s, and c_m are defined on the discrete space-time $\{(\vec{r},t) \in \mathbb{N}^2 \times \mathbb{N}\}$ and take any real positive value; $\sigma(\vec{r},t)$ is similarly defined on $\mathbb{N}^2 \times \mathbb{N}$ but takes only values 0 or 1 according to whether a cell is absent or present in $\vec{r}$ at time t. The simulation is synchronous, the state of each grid cell

is updated at a step of the simulation. The growth of the tumor and the coupled variation of the different forms of PAI-1 (internal, soluble, and matrix-bound) are implemented according to the same kinetic scheme as in Figure 3. Based on the qualitative analysis of this scheme, an additional simplification was made, considering that all tumor cells release the same amount of soluble PAI-1; with the notations of the previous subsection, this amounts to consider that c_i attains its stationary value $\bar{c}_i$ fast enough and $c_i = \bar{c}_i$ in all cells; accordingly, k_s is replaced by an effective coefficient $k_{s,\text{eff}}$ (with the relationship $k_{s,\text{eff}} = k_s \bar{c}_i$ to the previous model). As we have no experimentally supported expression of the function $f(c_i)$ (an intricate biochemical analysis would be necessary), the simplification $c_i = \bar{c}_i =$ constant strengthens the robustness of the simulation: the influence of f appears in the value of $k_{s,\text{eff}}$. Keeping an equation for c_i with an unreliable term $f(c_i)$ would be illusory. Omitting the argument $(\vec{r}, t)$ in all right hand side quantities c_i, c_s, c_m, Δc_s and σ, a basic step of simulation writes explicitly:

$$c_m(\vec{r}, t+1) = c_m + k_m(1-\sigma)c_s - k_i \sigma c_m \quad (6)$$

$$c_s(\vec{r}, t+1) = D(1-\sigma)\Delta c_s + k_{s,\text{eff}}\sigma + c_s\left(1 - k_m(1-\sigma) - k_d\sigma\right) \quad (7)$$

Denoting $\vec{r} = (x, y)$, the standard discretization of the Laplacian involved in describing the diffusion of soluble PAI-1 is:

$$\Delta c_s(x,y,t) = (1/4)\left(c_s(x+1,y,t) + c_s(x-1,y,t) + c_s(x,y+1,t) + c_s(x,y-1,t)\right) - c_s(x,y,t) \quad (8)$$

This implementation corresponds to a space-time discretization of the partial differential equations except what concerns the growth of the tumor, now described as a stochastic process involving discrete cells: at each time step, one cell is created at the periphery of the tumor and its location is chosen at random among the empty sites around the tumor. At the beginning of the simulation, $c_m = 0$ and $c_s = 0$ for each grid cell, and one cell is located at the center of the grid. We consider the internalization flux J_i as the control parameter of the mesenchymatous-amoeboid transition, namely the transition occurs in a given cell when its flux J_i overwhelms a threshold J_i^*. This overshoot is controlled directly by the concentration of matrix-bound PAI-1 at the location of the cell, established before a cell is created at this location. In the course of the simulation, we record the spatio-temporal variation of the internalization flux and the various concentrations. We expect a realistic metastatic effect if the mean-field internalization flux $\bar{J}_i \approx k_i \bar{c_m}$ is below J_i^*. The meaning is that, on average, the bifurcation threshold is not reached, otherwise most cells would experience the transition, at odds with experimental observations. In this case, only a non trivial localization could allow a few cells to encounter high enough levels of matrix-bound PAI-1. The resulting internalization would be enough to display a mesenchymatous-amoeboid transition. Figure 4 shows the concentration of matrix-bound PAI after a typical trajectory of 500 simulated steps on a 40×40 cell grid. In agreement with our qualitative analysis, matrix-bound PAI-1 is mainly located at the tumor outer boundary. Moreover, several peaks appear at the periphery of the tumor; their locations vary from one simulation to another, and their heights are highly variable form place to place, with a standard deviation of the order of their mean value. In agreement with our scenario, peaks are observed to correlate with "gulfs" on the tumor boundary. These gulfs favor the localized accumulation on a single site of PAI-1 produced by several neighboring cells.

