Novel technologies and recent advances in metastasis research

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ABSTRACT In this review we have attempted to summarize some of the recent developments in using novel technologies to unravel the molecular mechanisms of tumor progression, in particular the formation of tumor metastasis. In order to push forward the frontiers in cancer research, it is obvious that several fields have to be further developed and interconnected: (1) clinical, epidemiological and pathological studies which mainly use innovative technologies, including microarray technology and nanotechnology to determine as many parameters as possible, (2) the development of improved and suitable bioassays and better animal models and (3) the use of novel computation and bioinformatics methods to sample and integrate the exponentially growing sets of data coming from such investigations. Fashionable as scientists are, this new endeavor may be called systems biology.

KEY WORDS: cancer, gene expression profiling, metastasis, mouse models, technology

I. Tumor progression – an introduction

In the past we have learned that malignant cancers develop in multiple steps involving a number of genetic or epigenetic events. More recently, we have been alerted to the fact that such multiple events are not exclusively restricted to the tumor cells themselves. Rather there is a pivotal contribution of the tumor stroma to malignant tumor progression and metastasis. Stromal players may include fibroblasts, blood or lymphatic vessel endothelial cells, and infiltrating cells of the immune system. The major steps of tumor progression can be defined by a number of histopathological criteria: (1) hyperplasia: hyperproliferation of cells; (2) adenoma: encapsulated tumors with relatively normal cells and without infiltration of surrounding tissue; (3) carcinoma: invasive tumors where cells degrade the basal membrane and invade into surrounding tissue and (4) metastasis: the final stage in tumor progression when tumor cells disseminate either via the lymphogenic or via the hematogenic pathway to local lymph nodes and distant organs, respectively (Fig. 1). The mechanisms of cell dissemination from primary tumors, intravasation and survival in the lymphatic or blood stream, and metastatic colonization of the target organ (Paget, 1889), are a major focus of current cancer research. After all, metastatic spread is the actual cause of death in approximately 90% of all cancer patients.

Since the discovery of oncogenes and tumor suppressor genes, intense research in many laboratories all over the world has brought us to the point where we are starting to understand the main principles underlying molecular changes in the course of tumor progression (Hanahan and Weinberg, 2000). Owing to new technologies, the molecular picture of tumorigenesis has become more complex, and the process of tumor progression has to be envisaged as a network of simultaneous events within both tumor cells and tumor stroma. In this review, we have attempted to introduce some of the novel technologies that are currently used in cancer research and to describe selected experimental results that exemplify current efforts and advances in the field.

II. Experimental systems

Cell adhesion, migration and invasion assays

The first step in the formation of tumor metastasis is the dislodgment of tumor cells from the primary tumor and their subsequent invasion into neighboring tissue. In a macroscopic view, tumor cell invasion includes attachment to, proteolysis of and

Abbreviations used in this paper: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BAC, bacterial artificial chromosome; CML, chronic myelogenous leukemia; CT, computer tomography; CTGF, connective tissue growth factor; DMBA, dimethylbenzanthracene; ER, estrogen receptor; ERM, Ezrin-Radixin-Moesin; ES cells, embryonic stem cells; ESTs, expressed sequence tags; FGF, fibroblast growth factor; GFP, green fluorescent protein; IVVM, intravital videomicroscopy; LPTA, light producing transgenic animals; MAP, mitogen activated protein; MARCM, mosaic analysis with a repressible cell marker; MMP, matrix metalloprotease; MRI, magnetic resonance imaging; NCAM, neural cell adhesion molecule; PET, positron emission tomography; SOL, surgical orthotopic implantation; TPA, tetradecanoylphorbol-13-acetate; VEGF, vascular endothelial growth factor.

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migration through the basal lamina (Liotta, 1984). Cell migration and invasion are not precisely defined in the literature and are often used as synonyms. However, migrating cells are usually motile cells that do not degrade and invade the basal lamina, whereas invading cells do (reviewed by Staff, 2001). On the molecular level, the function of many different genes and factors is involved in these processes, including cell-cell adhesion molecules, cell-matrix adhesion molecules, proteases, and proteins involved in the regulation of the actin cytoskeleton. To investigate the specific function of such factors in cell migration and invasion, cultured tumor cells are often genetically manipulated, for example by forced expression of a gene (gain of function) or by ablation of gene function either by homologous recombination or by antisense RNA approaches (loss of function). Such genetically altered cells are then tested for gene expression-dependent changes in migration and invasion.

