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Review

New models towards assessing anti-cancer therapeutics

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Summary. Cancer is the subject of intense research around the world, but many questions about how the disease works remain unanswered. How exactly does cancer start and how do tumours grow? In fact, at present there are ten times more anticancer drugs being tested in clinical trials than there were 15 years ago. However, many of the new anticancer agents are predicted to show clinical benefit in only small subpopulations of patients. The cancer stem cell model could explain not only how some cancers work but also why patients suffer relapses, providing a good opportunity to gain insight into the reasons why agents work or, more commonly, don't work, before going into a clinical trial.

Key words: Cancer treatments, Targeted therapy, Cancer stem cells, Mouse models, Pharmacogenomics, Molecular image

Cancer and treatments

It is now widely accepted that cancer results from the accumulation of mutations in the genes that directly control cell birth or cell death (Hanahan and Weinberg, 2000). But the mechanisms through which these mutations are generated are the subject of continuing debate. In addition, the microenvironment of a tumour, including stromal and vascular endothelial cells, is important for the growth and persistence of the tumour (Hanahan and Weinberg, 2000). This entire constellation of the abnormal molecular biology of tumour cells and their microenvironment is the molecular signature of cancer.

It has been argued that an underlying genetic instability is absolutely required for the generation of the multiple mutations that underlie cancer (Loeb, 1991; Hartwell, 1992). It is only in recent years that the involvement of specific genes has been demonstrated at the molecular level in most cancers. Cancer cells arise from normal cells through the acquisition of a series of mutations in oncogenes and tumour suppressor genes (Hanahan and Weinberg, 2000). The current working hypothesis on the origin of cancer proposes that the accumulation of somatic mutations in certain functional pathways leads to deregulated proliferation (Jackson and Loeb, 1998; Lengauer et al., 1998). Genetic pathways need to be identified for the development of molecular and pharmacological therapeutics to treat and prevent cancer. However, cancer is a disease of the organism and not only the result of abnormal cell growth. This may explain why genetic diseases like cancer are underprovided in ethiologic treatments (Sanchez-Garcia et al., 2007), explaining that the relative survival considering the stage of diagnosis has not changed for the past three decades (Etzioni et al., 2003). Current observed decrease in cancer mortality is mostly the result of early detection and prevention rather than the consequence of effective therapeutics.

Much effort is currently being expended to target, during treatment, the mutated oncogenes and tumour suppressor genes that control neoplastic cell growth directly. The genetic instabilities that underlie cancer may provide equally valid therapeutic targets. Although such instabilities are not directly responsible for cancer's

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abnormal growth, they are likely to be genetically based and, therefore, permanent components of the cancer cell that distinguish it from all normal cells. Because instabilities reflect defects in cellular processes that maintain the integrity of the genome, they can be expected to generate sensitivities to particular chemical agents (Lengauer et al., 1998). In fact, one can argue persuasively that all chemotherapeutic compounds used at present are more toxic to cancer cells than to normal cells only, specifically because of the defective checkpoints that occur in the former cells (Hartwell, 1992).

Conventional chemotherapy

The era of chemotherapy began in the 1940s with the first uses of nitrogen mustards and antifolate drugs. Cancer drug development since then has been transformed from a low-budget, government-supported research effort to a high-stakes, multi-billion dollar industry (Chabner and Roberts, 2005).

Cytotoxic chemotherapy or radiotherapy of cancer is limited by serious, sometimes life threatening, side effects that arise from toxicities to sensitive normal cells because the therapies are not selective for malignant cells (Allen, 2002). Cancer cells share many common features with the normal host cells from which they derive. Most, if not all, cancer chemotherapeutics that are in common use at present- including doxorubicin, vincristine, cyclophosphamide, topotecan and paclitaxel- owe what little selectivity they have for cancer cells to their higher proliferation rates. This can lead to increased toxicities against normal tissues that also show enhanced proliferative rates, such as bone marrow, gastrointestinal tract and hair follicles. All these caveats result in the eventual failure of therapy; this is often accompanied by the development of drug resistance and metastatic disease (Allen, 2002).

Several approaches for improving the selective toxicity of anticancer therapeutics are being pursued at present.

Targeted therapy

While the attempts to improve the pace of discovery of cytotoxic agents proceeded in the late 1980s, molecular and genetic approaches to understand cell biology uncovered entirely new signaling networks that regulate cellular activities such as proliferation and survival (Jackson and Loeb, 1998; Lengauer et al., 1998). Many of these networks were found to be radically altered in cancer cells. An industrial revolution unfolded, based primarily on small biotechnology firms, as researchers set out to repair these molecular defects in cancer cells, beginning the era of "targeted therapy" (Chabner and Roberts, 2005).

The term "targeted therapy" refers to a new generation of cancer drugs designed to interfere with a specific molecular target (typically a protein) that is believed to have a critical role in tumour growth or progression. The identification of appropriate targets is based on a detailed understanding of the molecular changes underlying cancer. This approach contrasts with the conventional, more empirical approach used to develop cytotoxic chemotherapeutics- the mainstay of cancer drug development in past decades (Allen, 2002; Sawyers, 2004). The hope is that such therapeutics will selectively target tumour cells and leave normal cells untouched, thereby reducing the common side effects of current anticancer therapies such as radio- and chemotherapy (Sullivan and Kelloff, 2005). There is an increasing number of such targeted therapeutics being developed and made available for clinical use (Table 1).The new targets included growth factors, signaling molecules, cell-cycle proteins, modulators of apoptosis and molecules that promoted angiogenesis (Hanahan and Weinberg, 2000).

