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## Cancer micrometastasis and tumour dormancy

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Many epithelial cancers carry a poor prognosis even after curative resection of early stage tumours. Tumour progression in these cancer patients has been attributed to the existence and persistence of disseminated tumour cells (DTC) in various body compartments as a sign of minimal residual disease. Bone marrow (BM) has been shown to be a common homing organ and reservoir for DTC. A significant correlation between the presence of DTC in BM and metastatic relapse has been reported in various tumour types. However, only a portion of patients with DTC in BM at primary surgery relapse. Thus far, little is known about the conditions required for the persistence of dormancy or the escape from the dormant phase into the active phase of metastasis formation. Thereby, this peculiar stage of conceivably balanced tumour cell division and death may last for decades in cancer patients. Most likely, the ability of a dormant DTC to “be activated” is a complex process involving (i) somatic aberrations in the tumour cells, (ii) the interaction of the DTC with the new microenvironment at the secondary site, and (iii) hereditary components of the host (i.e., cancer patient). In this review, we will summarize the key findings of research on micrometastatic cancer cells and discuss these findings in the context of the concept of tumour dormancy.

**Key words:** Tumour dormancy; cancer micrometastasis; disseminated tumour cells; microenvironment; oncogenes and tumour suppressor genes; angiogenesis; immune surveillance.

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### INTRODUCTION

Solid tumours derived from epithelial tissues represent the majority of cancers, with approximately 2.7 million newly diagnosed cases every year in the European Union. Most deaths from these tumours are caused by the metastatic spread of the primary tumour to distant organs. The formation of distant metastases requires several functionally distinct events, including tumour cell intravasation, survival of a tumour in the blood stream or lymphatic system, homing of the tumour to a specific site, and finally outgrowth.

The traditional view has been that only a small fraction (metastatic subclone) of tumour cells showing up late in the progression of tumours has the ability to disseminate from the primary tumour to form metastases (1). According to this hypothesis, metastasis represents a late event with only a subset of cancer cells within a primary tumour being able to metastasize. Bernards & Weinberg proposed an alternative model, in which the metastatic capacity is gained early during primary tumour development and thus transmitted to most of the cells in a “metastatic” primary tumour (2). This model has been supported by genomic and mouse studies. Several gene expression profiling studies have shown that the micrometastatic and metastatic potential of various tumours is

closely related to specific gene expression patterns (3–6). Even though it is well accepted that primary tumours are heterogeneous, in the above-mentioned experiments the tumours were analysed “in bulk”, implying that the identified metastatic signatures were expressed in the majority of tumour cells rather than only in a small metastatic subclone. In addition, experimental mouse studies using xenotransplanted breast carcinoma cell lines showed that differences exist in the ability of individual primary lesions to form secondary tumours in different secondary organs (7, 8). Therefore, not only the potential of a tumour to form (micro)metastasis might already be genetically determined in primary tumours but also the organotropism of the tumour (9).

The fact that single circulating tumour cells (CTC) in cancer patients can be detected at early stages of tumour progression in peripheral blood and disseminated tumour cells (DTC) in regional lymph nodes, and in bone marrow (BM), implies that the metastatic propensity of cancer cells is manifested early on (10). Interestingly, BM seems to be a common homing organ for DTC derived from various types of malignant epithelial tumours, including tumours which do not typically form metastases in bone, such as colon cancer. This suggests that BM might be the preferred reservoir for metastatic tumour cells from where they may re-circulate into other distant organs. In most instances, DTC are present in BM samples of 15–40% of patients (up to ~70% in prostate cancer (11)), even in the absence of lymph node metastases (stage N0) or clinical signs of overt distant metastases (stage M0) (10). Several clinical studies have indeed shown significant correlation between the presence of DTC in BM and metastatic relapse in various tumour types (reviewed in (10)).

Only a portion of patients with DTC at primary surgery eventually relapse. Animal models indicate that a significant fraction of DTC may never develop into overt metastases but may die or remain in a dormant state (12). Thus far, little is known about the conditions required for escape from the dormant or latent phase into the active phase of metastasis formation. Thereby, this stage of conceivably balanced tumour cell division and death may last for decades in cancer patients (13). Most likely, the ability of a

dormant tumour cell to “be activated” and later to produce a secondary lesion is a function of both its interaction with the stroma at the secondary site and the combination of somatic aberrations and hereditary components influencing each process. It is accepted that only a small fraction of tumour cells are able to proliferate and self renew. These alleged cancer stem cells might also be the cells that are able to grow out in secondary sites. Indeed, recent findings suggest that DTC in bone marrow of breast cancer patients show cancer stem cells properties (14).