Two-dimensional (2D) assays

These assays include variations on the theme of simply culturing cells on plastic surfaces. By varying the coating of the plastic surface one is able to analyze the capabilities of cells to adhere to certain extracellular matrix compounds. On the other hand, cell-cell aggregation can be measured by preventing cell adhesion to the culture surface, for example by using bacterial plastic dishes and shaking. Cell-cell adhesion between cell types expressing particular adhesion molecules can be determined by culturing a particular cell type on a layer of another cell type.

A simple and inexpensive way of determining the migratory capability of cells is to culture cells to near confluency and then to introduce a defined wound by scratching for example with a pipette tip. The number of cells and the kinetics by which they migrate into the cell-free zone and close the “wound” can then be quantified. Classical in vitro assays of cell migration and invasion are Boyden chambers and Transwell filter chamber systems. They consist of upper and lower chambers separated by a filter to separate the two compartments. In migration assays, test cells are seeded into the upper chamber, a chemoattractant is added to the lower chamber, and cells that have traversed through the filter are quantified after an appropriate time span. To determine invasion, the filter membrane is coated with extracellular compounds such as matrigel (extracellular matrix from a tumor cell line (Albini et al., 1987)), in order to mimic the natural basal membrane. The extensive use of these assays has provided us with important knowledge about the composition and function of cell-cell and cell-matrix adhesions.

Three-dimensional (3D) assays

In order to better mimic the in vivo situation, new tissue culture systems have been developed where cells can be observed in three-dimensional matrices composed of extracellular-matrix macromolecules. For example, collagen and fibrin gels as well as matrigel have been widely used to study tumor-stromal interactions in cancer (Cukierman et al., 2002). Not surprisingly, the molecular mechanisms of cell adhesion and its functional contribution to cell behavior differ dramatically in three-dimensional matrices compared to two-dimensional cell cultures (Cukierman et al., 2001). Recent experimental data on cell growth and differentiation is also based on the use of culture systems in which cells are grown either alone or in co-culture with other cell types in spheroids, thereby recapitulating the three-dimensional growth of cells on their own extracellular matrix (Korff and Augustin, 1998).

Mouse models of tumorigenesis

For obvious reasons, the molecular analysis of carcinogenesis in patients can only be performed in a retrospective way, and genetic or functional experiments, that would clearly demonstrate the functional contribution of a gene of interest to tumor progression, are not possible. Hence, mouse tumor models have been instrumental to further our understanding of the complex processes involved in tumor progression (Macleod and Jacks, 1999; Herzig and Christofori, 2002). In particular, the investigation of tumor-stroma interactions and metastatic dissemination of tumor cells require studies in an intact organism. In order to “produce” cancer, a number of experimental approaches are employed, such as chemical carcinogenesis, xenograft or syngeneic transplantations of tumor cells into mice, or the genetic manipulations of the mouse genome. Of course, fundamental differences in anatomy, physiology and biochemistry between mice and humans raise a number of questions about equating mouse and human tumorigenesis. Yet, mouse models of tumorigenesis represent an important and valuable source of novel insights into the molecular principles of multistage carcinogenesis (Van Dyke and Jacks, 2002; Rangarajan and Weinberg, 2003).

Chemical carcinogenesis

Initiation of skin tumors is frequently done by applying the carcinogen 7,12-dimethylbenzanthracene (DMBA) to the skin, which invariably induces oncogenic activation of the c-H-Ras gene (Quintanilla et al., 1986). Subsequent treatment with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) will lead to full-blown cancer. This rather “old-fashioned” protocol is now being heavily used in combination with genetically modified mouse lines to reveal genetic modifiers in multistage skin tumorigenesis (Balmain, 2002).