One of the landmark events in the targeted revolution has been the development of Imatinib mesylate (Gleevec), a relatively simple structure that possesses all the desired factors of the "ideal" targeted compound (Mauro and Druker, 2001a). It was derived from a natural product by Novartis. Imatinib is a moderately potent inhibitor of the kinase BCR-ABL, the fusion protein product of a chromosomal translocation, named Philadelphia chromosome (Ph), which is involved in the pathogenesis of chronic myeloid leukaemia (CML) (Chabner and Roberts, 2005; Mauro and Druker, 2001b; Druker et al., 2001). Brian Druker and collaborators showed that when Imatinib is used to treat patients with chronic-phase CML, 90% seem to achieve complete haematological remission and many lose cytogenetic evidence of the malignant clone (Druker et al., 2001). However, BCR-ABL translocation can still be detected by PCR analysis in cells of most patients (Mauro and Druker, 2001a; Druker et al., 2001). In the acute leukaemic phase of CML, Imatinib induces brief remissions, and treatment leads to a rapid outgrowth of drug-resistant cells that display mutations in the catalytic kinase domain of ABL (Shah et al., 2002). The origin of CML begins in a hematopoietic stem cell (HSC), a target population that is largely quiescent (Spangrude et al., 1988). *In vitro* and *in vivo* studies in the last years have shown that these quiescent Ph+ HSCs, the origin of CML, are insensitive to Imatinib mesylate treatment (Graham et al., 2002; Chu et al., 2005), and are not eliminated in CML patients. Imatinib mesylate inhibits malignant primitive progenitor growth primarily through inhibition of their abnormally increased proliferation rather than selective induction of apoptosis (Bhatia et al., 2003). This suggests that BCR/ABL kinase activity may be required for abnormal proliferation and expansion of Ph+ cells in CML but may not be essential for preservation of primitive malignant cells. BCR-ABL kinase inhibition by Imatinib mesylate may therefore remove the proliferative advantage of Ph+ progenitors and their progeny cells allowing regrowth of coexisting Ph- cells

without eliminating all Ph+ primitive progenitors. In conclusion, although Imatinib mesylate therapy is able to suppress the oncogenic activity of wild-type BCR-ABL expression, the treatment is unable to destroy the Ph+ cancer stem target cell (Perez-Caro et al., 2009; Vicente-Dueñas et al., 2009).

The transition from cytotoxic drugs to targeted therapies represents an important advance, but the basic principles of cancer treatment and drug resistance, as developed in the period from 1950 to 1980, remain the same (Chabner and Roberts, 2005). Human malignancies seem to be a very diverse group of diseases, even within histological classifications, and quickly display their diversity when exposed to all forms of chemotherapy. The next decade will present the challenge of designing trials to combine targeted drugs and cytotoxics in a more effective manner. These trials will be aided by the use of genomics and molecular assays to identify subsets of patients that are most likely to respond to certain drugs, thereby avoiding the needless cost and toxicity of ineffective treatment (Chabner and Roberts, 2005).

All these targets work on targeting of proliferation controls in agreement with the current working hypothesis on the origin of cancer, which proposes that the accumulation of somatic mutations in certain functional pathways leads to deregulated proliferation (Jackson and Loeb, 1998; Lengauer et al., 1998). However, despite the promise of rational treatment, many researchers have serious doubts as to whether this is a viable approach (Sanchez-García et al., 2007; Sánchez-García, 2009). Many of the new classes of agents are predicted to show clinical benefit in only small subpopulations of patients, if at all, and target non-essential aspects of tumour maintenance and development. How can cancer treatment development be re-designed to select effective targeted anticancer drugs?

Limitation in our knowledge of human cancer:cancer stem cells

Human cancer as a stem cell disease

It is well-known that cancer is a clonal disease that initiates in a single cell whose descendants make up the tumour. However, the cell nature in which the initiating mutation takes place in human cancer has received little attention during the last decades (Cobaleda and Sánchez-García, 2009). An enduring idea in cancer biology is that tumours arise and grow as a result of the formation of cancer stem cells, which may constitute only a minority of the cells within a tumour but are nevertheless critical for its propagation. The concept of cancer stem cells dates back almost as far as the discovery of somatic stem cells in the haematopoietic system, and was firmly established experimentally in acute leukemias (Bonnet and Dick, 1997; Cobaleda et al., 2000; Shet et al., 2002; Sanchez et al., 1995; Sanchez-Garcia et al., 1999). Indeed, the key role of stem cells in human tumorigenesis is becoming an increasingly accepted phenomenon and has been the issue of recent reviews (Reya et al., 2001; Ruiz i Altaba et al., 2002). As stem cells and cancer cells share a great number of features, it would certainly make sense that human cancers derive from stem cells, especially as stem cells, like cancer

Table 1. Main new targeted drugs to treat cancer.