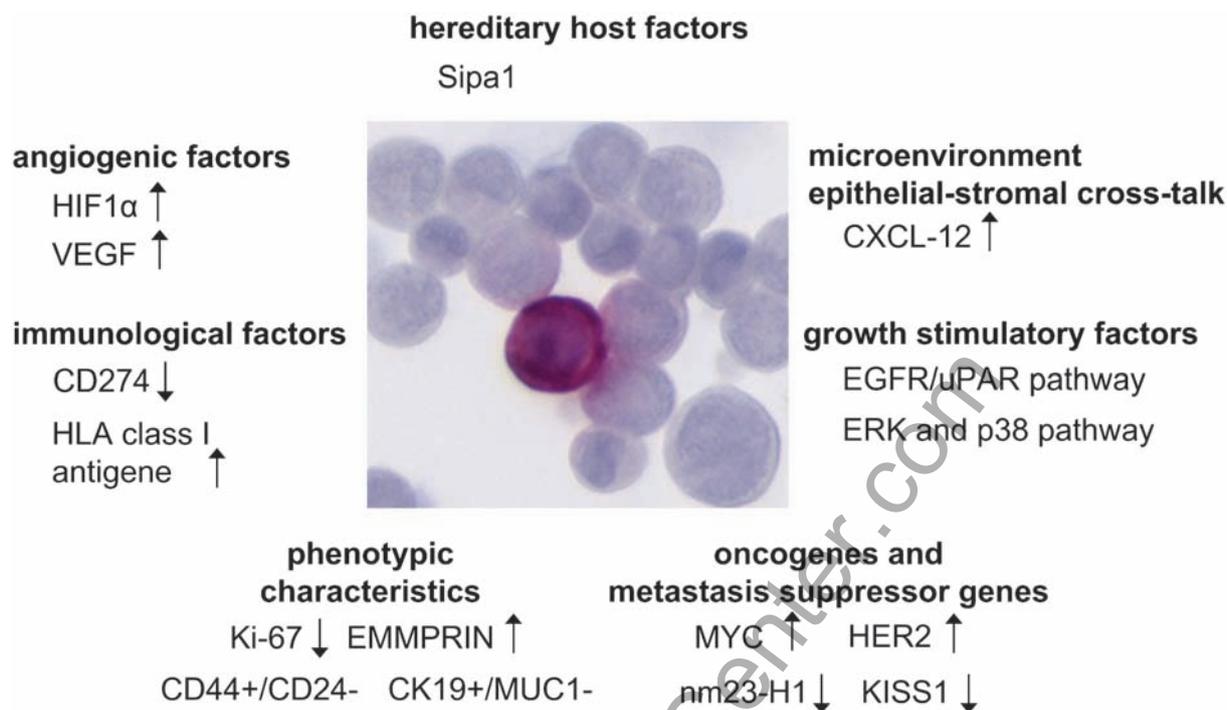
Here we summarize the latest findings from experimental and clinical research on micrometastatic cancer cells, and discuss the concept of tumour dormancy in the context of cancer micrometastasis.

#### CANCER MICROMETASTASIS AND DISSEMINATED TUMOUR CELLS (DTC)

Micrometastases were originally defined by pathologists as small occult metastases (<0.2 cm in greatest dimension). Sensitive techniques have now been developed that allow the detection of single DTC in the blood and bone marrow in a background of millions of normal haemopoietic cells. Currently, these single DTC are also called micrometastases. Even though there are apparent differences in the biology of true micrometastases and single DTC, the appearance of either type of micrometastases has been associated with poor prognosis (10).

#### DETECTION OF DTC

Following the first publication in 1981 (15) of the use of immunocytochemistry (ICC) for the detection of tumour cells in BM, numerous studies have been performed in various types of epithelial cancers (10). In order to be able to detect one tumour cell per million haematopoietic cells, highly sensitive assays are needed to locate and study DTC. Several different methods have thus been developed. Currently, the most widely used method is Ficoll-Hypaque density centrifugation followed by ICC or RT-PCR. Using this procedure red blood cells and granulocytes can be separated from mono-



*Fig. 1.* Potential factors controlling DTC dormancy. Picture showing a DTC found in BM, stained with the APAAP method (145).

nuclear cells (MNC) and possible DTC based on their buoyant density (10).

Owing to the rareness of DTC/CTC, several specific enrichment methods have been generated, including different types of density gradient separation, negative and positive cell depletion assays, using for example immunomagnetic particles, filtration and magnetic affinity cell sorting (MACS), laser scan cytometer and fluorescence-activated cell sorting (FACS)-based methods (16–21).

The immunomagnetic bead enrichment procedure enables the use 5–10 fold larger volumes of blood or bone marrow, compared with a direct ICC technique. The beads are coated with monoclonal antibodies that are directed either against the tumour cell (direct positive selection) or against the cells of non-interest (indirect negative selection). In the negative depletion a CD45 antibody is usually used, which depletes the majority of leukocytes, thereby negatively enriching tumour cells, if present. In positive depletion, the beads are pre-coupled with different epithelial cell surface antigens, including EpCAM, EGFR, HEA and/or HER2/ErbB2 or a mixture of these antibodies (22–25). Enrichment is usually followed by either ICC or RT-PCR.