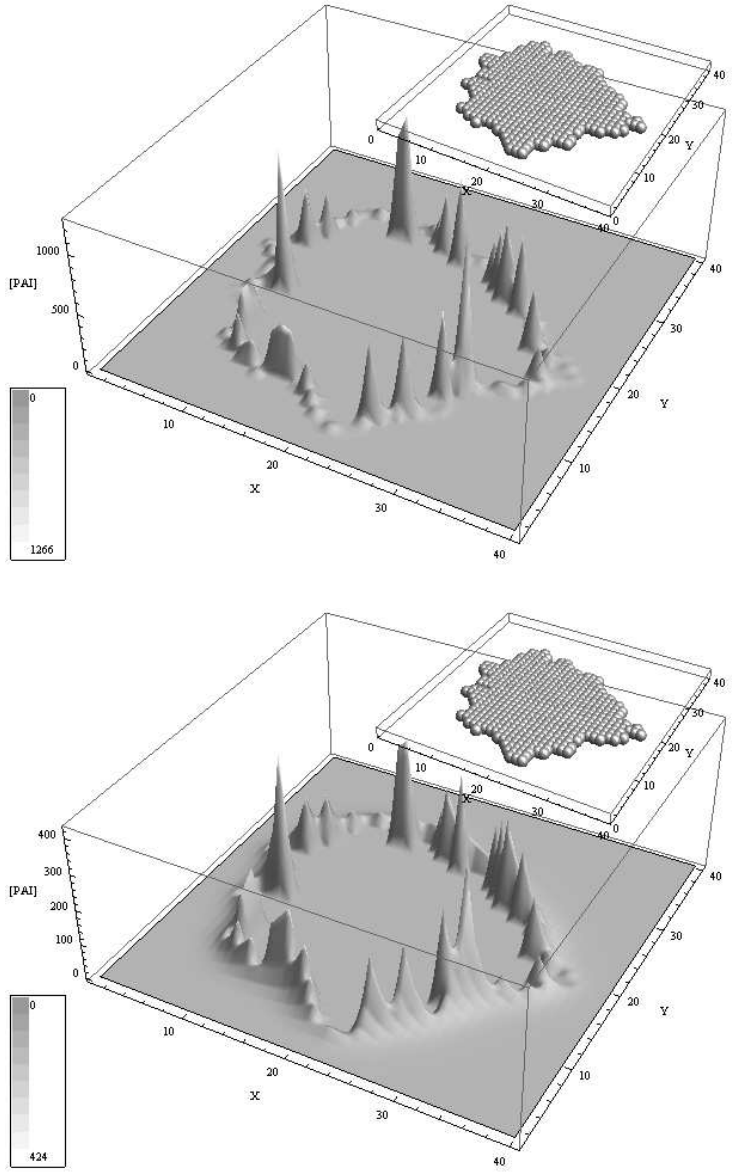

Figure 4: Concentration of matrix-bound PAI-1 (vertical axis) after 500 steps of simulation, starting from a null concentration in the whole unit square ($x \in [0,1]$, $y \in [0,1]$) and one tumor cell at the center, with $k_d = 0, k_i = 0.1$ and (left) $k_s = 1$, $k_m = 1$ or (right) $k_s = 0.5$, $k_m = 0.25$. The insets describe the corresponding shape of the tumor. In a wide range of values of k_m and k_s, matrix-bound PAI-1 displays a heterogeneous distribution, localized at the tumor boundary whose peaks correlate with the gulfs in the geometry of the boundary.

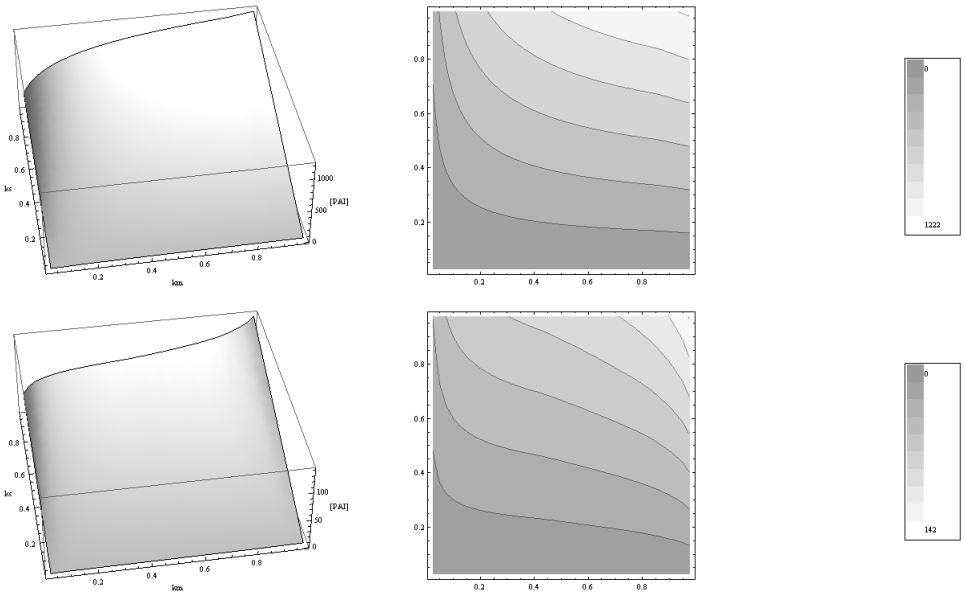

Figure 5: Sensitivity analysis of the cellular-automata simulation results. (left) mean and (right) standard deviation of the spatial distribution of matrix-bound PAI-1, with respect to kinetic rates k_m (horizontal axis) and k_s (vertical axis). Increasing k_i would only increase the internalization rate and decrease matrix-bound PAI-1 concentration wherever cells are present; altogether, this would localize peaks of concentration in the outer boundary of the tumor, without changing significantly their average height and standard deviation (see Figure 6).

The simulation shows that the interplay of transformation and degradation kinetics of PAI-1, the molecular diffusion of soluble PAI-1 collectively secreted by the tumor boundary cells in the extracellular medium, and the randomly changing geometry of the tumor can induce a localized accumulation of matrix-bound PAI-1 at a few places strongly dependent on the random growth history. Correlatively, it produces peaks in the flux J_i of internalization 1 by the cells of the matrix-bound PAI-1. The secretion of PAI-1 by the set of tumor cells is a collective effect. It allows the internalization flux J_i of a few single cells to reach values which would never be reached if the cells were functioning in isolation. Spontaneously, some cells might benefit from the PAI-1 secretion of the other ones. In particular, J_i might now locally reach large enough values $J_i > J_i^*$ to trigger the mesenchymatous-amoeboid transition of a few single cells and allow their amoeboid move away from the tumor. Due to the inherent stochasticity of tumor growth and boundary location, self-consistently coupled with PAI-1 reactions, this overshoot $J_i > J_i^*$ is basically a random event, occurring at history-dependent moments and locations.

Altogether, this numerical implementation supports the claim that only tumor boundary regions are involved in metastatic escape. It highlights an emergent situation leading to the spontaneous heterogeneous accumulation of matrix-bound PAI-1 at the tumor boundary,

which in turn promotes the amoeboid-mesenchymatous transition and the metastatic escape of a few single cells, selected in a random and history-dependent way. This mechanism accounts for both the possibility and the rarity of mesenchymatous-amoeboid transition events, and it relies on very few ingredients.