Cancer/Drug	Action	Clinical Benefit*	Preclinical trials in mouse model reproducing human cancer	Reference
Colorectal cancer				
Bevacizumab (Avastin®)	Angiogenesis inhibitor	?	NO	Venook, 2005
Cetuximab (Erbitux®)	EGFR inhibitor	?	NO	Venook, 2005
Irinotecan (Campo®)	Topoisomerasa I inhibitor	?	NO	Venook, 2005
Capecitabine (Xeloda®)	Inhibits DNA/RNA synthesis	?	NO	Venook, 2005
Oxaliplatin (Eloxatin®)	Inhibits DNA/RNA synthesis	?	NO	Venook, 2005
Multiple myeloma				
Bortezomib (Velcade®)	Proteasome inhibitor	?	NO	Chang et al., 2005
Lung cancer				
Gefitinib (Iressa®)	EGFR tyrosine kinase inhibitor	?	NO	Pal and Pegram, 2005
Erlotinib (Tarceva®)	EGFR tyrosine kinase inhibitor	?	NO	Pal and Pegram, 2005
Chronic myeloid leukemia				
Imatinib mesylate (Gleevec®, Glivec®)	Tyrosine kinase inhibitor	?	NO	Graham et al., 2002; Chu et al., 2005
Breast cancer				
Exemestane (Aromasin®)	Aromatase inhibitor	?	NO	Joensuu et al., 2005
Letrozole (Femara®)	Aromatase inhibitor	?	NO	Joensuu et al., 2005
Anastrozole (Arimidex®)	Aromatase inhibitor	?	NO	Joensuu et al., 2005
Tamoxifen	Oestrogen receptor antagonist	?	NO	Delozier, 2005
Fulvestrant (Faslodex®)	Oestrogen receptor antagonist	?	NO	Bundred, 2005

The absence of adapted preclinical trials in mouse models reproducing human cancer is the bottleneck preventing compounds from showing a clinical benefit on clinical trials. *: The symbol (?) means that sustainable long-term survival benefit has not been formally demonstrated yet.

cells, are immortal, poorly differentiated cell types that regularly express certain key survival genes (Greaves, 1996). Supporting this view, recent results suggest that a stem cell constitutes the target cell in human breast carcinomas and brain tumours (Al-Hajj et al., 2003). These results indicate that we must view cancer as a disease of cell differentiation rather than multiplication.

Implication of the stem cell model for development of cancer treatments

Cancers consist of heterogeneous populations of cancer cells that differ markedly in their ability to proliferate and form new tumours. While the majority of cancer cells have a limited ability to divide, a population of cancer stem cells that has the exclusive ability to extensively proliferate and form new tumours can be identified based on marker expression. Growing evidence suggests that pathways that regulate the self-renewal of normal stem cells are deregulated in cancer stem cells, resulting in the continuous expansion of self-renewing cancer cells and tumour formation (Al-Hajj et al., 2004; Clarke, 2005). This suggests that agents that target the defective self-renewal pathways in cancer cells might lead to improved outcomes in the treatment of these diseases (Al-Hajj and Clarke, 2004).

This observation has implications for the biology of tumour formation as well as the diagnosis and treatment of cancer. To treat cancer effectively, the CSCs must be eliminated (Sanchez-Garcia et al., 2007; Perez-Caro et al., 2009; Vicente-Dueñas et al., 2009). Otherwise, the tumour will rapidly reform if the therapy eliminates non-tumourigenic cancer cells but spares a significant population of CSCs (Al-Hajj and Clarke, 2004). Classically, treatments for cancer have relied on the ability to shrink tumours. Since in many cases the CSCs represent a minority cell population of the tumour (Al-Hajj et al., 2003; Lapidot et al., 1994; Singh et al., 2003, Matsui et al., 2004; Setoguchi et al., 2004), agents selectively killing the CSCs are likely overlooked in our current screening methods, which rely on rapid reduction of tumour size. If an agent spares a significant number of the CSCs, then the remaining cells could rapidly reform the tumour (Clarke, 2005) (Fig. 1). In addition to its effect on our understanding of the efficacy of our current therapies, the stem cell model for cancer is likely to affect the identification of future therapeutic targets. By directing expression analyses to an enriched population of tumorogenic cancer cells, the identification of novel diagnostic markers and novel therapeutic targets should be more effective. In addition, it is becoming apparent that treatments that directly target the pathways involved in maintenance of CSC would have a significantly greater chance of success (Castellanos et al., 2010; Sanchez-Garcia, 2010).

The efficacy of anti-tumor agents in clinical trials is commonly evaluated following RECIST (Response Evaluation Criteria In Solid Tumors) rules that define when cancer patients improve ("respond"), stay the same ("stable") or worsen ("progression") during treatments, which are based on tumor size. Since it is the bulk of tumor cells, non CSCs, which make up most of the tumor mass, efficacy mainly reflects the ability to kill those non CSCs. The identification of CSC biomarkers will certainly allow direct evaluation of the effect of anti-tumor agents on these minor populations. Therefore not tumor size reduction but instead complete response (CR) is a valid end point when associated with reduced recurrence rate. An agent that only targets CSCs is predicted to show only a moderate effect on tumor size but would have a dramatic effect preventing tumor recurrence; on the other hand, an agent that targets the bulk of tumor cells but not CSC self-renewal will initially show good clinical response but will not prevent recurrence. This trial may result in a failure because of evidence of tumor progression. It is necessary to introduce recurrence in the adjuvant setting to identify effective CSC targeting agents.

