Title: MOUSE MODEL OF A SUSTAINED CANCER CONDITIONALLY EXPRESSING AN ONCOGENE

Abstract: The invention relates, in particular, to leukaemia. In particular, the invention relates to transgenic non-human animals comprising in their genome a human oncogene which can be regulated exogenously by an effector substance. The invention further relates to transgenic non-human animals comprising in their genome the BCR-ABLP 190 gene. In addition the invention relates to the use of the transgenic non-human mammals of the invention for the discovery and development of compounds for the prevention and/or treatment of cancer.
MOUSE MODEL OF A SUSTAINED CANCER CONDITIONALLY EXPRESSING AN ONCOGENE

FIELD OF INVENTION

The invention relates, in general, to cancer development; more specifically, leukaemia. In particular, the invention relates to transgenic non-human animals that express BCR-ABLp190 in a controllable fashion.

BACKGROUND OF THE INVENTION

The relative decrease in cancer mortality is mainly the result of early detection and prevention rather than the consequence of effective therapeutics (Etzioni et al., 2003). Understanding the genetic pathways underlying cancer maintenance is the prerequisite for the discovery of molecular and pharmacological therapeutics to treat and prevent cancer. Much effort is currently being expended to target the mutated oncogenes and tumour suppressor genes that control neoplastic cell growth directly. However, many of the new classes of agents are predicted to show clinical benefit in only small sub-populations of patients, if at all, and target non-essential aspects of tumour maintenance. A very important cause of the inefficiency of the drug discovery and development process is the disparity between pre-clinical and clinical trials. An almost unanimously recognised factor leading to this disparity is lack of predictive animal models.

The main reason why current mouse models have failed in being predictive for clinical trials is that they do not accurately reproduce the human disease. In this sense, the ability to model cancer conditionally in the mouse has provided a robust methodology to dissect the molecular etiology of cancer (Huettner et al., 2000; Jackson et al., 2001; Meuwissen et al., 2001; Van Etten, 2001). These studies indicate that targeted inactivation of specific oncogenes can be effective in inducing sustained regression of tumours, although these models mainly fail to reproduce the human disease (Huettner et al., 2000; Jackson et al., 2001; Meuwissen et al., 2001; Van Etten, 2001; Perez-caro et al., 2005). However, most often, the anticancer effects seen in the pre-clinical state (on cells or animal models) cannot be replicated in humans using targeted therapies. There exists a greater need to bridge the gap between disease models and the clinic. What is needed is a validation of target compounds in models mimicking human cancer. Current pre-clinical
settings do not accurately predict clinical efficacy of potential drug compounds in humans.

Moreover, a complete understanding of the cancer process requires more detailed knowledge of the mechanisms maintaining neoplastic growth. The generation of mouse models mimicking human cancer pathology is a prerequisite not only for understanding the genesis of human cancer but also for the identification of molecular events responsible for cancer maintenance. New drugs must be designed targeting the mechanisms that are responsible for cancer maintenance, not for the initial event that transforms a normal cell into a cancer cell, because it is possible that the first alteration of the cancer cell will have no role in the subsequent steps of cancer development.

One of the landmark events in the targeted-therapy revolution has been the development of Imatinib mesylate (Gleevec™), a relatively simple structure that possesses all the desired factors of the "ideal" targeted compound (Savage et al., 2002). Imatinib mesylate is a moderately potent inhibitor of the kinase BCR-ABL\textsuperscript{p210}, the fusion protein product of a chromosomal translocation, named Philadelphia chromosome (Ph), that is involved in the pathogenesis of chronic myeloid leukaemia (CML) (Druker et al., 2001a; Mauro and Druker, 2001; O'Dwyer and Druker, 2001; Chabner and Roberts, 2005) and of the BCR-ABL\textsuperscript{p90} product which is mainly associated with B-cell acute lymphoblastic leukaemia (B-ALL) (Chan et al., 1987; Clark et al., 1987; Hermans et al., 1987; Kurzrock et al., 1987; Melo, 1996). It has been shown that when Imatinib mesylate is used to treat patients with chronic-phase CML, 90% seem to achieve complete hematological remission and many lose cytogenetic evidence of the malignant clone (Druker et al., 2001a). However, in the acute leukaemic phase of CML or in P190-B-ALL, Imatinib mesylate treatment is not effective (Shah et al., 2002).

The origin of CML and pl90-B-ALL begins in a hematopoietic stem cell (HSC), a target population that is largely quiescent (Cobaleda et al., 2000; Cox et al., 2004). \textit{In vitro} and \textit{in vivo} studies have shown that these quiescent Ph+ HSCs are insensitive to Imatinib mesylate treatment (Graham et al., 2002; Chu et al., 2005), with the cells not being eliminated in Ph+ patients. These clinical data suggest that BCR/ABL kinase activity may be required for abnormal proliferation and expansion of Ph+ cells but may not be essential for preservation of primitive malignant cells and clearly indicates that a
complete understanding of the cancer process requires more detailed knowledge of the mechanisms maintaining neoplastic growth.

A major obstacle to elucidating the mechanisms that are responsible for cancer maintenance and not just for the initial event that transforms a normal cell into a cancer cell is a lack of an appropriate animal model. Therefore, the generation of mouse models mimicking human cancer pathology is a prerequisite not only for understanding the genesis of human cancer but also for the identification of molecular events responsible for cancer maintenance.

SUMMARY OF THE INVENTION

The inventors have surprisingly overcome the problems associated with the animal models described in the prior art by developing a conditional transgenic animal model, which reproduces human cancer.

According to the present invention, there is provided a transgenic non-human mammal model of a sustained cancer comprising in its genome a human oncogene, wherein the expression of said oncogene can be regulated exogenously by an effector substance. Transgenic non-human mammals of this type are thus useful, among other goals, for studying cancer, in particular hematological malignancies as well as for evaluating potentially useful compounds for treating, diagnosing and/or preventing such pathologies. The animal is particularly useful as a model which faithfully reproduces human hematological malignancies. In these models, and cells derived therefrom, it has been found that inactivation of the oncogene is not able to eliminate the tumour-maintaining cells.

Unlike some previous animal models used to study cancer, the model disclosed herein uses a binary system (i.e. a single plasmid), closely replicates the pathology of the human disease and allows the translation of the target validation/selection/therapeutic response observed to human beings. The model described herein is the first demonstration that it is possible to reproduce human cancer in animals using regulated expression of an oncogene. Furthermore, these animals faithfully predict the human cancer response and can be used to select targets responsible for cancer maintenance and develop human therapeutic and diagnostic strategies.
Specifically, in the present invention, in order to investigate the function of BCR-ABLp190 during cancer development further, mice harbouring a tetracycline-repressible BCR-ABLp190 transgene were generated. A single-plasmid system containing the regulating and expression elements of the original binary tetracycline system was used to allow high induction and tight control of human BCR-ABLp190 gene expression by tetracycline. This system, which has the transactivator and the tet-operator minimal promoter driving the expression gene unit on a single plasmid, ensures the integration of the transactivator and reporter gene units in equal copy numbers in a direct c/s-configuration at the same chromosomal locus and prevents genetic segregation of the control elements during breeding. The system is described herein as the CombiTA-pl90 system. The mice produced did not exhibit morphological defects at birth but did develop B-ALL leukaemias similar to those associated with BCR-ABLp190 expression in humans.

In vitro studies have shown that BCR-ABLp190 confers resistance to cell death induced by the withdrawal of survival factors (Sanchez-Garcia and Grutz, 1995). The inventors have successfully confirmed the physiological relevance of CombiTA-pl90 suppression in vitro by assaying survival of Ba/F3 cells expressing CombiTA-pl90 after IL-3 withdrawal. The analysis of the BCR-ABLp190-expressing mice, with reference to known human phenotypes, identified that these mice develop a B-cell acute lymphoblastic leukaemia characterized by lymphoid blasts co-expressing myeloid markers. Thus, the CombiTA-pl90 mice expressing the BCR-ABLp190 chimeric gene product shows consistently the same phenotype as that with which this oncogene is associated in human pathology.

Modulation of BCR-ABLp190 expression in the model of the invention allows demonstration that the oncogene can initiate leukaemia when activated in both young and adult mice. However, the survival conferred by BCR-ABLp190, while reversible in vitro, can escape such control in vivo. These results are congruent with the poor efficacy of Imatinib mesylate treatment in P190-B-ALL. In agreement with this result, CombiTA-pl90 leukaemic mice did not show clinical improvement when treated with Imatinib mesylate and transitory expression of the oncogene was enough to initiate leukaemia development. Furthermore, the leukaemic phenotype seen in BCR-ABLp190-expressing mice could not be rescued by suppression of the BCR-ABLp190 transgene, indicating that BCR-ABLp190 is not required to maintain
leukaemia development. Moreover, gradual BCR-ABL inactivation resulted in the differentiation of blast cells into mature cells, indicating that oncogene level modulates differentiation of leukaemic cells.

The results presented herein confirm that this model is an accurate model of sustained hematological malignancies. The model suggest that BCR-ABLp190-induced tumourigenesis is not reversible through the unique inactivation of the gene defect initiating cancer development. The results suggest that the BCR-ABLp190 gene imposes a cancer stem cell imprinting context in which oncogene inactivation cannot change the epigenetic context. The inability of BCR-ABLp190 inactivation to eliminate cancer cell progenitors suggests that B-ALL patients may require a combination of Imatinib mesylate therapy with other therapeutic strategies.

These observations can be readily applied to other cancer-initiating gene defects when validated in a physiological disease state. Therefore, not only does the model described herein offer the opportunity to evaluate changes in the hematopoietic system in response to transgene repression, because it mimics the natural in vivo phenotype of cancer, in that inactivation of the causative oncogene is not able to eliminate the tumour maintaining cells, it also has important implications for the development of new cancer drugs targeting essential aspects of tumour maintenance.

In order to facilitate the understanding of the instant description, the meaning of some terms and expressions in the context of the invention are explained below.

The term "subject" as used in this description refers to members of mammal species, and includes, but is not limited to, domestic animals, rodent, primates and humans; the subject is preferably a human being, male or female, of any age or race.

The term "sample", as used herein, can be any biological sample from a subject, such as a liquid sample, for example, blood, serum, etc., or a solid sample, such as a tissue sample, etc. The sample can be obtained by any conventional method, including surgical resection in case of solid samples. The sample can be obtained from a subject previously diagnosed, or not diagnosed, with a hematological malignancy, or also from a subject undergoing treatment, or who has been previously treated, for a hematological malignancy such as leukaemia. In an embodiment, the sample is a liquid or solid biological sample from the blood or bone marrow of the subject.

The term "cancer", as used herein, refers to epithelial cancer or mesenchymal cancer.
The term "epithelial cancer", as used herein, refers to a cancer of which tumour cells are the cells that line the internal and external surfaces of the body. The term "mesenchymal cancer", as used herein, refers to a cancer which tumour cells develop into connective tissue, blood vessels and lymphatic tissue, for example sarcomas and hematological malignancies.

The term "hematological malignancy", as used herein, refers to one of a type of cancer that affects blood, bone marrow and lymph nodes. As the three are intimately connected through the immune system, a disease affecting one of the three will often affect the others as well. Illustrative, non-limitative examples of said hematological malignancies include lymphomas, leukaemias, such as, for example, chronic myeloid leukaemia, B-cell acute lymphoblastic leukaemia, T-cell acute lymphoblastic leukaemia, acute myeloid leukaemia, chronic myeloid leukaemia and lymphoproliferative syndromes.