Xenograft transplantation studies in nude mice

Tumorigenicity and metastatic potential of cancer cells and established cell lines is often studied in immunodeficient or syngeneic mice by subcutaneous, orthotopic or intravenous injections (reviewed by Kubota, 1994). Orthotopic injections of cell suspensions into the organ of the tumor cells’ origin are often necessary for tumors to metastasize, because the subcutaneous microenvironment is often too different from the original cellular environment (Fidler et al., 1990). Along these lines, surgical orthotopic implantation (SOI) of tumor fragments yields metastatic rates and sites in the transplanted mice that better reflect the clinical pattern (Hoffman, 1999). The capability of tumor cells to specifically colonize distant organs is frequently determined by injection of tumor cells into the tail vein or into the heart ventricle.

Genetically modified mice

Although the mere overexpression of an oncogene or the ablation of the function of a tumor suppressor gene in most cases did not generate mouse models that reliably recapitulated human cancer, recent years have witnessed dramatic progress in ‘rebuilding’ human cancer in the mouse. Reproducible mouse models of multistage carcinogenesis are amenable to genetic and physiological manipulation and have allowed many important
proof-of-concept experiments, i.e. experiments that clearly demonstrated the causal function of a gene in tumor development. The first mouse models of cancer were inbred isogenic strains prone to specific cancer and mouse mutants generated by random germ-line mutagenesis or insertional mutagenesis with retroviruses (reviewed by Jonkers and Berns, 2002). The methodology to introduce oncogenes into the mouse germ line (transgenic mice) or to ablate gene function by homologous recombination in embryonic stem (ES) cells (knock-out mice) has enabled researchers to study the functional contribution of genes to tumor progression. Recent developments in this methodology allow the introduction of whole gene loci into transgenic mice (bacterial artificial chromosome (BAC) transgenics) and to replace mouse genes with their human counterparts or with mutated versions thereof (knock-in mice). Finally, it is now possible to induce or ablate gene function at will in specific tissue or at certain time points (inducible transgenes and conditional knock-outs, respectively (reviewed by Lewandoski, 2001; Jonkers and Berns, 2002):

1. Conditional knock-out (permanent, irreversible): Two systems have been developed to ablate gene function in an inducible manner, the phage Cre/loxP and the yeast Flp/Frt systems (Sauer and Henderson, 1989; O’Gorman et al., 1991). Recombination sites are introduced into the mouse genome by homologous recombination in ES cells so as to flank the region of a gene that is important for the function of the gene’s product. Mice carrying these recombination recognition sites are usually unaffected. Yet, upon introduction of the appropriate recombinase, Cre for the LoxP sites and Flp for Frt sites, either by tissue-specific transgene expression or by viral delivery, the recombinase will “excise” the flanked regions by conservative recombination between the recognition sites, thereby ablating gene function in a particular tissue or at a certain time point.

2. Inducible transgene expression (transient, reversible): The generation of transgenic mice in which the expression of the transgene can be induced in tissues of interest and at different time points, is based on the bacteria-derived tetracycline-inducible system (Gossen and Bujard, 1992). Two mouse lines are usually generated; one line expresses the gene of interest under the control of a Tet-operator promoter region that binds and responds to the tetracycline-dependent transcriptional regulator (Tet-activator). The second mouse line expresses the Tet-activator protein which specifically binds the Tet-operator sequence. The Tet-activator protein has been engineered to transactivate either in the absence of tetracycline (Tet-Off system) or in the presence of tetracycline (Tet-On system). Appropriate choice of the promoter expressing the Tet-activator will decide in which tissue or in which cell type expression of the gene of interest will be modulated. The two mouse lines are then intercrossed to generate bitransgenic mice. The more stable Tetracycline analog Doxycycline is provided in the drinking water or by other means, resulting in repression of gene expression in case of the Tet-Off system and activation of gene expression in the Tet-On system.

**Drosophila as a model for tumor invasion and metastasis**

Recent experiments with *Drosophila* mosaic mutants have demonstrated that the loss of cell polarity is not only a consequence of cancer, but can directly cause increased proliferation and tumor development (Brumby and Richardson, 2003; Pagliarini and Xu, 2003). Namely, loss of the cell polarity gene *scribble* in RasV12-expressing cells disrupted apico-basal polarity which led to progressive invasion of these cells into neighboring structures. The recently developed MARCM (mosaic analysis with a repressible cell marker) technique used in these studies (Lee and Luo, 2001) enables researchers to produce tissue specific mutants in a context of normal tissue, which resembles the tumor situation in humans. Lately, it has been demonstrated that several key aspects of mammalian metastasis, such as the loss of E-cadherin expression and degradation of the extracellular matrix, are recapitulated in the *Drosophila* model (Pagliarini and Xu, 2003). Therefore, future experiments with the genetic model organism *Drosophila* will certainly also contribute to cancer research.