3.3.2 Agent-Based Simulation

A cellular-automata model, although introducing cell discreteness and some stochasticity in partial differential equations, might *a priori* lack realism and suffer from over-simplification. A structural stability analysis helps delineate the validity of the model. Inferring possible additional contributions quantitatively and investigate their importance are difficult experimentally because of the rarity of the phenomenon. We rather challenge the hypotheses and simplifications underpinning the cellular-automata simulation, for instance a minimal number of species, effective kinetics and drastically simplified cellular processes, by considering a biologically more detailed agent-based model.

Three kinds of entities are now considered: tumor cells, PAI-1 molecules in their three different forms, and additional vitronectine molecules bound to the extracellular matrix and promoting soluble PAI-1 matrix binding upon encounter. Moreover, cells are modeled as autonomous entities evolving in a continuous space and endowed with more realistic behaviors: by contrast to the cellular-automata simulation, cell modeling is now dissociated from the topology of underlying space. Cells can be in one of the following states:

- *Active:* an active cell may proliferate, internalize PAI-1, and release soluble PAI-1; cells are created in this active state.
- *Quiescent:* cells become quiescent if they lack basic nutrients and proliferation is paused; however, they continue to release and internalize PAI-1 molecules.
- *Necrotic:* if environmental conditions are even harsher, cells become necrotic and die.

PAI-1 molecules are modeled by accounting for discreteness and stochasticity of molecular events and diffusion, thus no longer relying on the mean-field description in terms of smooth deterministic concentration fields considered in both partial differential equations and cellular-automata. Molecules of vitronectine are described on average, in terms of a local concentration of molecules in each cell of a regular grid. Molecules of PAI-1 are modeled as autonomous agents produced and released by cells. These molecules can be in one of the aforementioned states: internal, soluble (either active or inactivated) or matrix-bound (when entering a cell-free region with a sufficient concentration of vitronectine so that it encounters a vitronectine molecule almost surely and binds to it); in the soluble state, they diffuse according to a random walk into the extracellular medium. Molecules of vitronectine have neither active behavior nor diffusive motion (for this reason are not modeled individually) but their concentration in a given grid cell is updated each time a PAI-1 molecule gets bound to the matrix in this grid cell.

Starting from a single active mesenchymatous cell, an homogeneous distribution of vitronectine, and no molecules of matrix-bound PAI-1, the agent-based simulation evolves toward a final state in which the initial cell has generated a full-grown tumor, surrounded by an irregular accumulation of molecules of matrix-bound PAI-1. This observation *in-silico*

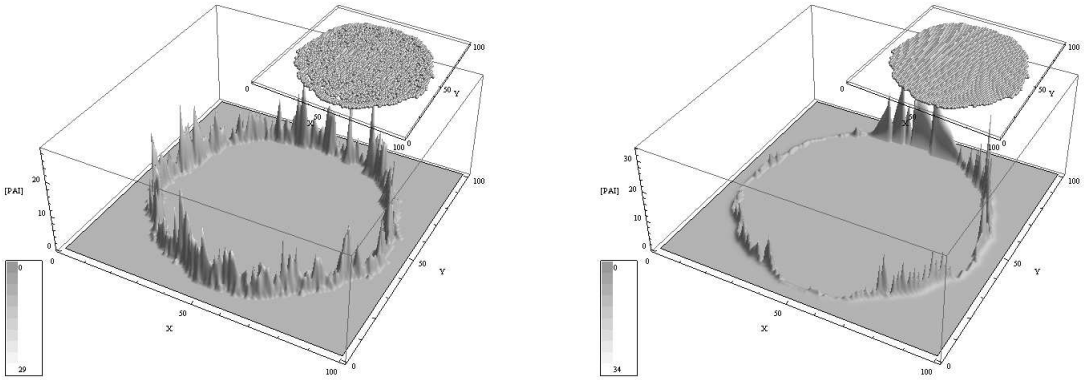

Figure 6: Comparison of the (left) agent based and (right) cellular-automata simulation results. Both simulations support the spontaneous heterogeneous accumulation of matrix-bound PAI-1 at the tumor boundary. In the agent-based simulation, tumor growth results from several physiological reactions whereas it follows from a random choice of a dividing cell in the cellular-automata simulation. Here this choice is constrained by enforcing the final shape in order to obtain a tumor geometry comparable to that obtained in the agent-based simulation. The difference with Figure 4, in which the simulation follows a plain random growth as described in § 3.3.1, shows that both tumor growth history and geometry matter.

holds true for a wide range of kinetics and parameter settings, a fact which testifies for the robustness of the modeling. It may also indicate a robustness of the phenomenon itself with respect to change in the surrounding conditions or in any metabolic or signaling factor influencing the parameters. Our numerical results suggest that:

- *the amount of matrix-bound PAI-1 in a given location on the tumor border varies greatly in time*; this is because of an alternation of phases during which PAI-1 accumulates by binding to a cell-free extracellular matrix (through forming a complex with vitronectine) and phases during which, because of tumor growth, one or more cells come in contact and internalize matrix-bound PAI-1; vitronectine molecules do not appear here as a limiting factor, that justifies *a posteriori* their omission in the two models with partial differential equations and cellular automata;

- *the amount of matrix-bound PAI-1 varies greatly from one point to another along the tumor border*; this is because the tumor growth is not spatially homogeneous and does not occur simultaneously all around the tumor;

- *the amount of matrix-bound PAI-1 is inversely correlated to the speed of the tumor growth*; this is because the slower the proliferation, the longer the phases during which matrix-bound PAI-1 can accumulate; this suggests that the environment of the tumor, by differentially influencing tumor growth (for instance if the medium is more dense on one side of the tumor), may play a major role in the initiation of amoeboid escape.