The main approach to identifying DTC/CTC is ICC with monoclonal antibodies against epithelial or tumour-associated antigens (26). Cytokeratins (CK) are currently the most widely accepted protein markers for the detection of epithelial tumour cells in mesenchymal tissues such as BM, blood or lymph nodes (10). Cytokeratins are cytoskeleton proteins expressed in epithelial cells but usually absent in haematopoietic cells. Several different cytokeratins exist with different specificities against different epithelial tissue. Therefore, usually a pan-cytokeratin antibody is used in order to increase the sensitivity of detection. However, different staining techniques, as well as the use of different antibodies, can result in specificity variations. The need for standardization of the immunocytochemical assays is widely recognized and addressed in many research projects (27, 28) ([www.dismal-project.eu](http://www.dismal-project.eu)).

FACS- and MACS-based methods have also been used for the detection of CTC/DTCs. However, very few studies have been conducted in larger patient material and even fewer where these methods have been compared to other currently used methods (29). PCR-based methods have been shown to be technically even

more challenging than immunocytochemical assays in respect of sensitivity and specificity (30). In principle, the DNA or RNA in a sample can be amplified by PCR, so that single tumour cells can be detected in a heterogeneous population of cells. The major problem with DNA-based methods is the absence of common specific genetic aberrations such as mutations in solid tumours. The most promising approach is currently the detection of so-called tumour-specific expressed genes by quantitative RT-PCR. However, defining proper cut-offs discriminating normal and tumour cell expression is challenging, as many studies have shown that also normal haematopoietic cells might express these markers, e.g. cytokeratins at low abundance. Pre-analytical depletion of the disturbing normal cell fraction (e.g. granulocytes that express CK20) might solve this problem (31–33). Numerous transcripts have been evaluated as “tumour-specific” markers, such as CK18, CK19, CK20, Mucin-1, MAGE-A and carcinoembryonic antigen (CEA) (34–44). A multimarker approach with a panel of different markers may improve sensitivity for detection of DTC over single marker assays (45–47).

Recently, a new DTC detection system called EPISPOT (epithelial immunospot) was introduced. This system is an adaptation of the enzyme-linked immunospot (ELISPOT) and is based on the detection of specific marker proteins secreted or actively released by viable epithelial tumour cells. The proteins are caught on an antibody-coated membrane and visualized by a secondary set of enzyme- or fluorochrome-conjugated antibodies (48, 49). In contrast to other DTC detection technologies, the EPISPOT assay offers the advantage that only viable tumour cells will be detected. However, the drawback, as in RT-PCR, is that the tumour cells of interest cannot be morphologically identified and isolated for further analysis as the detection is based on fingerprints of secreted proteins and the cells are washed away before detection of these fingerprints.

The EPISPOT method relies on secretion of tumour-specific proteins. For breast cancer, mucin-1 (MUC1) and CK19 have been shown to be promising markers. The enumeration of both MUC1- and CK19-secreting cells allowed the detection of viable DTC in BM of 90% of breast cancer patients with overt distant metastasis

and 54% of patients with early stage tumours (14). Also in prostate, thyroid and colon cancer secreted proteins (e.g. prostate-specific antigen, thyroglobin and CK19) have been successfully used as markers for circulating tumour cells (14, 48, 49). These encouraging results provide hope that also in other epithelial tumours specific markers with high specificity and sensitivity could be found.

Although all these analyses of DTC in cancer patients have provided insight into the metastatic cascade of cancer in humans, they have clear limitations. Today, micrometastasis detection relies on detecting markers (protein or DNA or mRNA) that are not expected to be present in normal BM. However, none of the markers used today are really tumour specific, but instead are tissue or cell-type specific. Furthermore, at present, DTC cannot be detected in other distant organs because they are not as easily accessible as BM. These DTC may be biologically different from those in BM since the tissue microenvironment can influence both selection of the “fittest” DTC clones and gene transcription of the surviving DTC clones.

## CLINICAL RELEVANCE

Several studies have shown significant correlations between the presence of DTC in BM and metastatic relapse in various tumour types, suggesting that the founder cells of overt metastases might be among those DTC (10). For breast cancer, the most solid data base (n=4703 patients) on the prognostic relevance of DTC in BM is currently available (26). It is noteworthy that the presence of DTC in BM not only predicts the development of skeletal metastases but also the development of metastases in other organs such as lung and colon (26). In other tumour entities the data base on prognostic relevance is not as solid as in breast cancer, but single institution studies including colon, gastric, pancreas, lung, prostate, head and neck, and ovarian cancer, as well as melanoma, have documented a link between the presence of DTC at primary surgery and subsequent metastatic relapse (for different tumour entities see reviews (50–57)).

Besides the detection of DTC at primary diagnosis and surgery, it has been shown that

DTC can survive chemotherapy and hormonal therapy (56, 58–60) and persist in BM over many years post-surgery, often linked to an increased risk of late metastatic relapse (22, 61–65). The largest data source is again available for breast cancer. For example, in high-risk breast cancer patients (>3 involved axillary lymph nodes or extensive invasion of lymphatic vessels), the presence of tumour cells after therapy was associated with an extremely poor prognosis and pointed to a heterogeneous response to treatment (59). A European pooled analysis involving 696 breast cancer patients confirmed these findings; 16% of breast cancer patients had tumour cell persistence in bone marrow, and such presence was an independent prognostic factor for subsequent reduced breast cancer survival (66).