The term "leukaemia", as used herein, refers to a cancer of the blood or bone marrow characterized by an abnormal proliferation of blood cells, usually white blood cells (leukocytes). Illustrative, non-limitative examples of said leukaemia include acute lymphocytic leukemia (also known as acute lymphoblastic leukemia or ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), B-cell acute lymphoblastic leukaemia and T-cell acute lymphoblastic leukaemia.

The term "gene" refers to a molecular chain of deoxyribonucleotides encoding a protein. The term "DNA" refers to deoxyribonucleic acid. A DNA sequence is a deoxyribonucleotide sequence. The term "cDNA" refers to a nucleotide sequence complementary of a mRNA sequence. The term "RNA" refers to ribonucleic acid. An RNA sequence is a ribonucleotide sequence. The term "mRNA" refers to messenger ribonucleic acid, which is the fraction of total RNA which is translated into proteins. The term "protein" refers to a molecular chain of amino acids with biological activity.

The term "oncogene" refers to a modified gene, or a set of nucleotides that codes for a protein, that increases the potential malignancy of a tumor cell i.e. that increases the chance that a normal cell develops into a tumor cell. Preferably, the oncogene is a human oncogene, but other mammalian oncogenes may be used. Many oncogenes are known, and are linked to particular types of cancer. For example, BCR-ABLp210 is related to chronic myeloid leukaemia. BCR-ABLp190, TEL-AML1, E2A-HLF and E2A-Pbx1 are
related to B-cell acute lymphoblastic leukaemia (B-ALL). Further examples will be
known to those of skill in the art, including BCR-ABLp210, BCR-ABLp1 90, Slug, Snail,
HOX1 1, RHOM2/LMO-2, TALI, Maf-B, FGFR, c-maf, MMSET, BCL6, BCL1O,
MALT1, cyclin Dl, cyclin D3, SCL, LMO1, LM02, TEL-AML1, E2A-HLF, E2A-Pbx1,
TEL-ABL, AML1-ETO, FUS-DDIT3, EWS-WT1, EWS FLII, EWSRI-DDIT3, FUS-
ATFl, FUS-BBF2H7, K-RASv 12, Notch 1, etc. For example, comprehensive lists of
oncogenes are provided at: http://www.infobiogen.fr/services/chromcancer/
Genes/Geneliste.html; see also Cooper G. Oncogenes. Jones and Bartlett Publishers,

As the skilled person will appreciate, the exact sequence of any one of these genes that is
used may vary. Preferably, a mammalian oncogene is used, such as a mouse sequence,
more preferably a human sequence. However, the invention includes the use of variant
oncogenes so as to include, for example, polymorphic variants, mutants and other gene
5 types not considered wild type. For example, such genes could be used to investigate
differences in the oncogenic potency of such genes that might reflect differences in
patterns of disease and response to treatment across patient groups and so on.
Accordingly, reference to each of the genes referred to above includes within its scope,
references to variants of such genes, for example, that share significant homology or
sequence identity of 80%, 85%, 90%, 95%, 98%, 99% or more with the sequences
described above. The gene may be cDNA, or may comprise genomic sequence. cDNA is
preferred.

The term "BCR-ABLp190 gene" refers to the product of a chromosomal translocation
event called the Philadelphia chromosome (Ph). Parts of two chromosomes, 9 and 22,
10 swap places. The result is that part of the BCR ("breakpoint cluster region") gene from
chromosome 22 (region q11) is fused with part of the ABL gene on chromosome 9
(region q34). The nucleotide sequence of the human BCR-ABLpJ90 gene is known (see,
for example, NCBI, Accession number AJ 131467) and this is the preferred gene for use
in aspects of the invention referred to herein. Sequences of other oncogenes will be
The term "BCR-ABLp 190 protein" refers to the translation product of the BCR-ABLp 190 gene. The protein is a kinase. In particular the part of the ABL gene encodes a tyrosine kinase domain and, therefore, the BCR-ABLp 190 protein is a tyrosine kinase. In addition the BCR gene encodes a domain which is a serine/threonine kinase. The amino acid sequence of the human BCR-ABLp 190 protein is known (see, for example, NCBI, Accession number CAA 10377).

The term "transcription product of BCR-ABLp 190 gene" refers to the mRNA of BCR-ABLp 190 gene. The term "translation product of BCR-ABLp 190 gene" refers to BCR-ABLp 190 protein. The human BCR-ABLp 190 protein is a preferred oncogene according to the invention.

The invention is based on the discovery that mice harbouring a repressible BCR-ABLp 190 transgene are capable of mimicking human cancer.

In one aspect, therefore, the invention provides a transgenic non-human mammal model of a sustained hematological malignancy comprising in its genome a human BCR-ABLp 190 oncogene, wherein the expression of said oncogene can be regulated exogenously by an effector substance. In this model, subsequent inactivation of BCR-ABLp 190 is preferably not able to eliminate the tumour maintaining cells.

Herein, a non-human mammal that is termed "transgenic" comprises a transgene in its genome. According to a preferred aspect of the invention, said transgene may comprise a nucleic acid sequence encoding the BCR-ABLp 190 protein (i.e., said nucleic acid comprises the BCR-ABLp 190 gene), the expression of said transgene being exogenously regulated by an effector substance. Preferably the BCR-ABLp 190 protein is the human BCR-ABLp 190 protein (see, for example, NCBI, Accession number CAA 10377), encoded by the human BCR-ABLp 190 gene (see, for example, NCBI, Accession number AJ 13 1467), although other forms, such as the murine form, may also be of some utility.

In certain embodiments of the invention, it may be appropriate to use as the transgene a sequence encoding only a portion of the BCR-ABLp 190 protein, such as a fragment, or a variant of the BCR-ABLp 190 protein. By "fragments" we mean any portion of the full length BCR-ABLp 190 protein, including, for example, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or more of the full length sequence. For example, a fragment may include a specific domain or combination of domains within the protein structure. By "variants", we mean any variant of the BCR-ABLp 190 protein, such as, for
example, a mutant form comprising one or multiple (e.g. 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 35, 50, 75, 100 or more) insertions, deletions, substitutions and so on.

The transgenic non-human mammal provided by this invention possesses a genotype that confers a greater tendency to develop cancer when compared to the non-transgenic mammal. Preferably, subsequent inactivation of the causative oncogene is not able to eliminate the tumour maintaining cells.

Examples of cancers that are generated in transgenic models of the type disclosed herein include epithelial cancer, such as lung, prostate, intestine, breast, skin, pancreas and mesenchymal cancers including sarcomas and hematological malignancies. Preferably the transgenic non-human mammal provided by the invention possesses a genotype that confers a greater tendency to develop a hematological malignancy such as lymphomas, lymphoproliferative syndromes and leukaemia, such as, for example, acute lymphocytic leukemia (also known as acute lymphoblastic leukemia or ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL) and chronic myelogenous leukemia (CML). Preferably the transgenic models of the type disclosed herein develop B-cell acute lymphoblastic leukemia.

For example, all Combi-tTA-/?/90 mice generated herein (see examples) became unwell from approximately 5-7 months of age onward with clinical manifestations that included decreased physical activity, tachypnea, pilo-erection, shivering, and sustained weight loss, prior to sacrifice (Table 1). Macroscopic analysis showed that CombitTA-p/90 mice were pale with splenomegaly and hepatomegaly with pronounced white spots. The thymi and lymphoid nodes generally appeared normal. This examination is consistent with hematological disease. However, no tumours of other tissues were found in these mice, despite widespread activity of the promoter. These animals had elevation in the peripheral blood leukocyte counts and the histological analysis revealed marked leukaemic cell infiltration of hematopoietic (splenic white pulp) (Figure 3A) and nonhematopoietic tissues (liver sinusoids) in CombitTA-p/90 mice. Similar results were obtained with the two CombitTA-p/90 transgenic lines. There was no relationship between the level of BCR-ABLp1/90 transgenic expression and disease phenotype as there was found to be no minimum degree of expression required for the development of a leukaemic state. Therefore, these results show the generation of a new mouse model,
recapitulating the consequences of the BCR-ABL190 chromosomal abnormality. As a result of this, CombitTA-p/90 mice specifically develop acute leukaemias.

Table 1

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>Mice autopsied</th>
<th>Mice with tumour (%)</th>
<th>Age (months) at tumour onset</th>
<th>Tumour type</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS1353A</td>
<td>64</td>
<td>64 (100)</td>
<td>7-11</td>
<td>B-ALL co-expressing myeloid markers</td>
</tr>
<tr>
<td>IS1353B</td>
<td>54</td>
<td>54 (100)</td>
<td>5-9</td>
<td>B-ALL co-expressing myeloid markers</td>
</tr>
</tbody>
</table>

ONumber of mice dying or after the period of cancer. (c) Number of mice killed with cancer and percentage of tumour incidence.

The introduction of a DNA construct in which BCR-ABL1 190 is expressed in a way which allows regulation by an exogenous factor, into the genome of a model animal causes a genetic anomaly. In a particular embodiment, the genetic anomaly caused by the expression of the BCR-ABL190 chimeric transgene results in a hematological malignancy, in particular leukaemia. In which case, the descendants are analysed to evaluate the existence of activated genes and/or genes created by the genetic anomaly associated with the pathology in question.

The expression "non-human mammal", as used herein, includes any non-human animal belonging to the class of mammals. The non-human mammal is preferably a mouse but may be another mammalian species, for example another rodent, for instance a rat, hamster or a guinea pig, or another species such as a monkey, pig, rabbit, or a canine or feline, or an ungulate species such as ovine, caprine, equine, bovine, or a non-mammalian animal species. In a particular embodiment, the transgenic non-human animal provided by the invention is a murine animal. The term "murine" includes mice, rats, guinea pigs, hamsters and the like. In a preferred embodiment the murine animal is a rat or a mouse; most preferably the non-human mammal of the invention is a mouse.

Although the use of transgenic animals poses questions of an ethical nature, the benefit to man from studies of the types described herein is considered vastly to outweigh any suffering that might be imposed in the creation and testing of transgenic animals. As will be evident to those of skill in the art, drug therapies require animal testing before clinical
trials can commence in humans and under current regulations and with currently available model systems, animal testing cannot be dispensed with. Any new drug must be tested on at least two different species of live mammal, one of which must be a large non-rodent. Experts consider that new classes of drugs now in development that act in very specific ways in the body may lead to more animals being used in future years, and to the use of more primates. For example, as science seeks to tackle the neurological diseases afflicting a 'greying population', it is considered that we will need a steady supply of monkeys on which to test the safety and effectiveness of the next-generation pills. Accordingly, the benefit to man from transgenic models such as those described herein is not in any limited to mice, or to rodents generally, but encompasses other mammals including primates. The specific way in which these novel drugs will work means that primates may be the only animals suitable for experimentation because their brain architecture is very similar to our own.

This aspect of the invention aims to reduce the extent of attrition in drug discovery and development. Whenever a drug fails at a late stage in testing, all of the animal experiments will in a sense have been wasted. Stopping drugs failing therefore saves test animals' lives. Therefore, although the present invention relates to transgenic animals, the use of such animals should reduce the number of animals that must be used in drug testing programmes and decrease attrition rates in clinical assays in humans.