**The actual focus: cancer patients**

As mentioned above, the molecular dissection of tumor progression in cancer patients has been highly restricted to retrospective analyses. Yet, recent developments in endoscopic procedures and in imaging technologies have substantially improved the macroscopical

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**Fig. 1. Major steps in tumor progression and technical approaches to study them.**

Acquisition of unlimited proliferation potential and escape from apoptosis (survival) precede benign tumor formation. Ingrowth and formation of new blood vessels (angiogenesis) provides growing tumors with oxygen and nutrients. During progression to the malignant stage, tumor cells lose their differentiated phenotype and acquire migratory and invasive properties. Finally, angiogenesis and lymphangiogenesis provide a route for tumor cells to enter the circulation, to home to regional lymph nodes and/or to colonize distant organs. Techniques used to study different steps of tumor progression include animal models, gene and protein expression profiling and imaging (see text for details).
observation of cancer progression. Moreover, high-throughput analysis of gene expression in cancer tissue specimen are just starting to provide novel insights into human cancer (see below). Hence, in order to gain a realistic view of human tumorigenesis it is mandatory to combine the knowledge gained from experimental systems with the increasing insights into the parameters of human carcinogenesis.

III. Technology

Microarrays

The individual genetic set-up of each cancer patient, together with the variability of cancer types, indicates the need for a fast and reliable method to analyze as many biopsies as possible for changes in gene and protein expression and possibly in gene function. While the latter is a colossal task involving innovative approaches of systems biology, the former has been facilitated with the advent of array technologies.

cDNA microarrays

cDNA or oligonucleotide microarrays representing more or less the whole transcriptome are a powerful method to classify tumors according to their gene expression (molecular) signature (reviewed by Liang and Pardee, 2003). Molecular profiling of human cancer clearly helps to refine tumor classification that thus far was solely based on histopathological criteria. An example of such insights into novel subtypes of human cancer has been the use of DNA microarrays to distinguish acute myeloid leukemia (AML) from acute lymphoblastic leukemia (ALL) (Golub et al., 1999) or to subtype diffuse large B-cell lymphoma into two categories, with different prognostic importance for patients (Alizadeh et al., 2000). Subsequent studies using DNA microarray technology on different types of cancers have indeed demonstrated that it is possible to determine “poor prognosis signatures” when a high enough number of tumor samples is being analyzed (see below). It is expected in the near future that this technology will aid not only in diagnosis/prognosis but also in the identification of genes that are causally involved in the progression to tumor malignancy. A complementary approach in the analysis of genetic changes during tumor progression is the use of comparative genome hybridization on BAC-arrays (reviewed by Cowel and Nowak, 2003). Bacterial artificial chromosomes spanning the entire human genome are arrayed on glass slides and used for comparative hybridizations with DNA samples from tumor biopsies, revealing gene loci that are amplified or deleted during tumor progression.

Protein microarrays

Protein arrays contain a number of immobilized protein spots, proteins being either antibodies, cell lysates or recombinant proteins. To determine the protein content, the arrays are incubated with a tagged unknown biologic sample or labeled antibody (reviewed by Liotta et al., 2003). Similarly to DNA microarrays that reveal the gene expression profile at the mRNA level, this approach is thought to provide a “snapshot” of the protein content of cells or tissues at a certain time. This emerging technology is not yet fully in place, but it certainly will complement current proteomic approaches, such as the analysis of protein expression profiles by two-dimensional gel electrophoresis and subsequent high throughput mass spectroscopy analysis.

Multi-tissue arrays

Multi-tissue arrays are generated by placing many cylindrical tissue samples into a recipient paraffin block so that after sectioning, a slide containing up to a thousand of different tissues is produced (Kononen et al., 1998). This technique significantly accelerates tissue analyses by in situ technologies, since many tissue samples can be analyzed simultaneously on DNA, protein and RNA level by immunohistochemistry and in situ hybridization techniques (reviewed by Bubendorf et al., 2001). In cancer research, multi-tissue arrays are highly useful for correlating changes in gene expression with tumor progression in a large number of patient samples.