The identification of patients at increased risk for recurrence after completion of adjuvant chemotherapy is an application of great clinical relevance, since these patients might benefit from additional treatment. Bisphosphonates inhibit osteoclast precursor cells, modulate migratory and adhesive characteristics, induce apoptosis of osteoclasts and influence the microenvironment (67), and are thus used in the treatment of bone metastases. Small trials have been initiated to study the influence of bisphosphonates on DTC. In the study performed by Rack *et al.* (68) all 14 breast cancer patients receiving bisphosphonates were DTC negative after treatment, in contrast to 4 of 14 patients without bisphosphonate treatment showing DTC persistence.

HER2-targeted therapy by e.g. anti-HER2 monoclonal antibody (trastuzumab) may also be of value for primary breast cancer patients with HER2-positive DTC. Currently, all breast cancer patients are stratified for this targeted therapy by primary tumour analysis only. However, recent reports have shown that the genotype of persistent circulating tumour cells may change towards a HER2-amplified profile (13, 69, 70), suggesting that additional patients could benefit from HER2-directed therapies.

## MOLECULAR CHARACTERIZATION

It is still debated whether release of cells from the primary tumour is a selective process or rather represents a more unselective shedding of cells

into the circulation. Recent reports have, however, shown that early haematogenous dissemination of breast and lung tumour cells appears to be associated with a specific molecular signature of the primary tumour, suggesting that early haematogenous dissemination of DTCs is a specific process driven by a set of control genes (5, 6, 71). These findings challenge the traditional concept that tumour cells acquire their metastatic genotype and phenotype late during tumour development, and rather support the alternative concept that tumour cells acquire the genetic changes relevant to their metastatic capacity early in tumorigenesis (2), so that the metastatic potential of human tumours is encoded in the bulk of a primary tumour (4, 72). This concept could also explain the presence of DTC at an early stage of carcinogenesis.

Several studies have been conducted to characterize DTC by pheno- and genotype in order to identify biological features that might favour early dissemination. For example, using immunological double staining techniques, expression of different proteins has been detected, including Ki-67, p120, EGFR, HER2, transferring-R, EMMPRIN, uPAR and EpCam (reviewed in (10)). These studies have shown that most DTC and CTC do not express the proliferation antigen Ki-67, which could explain the partial resistance to chemotherapy (60). Furthermore, the presence or absence of the HER2 proto-oncogene appears to characterize an aggressive subpopulation with regard to invasive capabilities as well as impaired prognosis (73, 74). Also the presence of DTC that express the urokinase-type plasminogen activator receptor (uPAR) has been correlated with an unfavourable prognosis (75).

Immunocytochemical assessment of phenotype, meanwhile, has represented only one aspect of DTC research. Detailed molecular descriptions of single DTC found in BM at the genome level have revealed a high degree of genetic heterogeneity (76–78). Interestingly, some genomic aberrations seen in primary tumours could not be detected in DTC (78), indicating that the DTC may originate from small subclones within the primary tumour or that they undergo significant genetic changes after disseminating into the BM.

In summary, direct analysis of primary tumours, DTC and their surrounding microenvironment continues to provide new infor-

mation on the complex cascade of metastasis. New improved methods, such as whole genome amplification or expression profiling of single tumour cells, will provide even more information on the molecular characteristics of early DTC in cancer patients.

### CONCEPTS AND MECHANISMS OF TUMOUR DORMANCY

The mechanisms involved in tumour cell dormancy are complex and poorly understood. It is important to pursue the mechanistic processes because it is highly probable that dormant DTC will ultimately be the source of subsequent overt metastatic disease years to decades after primary tumour diagnosis (79, 80). The relative paucity of solid scientific findings in this field is due to several factors, both biological and practical.

One of the basic limitations is that we do not have a defined phenotype or molecular profile that identifies a dormant tumour cell. By definition, dormancy implies an ability to become activated. Therefore, it differs in several respects from senescence, which is often an irreversible state (81–83). One can speculate that the two processes are interrelated, since senescent cells can still have an active metabolism despite G1 arrest. However, dormancy might resemble quiescence more than senescence. Quiescence is the state where cells reside in G<sub>0</sub>–G1 arrest, a common finding in adult differentiated cells. Cells can also enter quiescence prior to differentiation (84). The lack of growth-promoting signals may initiate this process. Reversible quiescence is often cited as a feature of stem cells. It is highly likely that stem cells and/or tumour stem cells share many properties with dormant tumour cells (85, 86). Resistance to chemotherapy and ability to generate an overt metastasis upon reactivation are two prominent overlapping features.