If we assume that the genetic alteration responsible for cancer development takes place in the CSC, we can design mouse models based on this issue (Vicente-Dueñas et al., 2010). However we must focus on determining what the process responsible for the maintenance of the CSC phenotype is, because this will be the aim for the new identification of targets to design drugs against them. So, mouse models generated this way, will become the basic tool to design drugs in this context, against the maintenance of the CSC.

How to apply the new agents in appropriate clinical settings

Pharmaceutical companies are conducting more clinical trials than ever before and more patients are participating in these trials, though the number of drugs

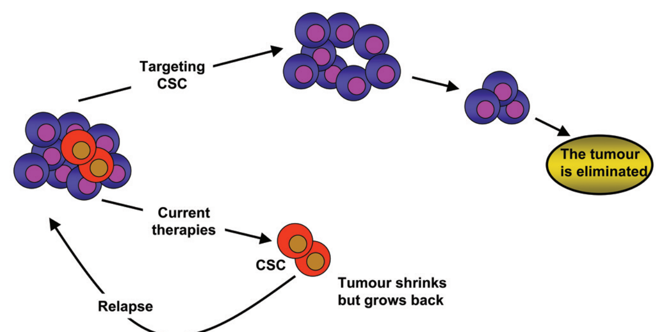

Fig. 1. Targeting cancer stem cells. Cancer is considered a proliferative disease (Jackson and Loeb, 1998; Lengauer et al., 1998) and current therapies are designed against these mechanisms. These therapies have not obtained a therapeutic benefit in the majority of cases (Graham et al., 2002; Chu et al., 2005).The tumour initially shrinks, but the self-renewing cancer stem cell regenerates the tumour. Recent results support cancer as a stem cell disease, so drugs that target the mechanism responsible for the maintenance of CSCs will eliminate the CSCs and thus have curative potential.

that have a real benefit has not increased. A very important cause of the inefficiency of the discovery and development process is the disparity between preclinical and clinical trials. Most often, anticancer effects seen in the preclinical state (on cells or animal models) cannot be replicated in humans. Almost unanimously recognized factors leading to this disparity are: lack of predictive animal models, absence of diagnostic and monitoring biomarkers and scarcity of properly oriented therapeutic strategies.

Thus, a clinical trial has become an assay to validate targets in a particular disease state, so that we need models mimicking human cancer in which to validate those targets. Current preclinical settings do not accurately predict clinical efficacy of that target in humans (Perez-Caro et al., 2009; Vicente-Dueñas et al., 2009). On the other hand, moving all targets forward through development is prohibitive in terms of cost and time and therefore rational choices between novel targets have to be made. The need for a good *in vivo* model can present a strong case for validation. However, modulation of many targets put forward for small molecules or biopharmaceutical development fails to reverse the disease phenotype in humans. Therefore, a greater need to bridge the gap between disease models and the clinic exists. Validation of targets in a disease state mimicking human disease will provide us the platform for high-value target validation.

There are many steps in developing a new putative agent and then using it as a drug (Table 2). First of all, we must select the targets needed for the maintenance of the CSC. These targets will be different from those that had transformed normal stem cell to CSC. If we can reverse the effect of the aberrant maintenance of CSC, we could probably treat the disease. To select these unknown targets, we can take advantage of mouse models based on CSC alterations that accurately reproduce the human disease from stem cells. The next step is validating these new targets in a disease state by mouse genetics tools plus molecular imaging and pharmacogenomics approaches. Targets validated this way should be tested on mouse models mimicking human disease pathology. After that, we must single out those animals that are sensible to the agents from the ones that are not. These results should guide us on deciding whether or not to use that agent on human clinical trials, so we are improving current preclinical trials because we can be more selective and effective. Taking these results to mouse models, we could define which models will respond to the agents through genetic profile analysis, so that we can use these data in human clinical trials. Keeping in mind preclinical assay results we must design human clinical trials (Table 2). So instead of selecting patients in a random manner, we should study the genetic profile of each patient and make homogeneous groups sharing similar genetic profiles. In this regard Pharmaco-genomics has a lot to say as we discuss later. The clinical setting proposed herein will reduce the number of agents that goes into clinical trials, being able to expend more effort on ones with a potential benefit.

Table 2. Steps on developing new agent in appropiate clinical settings.

a) To select targets for CSC maintenance.
b) To validate target in adisease state (by mouse genetic tools*).
c) To select responders vs. ne responders.
d) Design human clinical trials.

*: Molecular Image (same technology approach need to follow up human cancer); *: Pharmacogenomics.