Although the Applicant does not wish to be bound by this theory, it is postulated that transitory expression of BCR-ABLpI 90 in the transgenic non-human mammalian model of the invention is enough to initiate leukaemia development and expression of BCR-ABLp190 is not required to maintain leukaemia development. This suggests that there are other mechanisms responsible for the maintenance of the cancer pathology.

The invention, therefore, allows for the investigation of the mechanisms responsible for cancer maintenance.

The term "mechanisms responsible for cancer maintenance", as used herein, refers to epigenetic and/or genetic modifications produced in the cells of the transgenic non-human mammalian model of the invention by the initiating oncogenic event. The term "mechanisms responsible for cancer maintenance", encompasses any cellular mechanism which plays a role in maintaining the cancer pathology after the initiating oncogenic event. Such mechanisms may involve the upregulation or downregulation of one or more
genes; this includes an increase or decrease in the level of transcription products and translation products of the gene.

The term "initiating oncogenic event", as used herein, refers to the molecular event which causes the development of a cancer pathology. Preferably, the initiating oncogenic event is the expression of the transgenic oncogene. An example of an initiating oncogenic event is the expression of the \textit{BCR-ABLp190} gene.

The term "targets responsible for cancer maintenance", as used herein, refers to any gene or protein involved in the mechanisms responsible for cancer maintenance, subsequent to the initiating event. The term also includes any intermediate stage of gene regulation, including any transcription or translation product.

The term "effector substance", as used herein, refers to any substance which is capable of regulating the expression of the oncogene in the genome of the transgenic non-human mammal of the invention when said substance is administered. Suitable exogenously regulated expression systems are well known by persons of skill in the art and include the tetracycline system and Cre-Lox technology (see, for example, Maddison K., Clarke AR. 2005. New approaches for modelling cancer mechanisms in the mouse. J. Pathol. 205:181-193). Preferred effector substances include tetracycline and Adeno-Cre.

For the generation of the transgenic non-human mammal of the invention, a DNA construct containing the oncogene (transgene) is made. Preferably, this gene is introduced into the animal as a DNA construct, preferably comprising regulatory sequences. These regulatory sequences may be derived from humans, animals, prokaryotes or other species. In cases where the regulatory genes are not of human origin, the regulatory genes may be derived from the target animal, for example, the mouse. By regulatory genes is meant to include any promoter or enhancer sequences, 5' or 3' UTRs, poly-A termination sequences or other DNA sequences, that are necessary for transcription of the gene of interest. Transcripts used for insertion of human sequences are preferably terminated by a poly A motif. The invention may incorporate the endogenous promoter with the coding gene so that the fidelity of wild type expression is retained, developmentally, temporally and in a tissue-specific manner. By "endogenous promoter" is meant the promoter that naturally directs expression of the gene of interest. The endogenous promoter may thus be the endogenous human promoter, or may alternatively be the promoter that is endogenous to that introduced
gene in the transgenic animal subject. For example, in the case of transgenic mice, the expression of the human gene may be directed by the endogenous mouse promoter for that gene.

Preferably, said construct, hereinafter referred to as the DNA construct of the invention, thus comprises the oncogene cDNA, for example BCR-ABLpI90, under the control of an expression system exogenously regulated by an effector substance. Suitable systems will be clear to those of skill in the art. In a preferred embodiment, the exogenously regulated expression system may be based on the tet-off system (Clontech), i.e. the Combi-tTA (Combi-tTA) vector system of Schultze et al. (Nature Biotechnology 14: 499-503, 1996), or a modified version thereof.

A schematic representation of a preferred DNA construct is shown in Figure 1A. In this embodiment, the oncogene is preferably under the control of the tet-operator (tetO) minimal promoter. In the illustrated case, the expression of the oncogene is exogenously regulated by tetracyclin or its derivatives such as doxycyclin, and the effector substance according to the invention is preferably tetracyclin or its derivatives such as doxycyclin. Thus, the oncogene of the transgenic non-human mammal according to the invention is preferably silenced in the presence of tetracyclin and activated in the absence of tetracyclin.

In one embodiment, the original Combi-tTA vector as described by Schultze et al. (Nature Biotechnology 14: 499-503, 1996) is preferably modified by the following steps: 1) removal of the tetO-luciferase cassette from said original vector, and 2) introduction of a cassette comprising the tetO minimal promoter and the oncogene. Preferably said cassette should be introduced within the ampicillin resistance gene (referred to alternatively as Amp, bla, or the beta-lactamase gene) of the original vector disclosed in Schultze et al.

The inventors have improved upon the single-plasmid system of Schultze et al., (1996) containing the regulating and expression elements of the original binary tetracycline system to allow induction and tight control of gene expression by tetracycline in mice. The inventors found that the Schultze system requires some significant modification in order to allow a target gene to be efficiently expressed and appropriately silenced. For example, it has been found that without the modifications described herein, target gene expression is not silenced in the presence of tetracycline or, e.g., tetracyclin derivatives
such as doxycyclin, probably because of read-through from the other promoters (e.g. CMV and SV40) that are present on the Schultze plasmid. Accordingly, one, preferably two or more polyA sequences are introduced in flanking positions around the target gene to ensure that this read-through problem is resolved.

Additionally, it has been found useful to introduce a TATA sequence in order to improve expression of the target protein from this system. Preferably, this TATA sequence lies between the tetO sequence and the target gene sequence.

Preferably, said introduced cassette comprises a poly-A sequence, the tetO promotor, a TATA box sequence, the target gene, further two poly-A sequences, an ampicillin resistance gene, and a fourth poly-A sequence. These elements are preferably arranged on said cassette in the aforementioned order. This modified construct, as described above, may be used for expression of any target gene in a manner which is regulated by tetracycline, or its derivatives such as doxycyclin, and forms an independent aspect of the present invention. Accordingly, this aspect of the invention provides a DNA construct adapted for the expression of a target oncogene in a way which allows regulation by an exogenous factor, said construct comprising an origin of replication, at least one promoter, at least one sequence capable of mediating regulation by an exogenous factor, at least one transactivator sequence and a sequence encoding the target gene, wherein the sequence encoding the target gene is flanked on both sides by at least one polyA sequence. Preferably, the flanking polyA sequences are situated so as to prevent read-through from the promoter sequences, such as in the configuration set out in Figure 1. The construct may contain one, two or more flanking polyA sequences. It is not necessarily essential for the flanking polyA sequences to be directly contiguous with the sequence encoding the target gene. However, in a preferred embodiment, at least one polyA sequence is situated directly 5' and directly 3' to the sequence encoding the target gene.

By "polyA" sequence is meant a polyadenylation signal as known from eukaryotic genetics. Typically, polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. Examples of polyadenylation signals include those derived from SV40, although others will be known to those of skill in the
art. Such sequences comprise runs of adenosine nucleotides, preferably between 10 and
500 nucleotides in length, more preferably between 50 and 200 nucleotides.

Preferably, the promoter comprises an SV40 promoter and/or a CMV promoter, more
preferably both an SV40 promoter and a CMV promoter. Preferably the transactivator
comprises the viral VP16 transactivator domain. More preferably transactivator
comprises the viral VP16 transactivator domain fused to the ^/-repressor protein. However, other transactivator systems will be known to those of skill in the art

Preferably, the construct additionally comprises a promoter sequence, preferably a
TATA sequence, preferably situated upstream of the sequence encoding the target gene.

Preferably, the promoter sequence lies between the sequence capable of mediating
regulation by the exogenous factor and the sequence encoding the target gene.

Preferably, the sequence capable of mediating regulation by the exogenous factor is tetO,
or a functional equivalent thereof, and the exogenous factor is tetracyclin, or a derivative
thereof, such as doxycyclin.

Preferably, the sequence encoding the target gene is a gene implicated in predisposition
to cancer, including oncogenes, and BCR-ABLp 190. Other useful examples will be clear
to those of skill in the art.

A preferred embodiment of this aspect of the invention is a construct based on that
represented in Figure 1 herein for BCR-ABLp190. In a particularly preferred
embodiment, the oncogene is BCR-ABLp190.

The cassette for insertion into the Schultze et al. Combi-tTA vector, preferably within
the bla gene, in the construction of the transgenic non-human mammal according to the
present invention, is shown in Figure 1A. The final construct according to the present
invention is the Combi-tTA vector resulting from the insertion of said cassette
containing the BCR-ABLp190 gene, and is referred to herein as the Combi-tTA-pl90
vector. Most preferably, the orientation of the poly-A sequence, the tetO promotor, the
TATA box sequence, the BCR-ABLp190 gene, the further poly-A sequences, the
ampicillin resistance (beta-lactamase / bla) gene, and fourth poly-A sequence is as
shown in Figures 1A though, according to some embodiments, the Amp (bla) may also
be in the opposite relative orientation.
The DNA construct of the invention is next introduced into a non-human mammal, or into a predecessor thereof, in an embryonic state, for example, in the state of a cell, or fertilized oocyte and, generally, not later than the G cell state.

There are different means conceived in the state of the art by which a sequence of nucleic acid can be introduced into an embryo of an animal such that it can be incorporated genetically in an active state, all of which can be applied for the generation of transgenic non-human mammals of the invention. One method consists of transfecting the embryo with said sequence of nucleic acid as occurs naturally, and selecting the transgenic animals in which said sequence has been integrated onto the chromosome at a locus that as a result causes the activation of said sequence. Another method implies modification of the nucleic acid sequence, or its control sequences, before introducing it into the embryo. Another method consists of transfecting the embryo using a vector that contains the nucleic acid sequence to be introduced.

In a particular embodiment, the introduction of the DNA construct of the invention in the germ line of a non-human mammal is performed by means of microinjection of a linear DNA fragment that comprises the activatable gene into fertilized oocytes of a non-human mammal.

The fertilised oocytes can be isolated by conventional methods, for example, provoking the ovulation of the female, either in response to copulation with a male or by induction by treatment with the luteinising hormone. In general, a superovulation is induced in the females by hormonal action and they are crossed with males. After an appropriate period of time, the females are sacrificed to isolate the fertilised oocytes from their oviducts, which are kept in an appropriate culture medium. The fertilised oocytes can be recognised under the microscope by the presence of pronuclei. The microinjection of the linear DNA fragment is performed, advantageously, in the male pronucleus.

After the introduction of the linear DNA fragment that comprises the oncogene construct of the invention into fertilised oocytes, they are incubated in vitro for an appropriate period of time or else they are reimplanted in pseudopregnant wet nursing mothers (obtained by making females copulate with sterile males). The implantation can be performed by conventional methods, for example, by anaesthetising the females and surgically inserting a sufficient number of embryos, for example, 10-20 embryos, in the oviducts of the pseudopregnant wet nursing mothers. Once gestation is over, some
embryos will conclude the gestation and give rise to transgenic non-human mammals, which theoretically should carry the DNA construct of the invention integrated into their genome and present in all the cells of the organism. This progeny is the GO generation and their individuals are the "transgenic founders". The confirmation that an individual has incorporated the injected nuclear acid and is transgenic is obtained by analysing the individuals of the progeny. To do this, from a sample of animal material, for example, from a small sample from the animal's tail (in the event that it is, for example, a mouse) or a blood example, the DNA is extracted from each individual and analysed by conventional methods, for example, by PCR using the specific primers or by Southern blot or Northern blot analysis using, for example, a probe that is complementary to, at least, a part of the transgene, or else by Western blot analysis using an antibody to the protein coded by the transgene. The term "antibody" refers to a glycoprotein exhibiting specific binding activity to a particular protein, which is called "antigen". The term "antibody" comprises monoclonal antibodies, polyclonal antibodies, either intact or fragments thereof, recombinant antibodies, etc., and includes human, humanized and non-human origin antibodies. "Monoclonal antibodies" are homogenous populations of highly specific antibodies directed against a single site or antigenic "determinant". "Polyclonal antibodies" include heterogeneous populations of antibodies directed against different antigenic determinants. Other methods for evaluating the presence of the transgene include, without limitation, appropriate biochemical assays, such as enzymatic and/or immunological assays, histological staining for particular markers, enzymatic activities, etc.