Another limitation facing investigators is that to understand the mechanisms of dormancy, dormant tumour cells have to be studied, isolated either from clinical specimens or animal models. It has been speculated that these are very rare cells, often residing in niches of subsequent metastases such as the BM. As there are few defined cell surface markers, isolation of these cells is not a finite science. One can as-

sume that DTC present in BM of cancer patients for prolonged periods of time are in a dormant state, but there is limited proof. Functional studies would be persuasive, such as their growth as spheroids in culture or tumorigenicity in immune compromised mice, as evidence of stem-cell attributes. However, one can also argue effectively that if you expand the population of dormant tumour cells *in vitro* or *in vivo* they are then “activated” and beyond the dormant state. Would molecularly profiling these cells then provide a representative picture of tumour dormancy?

Despite these limitations and challenges, the field is not without a considerable number of hypotheses that address mechanisms associated with the development, maintenance and breaking of tumour dormancy. Much of the discussion that follows is based on experimental models. Verification of these findings in the clinical arena has yet to be achieved. Many of these experimental findings appear highly plausible and with further technological advances their validation in the clinic may not be that distant.

Current theories on potential mechanisms involved in tumour cell dormancy that are discussed in the following paragraphs include (a) loss of the aggressive phenotype through oncogene inactivation after release of tumour cells from the primary tumour (87), (b) lack of an appropriate angiogenic switch at the site of seeding (88), (c) lack of critical growth stimulatory factors or downregulation of their receptors at the site of seeding, (d) host polymorphisms imposing growth delay (89), (e) activated metastatic suppressor genes, stress genes or other transcriptional regulatory genes that inhibit proliferation (90, 91) and/or the need for additional genomic alterations post-seeding (76), (f) aberrations in adhesion factor signalling or interference in epithelial-stromal cell cross-talk (92–95), and (g) immunological factors (96–98). Several of these mechanisms have recently been reviewed by Aguirre-Ghiso (99, 100).

### ROLE OF ONCOGENES AND TUMOUR SUPPRESSOR GENES IN TUMOUR DORMANCY

The use of transgenic models makes possible the investigation of effects upon oncogene inacti-

vation and reactivation. The clear message from these experiments is that the outcome of oncogene inactivation is unpredictable and influenced by both genetic and epigenetic features of a cancer cell. The inactivation of a given oncogene typically results in growth arrest, differentiation and/or apoptosis. In some instances there is complete tumour regression, and in others reactivation of oncogene activity restores the neoplastic phenotype, but not in every cancer type (101–104). *MYC* inactivation and reactivation portrays this diverse biology. *MYC* activation can also result in tumour cells having stem-cell features. Thus in certain instances, the inactivation and reactivation of an oncogene can act like a switch converting a cancer cell to a more “normal” phenotype and then back again to a malignant phenotype. This process may well parallel quite precisely that involved in tumour dormancy. Of course, one key question is whether oncogene inactivation and reactivation occurs in humans.

Animal studies indicate that the process of metastasis is extremely inefficient as less than 0.1% of the shed tumour cells survive the stresses of dissemination, systemic seeding and the early phases of growth (105). Those that do survive and proliferate have also lost expression of metastasis suppressor genes (106, 107). This gene family, now encompassing between 10–20 genes, specifically regulates the seeding and growth of metastases but does not impact *in vitro* growth or *in vivo* growth of the primary tumour. However, there is evidence from a few studies that shed cells which retain certain active metastasis suppressor genes may enter a state of dormancy upon seeding of distant sites. An excellent review of tumour dormancy and the role of metastasis suppressor genes has recently been published (90). Here we discuss two seminal studies. In the first proof-of-principle study, Palmieri *et al.* demonstrated that reactivation of a metastasis suppressor gene (*nm23-H1*) *in vivo* is feasible, and this resulted in decreased evidence of lung metastasis in a breast cancer model (108). In the second study, Nash *et al.* identified *KISS1* as the first metastasis suppressor gene that induced prolonged dormancy (109). Several of the metastasis suppressor genes, including *MAPK* kinase 4 (*MKK4*) and *RHOGD12*, activate *JNK* and *p38* stress pathways (110). A resulting strong

*JNK* or *p38* signal can result in apoptosis of solitary DTC, but it is conceivable that a somewhat weaker signal may induce growth arrest and/or dormancy (111). Finally, metastasis suppressor genes are rarely mutated, so it is hypothesized that epigenetic events such as methylation may cause loss of function (112), and since epigenetic events are reversible, induction of tumour dormancy may be feasible. On the downside, despite common biological effects, metastasis suppressor genes appear to be cancer type specific and therefore manipulating metastatic suppressor genes may also mandate a cancer-specific approach.