Mouse models of human cancer

The generation of mouse models that accurately mimic human cancer must take into account two main criteria: i) the genetic/molecular alterations identified in human cancer and also ii) the target cells where the cancer-mutation takes place in humans. These target cells may be somatic stem/primitive cells, identifying them as the cells to be used as targets in the development of both mouse models and molecular and pharmaceutical therapeutics to treat and prevent human cancers (Pérez-Caro et al., 2005). This second criterion has not been taken into account in the design of current models of human cancer and may explain why many mouse models of cancer might be inadequate (Pérez-Caro et al., 2009; Vicente-Dueñas et al., 2009, 2010).

Numerous agents have shown exciting activity in preclinical models and yet have had minimal activity clinically. These disappointments have led to reasonable skepticism about the true value of both syngeneic and xenograft rodent tumour models in accurately identifying agents that will have important clinical utility (Peterson and Houghton, 2004). Whereas the development of newer techniques, including transgenic mouse models of cancer, offers the potential to develop more predictive models, the role of such mice in cancer drug development is not yet validated.

Biological criteria to confirm the same genotype-phenotype correlations in human and mice need to be used to validate the model, such as:

i) Similar histological features to the homologous human tumour.

ii) Progression through the same stages and equal systemic effects in the host.

iii) Same genetic pathways should be affected in tumour initiation and progression.

iv) Response to current cancer treatments should be similar to humans.

Current mouse models

Technical advances over the past two decades now allow investigators to introduce alterations in the mouse genome that constitutively or conditionally alter the expression of crucial genes, leading to the development

of particular tumours. These studies have provided tremendous insights into all aspects of cancer research and have further defined the biological functions of hundreds of genes. Genetically engineered mouse models (GEM) have helped to elucidate the molecular pathways involved in oncogenesis, to define the effects of particular mutations or gene deletions on cancer development, and have been useful for validating key genes as targets for therapy. More recently, these models have been used to test targeted therapies, cancer vaccines, preventive agents and combinations of chemopreventive and/or therapeutic agents (Green and Hudson, 2005). The selective use of GEM models has proved valuable for assessing the *in vivo* inhibitory activities and mechanisms of action of various cancer prevention agents at different stages of cancer development.

The evaluation of antitumour agents in immune-deficient mice transplanted with human tumours is the major model system for drug development. In its most simple iteration, tumours are grown subcutaneously, and the model allows rapid and quantifiable assessment of antitumour activity relative to mouse toxicity (Johnson et al., 2001). Logically, preference should be given to those agents that show the greatest antitumour activity in the preclinical setting, but these preclinical data are not predictive of drug activity in human studies. Xenograft models do not take into account CSC.

GEM models, which develop tumours over a relatively short time compared with the emergence over many years of many human cancers, offer several potential advantages over tumour xenograft or chemically-induced rodent cancer models. Unlike xenograft or chemically-induced models, chemo-preventive responses can be assessed for tumours that arise as a result of specific, engineered genetic lesions introduced into the mouse genome. Moreover, genetic and environmental factors can be controlled rigorously in mice, but remain poorly controlled variables in human trials. Whereas xenograft models generally represent an advanced stage of cancer and are not suitable for studies of early stages of tumorigenesis, cancer stage-specific responses to agents can be assessed in many GEM models in which lesions progress through predictable stages. Combinations of therapies and/or preventive agents, which are difficult to test in human trials, can readily be evaluated using GEM models to determine the biological consequences of interfering simultaneously with multiple oncogenic pathways.

The generation of mouse models of human cancer has become increasingly sophisticated and relevant for the pre-clinical testing of prevention agents (Vicente-Dueñas et al., 2010). Much recent attention has been devoted to determining how well the cancers of GEM models resemble human cancers. Initially, such comparisons were based on the biological properties and morphological characteristics of the mouse tumours (Cardiff et al., 2000; Boivin et al., 2003), but more recently, comparative genomic hybridization (Weaver et al., 2002) and gene-expression profiling have allowed molecular dissection and comparisons of genes that are aberrantly expressed in both human and mouse cancers (Desai et al., 2002; Hunter et al., 2003). This analysis allows researchers to compare how well different models replicate molecular aspects of oncogenesis, thereby helping to identify models that are particularly relevant for prevention studies.

The molecular dissection of human cancers continues to identify important genes, pathways and networks that are crucial to tumour progression. This information is being integrated into the new generation of GEM models. The use of strong promoters to drive the expression of oncogenes might bypass crucial mechanisms that are important in naturally occurring tumorigenesis, such as genomic instability and additional genetic and epigenetic changes. Promising new models have recently been developed and can be used for the preclinical testing of preventive agents