According to a preferred embodiment of the invention, the preferred transgenic non-human mammal thus generated is preferably obtainable by the procedures mentioned above using the Combi-tTA-pl90 vector. In this embodiment, the transgenic non-human mammal of the invention is referred to herein as a Combi-tTA-pl90 mouse.

In general, in transgenic animals, the inserted transgene is transmitted as a Mendelian characteristic and so it is not difficult to establish stable lines for each individual. If the GO individuals are crossed with the parent strain (retrocrossing) and the transgene behaves with Mendelian characteristics, 50% of the progeny will be heterozygotic for the inserted transgene (hemizygotic). These individuals constitute the G1 progeny and a transgenic line that can be maintained indefinitely, crossing hemizygotics of the G1 generation with normal individuals. Alternatively, individuals of the G1 generation can
be crossed among themselves to produce 25% homozygotics for the inserted transgene, 50% hemizygotics and 25% without the transgene provided the transgene does not affect the viability of the descendents.

The progeny of the transgenic non-human mammal of the invention, such as the progeny of a transgenic mouse provided by this invention can be obtained, therefore, by copulation of the transgenic animal with an appropriate individual, or by in vitro fertilization of eggs and/or sperm of the transgenic animals. As used in this description, the term "progeny" or "progeny of a transgenic non-human mammal" relates to all descendents of a previous generation of the transgenic non-human mammals originally transformed. The progeny can be analysed to detect the presence of the transgene by any of the aforementioned methods. The progeny of the transgenic non-human mammal of the invention, hereinafter referred to as the progeny of the transgenic non-human mammal of the invention, constitutes a further aspect of the present invention.

The invention also relates to a cell line comprising in its genome a human oncogene, wherein the expression of the oncogene can be regulated exogenously by an effector substance. Preferably, the oncogene is associated with a human cancer pathology and the inactivation of the oncogene does not eliminate the associated cancer pathology. In a particular embodiment, said cell line, is a murine cell line. The invention also relates to a cell line of the transgenic non-human mammal of the invention or of the progeny of the transgenic non-human mammal of the invention, to a primary cell of the transgenic non-human mammal of the invention or of the progeny of the transgenic non-human mammal of the invention or to a tissue sample of the transgenic non-human mammal of the invention or of the progeny of the transgenic non-human mammal of the invention. Said cell line, primary cell or tissue sample, contains a DNA construct of the invention in its genome, i.e., a DNA construct containing an oncogene such as the BCR-ABLp190 gene. In a particular embodiment, said cell line, primary cell or tissue sample is a murine cell line, primary cell or tissue sample.

The cell lines of the invention can also further comprising a reporter gene useful for spatiotemporal identification of the onset, progression, dissemination and further physiopathological processes, for evaluating the effect of therapies by molecular imaging techniques, for diagnostic assays, drug discovery and development processes,
for target identification and for improving the efficacy and reliability of all phases of the clinical development.

Reporter genes are nucleic acid sequences encoding directly or indirectly assayable proteins. They are used to replace other coding regions whose protein products are unsuitable or not amenable to the assay envisaged. Examples of suitable reporter genes that are known in the art and may be used in the present invention are selected from those genes encoding proteins including but not limited to: chloramphenicol-acetyltransferase, β-galactosidase, β-glucuronidase, luciferase, beta-galactosidase, green fluorescent protein, secreted alkaline phosphatase (SEAP), major urinary protein (MUP) or human chorionic gonadotrophins (hCG). It will be understood that the above list of suitable reporter genes is not exhaustive or exclusive and is not intended to limit the scope of the application. The skilled artisan may select another reporter system which will equally be applicable to the present invention.

The cell lines of the invention may be used as biomarkers for detecting the presence of a gene created and/or activated by a genetic anomaly associated with a human cancer pathology in a subject. They may also be used for assessing the risk or predisposition of a subject to develop a human cancer pathology in a subject, or for determining the stage or severity of a human cancer pathology in a subject. Furthermore, the cell lines of the invention can be used for monitoring the effect of the therapy administered to a subject having a human cancer pathology, or for designing an individualized therapy for a subject suffering from a human cancer pathology, or for designing human clinical trials, or for diagnosis of cancer and/or specific processes and effects of cancer development, like cancer dissemination; or for patient selection for personalized therapeutics; or for therapeutic monitoring and evaluation of therapeutic benefits; or for drug discovery and pharmacokinetics guidance.

The transgenic non-human mammal of the invention, the progeny thereof, the cell lines, primary cell and tissue sample provided by this invention, are useful for, among other applications, screening, searching, identifying, discovering, developing and/or evaluating compounds for the prevention and/or treatment of cancer. Furthermore, the transgenic non-human mammal of the invention, the progeny thereof, the cell lines, primary cell and tissue sample provided by this invention, are useful for evaluating potentially useful compounds which target the mechanisms and targets responsible for cancer
maintenance, said mechanisms and targets responsible for cancer maintenance being associated with the development of a sustained cancer.

Therefore, in other aspect, the invention refers to the use of the transgenic non-human mammal of the invention, or of the progeny thereof, or the cell lines, primary cell and tissue sample provided by this invention for identifying potentially therapeutic compounds for the treatment of a cancer, or for evaluating the efficacy of therapy administered to a subject suffering from said cancer, or for monitoring the evolution of said cancer, or for affecting, preferably preventing, cancer dissemination.

The invention also refers to the use of the transgenic non-human mammal of the invention, its progeny, or of a cell line, a primary cell or a tissue sample from the transgenic non-human mammal of the invention or its progeny in the screening, identification, validation, optimization and/or evaluation of potentially useful compounds (candidate compounds) for the prevention, treatment and/or diagnosis of a cancer, in particular a leukaemia selected from acute lymphocytic leukemia, acute myelogenous leukemia, chronic lymphocytic leukemia and chronic myelogenous leukemia, preferably for the prevention, treatment and/or diagnosis of B-cell acute lymphoblastic leukemia.

Therefore, in an aspect, the invention refers to a method for screening, searching, identifying, validating, optimizing, discovering, developing and/or evaluating compounds for the treatment and/or prevention of a cancer, in particular leukaemia or for repositioning known drugs or combinations of compounds, which comprises administering a candidate compound to a transgenic non-human mammal of the invention, or to its progeny, and monitoring the response.

The screening, searching, identifying, discovering, developing and/or evaluating of the candidate compound for the prevention and/or treatment of a cancer, in particular leukaemia, can be performed by administering the candidate compound to the transgenic non-human animal of the invention, at different doses, and evaluating the physiological response of the animal over time. The candidate compound can be administered to the transgenic non-human animal of the invention by any conventional and novel method, typically via oral or parenteral, depending, among other factors, on the chemical nature of the candidate compound. In some cases, it may be appropriate to administer the compound in question along with cofactors that enhance the effect of the compound.
In an embodiment, the invention refers to a method for identifying a target responsible for cancer maintenance, the method comprising comparing the level of one or more potential target(s) responsible for cancer maintenance between two groups, each group comprising a transgenic non-human mammal of the invention or the progeny thereof, or the cell lines, primary cells or tissue samples from the transgenic non-human mammal of the invention or its progeny, wherein the expression of said oncogene is upregulated in the first group; the expression of said oncogene is downregulated or normal in the second group; and comparing the levels of expression of said one or more potential target(s) responsible for cancer maintenance in the first group with the levels the second group. The expression of the oncogene in the second group may have been upregulated for a period of time prior to being downregulated.

In an embodiment, the above method comprises identifying, validating, optimizing and selecting a compound which inhibits or reduces targets responsible for cancer maintenance and mechanisms responsible for cancer maintenance. Preferably, the method comprises identifying, validating, optimizing and selecting a compound which inhibits or reduces targets responsible for cancer maintenance and mechanisms responsible for cancer maintenance after downregulation of the expression of the oncogene incorporated into the animal model, for example the BCR-ABLpI90 gene or its expression products (both transcription products and translation products, i.e., BCR-ABLpI90 mRNA or BCR-ABLp190 protein). In order to achieve this aim, the candidate compound is administered to a transgenic non-human mammal of the invention or to its progeny, wherein the level of the oncogenic expression products in a tissue/cell sample is known, and, subsequently, the level of expression products in said tissue is quantified, and a compound which is able to inhibit or reduce the malignant cells after downregulation of the expression of the oncogene, is selected.

In an embodiment, the invention comprises using a compound identified by any of the methods of the invention in the manufacture of a medicament for treating cancer.

In an embodiment, the above method comprises identifying, validating, optimizing and selecting a compound which inhibits or reduces the level of expression of the oncogene incorporated into the animal model, for example the BCR-ABLpI90 gene or its expression products (both transcription products and translation products, i.e., BCR-ABLpI90 mRNA or BCR-ABLp190 protein). In order to achieve this aim, the candidate
compound is administered to a transgenic non-human mammal of the invention or to its progeny, wherein the level of the oncogenic expression products in a tissue sample is known, and, subsequently, the level of expression products in said tissue is quantified, and a compound which is able to inhibit or reduce the level of the relevant expression product, e.g. BCR-ABLpI790 mRNA or BCR-ABLpl90 protein, is selected.

In another aspect, the invention refers to a method for screening, searching, identifying, validating, optimizing, discovering, developing and/or evaluating compounds for the treatment and/or prevention of a cancer, in particular leukaemia, or for repositioning known drugs or combinations of compounds, which comprises contacting a cell line, or a primary cell, or a tissue sample of the transgenic non-human mammal of the invention, or its progeny, and monitoring the response.

The screening, searching, identifying, validating, optimizing, discovering, developing and/or evaluating of the candidate compound for the prevention and/or treatment of a cancer, in particular leukaemia, can be performed by adding the candidate compound to a culture medium containing cells from a cell line or from primary cells or from tissue samples provided by the present invention, for an appropriate period of time, at different concentrations, and evaluating the cellular response to the candidate compound over time using appropriate biochemical and/or histological assays. In an alternative embodiment, cells may be used that are transfected with a construct that expresses an oncogene in a manner regulated by an exogenous substance. In a preferred embodiment, the Combi-TA-pl90 vector is used, as described herein. At times, it may be necessary to add the compound in question to the cellular culture medium along with cofactors that enhance the effect of the compound.