This agent-based simulation, aiming at a realistic account of the different factors involved, recovers several acknowledged results e.g. the appearance of a necrotic core while proliferation activity is highest close to the tumor boundary; it also shows a spontaneous and heterogeneous accumulation of matrix-bound PAI-1 in the outer shell of the tumor. Interestingly, when the simulation is performed with simpler rules, within a mean-field approximation in which the growing tumor is assumed to keep a circular shape and the secretion of PAI-1 is homogeneous, no such strong heterogeneities appear in the PAI-1 ring around the tumor (low variance in the ring concentration, data not shown): this confirms the result of the cellular-automata simulation that fluctuations in the shape of the tumor boundary as it grows, originating from the random cell divisions, play en essential role in the distribution of matrix-bound PAI-1 molecules.
These results, obtained with a simulation model that stands close to biological reality, strengthen those obtained with cellular automata model; the latter, being more abstract and minimal, better delineates the essential mechanism. Both simulations support the occurrence of a spontaneous heterogeneous accumulation of matrix-bound PAI-1 at the boundary of a growing tumor, where it favors the rare event of In both simulations, the fact that the standard deviation of the concentration of matrix-bound PAI-1 is of the same order of its mean is a very robust feature, observed in a wide range of the parameter values.

We have considered only a 2-dimensional lattice, mimicking a locally plane substrate. *In vivo*, amoeboid migration would rather occur through a porous 3-dimensional extracellular medium, with various effects of geometrical concentration. One advantage of the 2-dimensional simulation is to be directly comparable to *in vitro* experiments. Experimental setting monitoring 3-dimensional geometry and functionalized porous substrate are in their design stage still.

4 Insights from Bifurcation Theory

4.1 PAI-1-controlled Bifurcation Diagram

Our numerical simulations favor the explanation that both the metastatic escape and its rarity originate in the control by the internalization flux of matrix-bound PAI-1 of the switch between mesenchymatous and amoeboid states. We suggest now an integrated scenario accounting for the molecular, cellular, extracellular, and population features of metastatic escape and secondary tumor growth.

A first option could be to implement an extended agent-based simulation of the context-dependent transformations of *all* tumor cells, their migratory motions, their divisions, their interactions with their surroundings and metabolic changes as they move. Such an extensive simulation could include a wealth of ingredients presumably at work in the real system; its quality would be to use raw and elementary ingredients directly. It however does clarify neither the appearance of secondary tumors nor the major mechanisms. The parameters are too many to perform any sensitivity or structural stability analysis.

That is why we focus on the fate of *one* of the privileged cells having encountered a supra-threshold concentration of matrix-bound PAI-1 and experienced the mesenchymatous-amoeboid transition. The modifications of its microenvironment due to neighboring cells (including itself) will be tracked and taken into account *in an effective way*, through its

consequences on the concentration c_m of matrix-bound PAI-1 at the cell location or in a still more effective way through its consequence on the PAI-1 internalization flux J_i of the selected cell. The experimental investigation of the mesenchymatous-amoeboid transition, Figure 2, led us to formulate our basic claim that this cell change is not due to accumulated mutations but corresponds to a change in the physiological state of the cell, that is, to a *bifurcation in the intracellular dynamics.* The metabolic network formed with all the species and pathways connected somehow to PAI-1 or PAI-1-regulated reactions experiences a qualitative change of regime, with observable consequences on the cell state in the form of a bi-stable switch.

The generic instance of first-order bi-stability (that is, associated with a discontinuous transition between two stable states) is the S-shaped diagram (Ruelle, 1989) sketched on Figure 7. The horizontal axis represents the control parameter, here the concentration c_m of matrix-bound PAI-1 at the cell location or the internalization flux J_i related to c_m through Eq.(5). The vertical axis represents a quantitative feature discriminating the mesenchymatous and amoeboid states and affected in the mesenchymatous-amoeboid transition, notably:

- the *cell morphology* (observed shape, organization of the cytoskeleton, blebbing features appearing as J_i increases);
- the nature of the adherence points with or without integrins; the contribution of integrins decreases as J_i increases, replaced by a PAI-dependent mechanism (Czekay and Loskutoff, 2004);
- the force of adherence exerted by the cell, decreasing as J_i increases;
- the cell proteolytic activity (decreasing as J_i increases)
- the activation or inhibition of internal pathways related to PAI-1, typically the RhoA pathway, involving ROCK activation as J_i increases (Sanz-Moreno et al. 2008; McCarthy, 2009).

The S-shaped curve represents the possible states of a tumor cell in this plane, with bistability in a horizontal range $[{J_i}^{\min}, J_i^*]$. The lower branch corresponds to the mesenchymatous state and the upper branch to the amoeboid state. We are dealing with dynamic states, which are stable in the sense that they persist and correspond to stationary features. The intermediary dotted branch is unstable. This bifurcation diagram represents the abrupt mesenchymatous-amoeboid transition for $J_i = J_i^*$; the mesenchymatous state no longer exists for $J_i > J_i^*$. It also predicts the occurrence of a reverse transition, the amoeboid-mesenchymatous one, when J_i has decreased back to a value ${J_i}^{\min}$ smaller than J_i^*. This S-shaped bifurcation is termed a *subcritical bifurcation* to indicate that the amoeboid-state domain of existence and stability, in the control parameter space, overlaps the mesenchymatous-state domain of existence and stability, and covers a region $[{J_i}^{\min}, J_i^*]$ below the bifurcation threshold J_i^*. In biological terms, such a bifurcation diagram relies on the property that the threshold for triggering mesenchymatous-amoeboid transition is higher than the threshold required to simply maintain the amoeboid state once it is established. Reducing to a "plane" diagram (codimension one) is justified by the well-known generic irrelevance of stable components of the dynamics near the bifurcation point: the