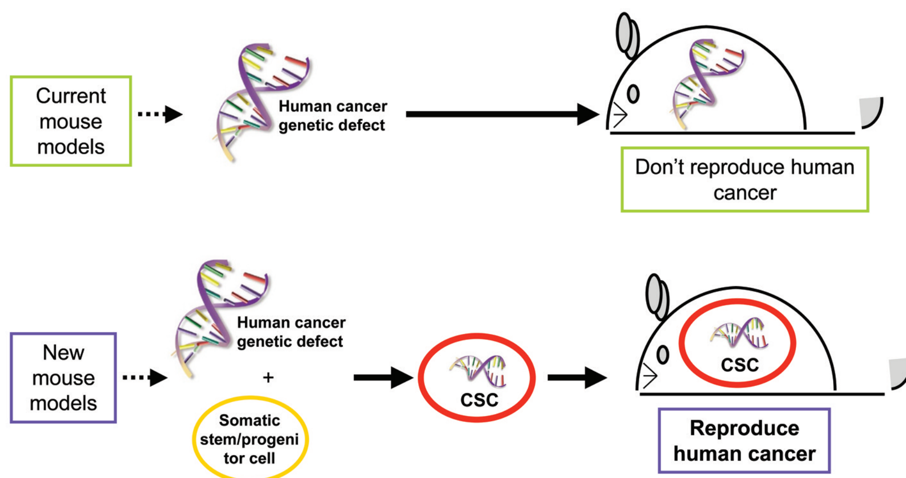

Fig. 2. Why do current mouse models not reproduce human cancer? Many available current mouse models only take into account the genetic alteration found in human cancers without considering the cellular context where it takes place. New mouse models that accurately reproduce human cancer express the genetic alteration responsible for cell transformation in the target cell where the cancer-mutation takes place in humans, the cancer stem cell (CSC). CSCs can be derived from normal stem cells that have acquired a genetic alteration. On the other hand, CSCs could arise from non stem cells that have gained the ability to self-renew and then became a stem cell.

(Perez-Caro et al., 2009; Vicente-Dueñas et al., 2009; Blasco et al., 2011).

The main reason why current mouse models have failed in being predictive for clinical trials is that all of them have only taken into account the genetic/molecular alteration identified in human cancer (Fig. 2). The target cells in which the cancer-mutation takes place in humans have not been taken into account. This may explain why many mouse models of cancer might be inadequate. In these models the mutation occurs in every cell of the organism or tissue, unlike the genetic lesions in human cancer that occur sporadically in single cells during prental or postnatal development.

How to model human cancer in mice?

To be able to model human cancer in mice we should identify the genetic alteration responsible for cell transformation and also identify the cell in which the alteration takes place. These models mimicking human cancer must be validated by comparative genomics and pathology. Live animal imaging will also enable the estimation of functional genomics and target validation.

We propose a new mouse model technology based on specifically expressing human genetic alterations in somatic stem cells, by means of stem cell-restrictive promoters. The application of this technology has generated the first mouse models of human cancer that accurately reproduce the human disease (Fig. 2) (Perez-Caro et al., 2009; Vicente-Dueñas et al., 2009). "Humanized" mouse models of mesenchymal cancer have been produced, in which the disease originates and evolves spontaneously in the correct environment, only in a few cells that accumulate further cancerous mutations once the initial, triggering oncogene is activated. Carcinoma models are also being developed using the same strategy. For these reasons, they are unique experimental tools for *in vivo* functional genomics, pharmacogenomics and drug discovery programs. They are also an invaluable source of biological materials (cells, tissues, fluids) with which to conduct *in vitro* genetic studies and pharmacological screenings.

Collection of tissues from models mimicking human cancer at time points that correspond with maximal clinical effect will provide the best opportunity to gain insight into the reasons agents work or, more commonly, don't work, before going into a clinical trial.

The problem of cancer heterogeneity

Cancer is an heterogeneous disease; indeed, patients that share the same apparent histopathological tumor lesion, present the same stage of the disease, and even receive the same therapy, could show a completely different evolution. Moreover, individuals under the same environment, and similar doses of carcinogens along their lives might or might not develop cancer, and the time of latency is variable, as occurs with heavy smokers or with people exposed to nuclear radiation (Carmichael et al., 2003; Aguirre-Ghiso, 2007; Slamon et al., 2001; Smigal et al., 2006; Winter and Hunter, 2008). This heterogeneity of cancer susceptibility, evolution and response to therapy is explained by the sum of effects of different low-penetrance genes (named modifier genes) that interact among them and with the environment (Balmain, 2002). Thus, cancer can be considered as a polygenic disease whose heterogeneous evolution would follow a model of quantitative genetics. The identification of those modifier polygenes is a difficult task mainly due to their weak individual effect. Genome Wide Association Studies (GWAS) in human populations have already identified good candidates, but the extrapolation of these results to different populations seems to be difficult due to heterogeneous human genetics and complex environmental interactions (Balmain, 2002; Hunter and Crawford, 2008).

Mice develop tumors that appear to be very similar to human cancer, and involve mutations with a similar spectrum, affecting the same genes and pathways as in human cancer (Balmain and Harris, 2000). So we can anticipate that part of the numerous Quantitative Trait Loci (QTL) containing tumor risk genes identified in the mouse could be relevant to the human scenario, and would serve to complement observations taken within human populations (Balmain, 2002). New cancer mouse models, together with the crosses between inbred mouse strains with homogeneous genetic background that show different grades of cancer susceptibility and evolution, can really simplify the identification of cancer modifier genes, because they permit a higher control of hereditary variation and environmental exposure (Hunter and Crawford, 2008). These mouse genetic studies permit the identification of QTL in which modifier genes are located. These QTL can include both types of modifier genes, those regulating intrinsic cellular characteristics (proliferation, apoptosis, DNA repair and others), other controlling extracellular factors (stroma, angiogenesis, immune system, endocrine factors, etcetera), and those that participate simultaneously in both functions.