Laser capture microdissection

This technique is being increasingly used for the isolation of homogeneous, morphologically defined cell populations, i.e. pure cell samples from tumor or stromal tissues (Going and Lamb, 1996; Fend and Raffeld, 2000). For example, the combined use of laser capture microdissection and DNA microarrays was recently used for generating in situ gene expression profiles of different stages of human breast cancer (Ma et al., 2003).

Imaging techniques

Much progress has been made in the use of animal model systems with the development of in vivo imaging methods. For example, GFP- or luciferase-labeled metastatic cells (using stable transfection) can be injected into nude mice and metastasis can be imaged by fluorescent optical imaging (reviewed by Hoffman, 2002) or by bioluminescence imaging (LightProducing Transgenic Animals, LPTA; reviewed by Jonkers and Berns, 2002). Intravital videomicroscopy (IVVM) has been developed for direct visualization of processes within living animals (MacDonald et al., 2002). Of course, the development of improved and novel imaging technologies for the diagnosis of cancer patients is a major issue in clinical cancer research. Positron emission tomography (PET) combined with computer tomography (CT), ultrasound imaging as well as magnetic resonance imaging (MRI) need to be further developed (reviewed by Laking et al., 2002). Other approaches to specifically detect and potentially even treat tumors and metastasis include the specific targeting of radioactive compounds to tumor cells (reviewed by Govindan et al., 2000; Chang et al., 2002).

IV. Novel approaches in metastasis research

In this section, we present a selection of recent reports that have significantly advanced metastasis research by using novel technologies.

Gene expression signatures of metastatic tumors

DNA microarray technology is now increasingly employed not only for the identification of genes involved in tumor metastasis but also for establishing expression profiles that may be useful for cancer diagnosis and the prediction of clinical outcome. For example, DNA microarray analysis of 65 surgical specimen from 42 individual breast cancer patients indicated that sets of different genes can be identified that relate to different physiological purposes and that tumors could be classified into different subtypes (Perou et al., 2000). Notably, tumors from one patient always looked more similar to each other in their expression profile than tumors from different individuals. In
another report, where 117 young breast cancer patients were analyzed, a specific expression profile pattern was identified that predicted with high probability that lymph node-negative patients would progress to metastatic cancer (van 't Veer et al., 2002). The genes highly expressed in tumors with the poor prognosis signature were all involved in tumor progression, such as the regulation of cell cycle, angiogenesis, invasion, and metastasis. This poor prognosis gene expression profile was then evaluated by analyzing tumor specimens from 295 breast cancer patients with known outcome, and, indeed, an impressive correlation between the poor prognosis signature and poor clinical outcome became apparent (van de Vijver et al., 2002).

In a similar approach, non-matching primary adenocarcinoma and metastasis of different tumor types, including cancers of the lung, breast, prostate, colon, uterus, and ovary were analyzed by gene expression profiling (Ramaswamy et al., 2003). Here, significant expression profiles for primary adenocarcinomas and for metastasis, respectively, were identified. Notably, some primary tumors exhibited the same 17 gene signature found in metastases and these tumors were associated with metastatic spread and poor clinical outcome. These data suggest that primary tumors that are prone to progress to metastasis can at least in part be identified based on their gene expression profile. In the long run, by establishing reliable expression signatures these studies will enable clinicians to exactly classify cancers in terms of appropriate therapy and clinical outcome. Unfortunately, these studies are often hampered by the unavailability of large numbers of appropriate samples from tumor metastasis. Major efforts towards establishing appropriate high quality tumor banks still need to be invested to reach this goal.