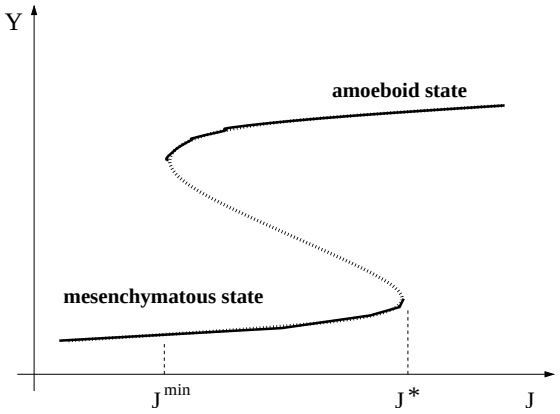

Figure 7: Generic bifurcation diagram of a bi-stable switch, here the transition between the mesenchymatous state (lower branch of the S-shaped curve) and the amoeboid state (upper branch) for a given cell as the internalization flux J_i of matrix-bound PAI-1 varies (horizontal axis); this flux is proportional to the concentration of matrix-bound PAI-1at the cell location and triggers several intracellular pathways altogether responsible for the transition. The vertical axis represents any quantitative feature discriminating the two states.

observed qualitative change is controlled by the parameter which is at the stability threshold, here the concentration of matrix-bound PAI-1, while other parameters and factors are inessential. Of course these arguments do not completely rule out the possibility of a non-generic, higher-dimensional and more complex bifurcation diagram but we have today no experimental clue indicating such a situation nor any inconsistency in our scenario requiring to envision a more complex diagram.

This bifurcation viewpoint integrates, within a dynamic framework, the exclusive proliferative and migratory capacities of the metastatic cells: the concentration of matrix-bound PAI-1 (which directly controls the internalization flux J_i) is critical in the choice between migration and proliferation, with a higher threshold for the transition from proliferation to migration than for the transition from migration to proliferation. Such a subcritical nature, supported by first-order nature of the transition from mesenchymatous to amoeboid states, suggests a protocol for observing the amoeboid-mesenchymatous transition at a decreasing concentration c_m, which is the quantity monitored in *in vitro* experiments (Malo et al., 2006).

The predictions of our study motivated systematic and quantitative experimental exploration of this transition and its control. The description in terms of a bifurcation diagram leads to predict its reversibility, that would provide a discriminating feature with explanation in terms of accumulated mutations as mutation-driven transitions are irreversible. As shown on Figure 8, the results of these experiments actually prove the existence of the reverse transition from amoeboid to mesenchymatous state (Cartier-Michaud et al., 2009). They provide an internal check of consistency of our scenario in supporting the bifurcation diagram on which it relies. In particular, they evidence both the reverse transition threshold

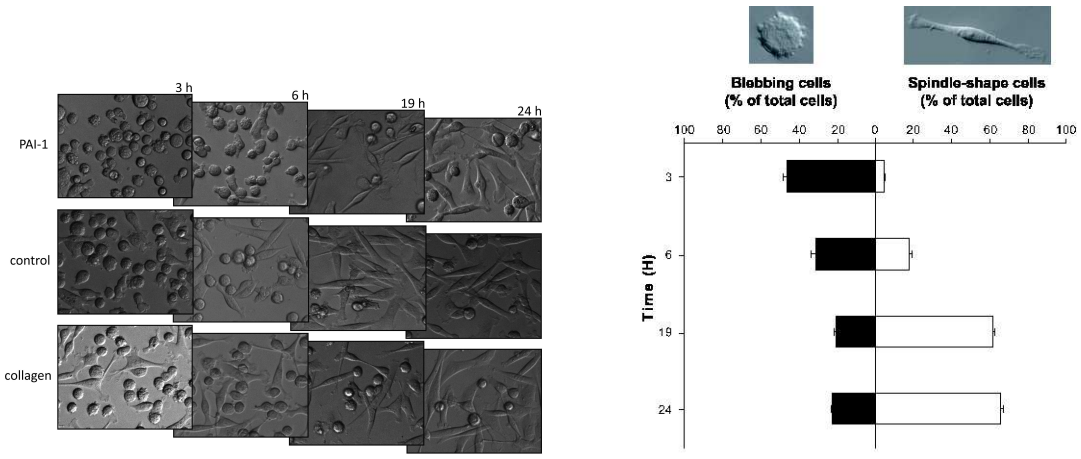

Figure 8: (left) Time-lapse photography of cells seeded on PAI-1 or collagen substrate; blebbing cells becoming elongated show the reversibility of the mesenchymatous-amoeboid transition. (right) The proportion of blebbing and spindle-shape MDA-MB-231 breast cancer cells seeded on a weakly PAI-1-enriched microenvironment (20 μg/cm^2, below the threshold of the mesenchymatous-amoeboid transition) is shown at successive time points (3, 6, 19, 24 hours). The proportion of blebbing cells (horizontal axis) decreases in favour of spindle-shape morphology indicating that a reverse amoeboid-mesenchymatous transition takes place.

$c_m^{\min} > 0$ and the fact that is is lower than the threshold for the direct transition $c_m^{\min} < c*$. The cell state is fully resilient and it recovers the mesenchymatous state at low matrix-bound PAI-1 concentration $c < c_m^{\min}$.

4.2 Metastatic Cycle

A cycle is usually associated with the subcritical bifurcation diagram sketched on Figure 7, and we shall now discuss its biological interpretation and conditions for its observation. We suggest a complete scenario of the recurrent hiving-fixation-growth cycle generating secondary tumors.