#### ROLE OF THE MICROENVIRONMENT IN TUMOUR DORMANCY

Once tumour cells disseminate, a host of pathways and factors need to be activated for these cells to adapt and flourish in their new microenvironment. As proliferation takes hold, one of the most important compensatory events is establishment of a neovasculature. Hypoxia-inducible factor (*HIF-1 $\alpha$* ) is a critical player in this process and is the initiator of several downstream genes associated with neovascularization (113), and upregulated *HIF-1 $\alpha$*  expression is also associated with dissemination of DTC in breast cancer patients (5). As tumour cells proliferate and gain mass, their need for oxygen exceeds that available in the surrounding tissue. This state of anoxia/hypoxia stimulates the tumour cells to produce *HIF-1 $\alpha$* . Without neovascularization or remodelling of the pre-existing vasculature, the resulting anoxia triggers apoptosis and cell death. The balance that exists between dividing cells and apoptotic cells due to anoxia has been associated with dormancy due to lack of neovascularization. The ability of tumour cells to avoid or escape angiogenic dormancy through activation of the neovascular programs is termed the angiogenic switch (114, 115). However, executing this angiogenic switch is often not a straightforward process and, as in many pathways, is tightly regulated and well balanced. Several genes serve as anti-angiogenic factors so it might be envisioned that early in the seeding process the genomic aberrations are relatively minor in the DTC (77), and this fa-

vours that the anti-angiogenic processes dominate and dormancy prevails. Subsequently, as additional mutations occur, the balance becomes altered and execution of the angiogenic switch, in part, allows escape from dormancy. While the multiple scenarios associated with angiogenic dormancy are logical, there is no compelling evidence as yet that this process is predominant in clinical metastases owing to the difficulties of acquiring these specimens for study or in the imaging of micrometastases.

Prior to the involvement of angiogenic considerations, the DTC must proliferate in their new microenvironment and evolve from single cells to micrometastases. It is well known that growth factors, including cytokines such as CXCL-12, play an essential role in the invasion, proliferation and progression of cancer cells (116, 117). Disseminating tumour cells are also able to secrete growth factors (e.g., FGF-2) relevant to their outgrowth *in vitro* (14, 118), which may allow stimulation of proliferation in an autocrine fashion. It is also well known that the growth factors in the tumour's new microenvironment may not resemble those at the primary tumour site. A non-permissive microenvironment could lead to the activation of stress signals that promote quiescence. If the necessary growth factors are not readily available within this new microenvironment, the tumour cell must adapt, become quiescent, or die. In a somewhat analogous situation, tumours dependent for survival upon known hormones or growth factors are treated by removal of these agents. Prostate cancer is a prime example where removal of androgens is a primary mode of treating recurrent disease. The majority of cells then undergo apoptosis, but a few may enter a dormant or quiescent state, which provides time for adapting to an androgen-depleted environment. This would again potentially force the tumour cells to adapt prior to proliferation. The remarkable plasticity of tumour cells in adapting to various stresses is well recognized but it is certainly conceivable that other inhibitory processes delay this process for long periods of time, augmenting the state of tumour cell dormancy.

One of the most critical issues within the new microenvironment is that of cross-talk between the newly seeded solitary tumour cell, stromal cells and adhesion factors (92, 119–121). Per-

haps it is possible for these solitary cells to undergo sufficient proliferation to be considered a micro-metastasis without significant cross-talk but this interaction is certainly necessary for further maintenance of proliferation and progression. For example, if the cross-talk is not sufficient, tumour cells can enter a phase of differentiation and growth arrest (93). In breast cancer this cross-talk may involve beta-1-integrins and EGF. Similarly, in head and neck carcinoma, the cross-talk is between u-PAR and alpha-5-beta-1-integrin (122), which set up a cascade of signalling and receptor activity, including focal adhesion kinase (FAK), EGF receptor, and the Ras-extracellular signal-related kinase (ERK). Interruption of this cross-talking network in this study and in another with squamous carcinoma cells resulted in suppression of tumour growth and dormancy (123, 124). In these studies and others, u-PAR is viewed as an important player (67, 75, 122, 123, 125, 126). Loss of u-PAR or interruption of the u-PAR pathway activates the p38 mitogen-activated protein kinase (MAPK) stress signalling pathway, which is associated with induction of dormancy (111, 127–129). There appears to be a balance between the ERK and p38 pathways that controls whether the tumour cell proliferates or enters dormancy—a high ERK to p38 signalling ratio favours proliferation whereas a lower ratio favours dormancy (127). Interestingly, several studies show dormancy was broken and growth resumed when there was inhibition of p38 signalling (111, 127, 128). Therefore from a clinical perspective, maintenance or induction of p38 signalling might offer a novel therapeutic strategy.

#### HOST SUSCEPTIBILITY AND TUMOUR DORMANCY

Recent evidence accumulated from a series of murine studies shows that polymorphisms can modulate the efficiency of tumour metastases (89, 130–132). The first set of investigations to support this hypothesis was a series of breeding studies that were carried out using the polyoma middle-T antigen transgene-induced metastatic mammary tumour model (132). Male mice bearing the transgene were bred with female mice of various different inbred strains and the

transgene-positive F1 female progeny were assessed for frequency of pulmonary metastases. The range was extraordinary and these results led to the identification of a metastasis modifier locus. One of the genes in this locus was *Sipa1*, a metastasis-promoting gene (131). Polymorphisms in the protein-protein interaction domain might result in loss of function. The consequence of lowered metastatic efficiency might then be induction of tumour cell dormancy.