Mouse models also offer a system for testing results obtained from human studies. Thus, moving back and forth between mouse and human systems is a good approach to identify the causal genetic variant of a candidate gene. Moreover, since environmental influences and way of life have a significant effect on tumour susceptibility, environment-genome interactions could be explored by the use of mouse models, and will eventually allow us to recognize how genes work together with environmental influences identified by epidemiological studies (Quigley and Balmain, 2009).

Identification of modifier genes in mouse models

Although the mouse is an effective tool to map QTL this strategy is not without problems (Hunter and Crawford, 2008). In particular, the deficiency of mapping resolution makes it difficult to identify those

modifier genes associated with complex genetic traits, such as cancer. New technological approaches have permitted us to relieve this caveat. The analysis of tumors using whole genome array comparative genomic hybridization (aCGH) and loss of heterozigosity (LOH) analysis by SNParrays, is helping to refine QTL regions. It is known that, similar to what happens with high penetrance susceptibility genes, tumors are marked by mutations on those genes implicated in susceptibility. The same would occur with at least part of the low-penetrance susceptibility genes that control intrinsic cellular activities. Low-susceptibility tumor genes would drive copy number changes in tumors in an allele-specific manner, therefore cancer resistance alleles would be lost along tumor progression as a result of deletion or recombination leading to loss of heterozigosity (LOH), whereas cancer risk genes would show gains through amplification. These types of allele specific somatic losses and gains can be exploited to identify modifier genes of cancer susceptibility and evolution (Ewart-Toland et al., 2005; Balmain et al., 2003). This strategy facilitated the recognition of Stk6 as a low-penetrance tumor susceptibility gene (Ewart-Toland et al., 2003).

The use of genome-wide expression arrays is also a useful technique to refine QTL. The observation that the majority of SNPs are located out of coding regions suggest the hypothesis that many QTLs were probably attributed to alterations in gene expression instead of to mutations within coding regions, as is the case of Kras2 in cancer induced by urethane (To et al., 2006). This idea has consequently resulted in the screening for genes in QTL that exhibit differential expression among the strains of interest (Arbilly et al., 2006). This strategy permits a fast screening of hundreds of possible candidate modifier genes (Hitzemann et al., 2002; Jansen and Nap, 2001; DiPetrillo et al., 2005). Genome wide expression arrays also permit the generation of co-regulated networks of transcripts from genes that are working collectively to modify a particular tumor phenotype of susceptibility and evolution. Each network would be constituted by those transcripts that significantly correlated with each other, and whose expression levels are controlled by common genetic loci. In a large part of those cases, it would be possible to identify the gene responsible for the QTL effect. The candidate gene would be affected in cis by that locus, whereas downstream genes would be influenced in trans (Mackay, 2001; Quigley et al., 2009; Quigley and Balmain, 2009).

Finally, the next generation of sequencing techniques, together with the culmination of the human (Lander et al., 2001; Venter et al., 2001) and mouse (Waterston et al., 2002) genome sequencing projects, will be invaluable tools in the refinement of QTL. Thanks to the conclusion of the human genome project it is possible to spot most genes inside a specified location. Additionally, thanks to next-generation sequencing, it will be possible to explore candidate genes without the presence of time-consuming genomic screening. The refinement of QTL has improved since the whole genome of more species has been sequenced (Chimpanzee Sequencing and Analysis Consortium, 2005; Lindblad-Toh et al., 2005). This has allowed recognition of evolutionary conserved sequence domains, and the genetic differences among some of the widely used inbred mouse strains (Frazer et al., 2007). The sequence accessibility throughout species would permit candidate gene recognition for all those traits which have already been localized in several species (Hunter and Crawford, 2008; DiPetrillo et al., 2005). In the forthcoming years, as a consequence of these technical innovations, we ought to refine several QTL containing mouse cancer risk alleles and their interactions, and analysed them in human populations (Balmain, 2002). Moreover, it is possible that with the advent of whole genome sequencing, positional cloning may turn out to be unnecessary and fine mapping of significant loci may result straight forward to their isolation. The combination of mouse genetics studies with new technologies provides a possibility to identify the whole genetic arena that controls tumor susceptibility, evolution and response to therapy. The knowledge acquired by means of these genetic studies will have a significant effect on medical sciences, and should certainly lead to improved prognosis and therapy of human cancer, leading to a more individualized clinical management of the disease.

Pharmacogenomics

The variability of human responses to drug treatments has prompted a range of studies that aim to identify the genetic basis of this variation: a research field that has been termed "pharmacogenetics" or "pharmacogenomics" (Goldstein et al., 2003; Weinshilboum, 2003). This field includes the study of allelic variants that underlie individual differences in clinical effectiveness, benign adverse drug reactions, and severe, typically uncommon, adverse reactions.

It is now evident that differences in the DNA sequence of genes involved with drug action can lead to interindividual differences in effectiveness and adverse reactions to therapeutic drugs. Pharmacogenomics raises the possibility that drug discovery and patient management could move from a "one drug fits all" approach to one in which therapy is tailored to patients' genomes. Genetically modified mice that mimic human variation in drug response can provide one of the tools to move the field towards these goals (Liggett, 2004).