History- and geometry-dependent heterogeneities in the concentration c_m of matrix-bound PAI-1 could build up at the boundary of a proliferating tumor. A mesenchymatous cell reaching one of these privileged locations would follow the lower branch up to the critical value c_m^* at which it switches to the upper branch into the amoeboid state. Once the cell has switched to the amoeboid state, it starts to migrate away from the tumor. Before entering a proliferative state, a migrating cell into the amoeboid state has first to stop and settle, and its metabolic and transcriptional states shift to a regime promoting growth and division. We saw that such a transition back to the mesenchymatous state could be promoted by the surrounding. The diffusion-limited concentration of soluble PAI-1 decreases as the distance from the tumor cells producing PAI-1 increases, and so does the concentration of its matrix-bound form. The escaping cell soon reaches regions where the concentration of matrix-bound PAI-1 is low. This lowers its internalization flux J_i. Moreover, due to a

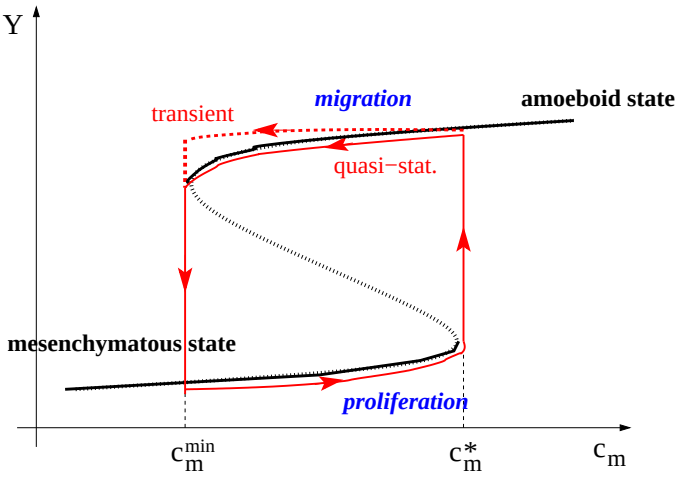

Figure 9: Metastatic cycle. The control parameter is the local and instantaneous value c_m of the concentration of matrix-bound PAI-1 at the cell location. The bold lines represent the stable states and the dotted one the unstable branch (bifurcation diagram as in Figure 7); the dashed line represents the typical path of the cell-state trajectory when the quasi-approximation is no longer valid and c_m varies before the cell has relaxed toward the stable branch.

negative self-regulation of the expression of PAI-1 at the transcription level, the secretion of PAI-1 is reduced in the amoeboid state (while being maximal in the mesenchymatous state). The migrating cells cannot by themselves modify their microenvironment into a track covered with a high quantity of matrix-bound PAI-1. Subsequently, the amoeboid state cannot be sustained and the cell switches back to the mesenchymatous state. It could then start proliferating again and generate a metastasis. As the secondary tumor grows, the same metastatic cycle could occur for a small fraction of its cells. Figure 9 represents how the metastatic cycle could originate in an intrinsic hysteretic cycle between two states for a single cell (bold lines). The point is that the control parameter c_m is not tuned from outside the cell but that its variations reflect the modifications of the cell microenvironment during proliferation and migration.

After a few metastatic cycles, the newly born metastasis is far enough from the original tumor and its cells descend from a minute fraction of the cells of the primary tumor, in agreement to *in vivo* observations. The metastatic escape of a single cell is in fact the consequence of a collective effect involving many cells, mainly through their contributions to the release of PAI-1 in the extracellular medium and fixation to the matrix: the consequence is observed at a single cell but originates at the cell population level. The probability that an escaping metastatic cell reaches blood circulation increases at each cycle, opening the way to other modes of metastatic processes.

In vitro experiments and the determination of quantitative bounds on the upper and lower thresholds should allow us to check the biological reality of the hysteretic cycle predicted here using generic arguments of bifurcation theory. Direct *in vivo* validation is today intrinsically

impossible yet, due to the too low probability of the escape and the too low fraction of cells involved in this cycle, yielding too low a signal-to-noise ratio. An additional difficulty is the limited duration in which the few emerging cells remain in the amoeboid state, although this transient stage with its associated migration appears to be a determinant event in the metastatic process.

4.3 Quasi-Stationary or Transient Cell States

We described the cell trajectory as a succession of well-defined states controlled by the local concentration c_m of matrix-bound PAI-1 or equivalently by the instantaneous value J_i of the cell internalization flux of PAI-1. This paradoxical description (a succession of stationary states), called "quasi-stationary approximation" (also "adiabatic approximation" in physics), is justified by the difference between the time scale of the modification of the microenvironment (and the ensuing modification of c_m and J_i) and the faster time scale of the cell response: the cell is almost always observed in a stationary state, following the stable branches of the bifurcation diagram as c_m or J_i slowly vary.

When this quasi-stationary approximation fails to be valid, for instance in the presence of memory or slow relaxation, the bifurcation diagram nevertheless provides bounds and qualitative guidelines about the trajectory of the cell state. Consider for instance an amoeboid cell reaching a region poor in PAI-1, with $c_m < c_m^*$. If a non negligible duration (compared to other relevant characteristic times) is required before the PAI-1 signal comes down, the cell relaxes to the mesenchymatous state with a delay: it first remains in a transient regime and its trajectory, represented by the dashed line on Figure 9, then deviates from the branches of stationary states forming the S-shaped curve. This trajectory presumably depends on the specific cell history, but lies above the upper branch. Whether the cell behavior is an adiabatic response following a stable branch of the bifurcation diagram or an out-of-equilibrium response depends on the relative values of the characteristic time of variation of the value c_m at the cell location (which corresponds to the characteristic time of migration toward the region poor in matrix-bound PAI-1 and depends on both the gradient of matrix-bound PAI-1 and the migration velocity) and the characteristic time of relaxation of the PAI-1-signaling activity triggered by the passage of the cell into a region rich in matrix-bound PAI-1.

It could be argued that a relaxation lag after the cell displays an amoeboid morphology and that c_m gone back under c_m^* is sufficient, by itself, to reproduce the metastatic cycle, without the existence of a stable amoeboid branch extending down to $c_m = c_m^{\min}$. During this lag, the cell could move far away from the tumor, before it recovers proliferative abilities and gives rise to metastasis. Because a stable amoeboid state was observed experimentally, the mesenchymatous-amoeboid transition cannot correspond to a mere transient excursion away from the stable mesenchymatous branch and the mesenchymatous-amoeboid transition would correspond in the bifurcation diagram to a jump to a degenerate amoeboid branch reduced to a point. Such a situation is non generic and it can be observed only for highly peculiar kinetics. The experimental observations of a discontinuous and reversible transition between two stable stationary states of the cell support the S-shaped of the bifurcation diagram of Figures 7 and 9.