### ROLE OF IMMUNE SYSTEM IN TUMOUR DORMANCY

The role of the immune system in tumour dormancy is somewhat debatable. There is no question that under appropriate conditions the immune system has the capacity to control tumour growth. In mouse leukaemia and lymphoma, several studies have demonstrated a relationship between the immune system and tumour dormancy (133–137). Using a sarcoma mouse model of primary chemical carcinogenesis, Koebel *et al.* recently demonstrated that adaptive immunity maintains occult cancer in a state of equilibrium (97). Even in some immunocompetent animals, however, stable lesions occasionally escaped from dormancy and these lesions had lost their immunogenicity. Commonly proposed mechanisms are that tumour cells lose expression of tumour-associated antigens and/or overexpress programmed cell death ligands (e.g. CD274) that allow escape from the immune response. Obviously, it is attractive to speculate that the immune system might be stimulated, e.g. vaccine therapies, to eradicate systemic dormant tumour cells in patients prior to the onset of overt clinical metastases. In contrast, the downside of current immunosuppressive chemotherapy and irradiation could be the escape of dormant tumour cells from immune control (98). The data, however, in patients are not yet compelling that the immune system has a significant involvement, especially in solid tumours, in maintaining dormancy through active surveillance or in allowing subsequent growth by exposure to evasion mechanisms acquired by the tumour cells. In some situations it has been speculated that the immune system might actually augment an escape of tumour cells from dormancy. Cytokines were previously mention-

ed as being very powerful growth factors. Inflammatory events might be envisioned as a source of cytokine release into the micro-environment and these cytokines may stimulate the activation of dormant tumour cells.

### LESSONS LEARNT FROM MATHEMATICAL MODELS

Using mathematical models Klein *et al.* recently concluded that tumour cell dormancy is a characteristic of nearly all CTC with metastatic outgrowth reliant on evolutionary processes and mutations following dissemination, often occurring very early in the growth of the primary tumour (138). They articulately discuss the pros and cons of these three models, summarized as (a) the stochastic model, which primarily relates tumour size to number of CTC but poorly explains tumour cell dormancy, (b) the linear progression model, which theorizes that most critical genetic changes occur at the primary tumour site and late in primary tumour growth, but presents many inconsistencies in relation to what is seen in clinical progression and also conflicts with the presence of long-term tumour cell dormancy, and (c) the parallel progression model, which is favoured by the authors as it accounts for the vast genomic diversity between the primary tumour, CTC, and metastatic sites, is consistent with early dissemination of tumour cells and is reliant on the primary tumour-secreting factors that promote solitary tumour cell growth at distant sites. They further speculate that interruption of these growth-promoting factors through removal or death of the primary tumour then forces adaptation of the disseminated tumour cells to the microenvironment of the distant site. This reiterates the discussions herein that micrometastases may exist in a dormant state where proliferation and apoptosis are finely balanced, perhaps in part due to inadequate neovascularization, whereas solitary tumour cell dormancy is associated with lack of proliferation or apoptosis with the cells experiencing cell cycle arrest.

Mathematical modelling of breast cancer recurrence also supports the existence of tumour dormancy and surgery-driven interruption of dormancy in breast cancer. In their recent article, Demicheli *et al.* summarized the evidence

that primary tumour removal can perturb metastatic homeostasis, and for some patients result in acceleration of metastatic cancer (139). This can be explained by the assumption that the primary tumour may release factors that block either (i) the conversion of single non-cycling DTC to proliferating micrometastases or (ii) angiogenesis required for a vascular micrometastatic foci to grow into overt metastasis (139).

### CHALLENGES OF RESEARCH ON TUMOUR DORMANCY

Tumour cell dormancy is of great importance and therefore the NCI convened a small workshop in July 2006 to discuss the challenges and research opportunities. A report detailing the findings of the workshop, including recommendations to the NCI, has recently been published (140). Of the multiple challenges confronting the field, three stand out and are briefly discussed here.

The first is the detection and characterization of dormant tumour cells. The detection, isolation and characterization of dormant tumour cells from bone marrow aspirates of patients who have endured a prolonged disease-free period present a considerable technical challenge. The identification of dormant tumour cells in these specimens is imprecise as there are no defined markers. Most investigators working with such clinical specimens from patients with epithelial tumours use immunomagnetic particle technologies targeting epithelial antigens (11, 24, 29, 141). These isolation techniques are labour intensive, sometimes involving the “plucking” of individual epithelial cells with a micropipette, with yields frequently being fewer than 20 putative dormant tumour cells for study. These studies have nearly all involved bone marrow specimens, but participants have been quick to note that efforts need to expand to other organ systems, such as the liver, where dormant tumour cells are also likely to reside. This most likely will only be an option at research “rapid autopsies” but it is believed that searching for and retrieving micrometastases could prove important for the study of tumour cell dormancy. Once the putative dormant tumour cells are isolated, the next challenge is molecular characterization. It should be re-

called that one is frequently only starting with 10–20 cells, and again this pushes the technological envelope. However, advances are being made and it is now possible to perform gene expression array studies and comparative genomic hybridization analyses on such few cells (142–144). Beyond molecular characterization, one would like to explore functional attributes, such as similarities between these cells and cancer stem cells. Just two functional aspects that should be explored are growth *in vitro* as spheroids and tumorigenicity when implanted into immunocompromised mice, but the limited number of cells to work with continues to haunt investigators in this field.