DNA microarray analysis is also increasingly being applied to identify genes involved in tumor progression. In addition to tumor biopsies from patients, matching pairs of non-metastatic and metastatic cell lines are frequently employed for these experiments. These human and mouse tumor cell lines are selected in vivo for low or high metastatic capabilities by repeated cycles of transplantation into immunocompromised or syngeneic mice (Fidler, 1973). In one report, genetic profiles of subcutaneously grown metastatic human and mouse melanoma cell lines were compared to profiles derived from subcutaneously grown, non-metastatic lines (Clark et al., 2000). This analysis revealed that almost all metastatic genes continued to be overexpressed in metastatic cells grown subcutaneously. Altogether, 32 genes and expressed sequence tags (ESTs) whose expression patterns positively correlated with the metastatic potential of tumor cells could be identified. Most of these genes encode cytoskeletal regulators or extracellular matrix proteins. The expression of several genes was consistently upregulated in all human and mouse metastasis analyzed, including the extracellular matrix component fibronectin, the small GTPase RhoC, thymosin β4, a small peptide that has been implicated in the regulation of angiogenesis, wound healing and metastasis and the proteolytic enzyme JPA. In subsequent functional experiments, RhoC was able to induce invasive and migratory behavior of tumor cells, a hallmark of metastatic capability. Conversely, a dominant negative version of RhoC suppressed this phenotype in metastatic cells, making this less-well studied member of the Rho family an interesting target for further investigations.

In another report, an established human breast cancer cell line was used to select for subclones that specifically metastasize to bone (Kang et al., 2003). Gene expression profiling revealed that overexpression of interleukin-11 (IL 11), connective tissue growth factor (CTGF), the chemokine receptor CXCR4, fibroblast growth factor 5 (FGF5), the multifunctional adhesion factor and metastasis-related protein osteopontin, and the matrix metalloproteinase MMP1 correlated with bone metastasis. Subsequent functional experiments demonstrated that these genes synergize in the induction of bone metastasis. In a similar approach, poorly or highly metastatic cell lines derived from a transgenic mouse model of rhabdomyosarcoma were used to identify genes that were differentially expressed (Yu et al., 2004). The membrane/cytoskeletal linker protein Ezrin and the transcription factor Six-7 were highly expressed in metastatic cells. Forced expression of these genes in poorly metastatic cell lines increased the metastatic potential, whereas interference with their function reduced the metastatic potential of highly metastatic cell lines. Interestingly, Six-1 appears to act directly upstream of Ezrin. In a separate report, interfering with Ezrin function also resulted in a decrease of metastasis in a osteosarcoma model (Khanna et al., 2004). Using single cell fluorescent imaging technology, it was shown that cells in which the function of Ezrin was suppressed underwent apoptosis when colonizing the lung. Apoptosis could be prevented by upregulating the MAP kinase pathway, indicating that Ezrin elicits an active survival signal in metastatic tumor cells. In this context, it should be noted that another member of the Ezrin-Radixin-Moesin (ERM) family, the neurofibromatosis type 2 tumor suppressor Merlin has also been implicated in tumor progression: heterozygous Merlin knock-out mice develop a variety of malignant tumors with a high incidence of tumor metastasis (Lallemand et al., 2003).

Together, such studies already have, and in the near future will, contribute to our understanding of the molecular mechanisms underlying tumor metastasis. These and other experimental approaches not described here will help substantially in identifying genes which are essential for the metastatic process in experimental model systems. However, these genes may not have exactly the same importance during the metastatic dissemination of cancer cells in patients.

Angiogenesis and lymphangiogenesis

Based on the fact that tumor cells frequently disseminate through the blood stream it has been tacitly assumed that tumor angiogenesis, the ingrowth and formation of new blood vessels in tumors, is a prerequisite for the formation of tumor metastasis. Moreover, micrometastases that have already seeded into distant organs clearly rely on angiogenesis to be able to grow out as life-threatening metastases. Finally, increased vessel density within tumors appears to correlate with metastatic disease in many instances. However, although the past years have brought major insights into the molecular details of how tumor angiogenesis is regulated (reviewed by Carmeliet, 2003), knowledge about the direct contribution of tumor angiogenesis to tumor metastasis remains rather anecdotal. A clean separation of tumor angiogenesis and tumor progression is also complicated by the fact that many transforming processes directly induce angiogenesis; for example, oncogenic Ras or Neu upregulate the expression of vascular endothelial growth factor (VEGF), a prototype angiogenic factor, and repress expression of angiogenic inhibitors, such as thrombospondin-1 (Viloria-Petit et al., 2003). Hence, it is not clear whether tumor angiogenesis would actively provoke tumor metastasis or whether a tumor that is reduced in size by anti-angiogenic therapy is still able to disseminate tumor cells to distant organs. Future investigations in mouse models as well as clinical
correlation studies will have to specifically address these questions.