No specific genetic mutation is necessary to our explanation of the metastatic cycle, where the passage from the mesenchymatous to the amoeboid state is a a bifurcation rather than an event triggered by a mutation. The reverse transition for the *same* cell when

external conditions have changed enough is an event that would be hard to observe if the transition were determined genetically. Its existence seems to be confirmed by our first experiments. Our scenario does not conflict with the accumulation of mutations associated with the metastatic process. We suggest that mutations observed in metastatic cells are neither specific to these cells nor sufficient to explain the move away from the tumor. In our scenario, tumor cells are genetically identical but made functionally different by their different microenvironments. The mutations arising as cancer develops and tumor ages are shared by a large fraction of tumor boundary cells. They are likely to influence the bifurcation diagram. Only a few candidates selected among the population of mutant cells by a dynamic and multilevel process move away. Our model leads to suggest a new experiment to test our prediction that cells in a nascent secondary tumor and cells at the outer boundary of the primary tumor have the same genome and mutation load. We here see how modeling allows to carry a qualitative scenario into experimental testable prediction.

4.4 Oncogenesis and Metastatic Catastrophe

Looking for the changes of metastatic features during cancer progression leads us to situate normal cells or early cancer cells (having just experienced the epithelial-mesenchymatous transition) in the above biifurcation viewpoint. The bifurcation diagram of Figure 7 can be put in a 3-dimensional space where the third axis Z represents the cell type. It represents the topological changes altering the S-shaped bifurcation diagram as the cell type is modified during oncogenesis, mainly by accumulating mutations: a relevant choice for Z is the total number of mutations. Thom (1975) established the possible generic changes in a bifurcation diagram seen as various singularities of the corresponding hypersurface (here a 2-dimensional surface, with S-shaped section, in a 3-dimensional space $\{(X, Y, Z)\}$). He termed "catastrophes" these generic singularities. In the present case, two situations can be encountered:
1) the bifurcation diagram, on Figure 7, is qualitatively the same for normal cells, early cancer cells, and advanced tumor cells. This means that both thresholds J_i^* and $J_i^{\min}$ both exist, keeping finite and distinct values when the cancerous stage of the cell changes;
2) no such bi-stability exists for normal cells, where a single stable state would change continuously as J_i increases. Genericity ensures that the bi-stability observed for cancer cells arises through a *fold catastrophe* (Thom, 1975), as represented on Figure 10.
The failure to observe the mesenchymatous-amoeboid transition for normal cells prevents one from discriminating the two cases. Perhaps the first case holds and the threshold for the mesenchymatous-amoeboid transition cannot be reached in normal or early cancer cells, either because the value J_i^* is larger than in cancer cells, or because the concentration c_m of matrix-bound PAI-1 cannot reach high enough values around normal cells, or because their rate of internalization k_i (such that $J_i = k_i c_m$) is too low. The rate k_i depends on the total number and the activity of uPA receptors, which are known to be far higher in cancer cells, all the more that cancer is advanced. During oncogenesis, k_i increases and PAI-1 is released by the cells, expectedly surrounded with higher c_m values. This is enough to explain how cancer cells can have higher internalization flux. The second case is described by a 3-dimensional extension of the bifurcation diagram into a fold catastrophe. The third dimension is associated with long-term evolution and accumulation of mutations, leading to metastatic potentialities in agreement with the cancer multistage theory presented in the

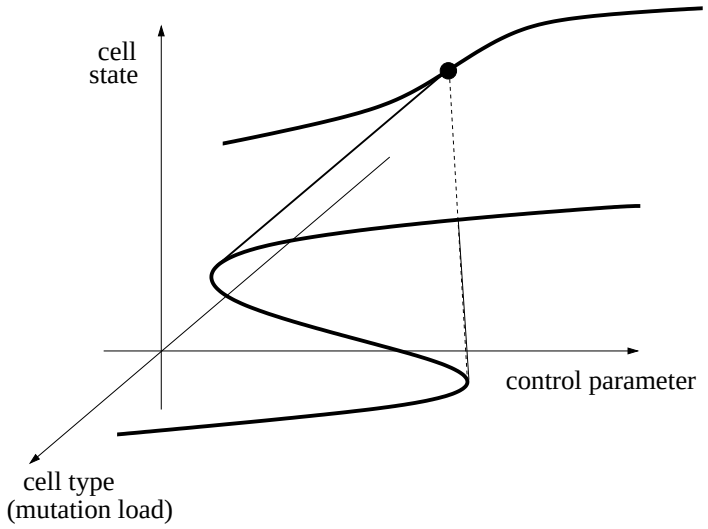

Figure 10: Unfolding of the bifurcation diagram of Figure 7 in a 3-dimensional space. The X-axis is the control parameter, namely the internalization flux J_i of matrix-bound PAI-1, controlled itself by the concentration c_m of matrix-bound PAI-1at the cell location. The Y-axis represents any measurable feature of the cell state discriminating mesenchymatous from amoeboid states. The Z-axis represents the cell cancerous stage, directly related to its mutation load. Bi-stability arising as the surface folds onto itself (fold catastrophe) occurs only for the cells having enough marked cancerous features.

paper by Gerstung and Beerenwinkel in this issue. A main result of catastrophe theory is the existence of only a few archetypal ways of fixed-point destabilization as the evolution law varies, here the 'fold' type represented on Figure 10. Right after the catastrophe, J_i^* and ${J_i}^{\min}$ are close to each other; they coincide at the catastrophe point. The difference between the two stable states in the bi-stable region is weak: a mesenchymatous-amoeboid transition can occur but the cell would show only a transient blebbing morphology, with no consequences on its fate. In particular, the cell remains in the amoeboid state too briefly to have a metastatic escape. Preliminary qualitative experiments (Cartier-Michaud et al., 2009) bring some evidence to such behavior. Further experiments are necessary to discriminate the two cases for the bifurcation landscape.