In one embodiment, the above method comprises identifying and selecting a compound which inhibits or reduces targets and mechanisms responsible for cancer maintenance after downregulation of the expression of the oncogene. In order to achieve this aim, the candidate compound is contacted with a cell line, or with a primary cell, or with a tissue sample of the transgenic non-human mammal of the invention, or its progeny, wherein the level of BCR-ABLpI90 expression products in said cell line, primary line or tissue sample is known, and, subsequently, the level of BCR-ABLpI90 expression products in said tissue is quantified, and a compound which is able to inhibit or reduce the malignant cells after downregulation of the expression of the oncogene, is selected.
In an embodiment, the above method comprises identifying and selecting a compound which inhibits or reduces the level of expression of BCR-ABL<sub>1p90</sub> gene or its expression products (both transcription products and translation products, i.e., BCR-ABL<sub>1p90</sub> mRNA or BCR-ABL<sub>p190</sub> protein). In order to achieve said aim, the candidate compound is contacted with a cell line, or with a primary cell, or with a tissue sample of the transgenic non-human mammal of the invention, or its progeny, wherein the level of BCR-ABL<sub>p190</sub> expression products in said cell line, primary line or tissue sample is known, and, subsequently, the level of BCR-ABL<sub>p190</sub> expression products in said tissue is quantified, and a compound which is able to inhibit or reduce the level of BCR-ABL<sub>p190</sub> expression products is selected.

When a compound inhibits or decreases the levels of the BCR-ABL<sub>p190</sub> expression products or reverts the effects of the increased expression of said gene or the activity of BCR-ABL<sub>p190</sub> protein, this compound becomes a candidate for cancer therapy, especially for treating and/or preventing a hematological malignancy, in particular leukaemia.

Illustrative, non limitative, examples of compounds which inhibit or decrease the levels of the mechanisms responsible for cancer maintenance and/or BCR-ABL<sub>p190</sub> mRNA include antisense BCR-ABL<sub>p190</sub> mRNA, ribozymes, triple helix molecules, small interference RNA (siRNA), small organic molecules etc.

Illustrative, non limitative, examples of compounds which inhibit or decrease the levels of the mechanisms responsible for cancer maintenance and/or BCR-ABL<sub>pWO</sub> protein include antibodies anti-BCR-ABL<sub>p90</sub>, enzymes or proteins which regulate the activity of the mechanisms responsible for cancer maintenance and/or BCR-ABL<sub>p90</sub> protein, small organic molecules such as small natural or synthetic organic molecules of up to 2000Da, preferably 800Da or less, peptidomimetics, inorganic molecules, peptides, antibodies, structural or functional mimetics of the aforementioned etc.

In another aspect, the invention refers to the use of a compound which inhibits or decreases the levels of the targets or mechanisms responsible for cancer maintenance and/or BCR-ABL<sub>p190</sub> expression products or reverts the effects of an increased level of BCR-ABL<sub>p190</sub> expression products in the manufacture of a pharmaceutical composition for prevention and/or treatment of a leukaemia. Illustrative, non limitative, examples of said compounds include antisense BCR-ABL<sub>p190</sub> mRNA, ribozymes, triple helix
molecules, small interference RNA (siRNA), antibodies anti-BCR-ABLp190, enzymes or proteins which regulate the activity of BCR-ABLp190 protein, etc.

The following examples illustrate the invention and should not be considered to limit the scope thereof. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA technology and immunology, which are within the skill of those working in the art.


Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

All documents cited herein are hereby incorporated by reference.

15 **BRIEF DESCRIPTION OF THE FIGURES**

**Figure 1. CombitTA-p190 construct, expression and its effect on the survival of the Ba/F3 cells deprived of growth factor.** A) Schematic representation of the CombitTA-p190 vector used in this study. B) Analysis of the tetracycline (Doxycycline) dependent CombitTA-p190 expression by Northern-blot in Ba/F3 cells (-Dox, +Dox in the medium). Actin was used to check the RNA integrity and loading. C) Survival of Ba/F3 cells expressing CombitTA-p190 in absence of IL-3. Cells growing exponentially in IL-3 supplemented media were adjusted to 5 x 10^5 cells/ml on day 0 and cultured after removal the IL-3. The cell number of viable cells is shown for Ba/F3+BCR-ABLp190 cells grown in the absence of IL-3 (Sanchez-Garcia and Grutz, 1995), Ba/F3 cells grown in the absence (Ba/F3-IL3) and in the presence of IL-3 (Ba/F3+IL3), CombitTA-p190-transfected Ba/F3 cells grown in the presence (+tet) or in the absence (-tet) of doxycycline in IL-3 free medium (the time of treatment with doxycycline was 48 hours), and CombitTA-p190-transfected Ba/F3 cells grown in the absence of IL3 and doxycycline (-tet) and treated with 10 µg/ml of STI571 for 48h.

**Figure 2. CombitTA-p/90 transgene expression.** A) Identification of the transgenic mice by Southern analysis of tail snip DNA after EcoRI digestion. The cDNA for human
ABL was used for detection of the transgene. CombitTA-p190 and the endogenous c-Abl are indicated. B) Expression of CombitTA-p190 was demonstrated by real time PCR in tissues derived from both lines (BM, bone marrow; PB, peripheral blood; wt, wild-type). The mean Ct values of triplicate assays are presented. Doxycycline was given at 4mg/mL for 4 weeks.

**Figure 3. B-cell acute lymphoblastic leukaemia development in CombitTA-p190 mice.** A) Hematoxiline/eosin (H/E) stained sections of the spleen and liver of CombitTA-p190 mice showed leukaemic cell infiltration undetected in control mice. Peripheral blood smear showed the presence of lymphoid blast cells. B) Phenotypic characteristics of leukaemias in CombitTA-p190 mice. Cells from peripheral blood of CombitTA-p190 and control mice were analyzed by flow cytometry. Cells were identified with combinations of specific antibodies. C) Murine blast cells co-expressed B-cell and myeloid markers. Cells from peripheral blood (PB), bone marrow (BM) and spleen of CombitTA-p190 and control mice were analyzed by flow cytometry with combination of the specific myeloid (Mac1) and B-cell lymphoid (B220) markers. D) Gradient of expression of B-cell (B220) and myeloid (Mac1) markers in blast cells of CombitTA-p190 mice.

**Figure 4. STI571 does not prolong the survival of CombitTA-p190 mice.** Mice were randomized to treatment with either STI571 or placebo. The survival curve depicts the percentage of animals alive at the indicated time point. The numbers of mice in each arm (N) is also shown.

**Figure 5. Phenotypes in CombitTA-p190 mice after suppression of BCR-ABLp190 expression by tetracycline treatment.** A) CombitTA-p190 mice were evaluated for disease progression by flow cytometry prior to administration of doxycycline. Forty mice were selected when the percentage of of blast cells (B220 positive-cells co-expressing myeloid markers) was higher than 10% (being the mean value 47±4.9%). The ratio between \( BCR-ABL^{190/abl} \) determined by real time PCR and the percentage of blast cells (B220 positive-cells co-expressing myeloid markers) is indicated before and after dox treatment for 10 weeks (PB, peripheral blood; BM, bone marrow). B) Flow cytometry phenotypic characteristics of cells from peripheral blood (pb) and bone marrow (bm) in both lines of CombitTA-p190 mice after suppression of BCR-ABLp190 expression by tetracycline treatment (4 mg/ml) for 10 weeks. (ND. Not detected).
Figure 6. B-cell differentiation in CombitTA-pl90 mice after downregulation of BCR-ABL expression by doxycycline treatment. Total number of B-cells, ratio of IgM-TIgM+ cells, and the ratio between $BCR-ABL^{+/\text{abl}}$ determined by real time PCR was measured in control, untreated CombitTA-pl90 mice, and CombitTA-pl90 mice under doxycycline treatment. A representative flow cytometry analysis and representative H/E stained sections of liver and spleen are shown per each group of mice. (ND. Not detected).

EXAMPLES

Example 1: Materials And Methods

Generation of transgenic mice.

The cDNA for human $BCR-ABLp^{190}$ was cloned into the Combi-tTA vector (Schultze et al., 1996; Perez-Mancer a et al., 2005a; Perez-Mancera et al., 2005b). Linear DNA fragments for microinjection were obtained by $N_{ol}$ digestion and injected into CBA x C57BL/6J fertilized eggs. We identified transgenic mice by Southern analysis of tail snip DNA after $EcoRl$ digestion as described (Garcia-Hernandez et al., 1997). We used a fragment of 863bp obtained for the human ABL cDNA for detection of the transgene. Two founder lines were obtained: IS1353A, and IS1353B and both lines showed germline transmission of the transgene (Table 1). Founder mice were crossed to the C57BL6 mice for five generations to establish co-isogenic transgenic mice. Similar phenotypic features were seen in all assays for both of the CombitTA-$BCR-AB{190}$ transgenic lines generated.

Histological analysis.

Mice included in this study were subjected to standard necropsy. All major organs were closely examined under the dissecting microscope, and samples of each organ were processed into paraffin, sectioned and examined histologically. All tissue samples were taken from homogenous and viable portions of the resected sample by the pathologist and fixed within 2-5 min of excision. Haematoxylin-and eosin-stained sections of each tissue were reviewed by a single pathologist (T.F.). For comparative studies, age-matched mice were used (wild-type or CombitTAp90 mice in the continuous presence of tetracycline).
Cell lines used include Ba/F3 (Palacios et al., 1985) and Ba/F3 cells expressing the human proteins BCR-ABL$^{190}$ (Ba/F3+p190), BCR-ABL$^{210}$ (Ba/F3+p210) (Sanchez-Garcia and Grutz, 1995) and CombitTA-p190. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). When required, 10% WEHI-3B-conditioned medium was added as a source of IL-3.

**Cell transfection and cell survival** assay.

Ba/F3 cells were transfected by electroporation (960 µF, 220 V) with 20 µg of either CombitTA-1/2/90 or empty vector (CombitTA). The neomycin-resistant pool of cells (Ba/F3+CombitTA-p790) was analysed by RT-PCR for CombitTA-1/2/90 expression in the presence and in the absence of tetracycline (20 ng/ml). These cells were resistant to IL-3 withdrawal during growth in the absence of tetracycline. The TET OFF repression system was analysed in Ba/F3+ CombitTA-p90 cells in presence or absence of tetracycline by Northern-blot, using the total RNA of these cells. Cells were screened for resistance to IL-3 withdrawal and cell viability was determined by trypan blue exclusion.

**Northern blot** analysis.

Total cytoplasmic RNA of different cells was glyoxylated and fractionated in 1.4% agarose gels in 10 mM Na2HPO4 buffer (pH 7.0). After electrophoresis, the gel was blotted onto Hybond-N (Amersham), UV-cross-linked, and hybridised to $^{32}$P-radiolabeled full-length ABL cDNA probe. Loading was monitored by reprobing the filter with a β-actin probe.

**Genomic DNA** analysis.

DNA was prepared from mice tails. DNA was digested with restriction endonucleases as described (Garcia-Hernandez et al., 1997), separated by electrophoresis in 0.8% agarose, transferred to Hybond-N (Amersham). Filters were UV cross-linked and hybridised to a $^{32}$P-radiolabeled full-length ABL probe.