In contrast to blood vessel angiogenesis, in the past few years we have seen a number of experimental results that convincingly implicate the neglected sibling of blood vessels, lymphatic vessels, in tumor metastasis. The data clearly demonstrated that upregulated lymphangiogenesis during tumor development resulted in the dissemination of tumor cells to regional lymph nodes. In many instances, the expression of the lymphangiogenic factors VEGF-C and to a lesser extent VEGF-D correlated with increased tumor lymphangiogenesis and lymph node metastasis (reviewed by Altalal and Carmeliet, 2002; Cassella and Skobe, 2002). Transplantation of established human, mouse, or rat tumor cell lines that by stable transfection expressed high levels of VEGF-C or VEGF-D resulted in lymphangiogenesis concomitant with tumor development and lymph node metastasis (Skobe et al., 2001; Stacker et al., 2001; Krishnan et al., 2003). Finally, transgenic expression of VEGF-C in a mouse model of pancreatic β cell carcinogenesis (Rip1Tag2), also resulted in upregulated lymphangiogenesis and lymph node metastasis to regional pancreatic lymph nodes (Mandriota et al., 2001). In contrast, transgenic expression of the blood vessel angiogenic factor VEGF-A in the Rip1Tag2 tumor model resulted in an early onset of tumor angiogenesis and with it in accelerated tumor development, but not in metastasis formation (Gannon et al., 2002). These data indicate that lymphangiogenesis is one of the processes mediating lymph node metastasis. Hence, lymph node metastasis may involve mechanisms of tumor cell dissemination that differ from the processes underlying the formation of metastasis in distant organs.

**Rebuilding human cancer in mice**

In the past 20 years, many mouse models of tumor development have been established. Yet, while a few mouse lines nicely recapitulated multistage tumor progression seen in human cancer, most mouse models did not mimic human cancer close enough to be useful for further studies. For example, while most mouse models have relied on overexpression of H-Ras and N-Ras, K-Ras is the Ras family member found to be activated in approximately 30% of human cancers. Moreover, transgenic overexpression does not resemble the expression levels of oncogenes from their endogenous promoters. Finally, transgenic expression of oncogenes or ablation of tumor suppressor genes in knock-out mice usually occurs in all cells of a given tissue, whereas in sporadic cancers of patients a single cell is undergoing neoplastic transformation in a field of unaffected cells. Hence, in the past few years a major effort has been undertaken to rebuild human cancer in mice, and major progress has been achieved by recapitulating the molecular processes observed in human cancer.

Using targeted recombination in ES cells, mice have been generated that carry an activated K-Ras (G12D) gene at its normal locus but in a silent configuration. Only upon sporadic recombination between or within chromosomes activated K-Ras will be expressed. Similar to patients, these mice are predisposed to the sporadic occurrence of a variety of tumor types, predominantly in the lung (Johnson et al., 2001). In a different approach, mice were generated that carried an activated K-Ras gene either in its original chromosomal locus or as a transgene, but with a floxed Stop element to prevent its expression. Upon adenoaviral delivery of Cre recombinase to the lung, excision of the Stop element resulted in K-Ras expression at endogenous levels and thus in the development of lung adenocarcinoma after short latency (Jackson et al., 2001; Meuwissen et al., 2001). Removal of the Stop element by expression of the Cre recombinase in pancreatic progenitor cells led to the progressive development of preinvasive and invasive ductal pancreatic cancer and metastasis to the regional pancreatic lymph nodes, the liver and other sites (Hingorani et al., 2003). In a similar experiment, mice developed only intraepithelial lesions in the pancreas, yet concomitant inactivation of the Ink4a/Arf tumor suppressor provoked the development of ductal adenocarcinoma and multiple metastasis (Aguirre et al., 2003). Inducing activated K-Ras expression in mature exocrine cells or in the ductal epithelium of the pancreas also led to the development of ductal adenocarcinoma of the pancreas (Brebeck et al., 2003; Grippo et al., 2003). In contrast, systemic recombination-induced expression of K-Ras(G12V) initially failed to induce neoplastic lesions. Only in a subset of mice late onset malignant transformation of lung bronchiolo-alveolar cells leading to adenoma and adenocarcinoma was observed, indicating that expression of K-Ras may depend on the cellular context to be sufficient for full carcinogenesis (Guerra et al., 2003).