Once the proper bifurcation diagram is established, another matter is to describe the joint dynamics of the cell physiological state (variable Y), control parameter (variable X), and cell genetic state (variable Z), in order to obtain a scenario at the cell population level with possible collectively-driven and out-of-equilibrium behavior at the cell level. For instance, in any bi-stable situation, the time variation of J_i would determine the observed behavior entirely, depending on whether J_i reaches J_i^* or not. If it reaches J_i^* , does it reach ${J_i}^{\min}$ back or not, leading either to a restricted back-and-forth motion on a single stable branch or to a full cycle? Time scale and the range of variation of J_i determine whether the motion follows adiabatically the stable branches or behaves out of equilibrium. In any case, a therapeutic target can be to modify the bifurcation diagram so as to weaken its metastatic possibilities and the efficiency of the metastatic cycle.

5 Conclusion

Our scenario of metastatic process underlines a novel idea: although the accumulation of mutations plays an essential role in the metastatic process, the actual determination of the cell experiencing a metastatic escape is the consequence of a complex sequence of stochastic and multiscale events involving a whole population of "potentially metastatic" cells all displaying the same mutation load. Four models (multi-agent simulation, cellular automata, bifurcation theory, catastrophe theory – partial differential equations are useless) are jointly required to capture the interplay between different levels, as well as the role of stochasticity and system history, and turn biological hypotheses into numerically and experimentally testable predictions. Because detailed factors and values of kinetic rates are not available experimentally, we chose to remain at a semi-quantitative level, which is sufficient to obtain robust proof-of-principles.

We first showed numerically that collectively induced heterogeneities in the concentration of matrix-bound PAI-1 can develop and induce the transition of a few cells to the migratory-prone amoeboid state. Our scenario is based on an experimentally supported alternation of mesenchymatous and amoeboid states, corresponding respectively to the proliferation and migration for a few cells. They are selected among other cells of the population by a rare, stochastic, and history-dependent conjunction of molecular events. This scenario is a cellular version of hiving: amoeboid migration involves a very small number of cells and is a very transient stage during which cells exchange their proliferative capacities against migratory abilities. We suggest that all cells of the outer shell of the tumor contribute

to this process because their mutualized production of PAI-1 is necessary to trigger the transition to the amoeboid state of a few cells. All cells of the outer shell of the tumor have the same metastatic potentiality on genetic grounds, but only those encoutering a localized accumulation of matrix-bound PAI-1 will be in position to express this potentiality. A single species, PAI-1 would coordinate events at different scales, from molecular determinants up to the consequences for the tissue. The metastatic cycle would involve both intra-cellular and extracellular processes, as well as a collective modification of the microenvironment, in agreement with recent observations (Albini et al., 2008; Bidard et al., 2008). Modeling thus requires to consider *jointly* the different levels, to focus on their interplay, and to explicitly describe the circular loop between the individual cells and the population so as to account for both the observed rarity and robustness of the metastatic process. Amoeboid migration is too rare and transient to be easily observed *in vivo* or *in vitro* but it is likely to have dramatic consequences on metastatic spreading. The benefit of modeling, in such a situation where the biologically determinant event cannot be detected due to its too low relative weight and frequency, deserves to be underlined.

The acknowledged reaction-diffusion modeling with partial differential equations, currently used in growth and cell population studies, here appears to be irrelevant, leading us to develop numerical simulations and a minimal model in the framework of bifurcation theory. The present study underlines the distinction between a scenario and its mathematical or numerical implementation: our different modeling approaches are based on the same mechanisms and couplings, but differ in the framework used for their implementation. In our scenario, cells modify their microenvironment, which in turn influences the state of some cells through the activation of specific signaling pathways. Such multiscale couplings, both bottom-up and top-down, are the hallmark of complex systems. Our modeling approach proposes guidelines to handle such systems. It has to involve several different frameworks (here partial differential equations, cellular automata and agent-based simulations, bifurcation theory) so as to validate the determination of essential variables, parameters and mechanisms retained in the explanation. Moreover, complementary models have to be developed at several levels (here, bifurcation theory at the cell level, simulation at the population level). Each one implements specifically a given set of working hypothesis to be tested in view of the model outcome and predictions. They are matched, bottom up, using effective variables and parameters and, top down, using effective inputs and boundary conditions (Lesne, 2008b).

Because the metastatic process originates from a coordinated alternation of proliferation and migration, it articulates single cells and the cell population. It is difficult to observe this process not only *in vivo*, but also *in vitro*. Only partial knowledge can be gained, each piece coming from a dedicated experiment. Modeling is here essential to bridge different experimental investigations. Our scenario accounts for available biological facts and suggest a protocol for investigating the mesenchymatous-amoeboid and amoeboid-mesenchymatous transitions with normal cells and cancer cells taken at different stages after the epithelial-mesenchymatous transition. The purpose is to reconstruct the multivariate bifurcation diagram and discriminate the two possible transformations of the bifurcation diagram along the progression of the cancer.

In our scenario, reducing matrix binding of PAI-1 would modify the migratory abilities of the amoeboid state or some other factor so as to decrease k_i or increase the threshold J_i^* of the mesenchymatous-amoeboid transition; reducing PAI-1 synthesis is not an option because it might have undesired side effects due to other intracellular roles of this molecule. It also

suggests to reduce the matrix binding ability of PAI-1, targeting the microenvironment rather than the tumor cells themselves (Whiteside, 2008).

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