**Real-time PCR quantification.**

To analyze expression of CombitTA-p90 in mouse cell lines and mice, RT was performed according to the manufacturer's protocol in a 20-µl reaction containing 50 ng
of random hexamers, 3 µg of total RNA, and 200 units of Superscript II RNase H-
reverse transcriptase (GIBCO/ BRL). Real-time quantitative PCR was carried out for the
quantitation of CombitTA-/p/90. Fluorogenic PCRs were set up in a reaction volume of
50 µl using the TaqMan PCR Core Reagent kit (PE Biosystems). cDNA amplifications
were carried out using the same primers in a 96-well reaction plate format in a PE
Applied Biosystems 5700 Sequence Detector. Thermal cycling was initiated with a first
denaturation step of 10 min at 95°C. The subsequent thermal profile was 40 cycles of
95°C for 15 s, 56°C for 30 s, 72°C for 1 min. Multiple negative water blanks were tested
and a calibration curve determined in parallel with each analysis. The Abl endogenous
control (PE Biosystem) was included to relate CombitTA-p/90 to total cDNA in each
sample. The sequences of the specific primers and probe were as follow: \textit{BCR-ABLp}^{190},
sense primer 5'-CCGCAAGACCGGGCAGAT-S', antisense primer 5'-
CAGATGCTACTGGCCGCTGA-3' and probe 5'-TGGCCCAACGATGCGAGGG-
3'; c-Abl, sense primer 5'-CACTCTCAGCATCACTAAAGGTGAA-S', antisense
primer 5'-CGTTTGGGCTTCACACCATT-3', and probe 5'-
CCGGGTCTTGGGTTATA ATCACAATG-3'.

Flow cytometry.

Nucleated cells from total bone marrow (flushing from the long bones), peripheral blood,
thymus, liver and spleen cell suspensions were made. In order to prepare cells for flow
cytometry, contaminating red blood cells were lysed with 8.3% ammonium chloride and
the remaining cells were then washed in PBS with 2% FCS. After staining, all cells were
washed once in PBS with 2% FCS containing 2 µg/mL propidium iodide (PI) to allow
dead cells to be excluded from both analyses and sorting procedures. Monoclonal
antibodies were obtained from Pharmingen and included: CD45R/B220, CD19, Ly51,
CD43, IgM and IgD for B lineage staining; CD4, CD8 and CD3 for T cell lineage;
CD1 Ib and Gr1 for myeloid lineage and Seal for stem cell. Single cell suspensions from
the different tissue samples obtained by routine techniques were incubated with purified
anti-mouse CD32/CD16 (Pharmingen) to block binding via Fc receptors and with an
appropriate dilution of the different antibodies at room temperature or 4°C, respectively.
The samples and the data were analysed in a FACSscan using CellQuest software (Becton
Dickinson). Specific fluorescence of FITC and PE excited at 488 nm (0.4 W) and 633
nm (30 mW), respectively, as well as known forward and orthogonal light scattering
properties of mouse cells were used to established gate. For each analysis at least a total of 5,000 viable (PI-) cells were assessed.

Tumorigenicity assay.

To test the tumorigenicity of the various CombitTA-pl90 leukaemias, 4- to 6-week-old athymic (nude) male mice were injected subcutaneously on both flanks with $10^6$ cells resuspended in 200 µl of phosphate-buffered saline (PBS). The animals were examined for tumour formation for up to 3 weeks.

**STI571 (Gleevec)**

For the animal studies, stock solutions of 5 mg/mL and 10 mg/ml were prepared fresh in water, sterile filtered and administered to mice in a volume of 250 µl by gavage twice a day. Mice were started on STI571 or placebo (the same volume of diluent water) beginning on day after leukaemia was confirmed (day 0) by means of an STI571 regimen of 50 mg/kg every morning and 100 mg/kg every evening by gavage. STI571 was administered in a volume of 250 µl sterile water by means of straight or curved animal feeding needles. Mice tolerated the therapy well and no interruption of therapy was necessary. Mice were followed clinically 3 times a week, and periodic peripheral blood counts were obtained by tail vein blood draw as indicated. For the survival analysis portion of this study, the death endpoint was determined either by spontaneous death of the animal or by elective killing of the animal because of signs of pain or suffering according to established criteria (Wolff and Ilaria, 2001).

*Example 2: Results*

**CombitTA-pl90 mice**

In order to determine the role of BCRABLpl90 in maintenance of leukaemia, we generated transgenic mice using the Combi-tTA system, in which the expression of BCR-ABL190 gene could be exogenously regulated. This system, which has the transactivator and the tet-operator minimal promoter driving the expression gene unit on a single plasmid (Schultze et al., 1996), ensures the integration of the transactivator and reporter gene units in equal copy numbers in a direct cw-configuration at the same chromosomal locus and prevents genetic segregation of the control elements during
breeding (Schultze et al., 1996; Perez-Mancer a et al., 2005a; Perez-Mancera et al., 2005b). Insertion of the human *BCR-ABL* 
\(^{190}\) gene under the control of the tetO-minimal promoter yielded the plasmid CombitTA-/?\(^{90}\) (Figure 1A). This was analysed in a cell system, using a murine haematopoietic precursor Ba/F3 cell line (Palacios et al., 1985).

In the absence of tetracycline, the tet-repressor protein (fused to the viral VP16 transactivator domain) binds to an engineered tet-operator minimal promoter and activates *BCR-ABL* 
\(^{90}\) transcription (CombitTA-p/PO). In the presence of the tetracycline, binding is abolished and the promoter silenced (Figure 1A). CombitTA-
\(^{90}\) expression was determined in transfected Ba/F3 cells after culturing for 2 days in the presence or absence of tetracycline. CombitTA-p/90 was detected in Ba/F3 cells without tetracycline by Northern blot, but not in cells cultured with tetracycline (20 ng/ml) (Figure 1B). The physiological relevance of the CombitJA-BCR-ABL 
\(^{90}\) suppression was confirmed in vitro by assaying survival of Ba/F3 cells expressing CombitTA-/?\(^{90}\) 24 h after IL-3 withdrawal (Figure 1C). Normally, *BCR-ABL*

expression protects Ba/F3 cells from apoptosis following IL-3 withdrawal (Sanchez-Garcia and Grutz, 1995) and, as we demonstrated, the level of CombitTA-p/90 expression was sufficient in Ba/F3 cells to prevent cell death. The sensitivity to IL-3 removal was restored by the addition of tetracycline (Figure 1C).

We generated two founder transgenic lines for CombitTA-/?\(^{90}\) (1353A, and 1353B) (Figure 2A). Both lines showed germline transmission of the transgene (Table 1). In both lines, the CombitTA-/?\(^{90}\) expression was detected in all tissues analyzed (Figure 2B). The CombitTA-/?\(^{90}\) expression was the result of transactivation as the suppression of expression to undetectable values was confirmed when mice were supplied with tetracycline in their drinking water (Figure 2B).

**No morphological abnormalities in CombitTA-/?\(^{90}\) mice.**

Cohorts of CombitTA-p/90 mice were generated to analyse the effect of the *BCR-ABL* 
\(^{190}\) expression in vivo (Table 1). A total of 115 transgenic animals (61 mice corresponded to line 1353A and 54 mice to line 1353B) and 37 control animals were used in this study. Both transgenic lines were analysed in detail and similar phenotypic features were seen in both lines. CombitTA-p/90 mice were born alive without overt morphological abnormalities, and were fully fertile with no differences apparent in the progeny. Autopsy of pups, including extensive histological analysis, revealed no
abnormality of the lung, heart, liver, kidneys, skin, brain or gastrointestinal tract of CombitTA-p/90 mice, indicating that this level of over-expression of BCR-ABL
190 does not perturb normal embryonic development.

**CombitTA-p/90 mice develop acute leukaemias**

We next analysed whether the CombitTA-p/90 mice developed cancer. All CombitTA-p/90 mice analyzed became unwell from approximately 5-7 months of age onward with clinical manifestations that included decreased spontaneous movements in the cage, increased respiratory rates, piloerection, shivering, and animals displayed a sustained loss of body weight (Table I). This condition persisted and animals were sacrificed. Macroscopic analysis showed that CombitTA-p/90 mice were pale with splenomegalia and hepatomegalia with pronounced white spots. The thymi and lymphoid nodes generally appeared normal. This examination is consistent with hematological disease. However, no tumors of other tissues were found in these mice, despite widespread activity of the promoter. These animals had elevation in the peripheral blood leukocyte counts and the histological analysis revealed marked leukaemic cell infiltration of hematopoietic (splenic white pulp) (Figure 3A) and nonhematopoietic tissues (liver sinusoids) in CombitTA-p/90 mice. Similar results were obtained with the two CombitTA-p/9790 transgenic lines. There was not relationship between level of BCR-ABL
190 transgenic expression and disease as there was not a minimum degree of expression required for the development of a leukemic state. Therefore, these results show the generation of a new mouse model, recapitulating the consequences of the BCR-ABL
190 chromosomal abnormality. As a result of this, CombitTA-p 190 mice specifically develop acute leukaemias.

**CombitTA-p/90 mice specifically develop B-cell acute lymphoblastic leukaemias.**

In human pathology the BCR-ABL1® chimeric product is associated with B-cell acute lymphoblastic leukaemia (B-ALL) (Chan et al., 1987; Clark et al., 1987; Hermans et al., 1987; Kurzrock et al., 1987; Melo, 1996), therefore, we next defined the acute leukaemic disease generated in our CombitTA-p/90 mice. Detailed analysis of the leukaemic cells in the CombitTA-p/90 mice established the diagnosis as B-cell acute lymphoblastic leukaemia. Haematoxilin/Eosin staining showed that the leukaemic cells had a lymphoid morphology (Figure 3A). The majority of peripheral blood mononuclear cells from
CombitTA-p/90 mice had a B-cell precursor immunophenotype (B220+, SIg-) (Figure 3B). A small percentage of cells were positive for CD3, "myeloid", and Slg (Figure 3B). This may represent the staining of residual T cells, myeloid and mature B cells, respectively. Different tissues from the CombitTA-/?/90 mice leukaemic mice were analyzed for immunoglobulin heavy chain (IgH) gene rearrangement. Monoclonal patterns were not observed in tissues from the mice examined by Southern-blot analysis (data not shown), although the tissue samples were heavily infiltrated with leukaemic cells, as previously reported (Griffiths et al., 1992). One interpretation of this observation could be that the leukaemic cells were very primitive or "pro"-B cells still retaining a germline configuration of IgH genes (Palacios et al., 1987; Hardy et al., 1991). Alternatively, the leukaemic cells in these cases might be polyclonal with respect to IgH rearrangements, as described for some cases of lymphoblastic leukaemia in humans (Ford et al., 1983). Moreover, we observed that murine blast cells co-expressed B-cell and myeloid markers (Figure 3C-D), a characteristic feature of BCR-ABLp 190 human leukaemias (Tabernero et al., 2001). These blast cells co-expressing both myeloid and B-cell markers were detected in all mice studies although with a different co-expression pattern (Figure 3D). As these cells are not normally present in the peripheral blood of control mice, they can be used to monitor disease development.

To test the malignant potential of cells from the CombitTA-/?/90 mice, 1 x 10^6 peripheral blood blast cells were injected subcutaneously into twelve 40-day old nude mice. All mice developed progressively growing tumours within 5-7 weeks of transplantation. The transplanted cells developed into the same kind of leukaemia (data not shown). Overall these data define that CombitTA-/?/90 mice develop a disease similar to the human situation, where BCR-ABLp 190 is associated with a B-cell acute leukaemia co-expressing myeloid markers (Tabernero et al., 2001). Therefore, our mouse model reproduces the same phenotype with which this fusion gene is associated in human pathology. These results validate this mouse model as ideal to study the biology of the BCR-ABLp 190 oncogene.