Finally, another approach to rebuild cancer in the mouse is the use of transgenic mice that express TVA, the receptor for avian leukosis virus, in specific tissues. RCAS vectors, derived from avian leukemia virus, are then used to infect the corresponding tissue and to deliver genes of interest. For example, expression of polyoma middle T-antigen by this method in the exocrine pancreas resulted in a variety of tumor types with acinar and ductal differentiation (Lewis et al., 2003). Notably, in the same approach, expression of c-Myc resulted in endocrine tumors, also underlining the cell context specificity of specific oncogenes. This experimental approach is now being used for many other tissue types.

Inducible systems of gene expression in mice enabled researchers to further pinpoint causal roles of particular genes/pathways for specific cancers but also to follow mechanisms underlying tumor regression in vivo. There is now a growing literature showing that maintenance of different tumor types depends on a specific oncogene and that tumors regress upon turning the specific gene expression off, some of which are summarized here.

One of the first reports on the role of oncogenes in the maintenance of tumor progression came from experiments where tetracycline-inducible H-Ras(V12G) was specifically expressed in melanocytes of Ink4a knock-out mice resulting in the development of melanoma (Chin et al., 1999). All melanoma induced by oncogenic Ras regression after switching off Ras expression, and repeated Ras induction resulted in the rapid recurrence of tumors at the previous tumor sites. Ras was also used to study lung carcinogenesis. Tetracycline-inducible expression of oncogenic Ki-Ras(G12D) in pneumocytes induced the formation of adenomas and adenocarcinomas of the lung within two months (Fisher et al., 2001). Deactivation resulted in apoptotic regression of the tumors which were undetectable after one month. Notably, full regression was also observed in the absence of the tumor suppressor gene products p53 or Ink4a/Arf, indicating that also in this case tumor maintenance is fully dependent on oncogenic Ras.

Similar tetracycline-inducible expression of c-Myc in the hematopoietic lineage gave rise to malignant T cell lymphomas and acute myeloid leukemia. Also here, dramatic tumor regression was observed upon inactivation of the transgene (Felsher and Bishop, 1999). With lesser frequency, the same mice also developed osteogenic sarcomas, which also regressed upon deactivation of the oncogene. In one case, even a brief inactivation of oncogene function resulted in a sustained loss of the neoplastic phenotype (Jain et al., 2003).
The majority of human solid cancers originate from epithelial cells where the cell-cell adhesion molecule E-cadherin plays a critical role in the formation and maintenance of epithelial structures. In most cancers of epithelial origin, E-cadherin-mediated cell-cell adhesion is lost concomitant with the progression towards tumor malignancy. Using a transgenic mouse model of β cell carcinogenesis (Rip1Tag2), it has been shown that the loss of E-cadherin-mediated cell-cell adhesion is causally involved in the progression from adenoma to carcinoma in vivo (Perl et al., 1998). Forced expression of wildtype E-cadherin throughout tumorigenesis resulted in cessation of tumor progression, whereas interfering with E-cadherin function by transgenic expression of a dominant negative form of E-cadherin caused an acceleration of the transition from benign to malignant tumors and the formation of metastasis.

Another cell adhesion molecule which may play an important role in tumor progression is neural cell adhesion molecule (NCAM), a member of the immunoglobulin-domain containing superfamily of genes. Consistent with the observation that NCAM expression was frequently downregulated in different types of human cancers, ablation of NCAM function during β cell tumorigenesis in Rip1Tag2 transgenic mice resulted in the formation of lymph node metastasis (Perl et al., 1999; Cavallaro et al., 2001). Notably, loss of NCAM in β tumor cells resulted in the loss of FGF receptor-mediated signal transduction and of β1 integrin-mediated cell-matrix adhesion (Cavallaro et al., 2001). Based on correlative expression studies in human cancer and also based on the potential signalling function of many cell adhesion molecules, more functional data need to be generated to understand their role during tumor progression.

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