The second high profile challenge was how to best model tumour cell dormancy. In the discussion above concerning possible mechanisms, nearly all were based on a few experimental models, whilst many more models are needed if we are to increase our understanding. With both experimental models and clinical specimens there are only a limited number of studies which can be done with the few cells acquired, so expansion *in vitro* or *in vivo* offers an attractive approach. But there is debate whether expansion then changes the molecular and phenotypic characteristics of dormancy. Identifying the factors which are involved in the “awakening” of dormant cells is urgently needed. There is also a need to improve imaging technologies for the study of tumour cell dormancy *in vivo*. Ideally the resolution should be down to at least the micrometastatic size and be able to detect these small clusters in organs such as liver and bone. Both the ability to detect and the ability to determine metabolic activity would be very helpful. As with knowledge from any model, the challenge is to then translate this information to the clinical setting. For example, in the discussion of possible involvement of tumour suppressor genes playing a role, there are considerable experimental findings, but to our knowledge no published reports demonstrating active tumour suppressor gene presence in dormant tumour cells isolated from patients who are in relapse for years. Such studies, perhaps involving collaboration between investigators with expertise in tumour suppressor genes and those isolating DTC, should be attempted.

The third challenge highlighted in the workshop if we are to advance our knowledge of tu-

tumour cell dormancy is the relative lack of investigators in this field. Of course, because of the challenges just described, it is not surprising that many investigators are sitting on the sidelines before becoming actively involved. However, with challenges come enormous opportunities. These involve technology development as well as basic scientific endeavours. It is to be hoped that the findings and recommendations from the workshop will stimulate the NCI to make available funding opportunities so that new investigators can enter this field.

### OPEN QUESTIONS AND FUTURE DIRECTIONS

To prevent the occurrence of metastases in distant organs, different adjuvant therapies (e.g. chemotherapy and hormonal therapy) with considerable side effects are currently administered to cancer patients. The decision to apply these therapies is today largely based on statistical risk assessment through clinical prognostic parameters such as tumour stage or tumour grade. However, only a relatively small fraction of patients benefit from adjuvant therapies, probably because of the presence of dormant DTC that are partially resistant to conventional chemotherapy (56, 58–60). Therefore, understanding the mechanism behind tumour cell dormancy and the development of new therapeutic approaches, which either target the drug-resistant non-mitotic tumour cells or alternatively induce or maintain the dormant cell state, are of the utmost clinical importance.

Even though in recent years much progress has been made in the characterization of DTC and the mechanism behind tumour cell dormancy, there remain many open questions. Some may not be answered until we are considerably more entrenched in clinical studies while others will require technological breakthroughs.

The first open question is whether tumour cell dormancy exists at the time of tumour shedding or whether the cell is responding to stress or an unfavourable microenvironment. One can envision both scenarios occurring, even within the same patient. For example, from the data presented on tumour suppressor genes, we might speculate that dormant tumour cells

could be shed if tumour suppressor genes were active or hypothesize that dormancy could be induced after shedding if the tumour suppressor genes were reactivated. Looking for activated tumour suppressor genes in CTC/DTC shed early in the disease process is critical but will also need to be assessed at the single cell level due to the heterogeneity of the CTC/DTC population. Alternatively, stress from seeding a new non-permissive microenvironment would appear to favour induction of dormancy by a variety of mechanisms discussed previously either occurring at the solitary cell level or after some initial proliferation.

Another question is whether dormant tumour cells are equivalent to, similar to, or distinct from cancer stem cells. It is a critically important question and there are advocates on both sides. Unfortunately, none have compelling clinically relevant data. Since there are debates in the stem-cell community as to whether cancer stem cells are distinct from normal stem cells and markers identifying each of their distinguishing molecular phenotypes are yet to be defined, it is unlikely that this question will be answered in the near future.

A third thought-provoking question is whether all relapse-free long-term survivors have niches of dormant tumour cells. By definition, dormancy implies the ability to be awakened. Coupled closely to this question is what awakens dormant tumour cells? Could it be an inflammatory event or an injury that favourably alters the soluble factors within the microenvironment or dormant tumour cell niche? More extensive epidemiological studies need to be conducted to associate late-term recurrence with adverse potentiating events. For this to be feasible, physicians and patients should be aware of and document such occurrences that may play a role. Of course, the most clinically relevant question of all is whether we can block the activating signals and thus prevent these clinically overt metastases which strike years after the patients and their physicians believe they are cured.

In conclusion, research on tumour cell dormancy will open a new avenue of investigation leading to a better understanding of tumour progression in cancer patients with important implications for improved diagnosis and treatment of minimal residual disease (146).

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