**BCR-ABV**<sup>190</sup> can initiate leukaemia in both young and adult mice

The BCRABL<sup>p90</sup>-B-ALL occurs mainly in adults (Seeker-Walker, 1991; Copeland et al., 1995). The above results support that BCR-ABV<sup>190</sup> expression is the responsible of the beginning of the leukaemia, but they do not demonstrate whether the disease can be
initiated if the genetic alteration is activated in adult cells. In order to address properly this question, we took advantage of the possibility of modulating the expression of BCR-ABLp190 in our conditional CombitTA-p/90 model. Thus, we randomized mice to oral treatment with doxycycline (4 mg/ml) or placebo beginning at stage EO-EO.5 (Table II) for 6 months. Every week mice were monitored for evidence of leukaemia both clinically and by serial peripheral blood count. Mice treated with placebo developed a B-ALL around 6 months of age, but the group of mice treated with doxycycline were healthy and did not develop leukaemia. During this period, BCR-ABLp190 expression was not detected by real-time PCR in the group of mice treated with doxycycline. After the tetracycline treatment for 6 months, doxycycline was removed from the drinking water and BCR-ABLp190 expression was restored (Table II). A similar B-ALL was detected after doxycycline withdrawal (Table II), indicating that BCR-ABLp190 can initiate a B-ALL in both young and adult cells.

Table II

<table>
<thead>
<tr>
<th>Mice</th>
<th>Doxycycline treatment (4mg/kg)</th>
<th>Disease (%)</th>
<th>BCR-ABLp190 expression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>NO</td>
<td>B-ALL co-expressing myeloid markers (100)</td>
<td>Detected (100)</td>
</tr>
<tr>
<td>30</td>
<td>Yes</td>
<td>ND (IQO)</td>
<td>ND (IQO)</td>
</tr>
<tr>
<td></td>
<td>(from EO-EO.5 until 6 months old)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>After 6 months old, DOX was removed</td>
<td>B-ALL co-expressing myeloid markers (100)</td>
<td>Detected (100)</td>
</tr>
<tr>
<td>10</td>
<td>After 6 months old, DOX was removed just for 4 weeks and then treatment was re-established</td>
<td>B-ALL co-expressing myeloid markers (100)</td>
<td>Detected (100) after treatment withdrawal and ND (100) after once treatment was restored</td>
</tr>
</tbody>
</table>

(1) Disease was monitored by the presence of blast cells co-expressing both myeloid and B-cell markers. (2) BCR-ABLp190 expression was monitored by real-time PCR. %, percentage; ND, no detected.

Defining the duration of oncogene expression required to produce cancer: transitory expression of BCR-ABLp190 is enough to develop leukaemia

The next question we addressed was whether BCR-ABLp190 is able to generate leukaemia just by transient expression. For this aim we used some of the CombitTA-
p190 mice subjected to doxycycline treatment (4 mg/ml) until 6 months of age (Table II). Thus, after 6 months of age doxycycline was removed from the drinking water until we could detect expression of BCR-ABLp190 in peripheral blood cells of CombitTA-pl190 mice (Table II). Once BCR-ABLp190 expression was detected, we restored doxycycline treatment. Doxycycline treatment was able to suppress BCR-ABLp190 expression and these CombitTA-pl90 mice were monitored for evidence of leukaemia development by serial peripheral blood analysis. After five months blasts cells co-expressing both myeloid and B-cell markers were detected, similar to leukaemia developed by mice without silencing the transgene (Table II). During this time, BCR-ABLp190 expression was not detected by real-time PCR. These results indicate transitory BCR-ABLp190 expression is enough to develop leukemia and suggest its presence is not required to disease maintenance.

CombitTA-pl90 mice with leukaemia do not demonstrate marked clinical improvement with the tyrosine kinase inhibitor STI571

The above results support the view that BCR-ABLp190 expression is not required for leukaemia maintenance. This idea is in agreement with lack of efficacy of imatinib treatment in human P190-B-ALL (Druker et al., 2001b). To study the effect of STI571 on hematologic response and overall survival in our model, mice of identical age were randomized to receive either STI571 or the same volume of placebo (water) twice a day beginning on day after leukaemia was confirmed by peripheral blood analysis. Our STI571 treatment regimens were based on previous pharmacokinetics studies of STI in BCR-ABL tumour-bearing mice (Druker et al., 1996; le Coutre et al., 1999). Mice were monitored clinically and by serial peripheral blood count for evidence of leukaemia. STI571 did not prolong the survival of these mice (Figure 4), reminiscent of human P190-B-ALL.

Validating BCR-ABLp190 target in a disease state in Combita-pl90 mice: Irreversibility of alterations induced by BCR-ABLp190

A critical step in understanding how malignant transformation is mediated by cancer-associated oncogenes (such as BCR-ABL) is to identify target genes and pathways, which allow cells to grow outside their normal environment (Sanchez-Garcia, 1997). Although we have shown that BCR-ABLp190 is sufficient to generate leukaemias and that the
growth of these cells becomes independent of BCR-ABLp 190 expression, these results do not imply a specific requirement for BCR-ABLp190 in the maintenance of malignant phenotype. In order to address this question we need to validate the target (BCR-ABLp 190) in a disease state mimicking human disease.

To assess this, forty leukaemic CombitTA-pl90 mice were evaluated for disease progression by flow cytometry prior to and following administration of tetracycline (4 gr/L in the drinking water for 10 weeks, a dose sufficient to suppress BCR-ABLp 190 expression) (Figure 5A). None of the CombitTA-pl90 mice treated exhibited amelioration of the leukaemic phenotype despite complete CombitTA-pl90 suppression:

Flow cytometry analysis identified the persistence of leukaemic cells in both the bone marrow and the peripheral blood (Figure 5B). Thus, these results show that the alterations induced by BCR-ABLp 190 are irreversible.

**Level of BCR-ABL expression required to reverse B-cell differentiation block in CombitTA-pl90 mice.**

BCR-ABL expression is tightly regulated according to the concentration of doxycycline, although we have not found a minimum level of oncogene expression necessary to initiate a neoplastic phenotype. Thus, the next question we addressed was whether the level of oncogene expression was related to the differentiation level of blast cells. In untreated CombitTA-pl90 mice the total number of B cells is increased in the peripheral blood. This increase is due to the presence of undifferentiated B-cells as measured, for example, by the ratio of IgM- versus IgM+ cells in CombitTA-pl90 mice (Figure 6). To understand if the level of BCR-ABL could influence the level of differentiation of leukaemic cells in CombitTA-pl90 mice, we examined the expression of transgene-encoded BCR-ABL in the peripheral blood cells derived from CombitTA-pl90 (Figure 6). The expression level of transgene-encoded BCR-ABL with respect to the endogenous abl expression was decreased upon doxycycline treatment (Figure 6). This downregulation of BCR-ABL expression in leukaemic CombitTA-pl90 mice is progressively accompanied by an increase in the total number of B-cells through accumulation of blast cells (IgM- cells). However, when the ratio BCR-ABL/abl is close to zero (but not zero), B-cell differentiation is restored and liver and spleen alterations are not observed (Figure 6). If the BCR-ABL expression is not detected in the context of a leukaemia in CombitTA-pl90 mice after doxycycline treatment, the B-cell
differentiation block is re-established. These results suggest that the level of BCR-ABL modulates differentiation of leukaemic cells, allowing identification of a minimum level of oncogene expression necessary to revert the specific block in B-cell differentiation in the leukaemic cells.

5 Example 3: Discussion

A complete understanding of the cancer process requires more detailed knowledge of the mechanisms maintaining neoplastic growth. The generation of mouse models mimicking human cancer pathology is a prerequisite not only for understanding the genesis of human cancer but also for the identification of molecular events responsible for cancer maintenance. New drugs must be designed against the mechanisms that are responsible for cancer maintenance not for the initial event that transform a normal cell into cancer cell, because it is possible that the first alteration of the cancer cell will have no function in the subsequent steps of cancer development. Thus, first of all, we must select the targets needed for cancer maintenance. To select these unknown targets, we should take advantage of mouse models that accurately reproduce the human disease. In this study we have investigated the role of $BCR^A B^{pl9\circ}$ in maintenance of B-ALL.

$BCR-ABL^{b00}$ is mainly associated with B-cell ALL (Chan et al., 1987; Clark et al., 1987; Hermans et al., 1987; Kurzrock et al., 1987; MeIo, 1996). This chimeric molecule represents an ideal therapeutic target because it is unique to the disease state and it exists in the tumour cells but not in the normal cells of the patient (Sanchez-Garcia I, 1997; Cobaleda et al., 1998). Inhibition of chimeric gene expression by antitumour agents specifically kills the cancer cells without affecting the normal cells (Szczyliek et al., 1991; Skorski et al., 1994; Choo et al., 1994; Zao et al., 1997; Huettner et al., 2000). Thus, the presence of the chimeric molecule is necessary for the persistence of the tumour cells. However, these systems used are still limited in representing in vivo biology of the disease. Thus, in P190-B-ALL, imatinib treatment is not effective (Druker et al., 2001b; Shah et al., 2002), suggesting that BCR/ABL may be required for abnormal proliferation and expansion of tumour cells but may not be essential for preservation of primitive malignant cells. We have utilised the single-plasmid system containing the regulating and expression elements of the original binary tetracycline system to allow high induction and tight control of gene expression by tetracycline in mice (Schultze et al., 1996; Perez-Mancer a et al., 2005a; Perez-Mancera et al., 2005b)
to study the relevance of BCR-ABLp190 to human cancer development. In vitro studies have shown that BCR-ABLp190 confers resistance to cell death induced by the withdrawal of survival factors (Sanchez-Garcia and Grutz, 1995). The physiological relevance of the CombitTA-p190 suppression was confirmed in vitro by assaying survival of Ba/F3 cells expressing CombitTA-p190 after IL-3 withdrawal. The analysis of the BCR-ABLp190-expressing mice, with reference to known human phenotypes, identified that these mice develop a B-cell acute lymphoblastic leukaemia characterized by lymphoid blasts co-expressing myeloid markers. Thus, the CombitTA-p190 mice expressing the BCR-ABLp190 chimeric gene product shows consistently the same phenotype with which this oncogene is associated in human pathology (Tabernero et al., 2001). The possibility of modulating BCR-ABLp190 expression in our CombitTA-p190 model allows us to demonstrate the oncogene can initiate leukaemia when activated in both young and adult mice. However, the survival conferred by BCR-ABLp190, while reversible in vitro, can escape such control in vivo. These results are congruent with the poor efficacy of imatinib treatment in P190-B-ALL (Druker et al., 2001b; Shah et al., 2002). In agreement with this result, CombitTA-p190 leukaemic mice did not show clinical improvement when treated with ST1571 and transitory expression of the oncogene was enough to leukaemia development in CombitTA-p190 mice. Moreover, gradual BCR-ABL inactivation resulted in the differentiation of blast into mature cells, indicating that oncogene level modulates differentiation of leukaemic cells.