Molecular image

Clearly, one crucial addition to the drug discovery toolbox would be the development of novel and more predictive models of drug effect and toxicity. In particular, reporter animals could make a significant contribution to drug discovery programs by facilitating

target identification and improving the efficacy and reliability of all phases of preclinical drug development.

In vivo reporter gene and imaging technologies have the potential to contribute to the drug discovery pipeline in several areas (Herranz and Sanchez-Garcia, 2007). They provide systems that enable the study of the biochemical activity of a target in disease, and in response to a drug, to be monitored over periods of time, and offer more accurate methods of measuring pharmacodynamics and toxicity. Although reporter-gene technology is in its infancy, with further refinement reporter animals could become a valuable tool in the early stages of target and lead identification and preclinical drug development (Maggi and Ciana, 2005).

To help in testing these new drugs for safety and efficacy, and to aid clinicians in selecting from the various alternatives, diagnostic tools to detect the molecular signature of cancer have become more important (Sullivan and Kelloff, 2005). In this context, molecular imaging is a valuable tool for basic researchers and clinicians for several reasons. First, molecular imaging is, in essence, an *in vivo* assay. The greatest potential value of molecular imaging is its ability to report on the molecular state of a tumour in its normal milieu. Cancer cells do not behave *in vitro* the same as they do *in vivo*-as soon as cells are removed from the body, by biopsy for example, their pattern of gene expression changes, reflecting the dependence of the tumour cell on its microenvironment. This information may be a better indicator of the effects of targeted therapeutics than the molecular expression observed in the same tumour cells when cultured. Second, cancer-cell phenotypes change over time and if such changes occur during the course of therapy, they often result in resistance to the administered drug. Serial information from molecular imaging can therefore assist physicians in determining whether the current therapeutic choices are still relevant in treating the specific phenotype of the tumour. Third, cancer is often distributed at various locations within an organ or throughout the body. In vitro information from a single biopsy may not reflect the full heterogeneity of molecular changes that have taken place within the tumour cells of a patient. Fourth, molecular imaging-when used during therapeutic trials-can provide kinetic and dynamic data on a drug that cannot be obtained from a static biopsy (Green and Hudson, 2005).

Molecular imaging agents and methods have been developed for a variety of systems using different forms of energy. These include nuclear medicine methods-such as positron emission tomography (PET) and single photon emission computed tomography-magnetic resonance imaging (MRI), ultrasound methods, computed tomography (CT), and optical technologies. Increasingly, methods with complementary strengths are combined in clinical practice, such as the CT-PET systems that are now commercially available (Sullivan and Kelloff, 2005; Rudin and Weissleder, 2003).

In cancer therapy, *in vivo* imaging has become an increasingly important tool in helping clinicians select patients with the appropriate molecular phenotype for a given therapeutic, provide quantitative information about the optimum biological dose and timing of the therapy (as opposed to the present paradigm of administering the maximum tolerated dose) and assess appropriate biological end points, which may not necessarily be the reduction of tumour size.

We can use this new technology in our new mouse models, so that while we are testing drugs in them, we can study more aspects of the drugs than we are currently doing in human clinical trial.

Molecular imaging assays in intact living animals could be of further benefit in resolving biological questions raised by pharmaceutical scientists. Transgenic animals are useful in guiding early drug discovery by "validating" the target protein, evaluating test compounds, determining whether the target is involved in any toxicological effects of test compounds, and testing the efficacy of compounds to ensure that the compounds will act as expected in man (Massoud and Gambhir, 2003; Livingston, 1999).

Conclusions

There are many reasons why preclinical results do not predict human clinical trials; one of them is the use of inappropriate mouse models for preclinical assays. Numerous agents have shown exciting activity in preclinical models and yet have had minimal activity clinically. Herein we have purposed some ways to solve these obstacles.

The first thing to take into account is that the stem cell is the cell in which the initiating mutation occurs in human cancer. Mouse models in which technology is based on specifically expressing human genetic alterations in somatic stem cell will accurately reproduce the human disease. The generation of mouse models reproducing human cancer pathology will be a prerequisite not only for understanding the genesis and maintenance of human cancer, but also for the development of molecular and pharmacological therapeutics to treat and prevent human cancer. New drugs must be designed against the mechanisms that are responsible for the maintenance of the CSC, not for the initial event that transformed normal stem cells to CSC, because it is possible that the first alteration of the CSC will retain no function in the subsequent steps of cancer development. Targets selected in this context should be validated in a disease state by comparative genomics and pathology. Live animal imaging enables us to estimate functional genomics and target validation. Once the target has been validated, we must select those animals that are sensitive to the agents from those that do not respond. These results should guide us on whether or not to use that agent in human clinical trials so we can be selective and effective, rather than with the current preclinical trials. The last thing to do is to design human clinical trials by taking into account the results of the

preclinical assays. The clinical setting proposed herein will reduce the number of agents that goes into a clinical trial, being able to expend more effort on ones with a potential benefit.

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