However, this observation that BCR-ABLp190 inactivation does not stop tumour growth conflicts with previous studies showing that BCR-ABL is required for tumour persistence (Szczęsny et al., 1991; Skorski et al., 1994; Choo et al., 1994; Zao et al., 1997; Huettner et al., 2000). One possible explanation is that the effects of BCR-ABLp190 inactivation may depend on the mechanism by which BCR-ABL contributes to tumorigenesis, which is likely to vary according to the genetic and cellular context. When BCR-ABLp190 causes a B-ALL, as described here and observed in humans (Tabernero et al., 2001), its inactivation does not revert neoplastic growth. By contrast, when BCR-ABL just transforms a cell into tumourigenic, its inactivation would result in tumour regression (Szczęsny et al., 1991; Skorski et al., 1994; Choo et al., 1994; Zao et al., 1997; Huettner et al., 2000).

We conclude that BCR-ABLp190-induced tumourigenesis is not reversible through the unique inactivation of the gene defect initiating cancer development, an observation that
could be applied to other cancer-initiating gene defects when validated in a physiological disease state. But, what are the mechanisms of tumor relapse by which tumors evolve to escape oncogene dependence? Results presented here suggest this oncogene imposes a cancer stem cell imprinting context in which oncogene inactivation cannot change this epigenetic context. Similarly, other genetic changes like for example the p14/Arf tumour suppressor (Williams et al., 2006) and p53 (Wendel et al., 2006) could be responsible for the reduced response to BCR-ABL inhibition. The inability of BCR-ABLpI90 inactivation to eliminate cancer cell progenitors suggests that B-ALL patients will require to combine Imatinib mesylate therapy with other therapeutic strategies (Graham et al., 2002; Bathia et al., 2003; Towatari et al., 2004; Chu et al., 2005). Thus, this model could have important implications for the development of new cancer drugs targeting essential aspects of tumour maintenance.
REFERENCES


CLAIMS

1. A transgenic non-human mammal model of a sustained cancer comprising in its genome a human oncogene, wherein the expression of said oncogene can be regulated exogenously by an effector substance.

2. The transgenic non-human mammal model of claim 1 or claim 2, wherein the oncogene is associated with a human cancer pathology and wherein the inactivation of the oncogene does not eliminate the associated cancer pathology.

3. The transgenic non-human mammal of claim 1, wherein the oncogene comprises a nucleic acid sequence encoding the BCR-ABLp190 protein.

4. The transgenic non-human mammal according to any preceding claim, wherein said mammal is a mouse.

5. The transgenic non-human mammal according to any one of the preceding claims, wherein said oncogene expression is tetracycline-regulated.

6. The transgenic non-human mammal according to any of the preceding claims, wherein said non-human mammal suffers from a human epithelial or mesenchymal cancer.

7. The transgenic non-human mammal according to claim 6, wherein said a human epithelial or mesenchymal cancer is selected from lymphomas, leukaemias, sarcomas and carcinomas.

8. The transgenic non-human mammal according to claim 6, wherein said a human epithelial or mesenchymal cancer is B-cell acute lymphoblastic leukaemia.

9. The transgenic non-human mammal according to any of the preceding claims, obtainable by crossing a non-human mammal according to any of the preceding claims with another non-human mammal carrying a mutation in the gene encoding the BCR-ABLp190 protein.

10. The transgenic non-human mammal according to any of the preceding claims, further characterised in that said non-human mammal carries a mutation in the gene encoding the BCR-ABLp190 protein.

11. The progeny of a transgenic non-human mammal according to any of the preceding claims.
12. A primary cell or tissue sample which is derived from the transgenic non-human mammal according to any of claims 1 to 10 or of the progeny thereof according to claim 11.

13. A cell line comprising in its genome an oncogene, wherein said oncogene is characterised as in any one of claims 1 to 3.

14. A cell line according to claim 13 which is obtainable from the transgenic non-human mammal, its progeny, or the primary cell or tissue sample according to claims 1-12.

15. A cell line according to any one of claims 12-14, further comprising a reporter gene useful for spatiotemporal identification of the onset, progression, dissemination and further physiopathological processes, for evaluating the effect of therapies by molecular imaging techniques, for diagnostic assays, drug discovery and development processes, for target identification and for improving the efficacy and reliability of all phases of the clinical development.

16. Use of a cell line according to any one of claims 12-15 as a biomarker for detecting the presence of a gene created and/or activated by a genetic anomaly associated with a human cancer pathology in a subject, or for assessing the risk or predisposition of a subject to develop a human cancer pathology in a subject, or for determining the stage or severity of a human cancer pathology in a subject, or for monitoring the effect of the therapy administered to a subject having a human cancer pathology, or for designing an individualized therapy for a subject suffering from a human cancer pathology, or for designing human clinical trials, or for diagnosis of cancer and/or specific processes and effects of cancer development, like cancer dissemination; or for patient selection for personalized therapeutics; or for therapeutic monitoring and evaluation of therapeutic benefits; or for drug discovery and pharmacokinetics guidance.

17. Use of a transgenic non-human mammal according to any of claims 1 to 10 or the progeny thereof according to claim 11, or the cell lines, primary cells or tissue samples according to any one of claims claim 12 to 15, for screening, searching, identifying, discovering, developing and/or evaluating compounds for the prevention and/or treatment of cancer, or for evaluating the efficacy of therapy administered to a subject suffering from said cancer , or for monitoring the evolution of cancer.
18. A method for screening, searching, identifying, validating, optimizing, discovering,
developing and/or evaluating compounds for the prevention and/or treatment of
cancer, or for repositioning known drugs or combinations of compounds, which
comprises administering a candidate compound to a transgenic non-human mammal
according to any of claims 1 to 10 or to the progeny thereof according to claim 11, or
the cell lines, primary cells or tissue samples according to any one of claims claim 12
to 15 and monitoring the response.

19. The use or method according to claim 17 or claim 18, wherein said cancer is a
hematological malignancy selected from the group comprising lymphomas,
leukaemias, such as, acute lymphocytic leukemia (also known as acute lymphoblastic
leukemia or ALL), acute myelogenous leukemia (AML), chronic lymphocytic
leukemia (CLL), chronic myelogenous leukemia (CML), B-cell acute lymphoblastic
leukaemia and T-cell acute lymphoblastic leukaemia.

20. A method for identifying a target responsible for cancer maintenance, comprising
comparing the level of one or more potential target(s) responsible for cancer
maintenance in two groups, each group comprising a transgenic non-human mammal
according to any of claims 1 to 10 or the progeny thereof according to claim 11, or
the cell lines, primary cells or tissue samples according to any one of claims 12 to 15,
wherein:

(a) the expression of said oncogene is upregulated in the first group;

(b) the expression of said oncogene is downregulated or normal in the second
group; and

(c) comparing the levels of expression of said one or more potential target(s)
responsible for cancer maintenance in the first group with the levels in the
second group.

21. The method for identifying a target responsible for cancer maintenance according to
claim 20, wherein the expression of said oncogene in the second group has been
upregulated for a period of time prior to being downregulated.

22. A method for identifying a compound which modulates the activity of a target
responsible for cancer maintenance identified by the method of claim 20 or claim 21,
which comprises administering to a transgenic non-human mammal according to any
of claims 1 to 10 or to the progeny thereof according to claim 11, or the cell lines, primary cells or tissue samples according to any one of claims 12 to 15 a candidate compound and subsequently monitoring the response

23. A method for identifying a compound which targets the mechanisms that are responsible for cancer maintenance, which comprises administering to a transgenic non-human mammal according to any of claims 1 to 10 or to the progeny thereof according to claim 11, a candidate compound and subsequently monitoring the response

24. A method for identifying a compound which targets the mechanisms that are responsible for cancer maintenance, which comprises contacting a cell line, or a primary cell, or a tissue sample according to any of claims 12 to 15, with a candidate compound contacted and subsequently monitoring the response.

25. Use of a compound identified by the method of any one of claims 17-19 and 22-24 in the manufacture of a medicament for treating cancer.

26. The use according to claim 25, wherein the medicament is intended to be administered in combination with one or more other compounds which inhibit the initiating oncogenic event.

27. Use of a compound which inhibits the expression of the BCR-ABLp190 gene products or reverts the effects of BCR-ABLp190 expression in the manufacture of a pharmaceutical composition for prevention and/or treatment of a cancer.

28. Use according to claim 27, wherein said compound is selected from the group consisting of antisense BCR-ABLp 190 mRNA, ribozymes, triple helix molecules, small interference RNA (siRNA), antibodies anti-BCR-ABLp190, enzymes or proteins which regulate the activity of BCRABLP190 protein, and mixtures thereof.

29. The use according to any one of claims 25 to 28, wherein the cancer is a hematological malignancy selected from the group comprising lymphomas, leukaemias, such as, acute lymphocytic leukemia (also known as acute lymphoblastic leukemia or ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), B-cell acute lymphoblastic leukaemia and T-cell acute lymphoblastic leukaemia.
30. A pharmaceutical composition comprising a therapeutically effective amount of a compound which inhibits the expression of the BCR-ABLp190 gene products or reverts the effects of BCR-ABLp190 expression together with one or more pharmaceutically acceptable excipients and/or carriers.

31. Pharmaceutical composition according to claim 30, which comprises a vector encoding a compound that inhibits the expression of the BCR-ABLp190 gene products or reverts the effects of BCR-ABLp190 expression.

32. Pharmaceutical composition according to claim 30 or 31, wherein said compound which inhibits the expression of the BCR-ABLp190 gene products or reverts the effects of BCR-ABLp190 expression is selected from the group consisting of antisense BCR-ABLp190 mRNA, ribozymes, triple helix molecules, small interference RNA (siRNA), antibodies anti-BCR-ABLp190, enzymes or proteins which regulate the activity of BCR-ABLp190 protein, and mixtures thereof.

33. A kit for in vitro diagnosing a hematological malignancy in a subject, said hematological malignancy selected from the group comprising lymphomas, leukaemias, such as, acute lymphocytic leukemia (also known as acute lymphoblastic leukemia or ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), B-cell acute lymphoblastic leukaemia and T-cell acute lymphoblastic leukaemia being, or for determining the stage or severity of said hematological malignancy in a subject, or for determining the predisposition of a subject to develop said hematological malignancy, or for monitoring the effect of the therapy administered to a subject with said hematological malignancy, or for screening, searching, identifying, discovering, developing and/or evaluating compounds for the prevention and/or treatment of a hematological malignancy which comprises an antibody that specifically recognizes BCR-ABLp190 protein in a suitable packing, or primer pair designed to specifically amplify a nucleic acid having a sequence that is specific to BCR-ABLp190.
FIG. 1A

[Diagram showing genetic elements and their interaction with CombitTA-p190 (TET OFF)]

FIG. 1B

[Image showing the effect of Dox on BCR-ABL190 expression with probe ABL and β-actin]

FIG. 1C

[Graph showing the number of living cells (x10^3/mL) over time (hours) with different treatments: Ba/F3+BCR-ABL190, Ba/F3+IL3, Ba/F3+CombitTA-p190 (-tet), Ba/F3+CombitTA-p190 (+tet), Ba/F3+IL3, Ba/F3+CombitTA-p190(-tet)+STI571]
ON

BCR-ABL<sup>190k</sup>/abl in PB: 1.1±1.7
PB blast cells: >10%

OFF

4 ng/ml dox
for 10 weeks

4

BCR-ABL<sup>190k</sup>/abl in BM: ND
BCR-ABL<sup>190k</sup>/abl in PB: ND
BM blast cells: >15%
PB blast cells: >10%